TISSUEGNOSTICS

PRECISION THAT INSPIRES

TissueFAXS SL

User Manual

1. Table of Contents

2. Disclaimer

TissueFAXSplus is a microscope-based cell analysis system for cells in cryocut-, paraffin-sections and/or TMAs. It consists of the software modules "TissueFAXS" combined with either "TissueQUEST" & "HistoQUEST" or with "StrataQUEST" and is used for acquisition of images in brightfield and/or fluorescence and/or confocal and/or multiplexing and/or multispectral mode, for counting the number of positive and negative cells and/or for quantification of staining intensities and/or classification of histological structures. TissueFAXSplus is used for the standardization of tissue analysis in combination with immunohistochemical and immunofluorescence staining.

TissueFAXSplus does not give any direct diagnosis and there is the possibility that the tissue specimen does not provide enough information for a proper analysis and/or diagnosis. The cell analysis system only measures cellular parameters. Such measurement parameters must in any case be reviewed and validated by a qualified human professional with profound knowledge of the sample under investigation and its cellular constituents as well as profound knowledge of the cell analysis system and who has received special training in the operation of cell analysis methods. The cell analysis system can under no circumstances make a decision on the therapy of patients.

The results of the analysis are purely statistical values. Users must re-evaluate images and likelihood of the statistical data. Pure interpretation of statistical data is not allowed

Notes:

- TissueFAXS FLUO is similar to TissueFAXSplus but is for fluorescence samples only and must not be used with brightfield/immunohistochemical samples. TissueFAXS FLUO consists of the software modules "TissueFAXS" as well as "TissueQUEST" and/or "StrataQUEST FLUO".

- TissueFAXS HISTO is similar to TissueFAXSplus but is for brightfield/immunohistochenical samples only and must not be used with fluorescence samples. TissueFAXS HISTO consists of the software modules "TissueFAXS" as well as "HistoQUEST" and/or "StrataQUEST HISTO".

- TissueFAXS 200 and TissueFAXS SL are similar to TissueFAXSplus but for batch scanning of up to 200/120 brightfield/immunohistochenical and/or fluorescence slides. TissueFAXS 200 /

TissueFAXS SL consist of the software modules "TissueFAXS 200" / "TissueFAXS SL" as well as "HistoQUEST" and/or "TissueQuest" and/or "StrataQUEST".

- TissueFAXS SPECTRA is similar to TissueFAXSplus but is for multispectral imaging of fluorescence samples only. Scanning of brightfield/immunohistochenical samples on TissueFAXS SPECTRA is possible with the colour camera, if any, but MUST NOT / CANNOT be done with the multi-spectral camera. TissueFAXS SPECTRA consists of the software modules "TissueFAXS SPECTRA" as well as "TissueQUEST" and/or "HistoQuest" and/or "StrataQUEST".

Using the unmixing procedure must be done with care as tissue-derived autofluorescence may interfere with the algorithm and cause inconclusive or even faulty output.

Selecting and correctly assigning proper reference spectra is critical for the accuracy of the unmixing result and is the sole responsibility of the user. ***Selecting wrong reference spectra and/or assigning any reference spectra inappropriately will cause inconclusive / faulty output and thus generate false measurement results!***

- TissueFAXS CHROMA is similar to TissueFAXSplus but is for multi-channel imaging of fluorescence samples through narrow band-pass filters only. Scanning of brightfield/immunohistochenical samples on TissueFAXS SPECTRA is possible with the colour camera. TissueFAXS CHROMA consists of the software modules "TissueFAXS" as well as "TissueQUEST" and/or "HistoQuest" and/or "StrataQUEST".

- StrataQuest provides a software tool for machine learning for the automatic classification of tissue structures, including detection of tumor areas in tissue sections. The results generated by the software have to be verified by a qualified human professional in any case (negative as well as positive results). In case the software does not detect specific histological structures and/or tumor cells, a human professional has to verify this result by other means, as it is possible that certain biological patterns and/or special types of (cancer) cells may not be detected by the automatic detection function. Measurement errors may also occur due to the fact that the cell-environment in which the software has to operate in is highly variable.

We point out to the fact that the following circumstances/factors might influence and/or impair the result of the analysis to the level where the result rendered might be inconclusive or even faulty:

- Quality of the tissue sample: In this context, especially the age of the tissue sample is relevant. Long durations between the harvesting and/or staining of the tissue sample and the analysis as well as storage errors can tamper with the outcome of the analysis.
- Quality of the preparation of the tissue sample for the analysis and the materials used: In this context, especially the type and quality of the reagents and the capability/precision of the person handling the reagents can be relevant and may lead to inconclusive/faulty results (for example: dilution-errors concerning the reagents).

These factors (as well as the capability of the human professional performing the validation of the test results) lie solely within the responsibility of the user of the software. TissueGnostics does not take any responsibility for test results that are influenced by one of the above mentioned factors.

Each and any product shall be used only after training performed by TissueGnostics or authorized distributors of TissueGnostics. A list of authorized distributors is available at the TissueGnostics website.

https://tissuegnostics.com/global-network/distributors

The TissueFAXS system and its software applications are FOR RESEARCH USE ONLY.

By using only parts of the system, changing system components (hardware or software) or using the TissueFAXSplus instrument in any other than the intended way it was designed for, the CE-Declaration becomes ***invalid****.*

Notices about symbols and labels on product

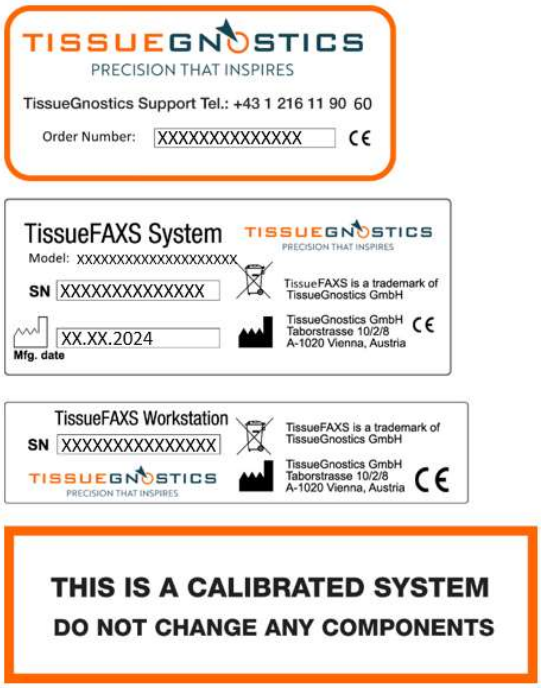
The following symbols appear on the labels of the product:

SN Serial number

WEEE symbol / separate disposal of electrical and electronic equipment

Manufacturer

CE mark

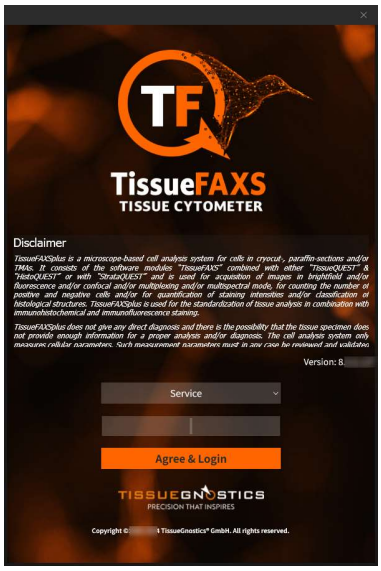


3. Getting Started with TissueFAXS

To start the application, press **TissueFAXS** icon on your desktop.

A login dialog will appear, where you can enter your login ID and password.

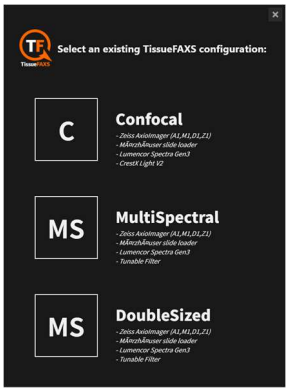
Read the **Disclaimer** regarding **TissueFAXS** application and click **Agree and Login**.



3.1. Choosing TissueFAXS Configuration

At this point, select an existing TissueFAXS configuration. The options listed here depend on your installed configurations.

In the image below, TissueFAXS possible configurations are shown.

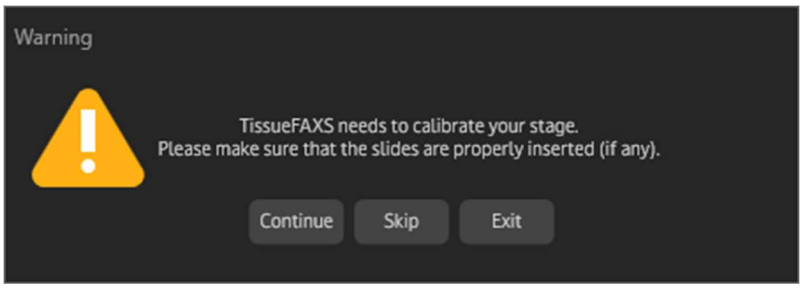


After selecting a configuration, a splash screen will be shown while the application is loading.

3.2. Device Calibration

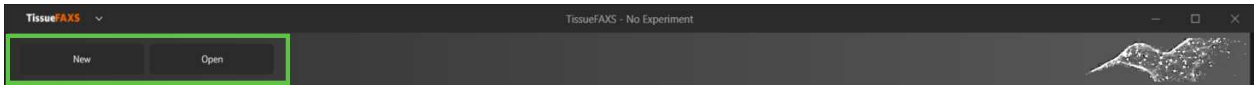
The first step of an image acquisition in TissueFAXS is to calibrate the stage when starting the application. This step may be skipped to simply view previously acquired images in TissueFAXS.

If acquisition is performed at a later time, there will be a prompt to calibrate the stage. For details, see **Chapter** Stage Calibration.

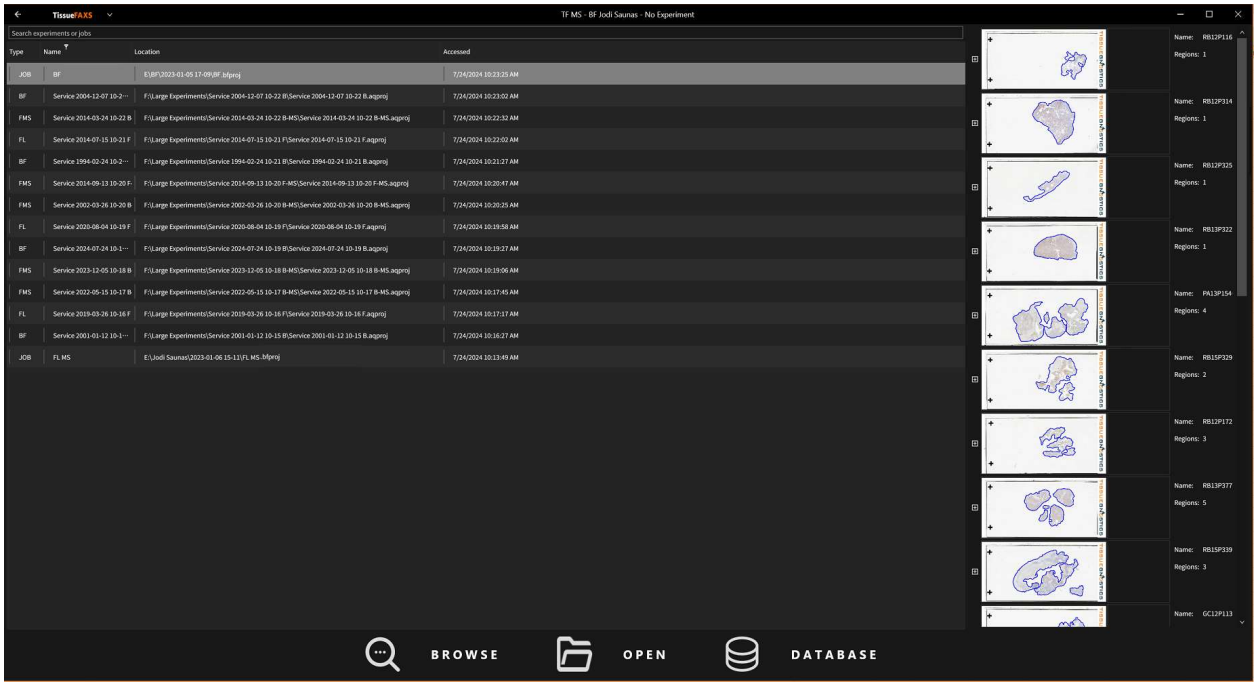


3.3. Opening Experiments

In order to use the full **TissueFAXS** functionality, either a new project must be created or an existing one must be loaded.



Open Existing Project

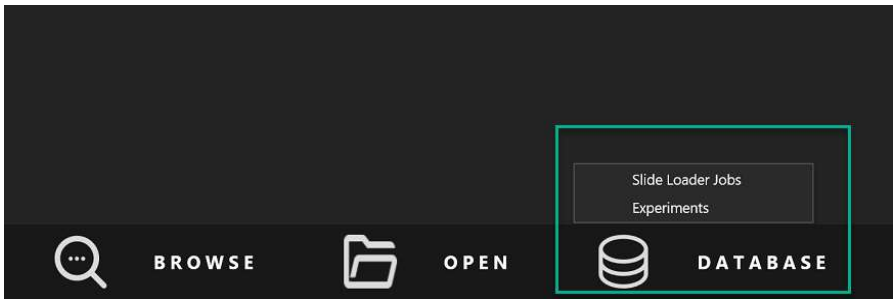


- You can either open a project from the recently opened list (if you already have previously opened projects in the list) or browse on your computer for a project.
- For each selected project from the list, a preview and a set of details are available on the right side.
- Use the **Search** feature in order to find a certain project in the list.

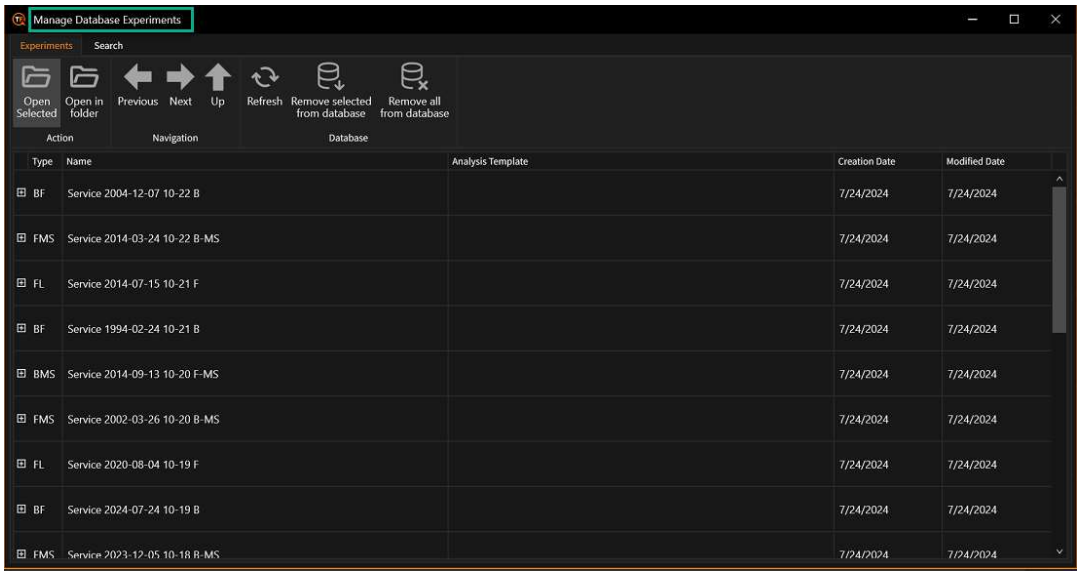
Use **Back** button to go back to **Start-up** screen.

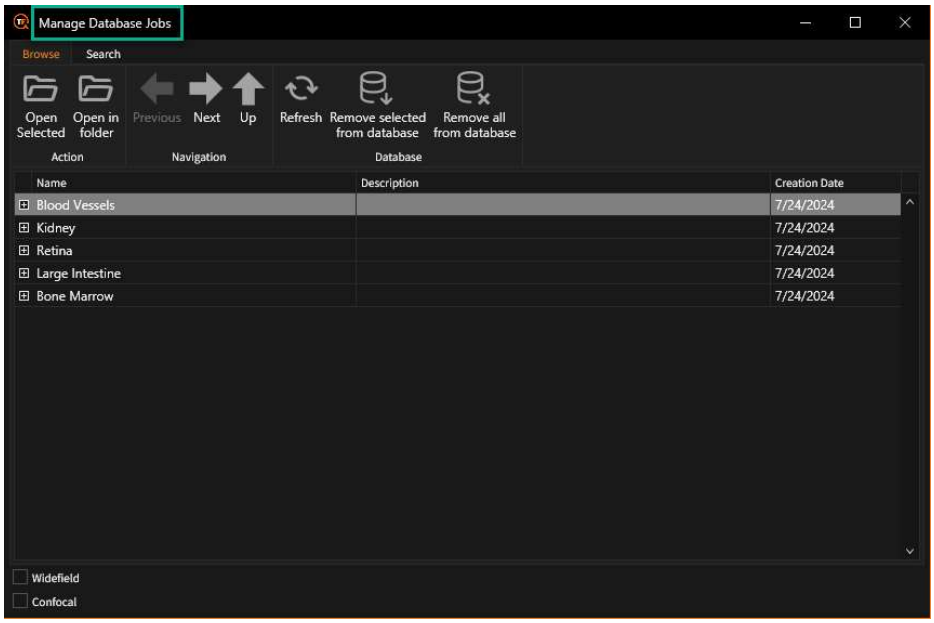
Open from Database

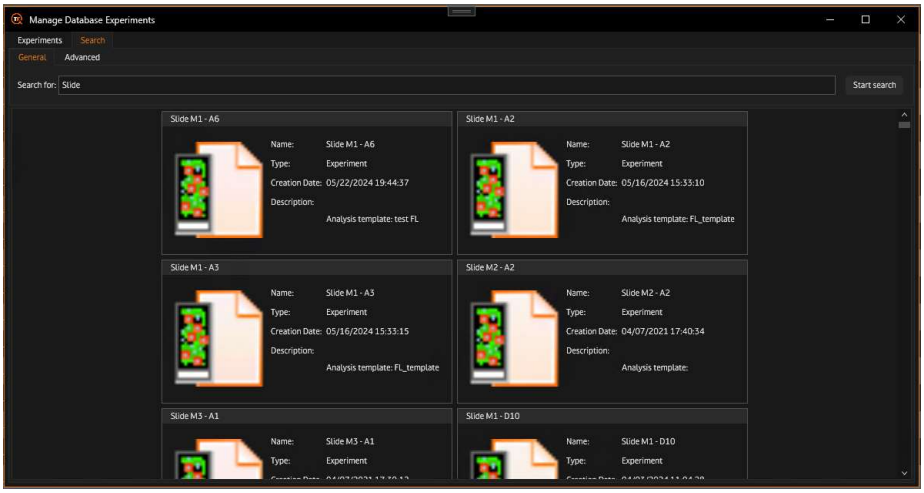
TissueFAXS stores in a database basic data about all the experiments that are created/opened to offer an easier search feature compared to manually browsing for experiments on the drive.



You can open from database either slide loader jobs or experiments.





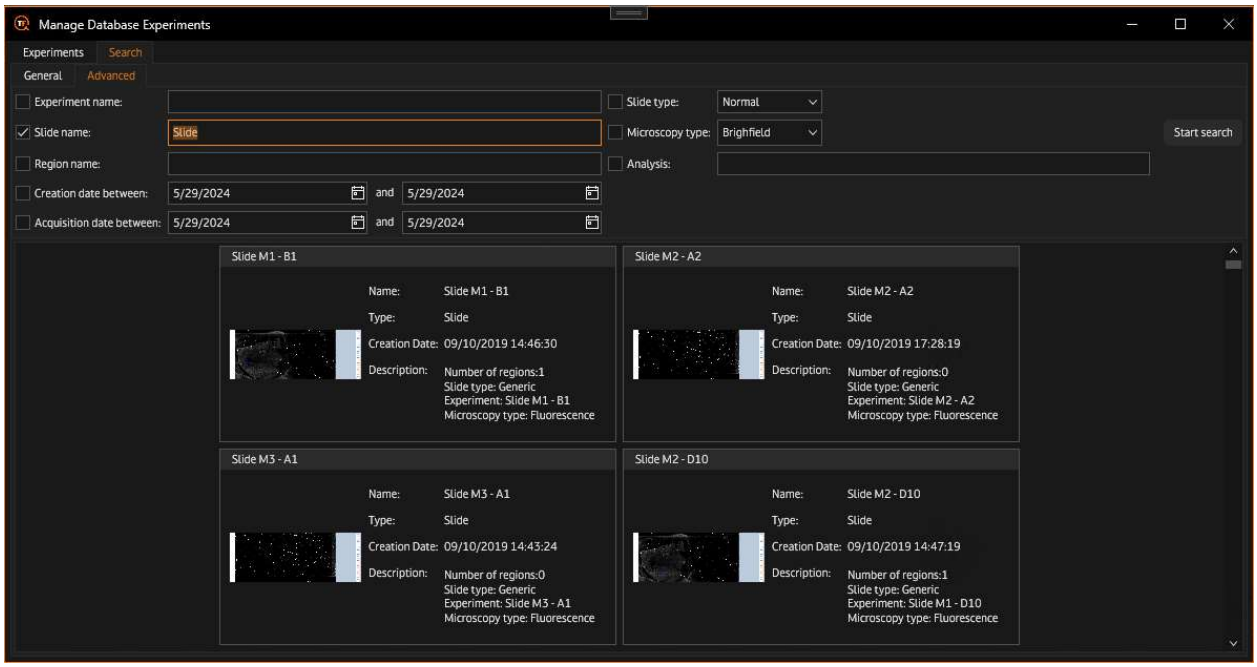


In **Manage Database Experiments** dialog, double click on a slide to open the corresponding TissueFAXS project or expand a job to see its details.

You can perform an advanced search in the database to find a specific project in a job.

Additional search types include:

- **General search**: enter the exact name of the slide in the upper field, then press **Find**: the requested job will be displayed.
- **Advanced search**: uses search criteria to find a specific slide – slide name, experiment name, creation/acquisition date, slide type, and microscopy type. After filling in one or more criteria, press the **Start search** button to see results.



3.4. Generating New Experiments

To acquire images in **TissueFAXS**, you have to create a new experiment or open an existing one. All images, settings and data are saved and embedded in your experiment files and folder.

Without having an experiment, you can only preview slides or capture single images from the camera window

Open experiment

Choose to open an experiment from the recently accessed list of experiments.

If the experiment you need is not in the list, browse for it on your computer or in database.

Create new experiments

In New Experiment window it is possible to:

- Create **custom** Brightfield/Fluorescence experiments;

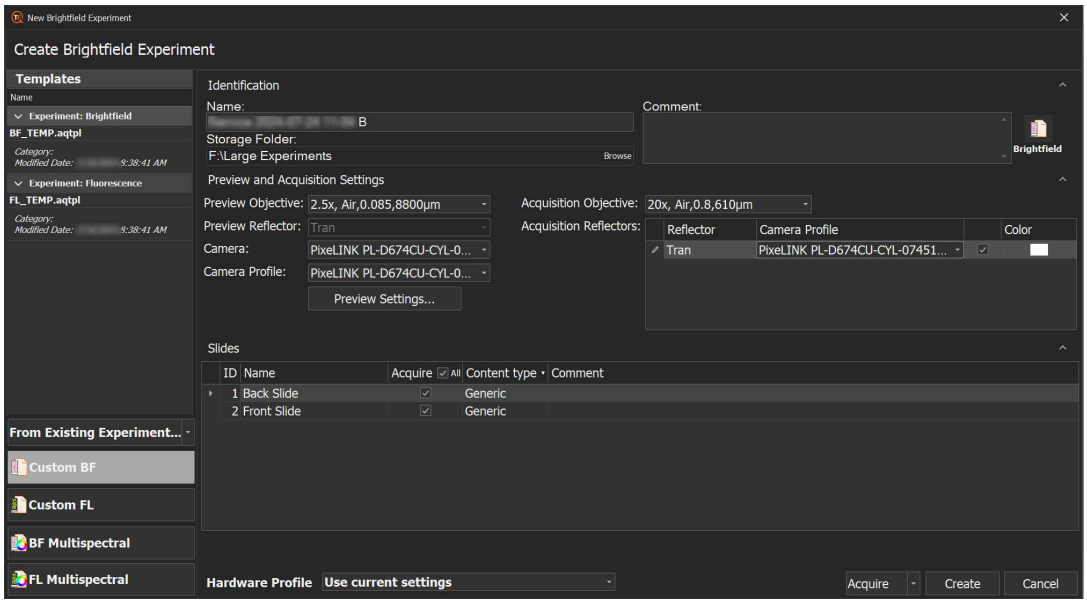
- Create new experiments using an existing **template** or experiment.

Experiment types

- **Brightfield experiments** have acquisition with transmitted white light which passes through the sample and the objective projects the image on a color camera.
- In **Fluorescence experiments** the images are created from reflected light by using an LED/laser lamp and a set of filters to excite fluorophores in the sample, which absorb the light and emit it at a longer wavelength to a monochrome camera (monochrome cameras are more sensitive and have higher bit depth output than color cameras).

1. Create a new custom brightfield experiment

From the left side of the **New Experiment** panel, choose **Custom BF**. The following panel will open:



Some of the fields that will appear on the right side of the **New Experiment panel can be customized and the data modified, if desired:**

- Experiment name;

- Storage folder;

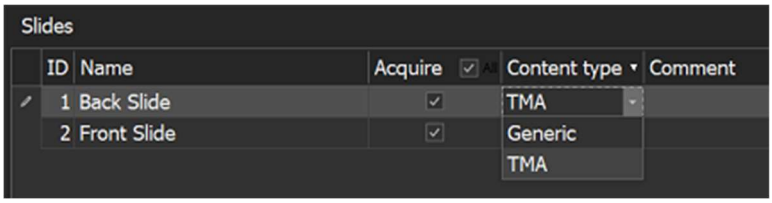
- Comment (optional);

- Preview and acquisition settings: objectives, reflectors, camera and camera profile (if any), preview settings.

TMA support

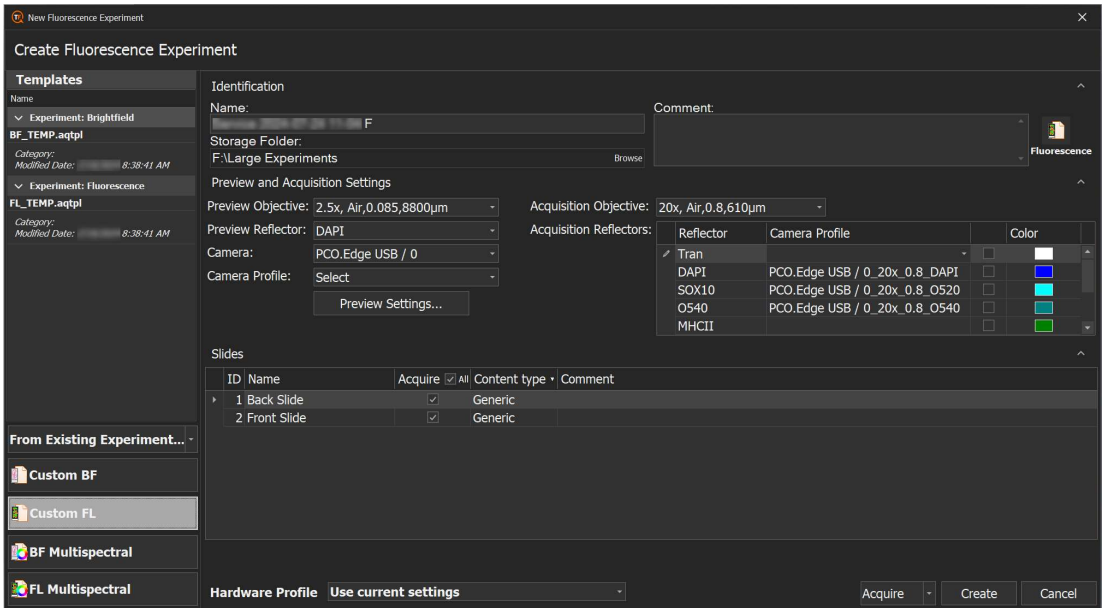
Although the TMAs can be treated and acquired as regular regions, **TissueFAXS** offers enhanced support for these types of samples/slides to better facilitate work and obtain higher quality results.

The content type of the slide must be set to **TMA**, in order to define TMA blocks and spots on a slide.



2. Create a new custom fluorescence experiment

From the left side of the **New Experiment** panel, choose **Custom FL**. The following panel will open:



Some of the fields that will appear on the right side of the **New Experiment** panel can customized and the data modified, if desired:

- Experiment name;

- Storage folder;

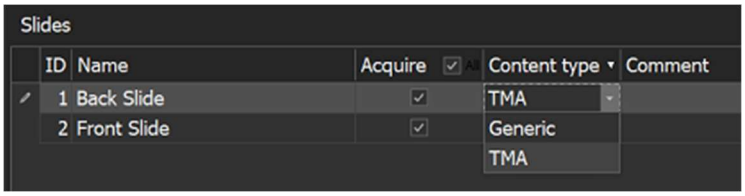
- Comment (optional);

- Preview and acquisition settings: objectives, reflectors, camera and camera profile (if any), preview settings.

TMA support

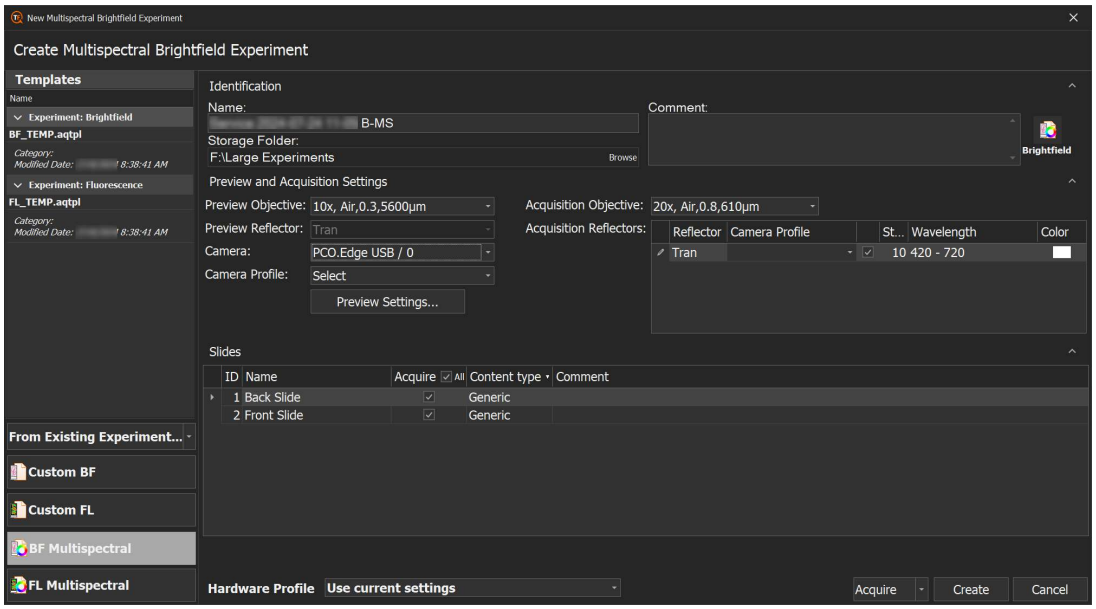
Although the TMAs can be treated and acquired as regular regions, **TissueFAXS** offers enhanced support for these types of samples/slides to better facilitate work and obtain higher quality results.

The content type of the slide must be set to **TMA**, in order to define TMA blocks and spots on a slide.



3. Create a new brightfield multispectral experiment

From the left side of the **New Experiment** panel, choose **BF Multispectral**. The following panel will open:



Some of the fields that will appear on the right side of the **New Experiment** panel can be customized and the data modified, if desired:

- Experiment name;

- Storage folder;

- Comment (optional);

- Preview and acquisition settings: objectives, reflectors, camera and camera profile (if any), preview settings;

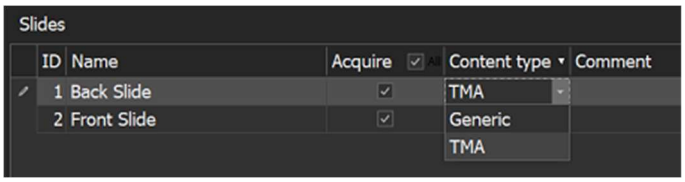
- Select a Step value and a color for Transmission.

Note: In brightfield experiments no selection can be made for reflectors. The channel Transmission is selected by default (it is specific for the Brightfield Experiments).

TMA support

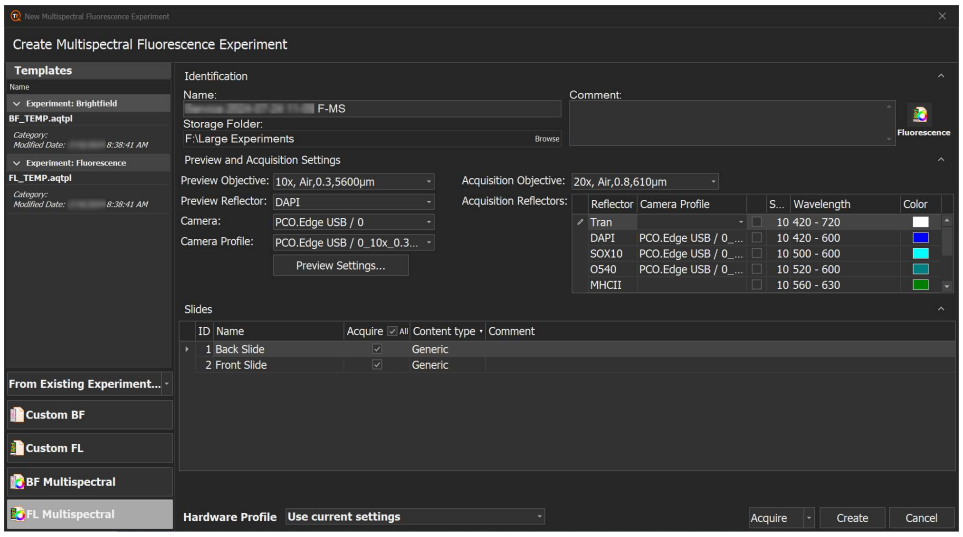
Although the TMAs can be treated and acquired as regular regions, **TissueFAXS** offers enhanced support for these types of samples/slides to better facilitate work and obtain higher quality results.

The content type of the slide must be set to **TMA**, in order to define TMA blocks and spots on a slide.



4. Create a new fluorescence multispectral experiment

From the left side of the **New Experiment** panel, choose **FL Multispectral**. The following panel will open:



Some of the fields that will appear on the right side of the **New Experiment** panel can be customized and the data modified if necessary:

- Experiment name;
- Storage folder;
- Comment (optional);
- Preview and acquisition settings: objectives, reflectors, camera, and camera profile (if any);
- Step values, wavelength and color for the reflectors:

- **Step**: the step value represents the difference in wavelength between two consecutive Lambda stack images.

- **Wavelength**: adjust the wavelength range for the Lambda stack per reflector.

***Example**: If DAPI wavelength range is 420-495 and a step of 20, the lambda images from acquisition will be at 420-440-460-480-495.*

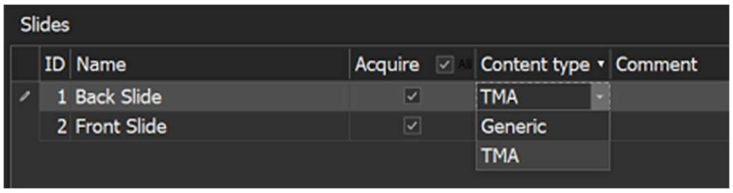
- Select a wavelength **Step** value and a color for selected channel.

Note: In brightfield experiments no selection can be made for reflectors. The channel Transmission is selected by default (it is specific for the Brightfield Experiments)

TMA support

Although the TMAs can be treated and acquired as regular regions, **TissueFAXS** offers enhanced support for these types of samples/slides to better facilitate work and obtain higher quality results.

The content type of the slide must be set to **TMA**, in order to define TMA blocks and spots on a slide.



Details on Preview Settings

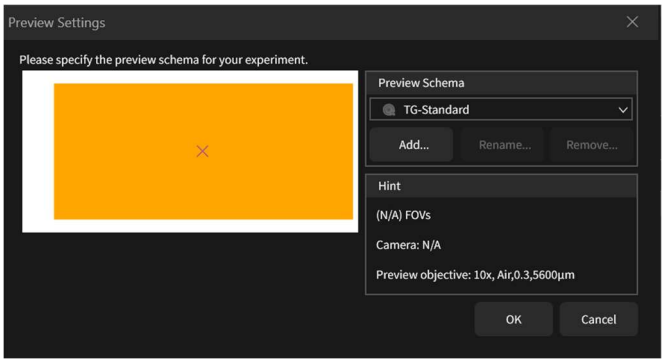
These settings apply to all the experiment types described above.

Settings for the preview operations can be set by clicking on the **Preview Settings** button. Choose a **preview schema** from the list and select the position of the label from the slide (up or down). This slide label setting will remain the same for the entire experiment. It can only be changed later for single slides.

In addition, a **profile** with predefined settings from the dialog may also be selected.

All settings made here can be changed later. Only the experiment type cannot be changed.

The preview area size can be adjusted by clicking on the **Preview Settings** button. The preview area (or yellow box) can be manually adjusted with the mouse or by selecting a pre-defined schema from the drop-down menu.



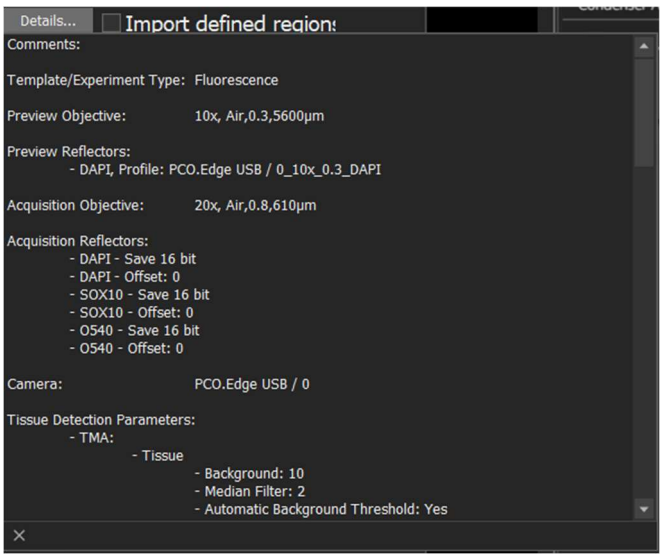
Preview Schemas can be named and saved for future projects. This is useful when working with multiple slides which frequently have the region of interest (tissue) in the same specific location. Defining the preview schema over the region of interest will save time. Otherwise the microscope will scan the entire slide in order to preview it.

The **Hint** area displays the preview schema size in terms of number of fields of view (FOVs). Additionally, the type of camera and objective used for preview is shown.

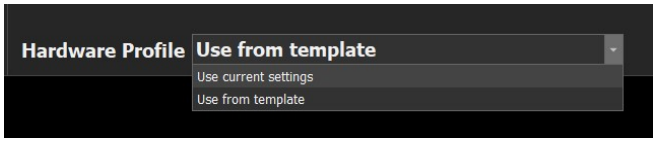
In the **Label Position** area, it is possible to specify how the slide is oriented on the stage. This is not a permanent setting. For instance, even if the slides have the labels oriented down, this can be changed later in the project for individual slides or for multiple slides. To do this later, just right click on the slide(s) and change its label position.

- In the **Slides** section, there is an option to edit slide's name, set the content type (generic or TMA), or add a comment.

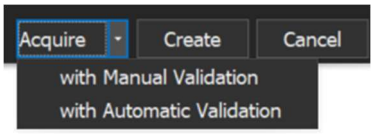
- Details about the template can be accessed by pressing **Details** button:



- Select desired **hardware profile**: use current settings or use the settings from the template:



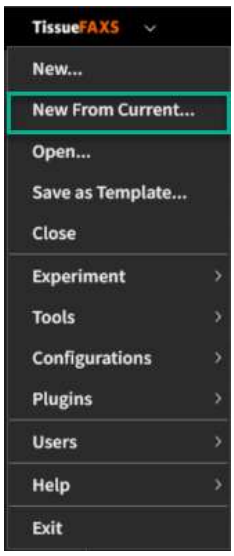
- Choose whether to acquire the slides or just create the new experiment. If to acquire, indicate whether the acquisition will be manual or automatic. By default, **Manual Validation** is selected (see Chapter Workflow).



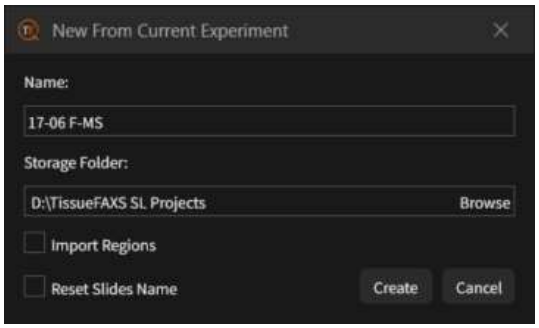
3.4.1. New from Current Experiment

You can create a new experiment based on the currently opened experiment.

Go to TissueFAXS main menu and select **New from Current**.



A popup will open where you can make the following **settings**:



- Choose a **name** for the new experiment;
- Choose a **storage** folder;
- Decide if you want to **import the regions** existing in the parent experiment;

- Reset **slide names** (the slides will get application's default names. i. e. "Slide 1", "Slide 2").

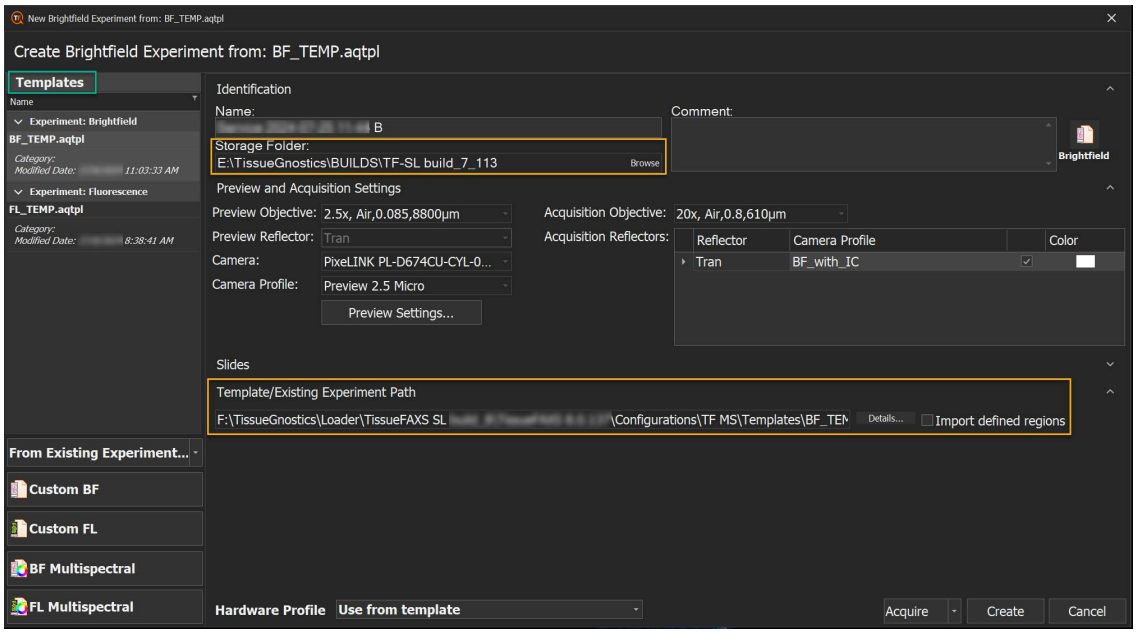
After you are done with the setting, press **Create**.

The new experiment will automatically open.

3.5. Experiment Templates

Experiment templates are settings combinations saved as templates for further use.

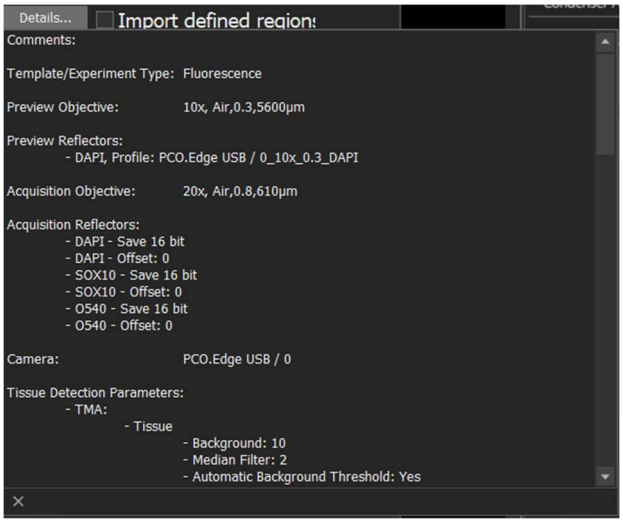
They can be found in the **New Experiment** window, grouped in default categories or custom defined categories:



Note: After selecting a template, the **Settings** section will be collapsed.

When selecting a template, you will access all the settings of the respective template: storage location, preview and acquisition settings.

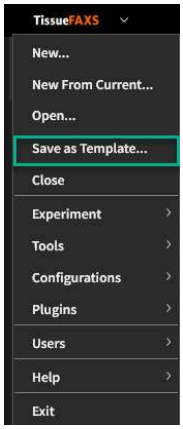
Also, you can see the acquisition details of the template (experiment type, preview/acquisition objective/reflectors, camera, tissue detection parameters, details regarding scan, stitching and focus, etc.) and import defined regions, if any.



Creating New Templates

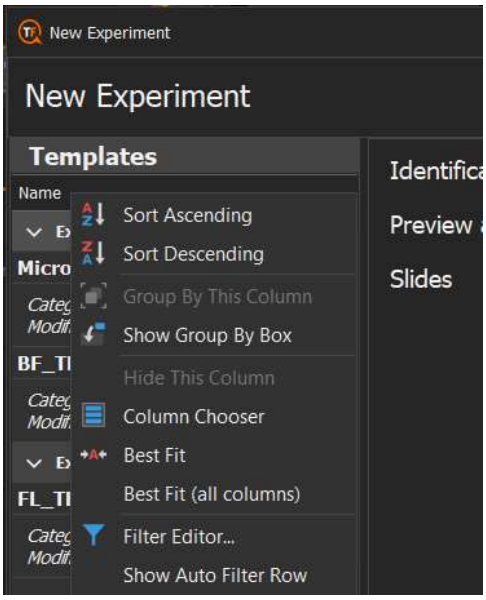
To create a template based on a currently opened experiment, Go to **TissueFAXS main menu** and choose **Save as Template**.

Browse for a location for the new template and give it a name. The new template will now be present in the **Templates** list.



Sorting Templates

To access the sorting feature for the templates list, right click on the **Name** field.

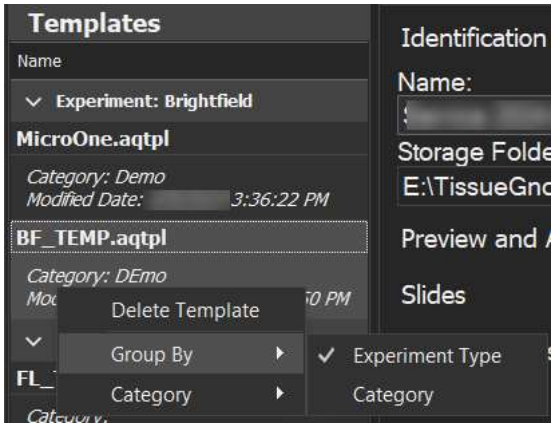


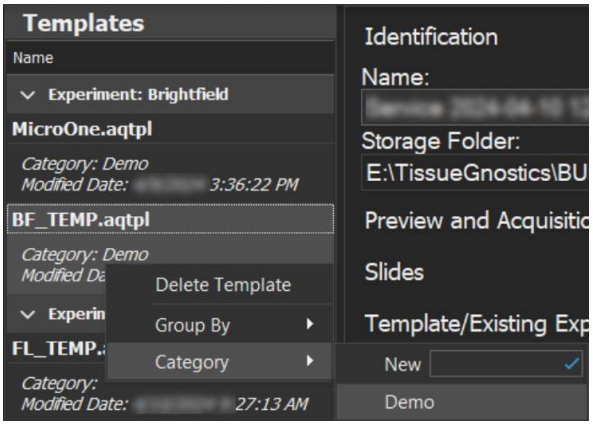
The following options are available:

- Sort Ascending/Descending
- Clear Sorting
- Group by this Column
- Show Group by Box
- Column Chooser
- Best Fit
- Best Fit (all columns)
- Filter Editor
- Show Auto Filter Row

Templates Contextual Menu

To access the contextual menu for a template, right click on the name of the respective template.



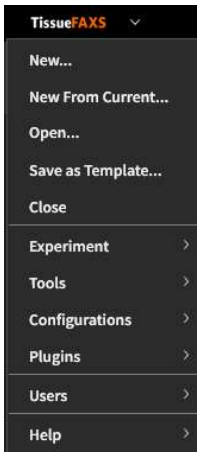


The following options are available:

- **Delete Templates**;
- **Group by Experiment Type/Category**: you can add templates to categories, for a better management, but also for easy search and a cleaner look;
- **Create Category**: creates new template category.

3.5.1. Save as Template

Save as Template option saves the current project as a template for further use (for new2-slide experiments or for jobs). This option can be found in **TissueFAXS main menu**.



Save As dialog will open, for selecting a name and a storage folder for the new template.

The new template will inherit the following from the initial project:

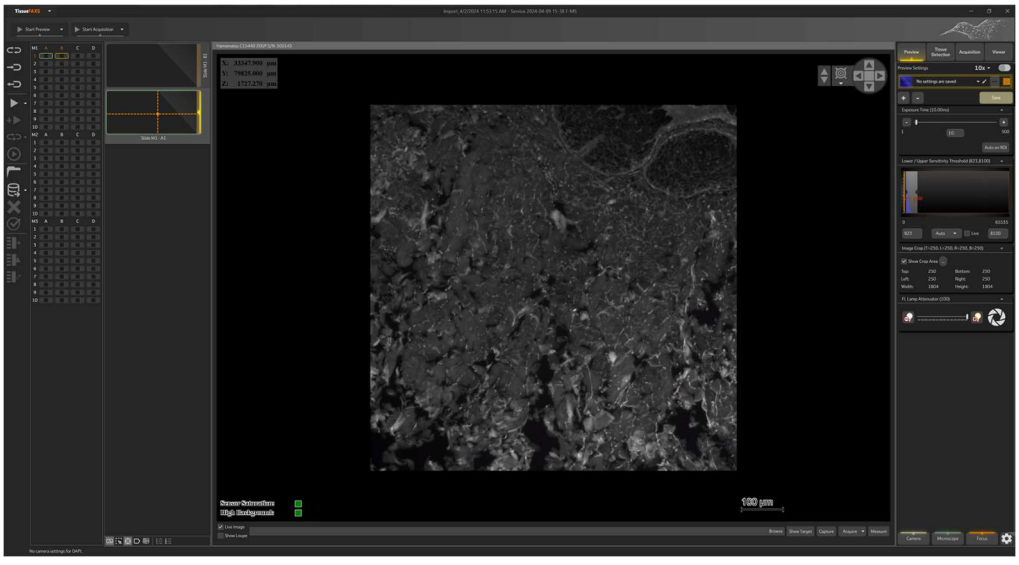
- Experiment type: brightfield or fluorescence;
- Preview objective;
- Preview reflector;
- Preview schema;
- Camera;
- Camera profile;
- Acquisition objective;
- Acquisition reflectors;
- Tissue Detection/TMA detection settings;
- Hardware profile, which includes most of the settings from Options the form.

The template will be available for creating new experiments in the future.

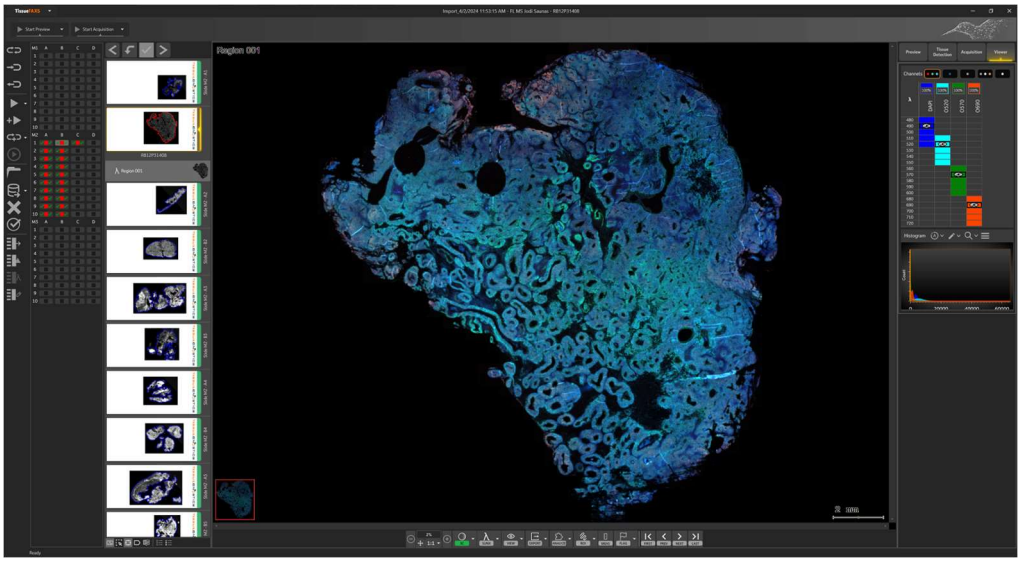
3.6. TissueFAXS Main Window Overview

Once you create or open an experiment, **TissueFAXS main window** will open.

If creating a new experiment, you can begin by choosing your settings for the preview and acquisition. If you already have a camera profile, you can directly start the preview and acquisition.



If you have opened an existing project, you will see all its details.



The two main actions in TissueFAXS (when a project is already created) are represented by the two buttons in the upper left side of the window:

- Start Preview
- Start Acquisition

The viewer at the center of the window shows the current item you are focusing on, be it live image, acquired slide or region. Below the viewer there is a toolbar with specific viewer options and features.

The left side shows:

- The loader and the status of its slides, if any, plus the job-related controls
- Virtual images of the slides present on the stage. If you define regions, annotations etc. on a slide, they will appear listed beneath the parent slide.

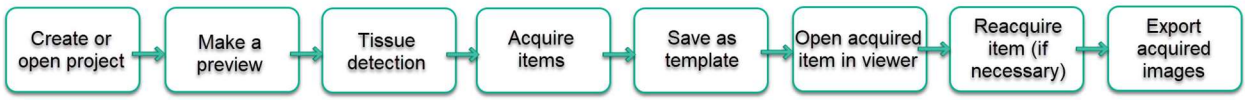
The right side is organized in tabs that reflect the order of actions in a regular experiment workflow:

- Preview
- Tissue Detection
- Acquisition
- Viewer

4. Experiment Workflow

A brief workflow with TissueFAXS implies two stages:

Stage one: create a new project and use it to generate a template. Further on, the job will be created based on this template.



1. Open an existing project or create a new one.

2. Make a preview: select the slide(s) you want preview (or part of a single slide), adjust the preview settings (camera, microscope, focus), then start the preview.

3. Once the preview is done, proceed with tissue detection. This means the tissue areas on the slide will be automatically detected by TissueFAXS, but you can also manually adjust the detection or draw regions for finer results.

4. The regions detected in the previous step can now be acquired. Select items to be acquired, adjust the preview settings (camera, microscope, focus), then start the acquisition.

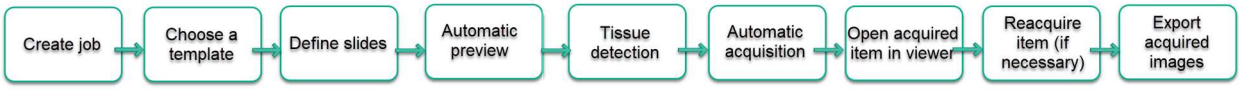
5. Save project as template for further use.

6. Acquired items can now be opened in the Viewer. You can refine your visualization experience with the digital images by using the viewer features (magnification, overlay, measurements, illumination adjustments, etc.).

7. If necessary, an item can be reacquired entirely or partially (using the Flags feature).

8. Export acquired images.

Stage two: create a new job based on the previously created template.



1. Create new job using the New Job button.

2. In the Job Properties panel, choose a previously created template.

3. Define the slides you want to include in the job.

4. After you are done selecting the settings, press Create to create the new job. Automatic preview process will begin.

5. Automatic tissue detection activates. You can manually intervene if you want to refine the detection.

6. Automatic acquisition activates: all the detected structures will be acquired.

7. Acquired items can now be opened in the Viewer. You can refine your visualization experience with the digital images by using the viewer features (magnification, overlay, measurements, illumination adjustments, etc).

8. If necessary, an item can be reacquired.

9. Export acquired images.

Further on, each of these steps is extensively explained in **Chapter** Job Workflow.

4.1. Image Preview

The first main action within TissueFAXS is called a **preview**. The preview means you will create an overview image of your sample/slide very quickly, using a low magnification objective.

This will help you know your sample. like having a map, and decide the areas that will be further acquired in high resolution.

Also, the slide label will be scanned (for details see Chapter Slide Labels).

Preview Workflow

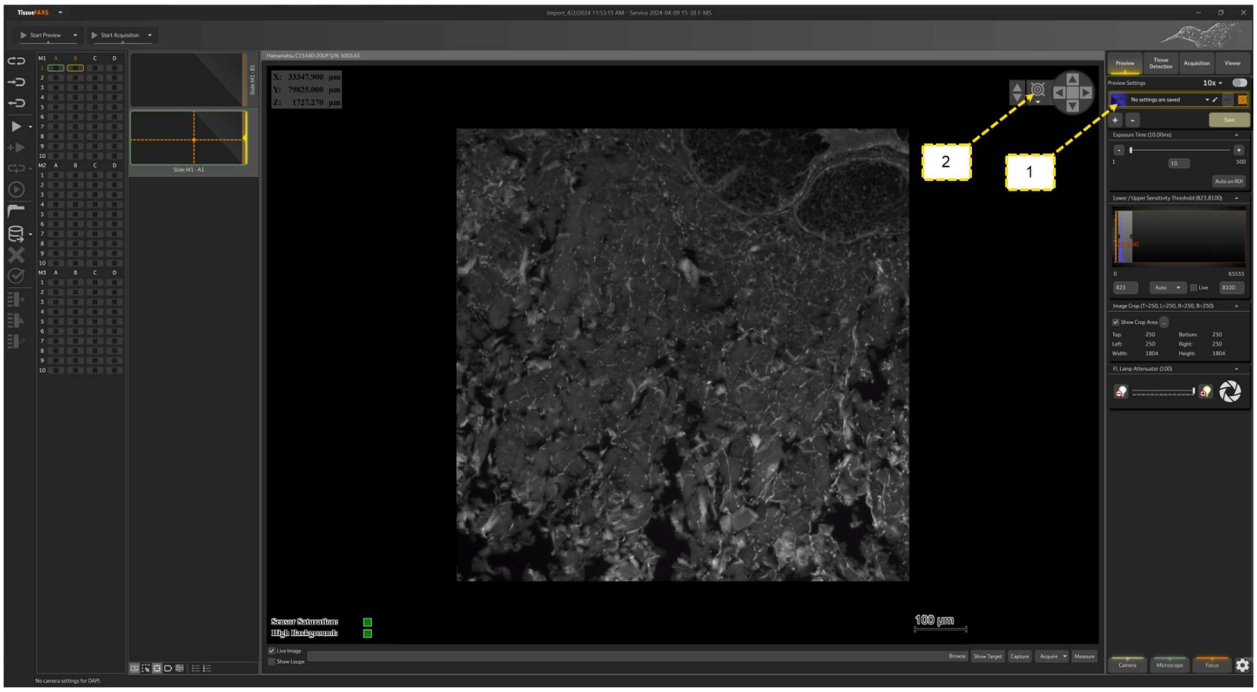
1. Create a new experiment (see **Chapter** Generating New Experiments).

2. To visualize the preview live image, press on the **channel** (1) to open the optical path.

3. Now you have to adjust the preview settings. Go in the **Preview Tab** and modify the settings depending on the specific of the current sample.

Note: Press **Save** button to save preview settings.

4. To focus the image, press **Focus** button over the tissue (2). For details see **Chapter** Stage.



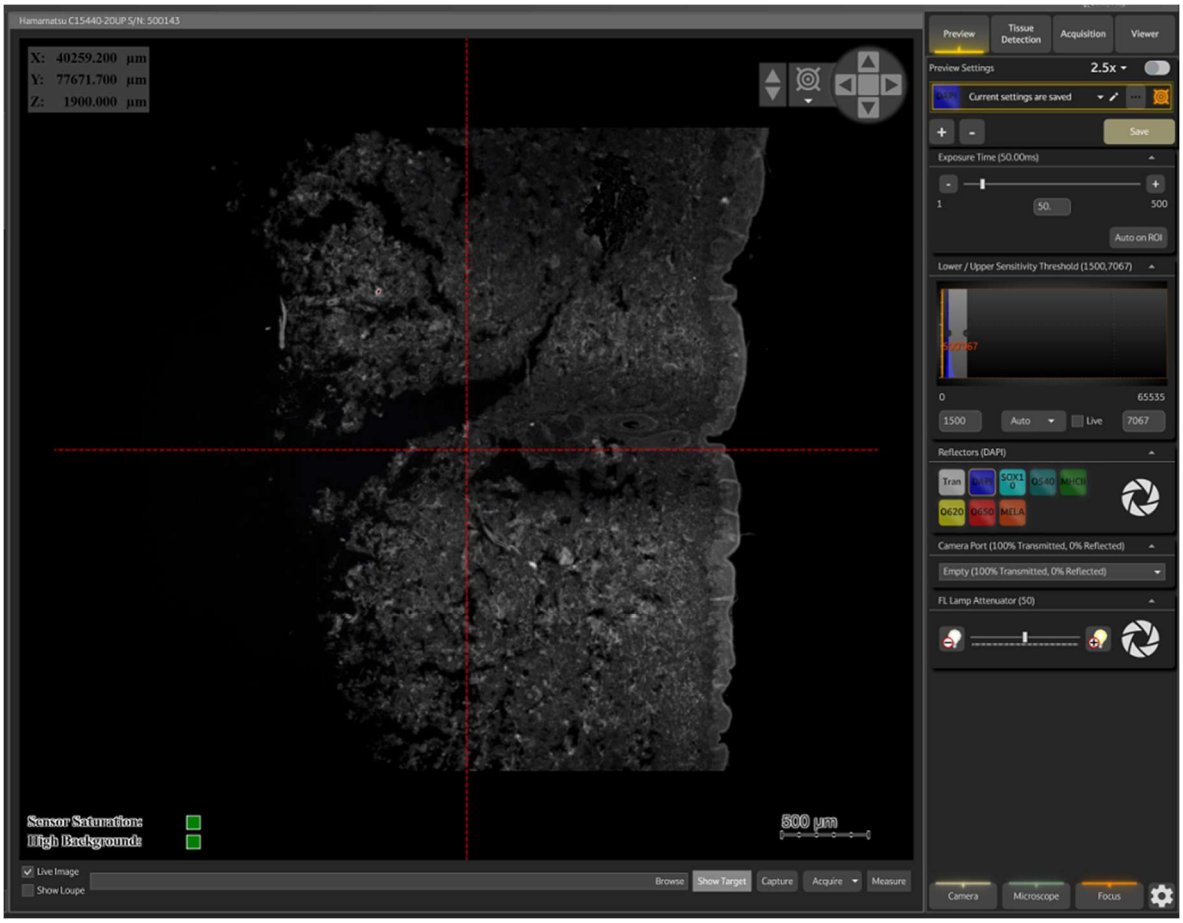
5. Press **Start Preview**. You will be asked to select the type of focus to be used: you can use autofocus or the current Z position (if you already have the sample in focus).

Note: If the preview settings are fine, you can save them as default camera profiles for further use (see **Chapter** Camera Controls), and then you can skip Step 2 and Step 3.

4.1.1. Preview Settings

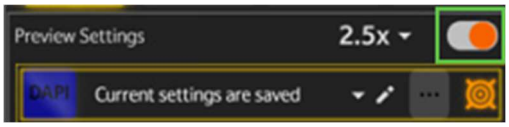
Preview Settings

Preview settings can be adjusted from the **Preview** tab, illustrated below.



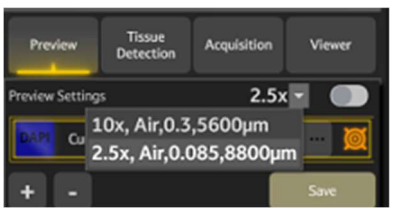
All the settings from the **Preview** can be automatically saved per experiment for further use, by enabling the **Auto save** button.

You can see the **status** of settings on the preview acquisition channel (settings saved/not saved etc.).



You can visualize the default preview objective, but it can be changed from the dropdown menu shown below.

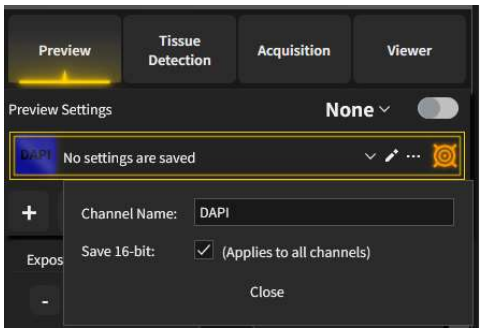
Note: The physical preview objective will switch depending on the selection made here.



All settings can be saved in a channel **profile** for further use. These profiles are available on the preview dropdown window. For more details see **Chapter** Camera Controls.

For FL experiments, extra settings are available when pressing **More Options**.

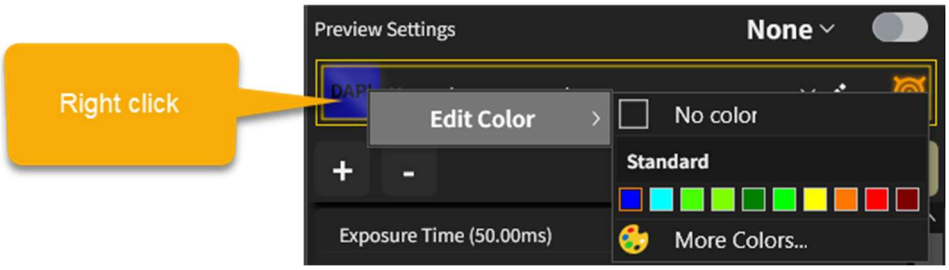
- **Edit channel name**;
- **Save 16bit** (check to apply to all channels).



Primary Focus: the focus will be made on the currently selected channel.

Add/Remove reflector: will add or remove channels from preview acquisition.

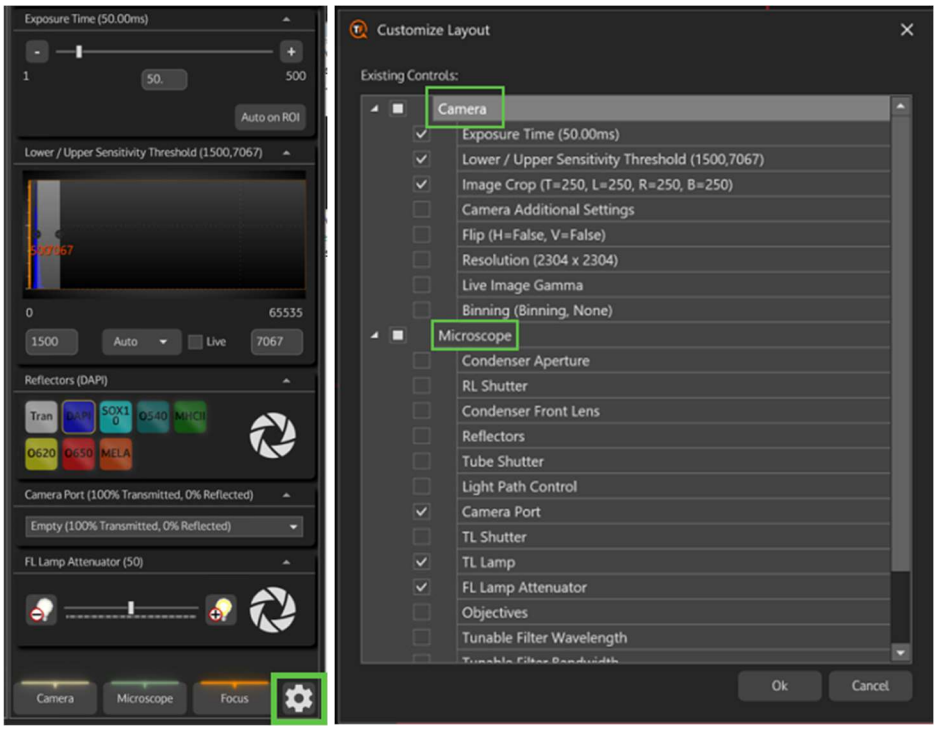
Editing color is possible if right clicking on the reflector name.



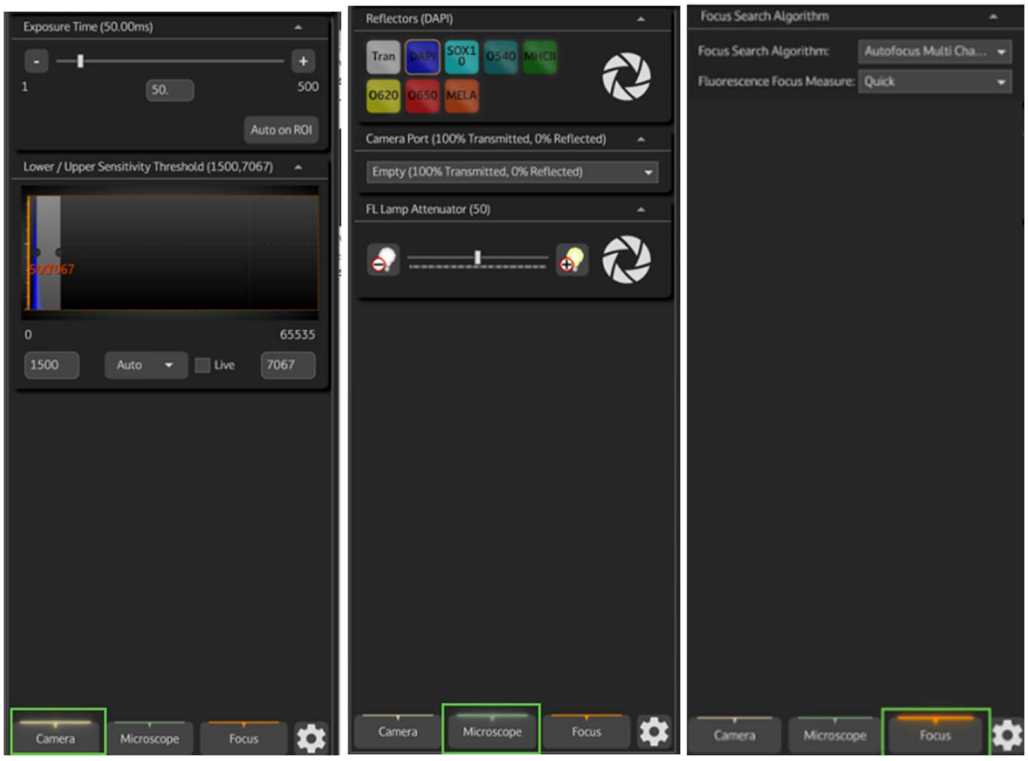
Save: press this button to save the settings for the current channel.

Controls Layout

By default, specific controls populate this section, depending on the experiment type (BF, FL, MS, Confocal). However, you can add or remove controls using **Customize Layout** window.



You can show/hide controls for camera by selecting /unselecting the corresponding tab.



4.1.2. Starting a Preview

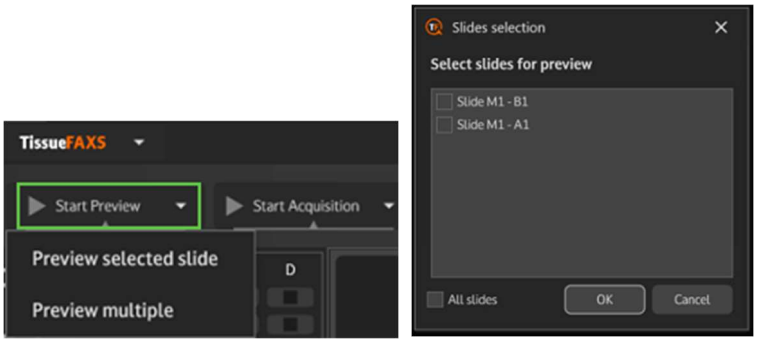
You can start a preview in two ways:

1. **Start Preview button**:

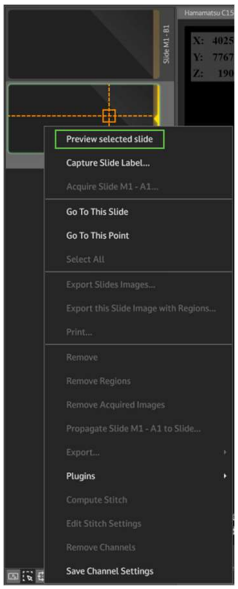
- If pressing directly **Start Preview**, the preview will be made on the default preview schema;

- Preview selected slide

- Preview multiple: choose what slides you want to preview.



2. **Slide contextual menu**: will start the preview for selected slide.



4.2. Tissue Detection

Tissue Detection is a TissueFAXS feature that automatically detects the structures present on a sample so that they will be further acquired.

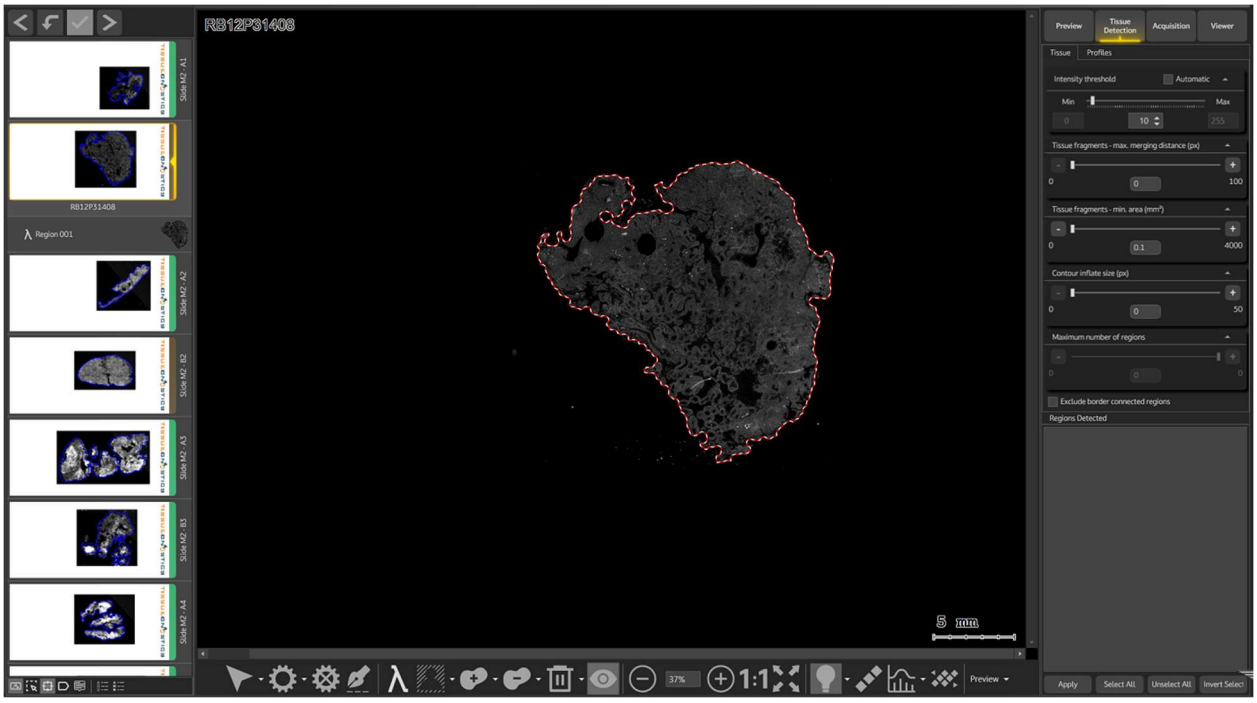
It has **two modes**: generic ROI detection and TMA detection.

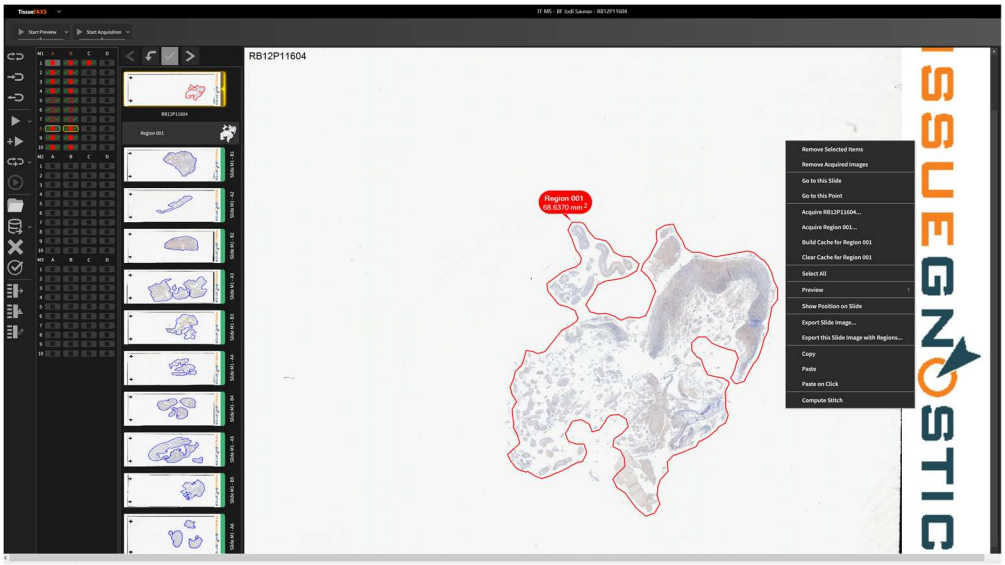
4.2.1. Generic ROIs

Automatic Tissue Detection of ROIs

After TissueFAXS will finish the preview, the detection can be made by opening the slide in tissue detection mode. The application will automatically run the detection using the default parameters.

Note: If a region was previously added to the experiment, the detection will not run automatically, you have to press **Run** button.



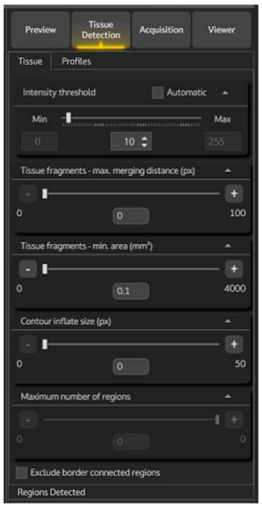


Manual Tissue Detection

If the automatic results of the detection need to be refined, go to **Tissue Detection** tab and manually adjust the settings until you obtain a good detection.

There is a set of parameters that can be adjusted to refine the results of automatic detection.

Tip: To begin a detection, set all parameters to "**0**". Then, one by one, starting with the first parameter, begin increasing the values until you obtain a neat detection.



- **Intensity Threshold**: is used as a threshold value to separate background (black) from tissue (white). If **Automatic** is enabled, the Intensity Threshold will be automatically computed on the gray image in the range selected by the user. The determined value will be shown in the edit box in the middle. A value can be entered by disabling Automatic and inputting the desired value in the edit box. Use the Gray indicator on the gray image to determine the pixel intensity in the image.

- **Lower**: the lower limit of the intensity range. It must be higher than the background values and lower than the dimmest nuclei that has to be detected.

- **Upper**: the upper limit of the intensity range. A lower value forces a lower threshold value. Must be higher than the average nucleus mean intensity.

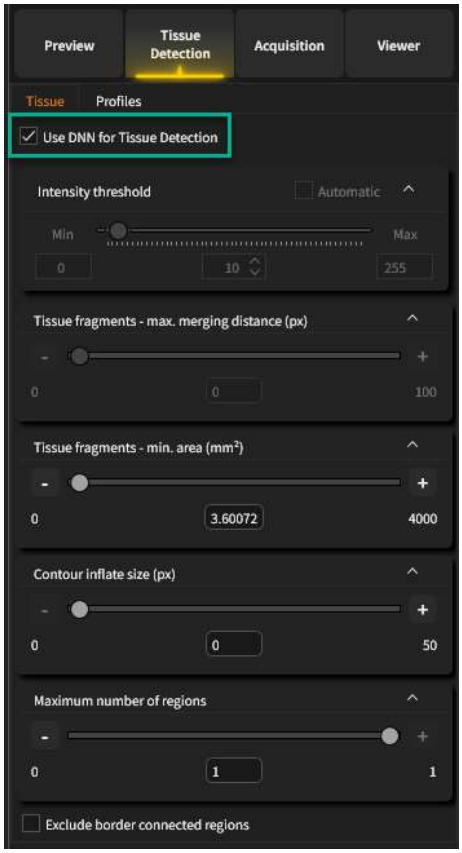
- **Tissue Fragments - max. merging distance**: try to increase this parameter if the region is split into multiple small subregions. The result will be a single region containing smaller subregions;
- **Tissue Fragments - min. area**: is the smallest area a tissue region can have. All regions with a smaller area will not be considered;
- **Contour Inflate Size**: the initially detected region will be inflated with a specified number of pixels, in order to include the edges of the tissue and a small surrounding area;
- **Maximum number of regions**: sets a maximum number of regions to be detected;
- **Exclude border connected regions**: regions touching the border are excluded from detection

Tissue Detection using DNN (Deep Neural Network)

This detection type uses a trained deep neural network model to perform ROI detection.

Note: Nuclei (Deep Learning) requires TissueGnostics Python Environment to be installed on the computer.

The following two **parameters** are specific to this detection type:



- **Tissue Fragments - min. area**: is the smallest area a tissue region can have. All regions with a smaller area will not be considered;
- **Contour Inflate Size**: the initially detected region will be inflated with a specified number of pixels, in order to include the edges of the tissue and a small surrounding area.

Detection Profiles

For an easier workflow, you can create detection profiles that work like detection templates.

Profile options:

- **Default**: allows the current set of parameter values to be stored for future detection.
- **Restore Defaults**: restores all the parameters to the last saved default parameters.
- **Profile**: saves the current parameter settings for future use.
- **Load Profile**: loads a previously saved profile from the existing list (by double clicking on it).

Tissue Detection contextual menu: see **Chapter** Tissue Detection Contextual Menu.

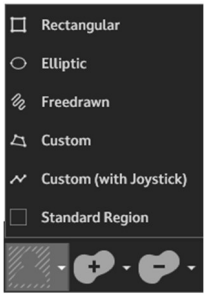
Tissue Detection Toolbar

Tissue detection specific controls are located within a toolbar in the lower area of the viewer

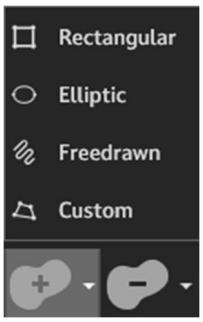
- **Select**: selects a rectangular detection area on the tissue. To remove the selection, press **Clear Selection**.

- **Run**: runs detection on selected area. If no selection is made, the detection will run for all the slide. From the arrow menu you can disable **Auto Tissue Detection**.

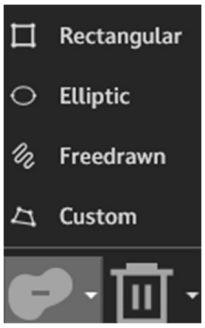
- **Clear**: clears all detected regions from the defined selection. If there is no defined selection, it will clear the detected regions from the full slide preview.
- **Edit**: enables/disables region editing mode. For details see the end of this chapter.
- **Multi-Spectral Mode On/Off**: if Multispectral mode is *On*, all created regions will be multispectral. If it is *Off*, all the new regions will be regular fluorescence regions.
- **User Defined Regions**: enables various shaped regions drawn by the user that will be included in the detection.



- **Add Extension Areas**: enables rectangular/elliptic/free-drawn areas drawn by the user in addition to the detection algorithm results (for example - TissueFAXS has automatically detected a region and you want to manually expand it).

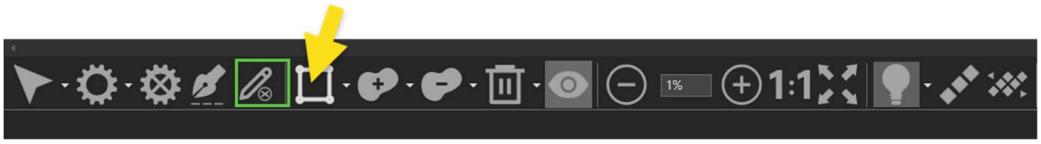


- **Add Excluded Areas**: enables rectangular/elliptic/free-drawn areas drawn by the user that will NOT be included in the automatic detection of regions.

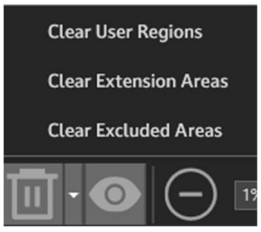


- **Exit Drawing Mode:** when in drawing mode for any types of regions, you can exit by pressing this button or by pressing Esc key on the keyboard.

Note: When in drawing mode, tissue detection contextual menu is not available.



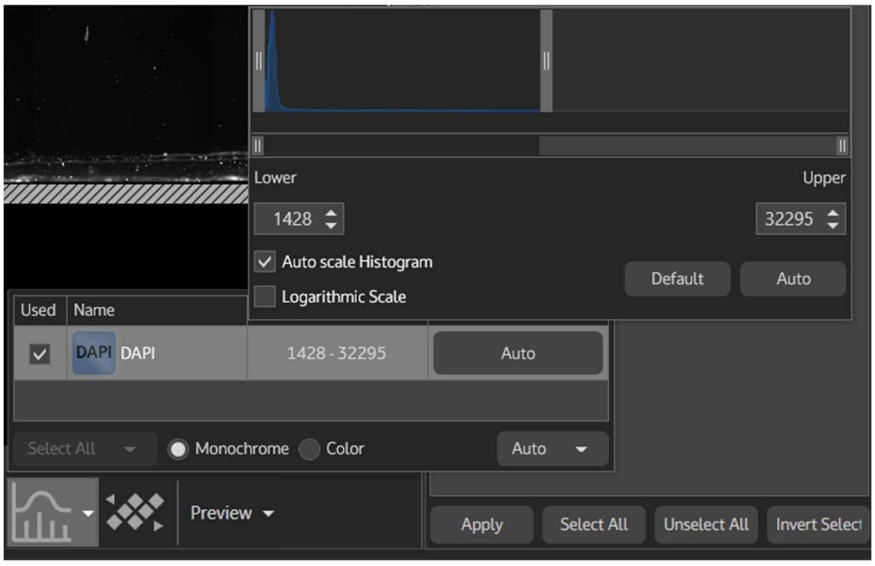
- **Clear All:** clears all the items added by the user.



- **Show All:** shows/hides all regions, extensions and excluded areas;
- **Zoom in/Zoom out;**
- **Best Fit;**
- **Original;**
- **Illumination Correction** (please see Chapter Illumination/Shading Correction);

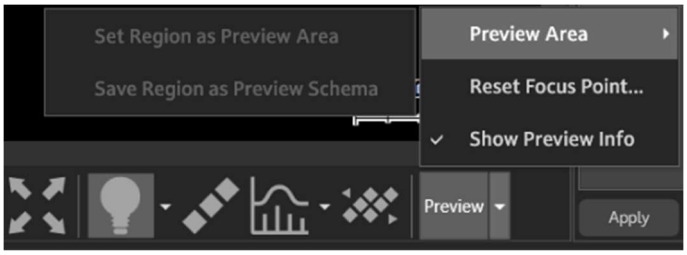
Note: Illumination correction affects the detection results as detection is done on the displayed image.

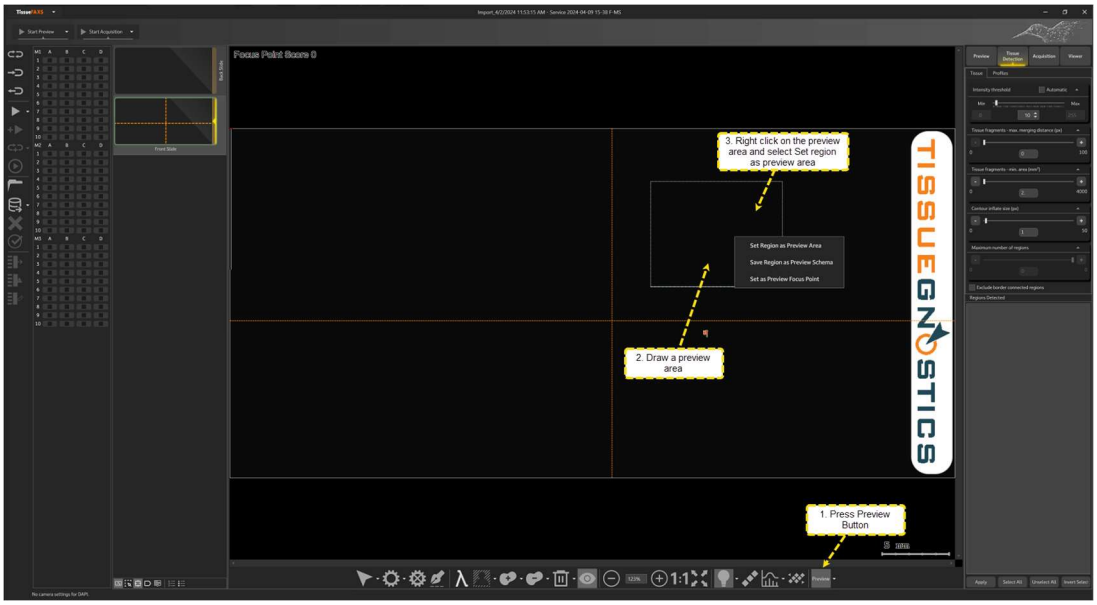
- **Show RGB color;**
- **Slide Overlay (**see **Chapter** Overlay);



- **Turn on/off gray image;**
- **Preview;**

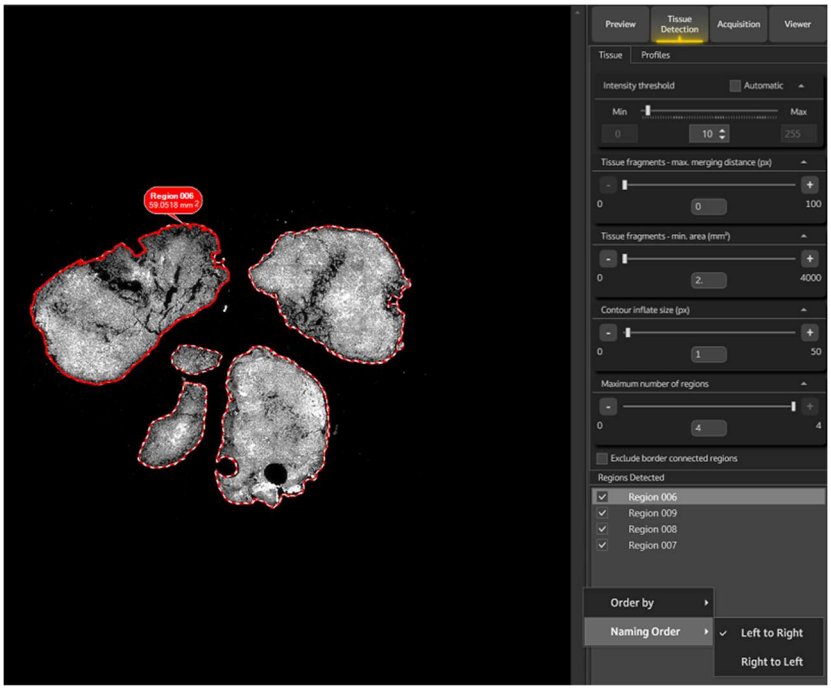
You can use the default preview schema or define a new one from the **Tissue Detection Preview** button, as shown below.

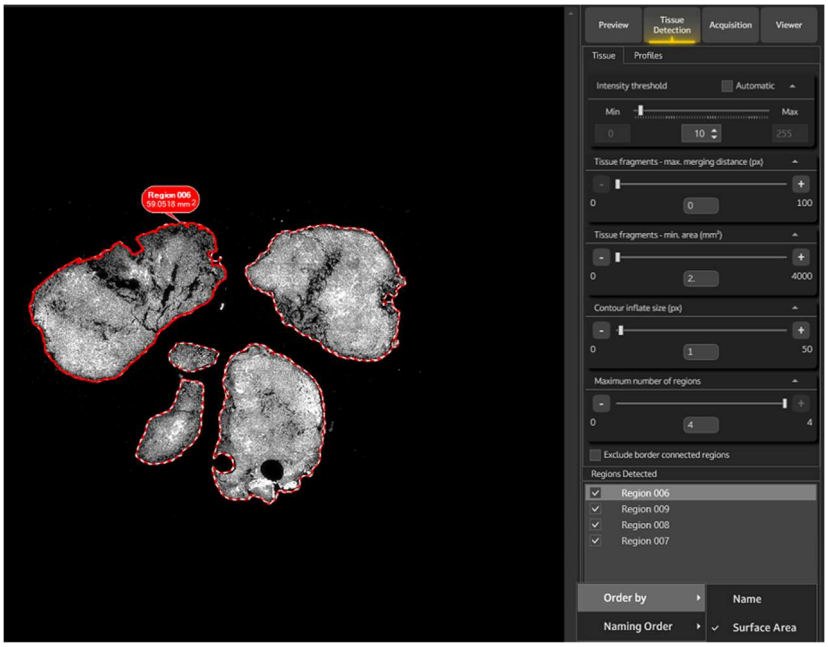




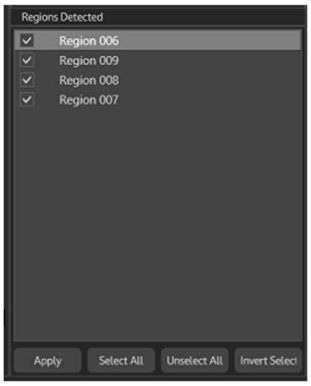
Detected ROIs Manager

- The region resulted from the tissue detection will be shown in a list. Check the ones you want to acquire, then press **Apply**.
- Detected regions can be ordered by name or by surface area.
- The naming order can be made from left to right or from right to left.



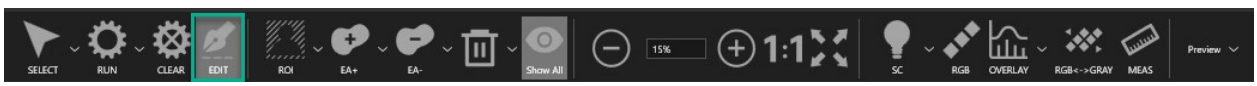


- For the detected regions, you have a set of selection controls: **Select All, Unselect All, Invert Selection**.



Edit ROIs

To edit a region, press **Edit** button from the **Image Viewer** toolbar, then choose one of the options described below:



Any region can be modified (resized, moved), as long as no images have been acquired for them.

- **Moving a ROI** requires 2 steps:

1. Select the region by right clicking an inner point of its shape;

2. Use the arrows from the keyboard to move the region to desired location.

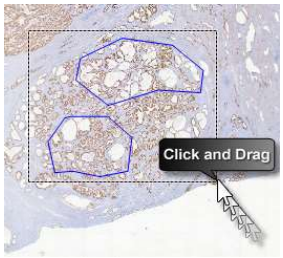
- **Resize or reshape ROI**:

Each region has special points that can be dragged for resizing or changing its shape, depending on the region type:

- For **Custom Regions**, select one by one the points that you want to move (they appear marked by a little blue highlight). All desired points must be modified one by one to reshape the region, by dragging them with the mouse in the desired direction.
- For **Rectangular Regions**, the entire rectangle is resized when the selected point is moved inwards or outwards (it appears marked by a little blue highlight).
- **Circular Regions** are resized by selecting a point on a region's contour and dragging inwards or outwards (it appears marked by by a little blue highlight).
- **Copy and Paste ROI**

• **Copy Region**: this operation can be done by choosing the **Copy** option from the region contextual menu or by using the Ctrl+C combination from the keyboard.
• **Paste Region**: this operation can be done by choosing the **Paste** option from the region contextual menu or by using the Ctrl+V combination from the keyboard.

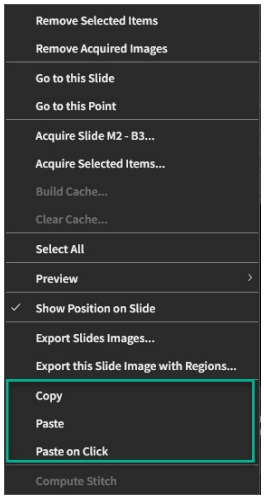
It is possible to select multiple regions to be copied, by using the mouse selection.



Copy/Paste multiple selection of regions

- By using ROI's contextual menu in the Image Viewer

You can copy multiple ROIs from a slide and paste them on another slide. Select the ROIs using Ctrl key and the mouse.

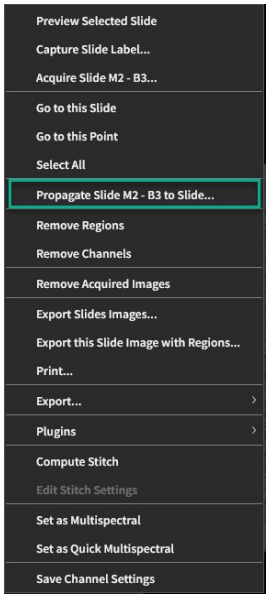


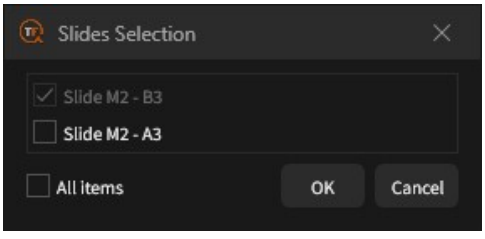
Go on the destination slide and use one of the two Paste options:

- **Paste**: will paste the copied regions at the same coordinates as in the origin slide, basically creating duplicates;

- **Paste on Click**: click on the destination slide where you want to paste the regions.

- By using slide's contextual menu: chose to **propagate** the regions from a slide to another slide(s) at your choice. Use **All Items** option to propagate regions on all existing slides.

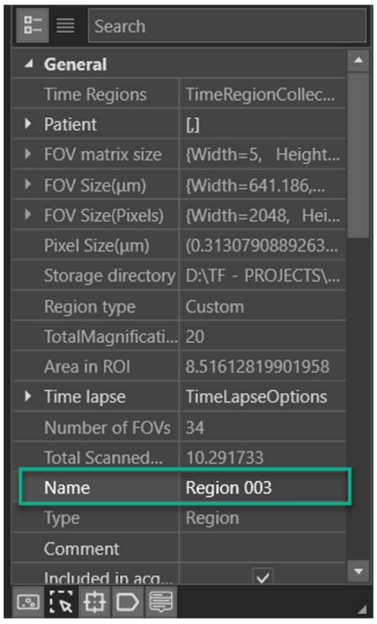




Rename ROI

The regions resulted from scanning can be renamed.

In the **Experiment Properties** section, select the name field, enter a new name, then press **Enter**. Please note that for some items renaming might take longer.



The region will be renamed.

4.2.2. TMA

TMAs Detection

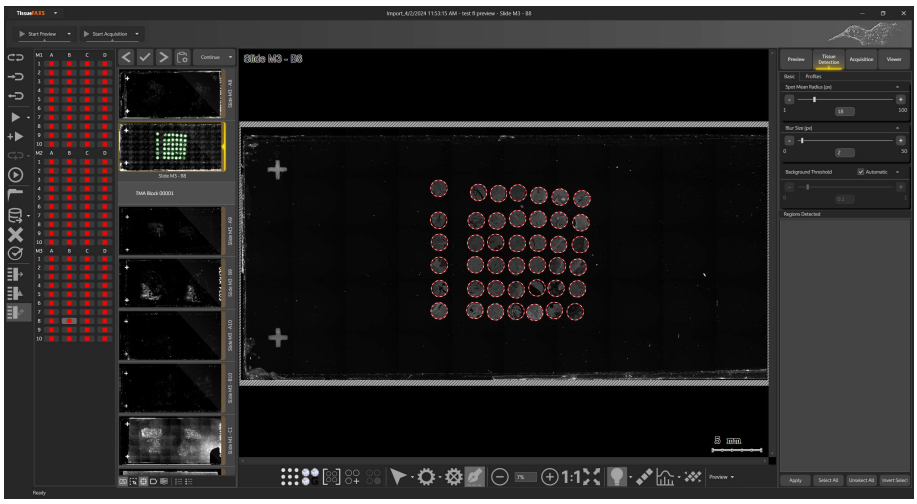
TissueFAXS provides automated tissue detection for TMA structures, reducing considerable time and effort.

It consists of an algorithm based on contrast and uniform illumination. Therefore, illumination correction might need to be applied before detection of the TMA spots. this can be done by using the **Illumination Correction** button.

After applying the illumination correction, **TissueFAXS** can automatically detect the TMA structures on a slide.

When the automatic detection of TMA structures is initiated, the below dialog will appear. In this dialog, the user can restart the TMA detection using different parameters. This operation can be done for the whole slide or over a specific selected area. For best results, the area selected for detection should contain mostly tissue. The coverslip borders and other artefact structures should be avoided.

TMA detection has some particular features, different from regular regions.



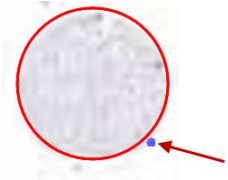
By default, the detection is run on the entire preview image. To refine the results, run the detection on a smaller area

If you set the slide type as **TMA**, the detection toolbar will have some TMA specific controls:

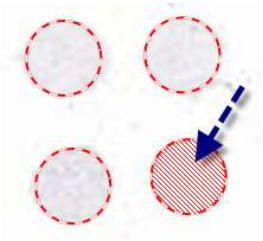
- **Define New Block:** to define a new TMA block, press New Block, then define the TMA block size in the form that pops up. While holding down the Shift key, draw the new block with the mouse on the sample.
- **Galileo Data Import**: opens a Galileo XML file (the file contains data regarding Galileo TMAs).
- **Group as New TMA Block:** When working with TMAs, it might become necessary to group certain spots within a block (they share similar properties, they contain certain types of cells, etc.). TMA spots can be grouped as follows: press Ctrl + A to select all detected spots or Ctrl + left click to select individual spots/blocks. After the selection, press **Group as New TMA Block**.

Note: To select a TMA block, click the space between spots.

- **Define New TMA Spot**: to define a new TMA spot, press the button and begin drawing the spot on the sample. To adjust the size and shape of the spot and to move the contour, use the blue area on the edge of the spot.



- **Set as Placeholder**: **place holders** are virtual TMA spots which replace missing tissue from some positions on the matrix structure of the TMA block. In the image below, the **place holders** appear filled with color. Select the desired TMA spot with the mouse, then press **Set as Placeholder**. Placeholders are not included in acquisition.



Notes

- **Edit** button must be used to edit the size of, and distances between TMAs.

- TMA block size dialog automatically opens after pressing the **New Block** button.

TMA Detection Parameters

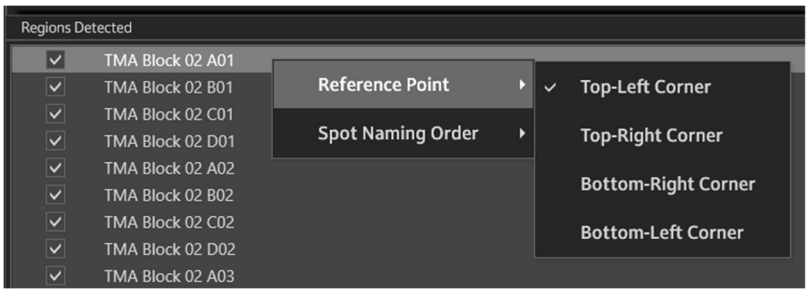
- **Spot Mean Radius**: Represents the radius of the TMA spots. When set to 0, it will be auto-detected. In case of many broken / incomplete TMA spots or poor contrast (mostly in FL samples), it is indicated to be manually set.

- **Blur Size**: Blurring filter size (higher the value, higher the blur level). It is indicated when noise is present in the image and also for eliminating small details from TMA spots (relatively blurred TMA spots will have a better detection)
- **Background threshold**: Used to discriminate TMA spots from background. When set to 0, the threshold value will be auto-detected. It is recommended to be manually set when the contrast is poor or more than 2 population are visible (ex: background, TMA spots and other marker areas inside the TMA spots - automated threshold might detect the value between TMA spots and marker area inside TMA spots).

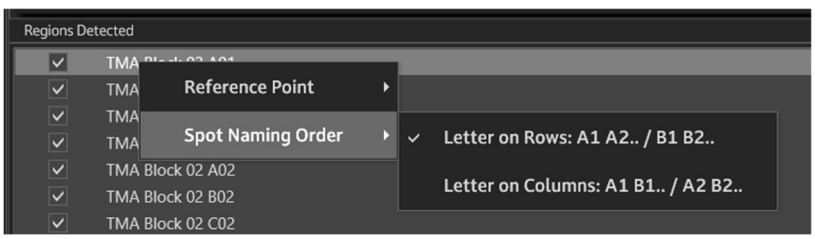
Naming order for TMA spots

Use the naming order feature for easier management of the TMA spots. It can be accessed by right clicking inside **Regions Detected** field.

- In the **Rename** field (only available if a detected item is selected), the TMA spot must be selected from the dropdown menu to be renamed.
- Choose a **Reference Point**: The reference point is used as reference (A01 position) for naming subsequent detected spots. Choose a suitable reference point from the given list: top-left, top-right, bottom-right and bottom-left.



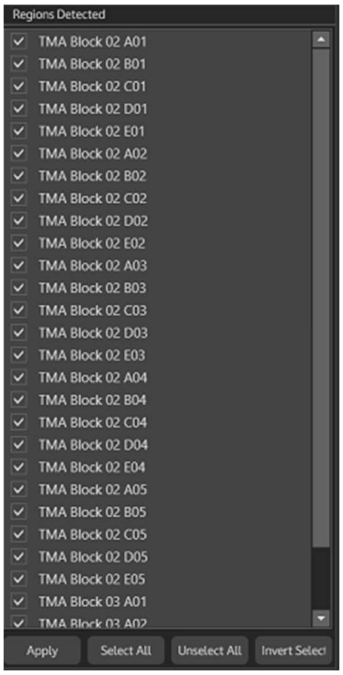
- Select **Spot Naming Order**: select the method for naming the spots: letter on rows or letter on columns.



Note: Detection must be re-run after changing a reference point or spot naming order for the changes to be applied.

Detected TMA manager

- The TMAs resulted from the tissue detection will be shown in a list. Check the ones you want to acquire, then press Apply.
- Detected TMAs can be ordered by name or by surface area.
- The naming order can be made from left to right or from right to left.



- For the detected regions, you have a set of selection controls: Select All, Unselect All, Invert Selection.

Edit TMA

Selection of TMA blocks and spots

The TMA blocks and spots can be selected as below:

- select an individual TMA spot by clicking on it with the left-mouse button;
- select an individual TMA block by clicking in the free area between the TMA spots with the left-mouse button;
- select portions of the TMA block by drawing a rectangle that surrounds the desired TMA spots and blocks;
- select multiple TMA spots by holding CTRL key;

- select the entire content of the slide by pressing the **CTRL**+ **A** keys.

Multiple selections with rectangle

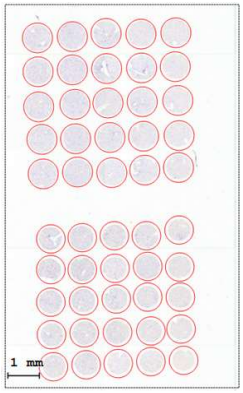
Multiple selections with CTRL

Select all

Resize the TMA spots

The TMA spots can be resized as follows:

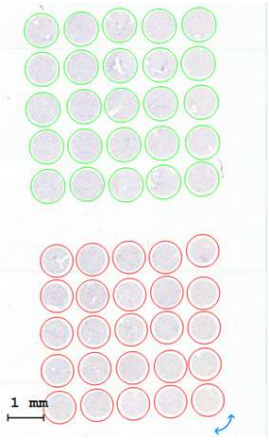
- select the desired TMA blocks or spots;
- use **CTRL**+ **+** keys to increase the size of the TMA spots;
- use **CTRL**+ **-** keys to decrease the size of the TMA spots.



Rotate a TMA block

The TMA blocks can be rotated as follows:

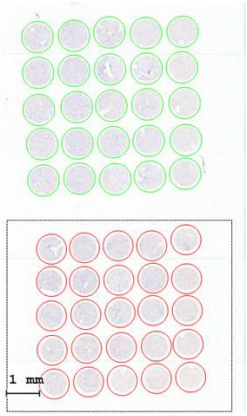
- place the mouse cursor next to the lower-right corner of the TMA block;
- press the left-mouse button on the blue arc that appears next to the lower-right corner of the TMA block and rotate the TMA block using the mouse;
- release the left-mouse button when finished.



Adjust the distance between TMA spots

The distance between TMA spots can be adjusted as follows:

- select the desired TMA spots;
- increase the distance on the X axis using **CTRL**+ → keys;
- decrease the distance on the X axis using **CTRL**+ ← keys;
- increase the distance on the Y axis using **CTRL**+ ↓ keys;
- decrease the distance on the Y axis using **CTRL** + ↑ keys;



Moving TMA blocks and spots

The TMA blocks and spots can be moved as follows:

- select the desired TMA blocks and spots;
- move the selected objects using the mouse or ←,→,↑,↓ arrow keys.

Deleting TMA blocks and spots

The TMA blocks and spots can be deleted as follows:

- select the desired TMA blocks and spots;
- press the right-mouse button and select **Remove selected items** in the menu that appears or press the **Delete** key.

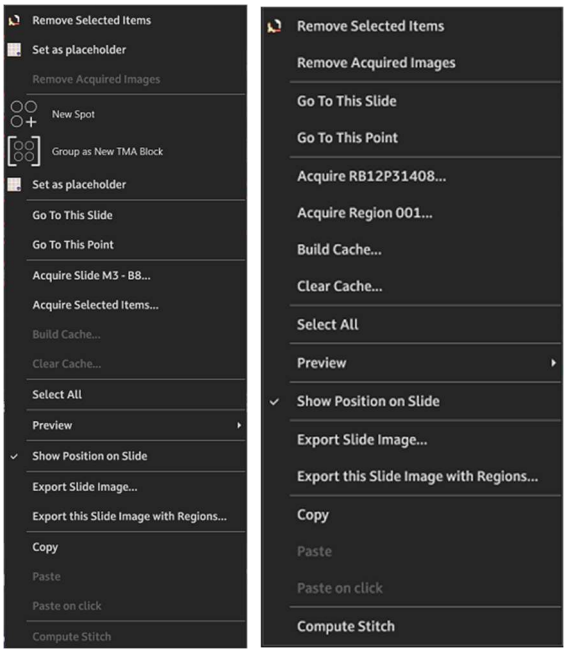
Grouping TMA spots

When working with TMAs, it might become necessary to group certain spots within a block (they share similar properties, they contain certain types of cells, etc.). TMA spots can be grouped as follows:

- select the desired TMA spots;
- press the right-mouse button and select **Group TA Spots** in the menu that appears.

4.2.3. Tissue Detection Contextual Menu

The regions generated by tissue detection have a contextual menu with specific options. TMAs have some extra options (see **Chapter** TMA).



- **Remove selected items**;
- **Remove acquired images**: erases all the acquired images and associated files from the storage area. Note: this option is only available for acquired regions;
- **Go to this slide**: current objective moves to chosen slide;
- **Go to this point**: current objective moves to chosen point on slide;
- **Acquire slide ...;**
- **Acquire region ...;**
- **Build/Clear cache;**
- **Select all;**
- **Preview**: see **Chapter** Image Preview;
- **Show position on slide;**

- **Export slide image;**
- **Export slide image with regions;**
- **Copy/Paste:** will paste the copied regions at the same coordinates as in the origin slide;
- **Paste on click:** click on the destination slide in the position you want to paste regions copied from another slide;
- **Compute stitch.**

4.3. Image Acquisition

The core action within TissueFAXS is called **acquisition**. Acquisition means you will create high quality digital images of your sample (or sample regions).

Acquisition Setup Workflow

1. Create a **new experiment** (see Chapter Generating New Experiments).

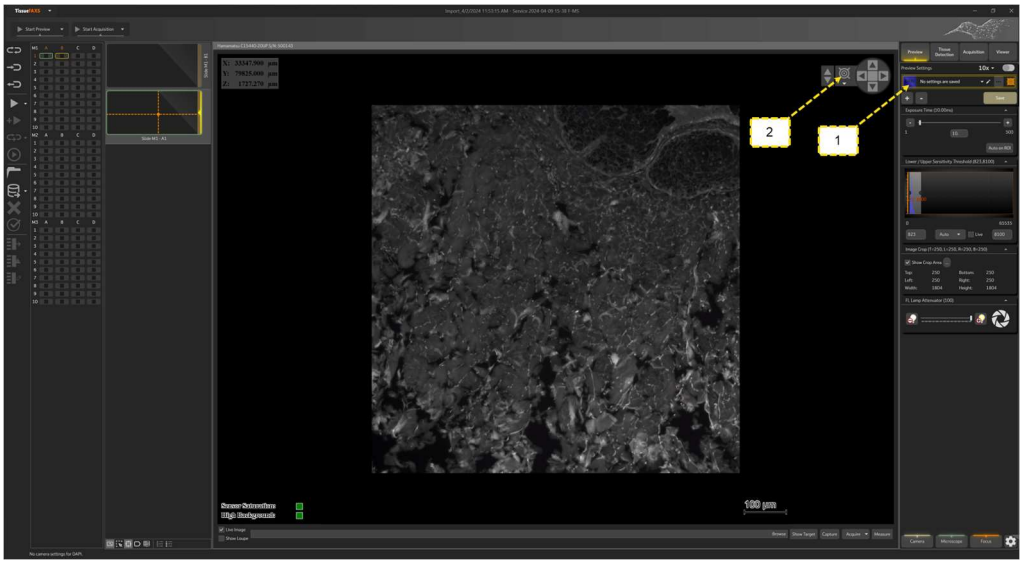
2. Acquire a **preview** (see Chapter Image Preview)

3. Autodetect or manually create **regions** on the sample (see Chapter Tissue Detection).

4. Now you have to adjust the acquisition **settings**. Go in the **Acquisition Tab** and modify the settings depending on the specific of the current sample.

Note: Press **Save** button to save acquisition settings.

5. To focus the image, press **Focus** button over the tissue (2). For details see Chapter Stage.



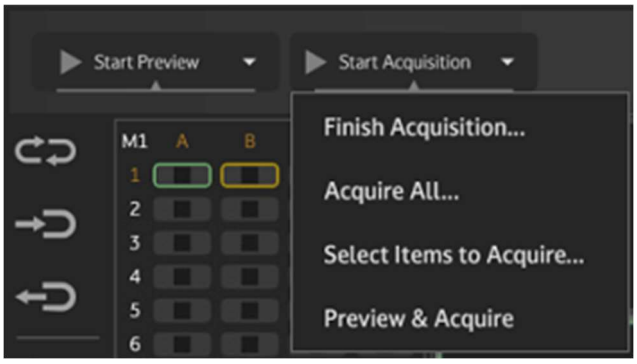
6. Press **Start Acquisition**.

Note: If the acquisition settings are fine, you can save them as default camera profiles for further use (see Chapter Camera Controls), and then you can skip Step 4 and Step 5.

4.3.1. Starting Acquisition

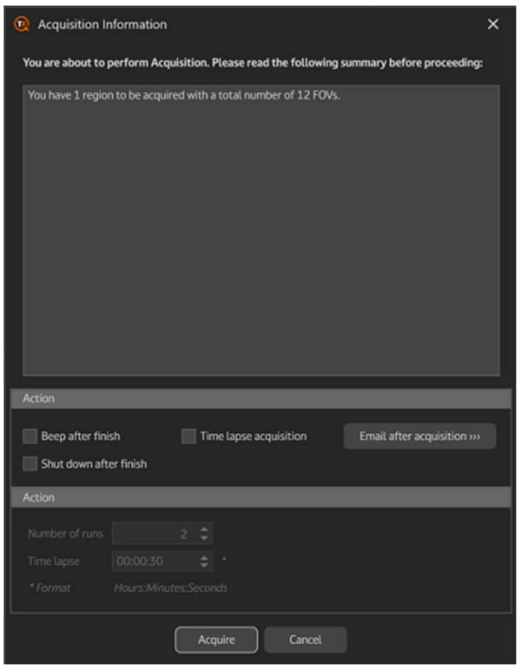
All the acquisition options can be found by pressing the arrow of the **Start Acquisition** button.

A dropdown menu like in the image below will appear:

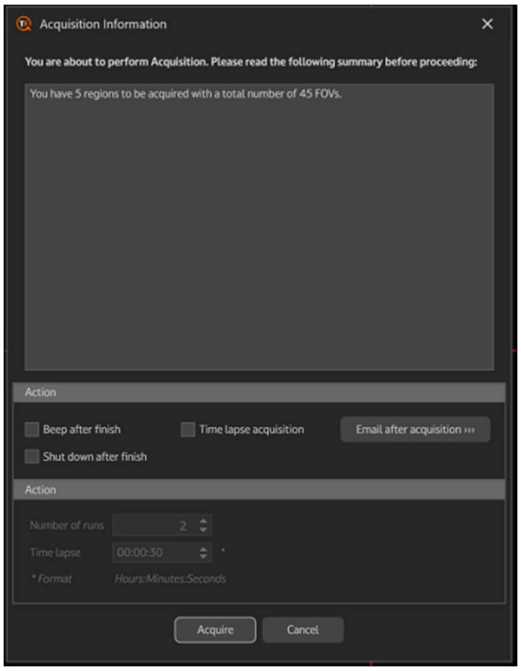


Acquisition Options

- **Finish Acquisition**: **TissueFAXS** will acquire only the unacquired regions from the experiment;



- **Acquire All...**: **TissueFAXS** will acquire all the FOVs of the experiment and will also erase any already acquired FOVs;



- **Select Items to Acquire...**: the user will select the items to be acquired (slides, ROIs).

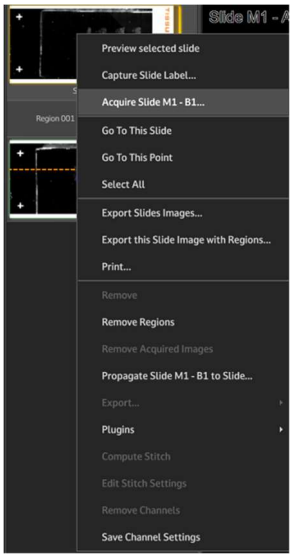
- **Preview and Acquire**: the user must select the slides for automatic preview and acquisition. Tissue detection for selected items can also be enabled by checking **Verify Tissue Detection**.

- If **Verify Tissue Detection** is *checked*, the user will manually validate the automatic detection.

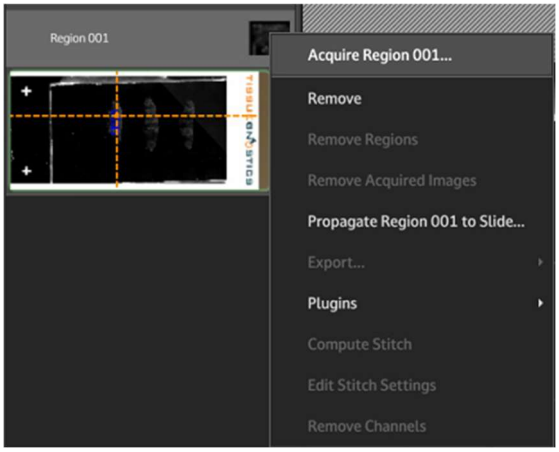
- If **Verify Tissue Detection** is *unchecked*, after the preview acquisition is finished, the Tissue Detection window will be displayed and a timer will appear. If the user does not intervene to stop the timer and perform manual modifications to the detection, the detection will be done automatically and the window will close. TissueFAXS will automatically proceed with the acquisition.

Other ways to acquire

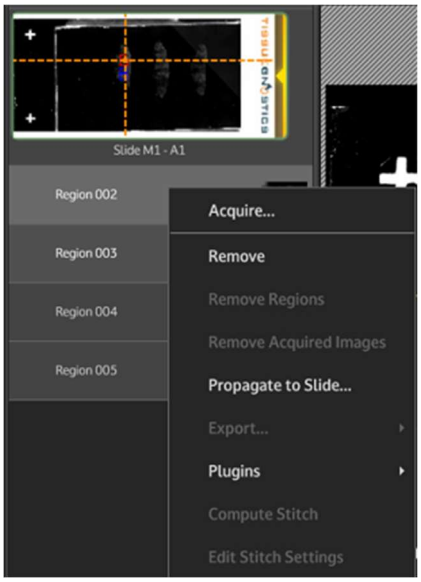
- Slide contextual menu



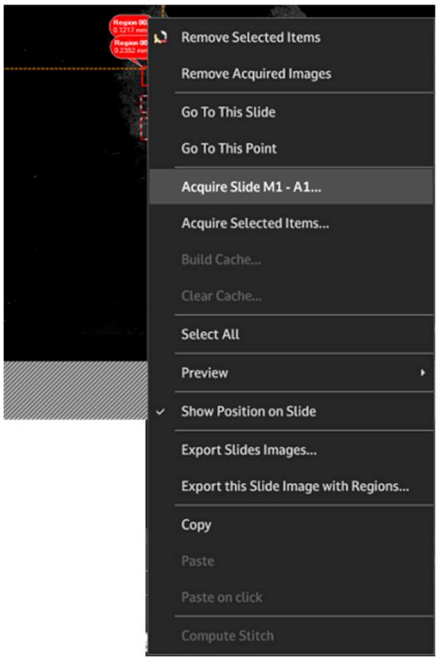
- Region contextual menu



- Multiple acquisition for more selected regions



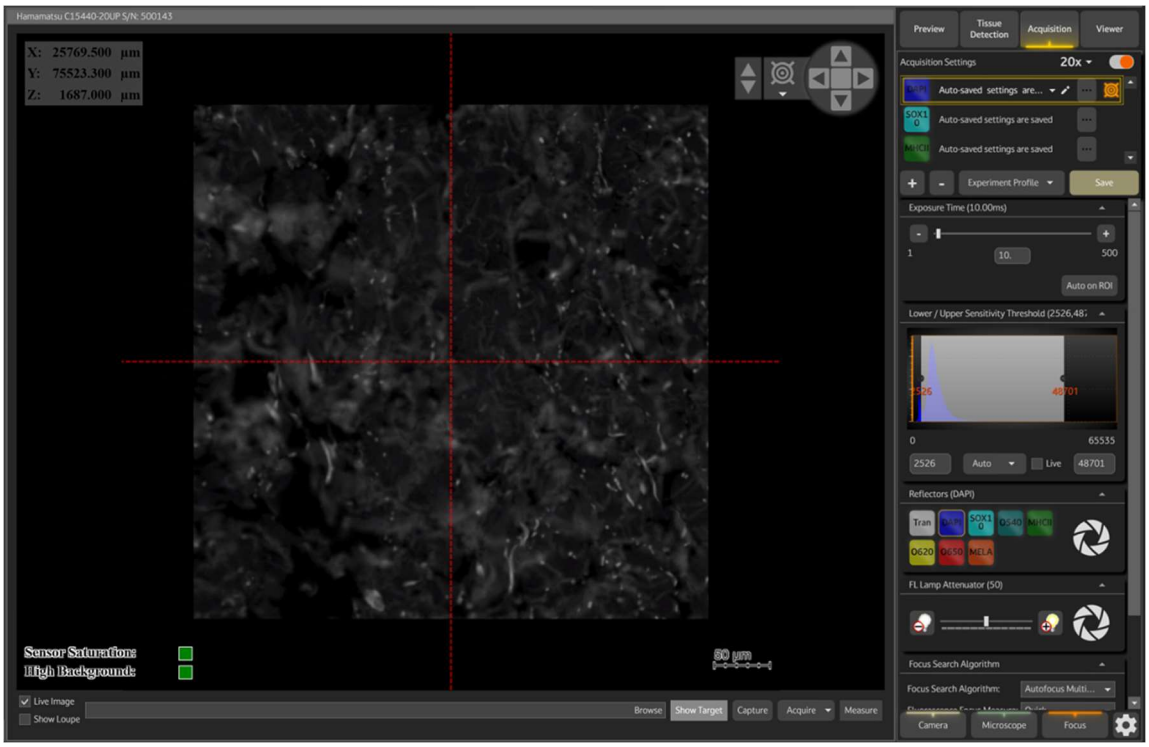
- Acquisition from the viewer in **Tissue Detection**



4.3.2. Acquisition Settings

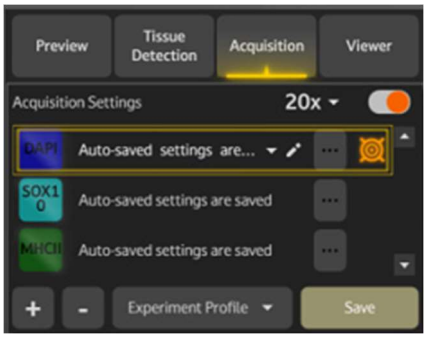
Acquisition Settings

Acquisition settings can be adjusted from the **Acquisition** tab, illustrated below.



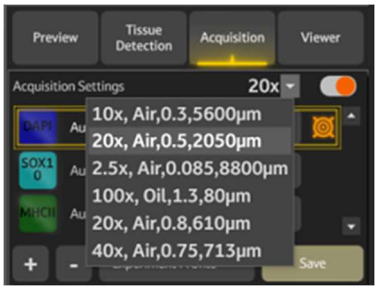
All the settings from **Acquisition** tab can be automatically saved per experiment for further use, by enabling the **Auto save** button.

You can see settings status on the acquisition channel (settings saved/not saved etc.).

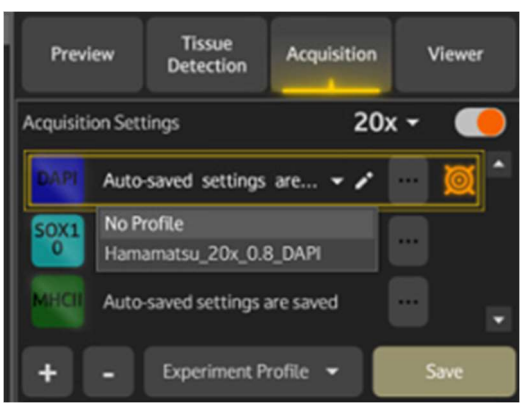


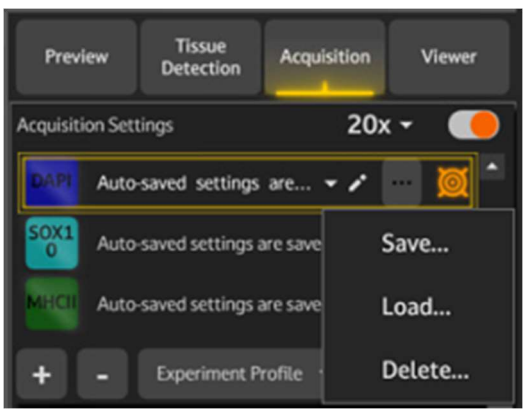
You can visualize the default acquisition objective, but, if necessary, it can be changed from the dropdown menu shown below.

Note: The physical acquisition objective will switch depending on the selection made here.



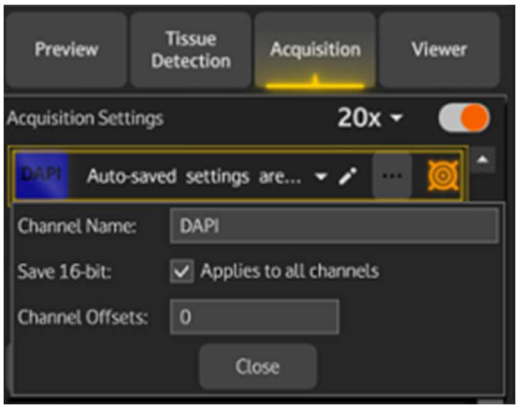
All settings can be saved in a camera profile for further use. These profiles are available on the acquisition dropdown window. For more details see Chapter Camera Controls.





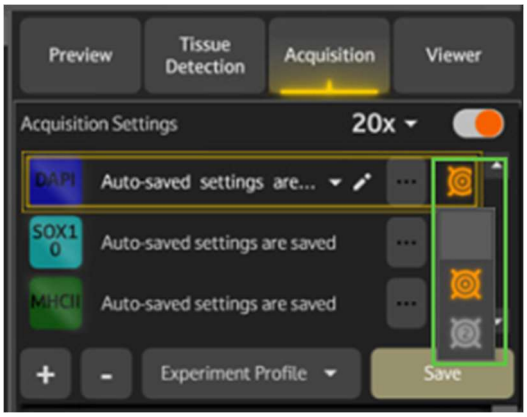
Extra settings are available when pressing **More Options**.

- **Edit channel name;**
- **Save 16bit** (you can choose to apply this to all channels);
- **Channel Offset**: by default, Channel Offset is populated with the template settings (if that's the case) or from already computed channel offsets (using **Compute Channel Offsets** option). For details see **Chapter** Adjust Channel Offsets.



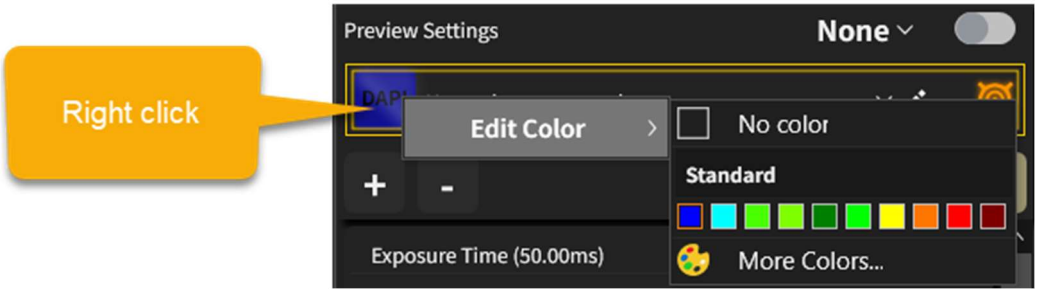
Primary Focus: the focus will be made on the currently selected channel.

You can also access a second focus channel, from the dropdown shown below (see below in this chapter).



Add/Remove reflector: will add or remove channels from acquisition.

Editing channel color is possible if right clicking on the reflector name.

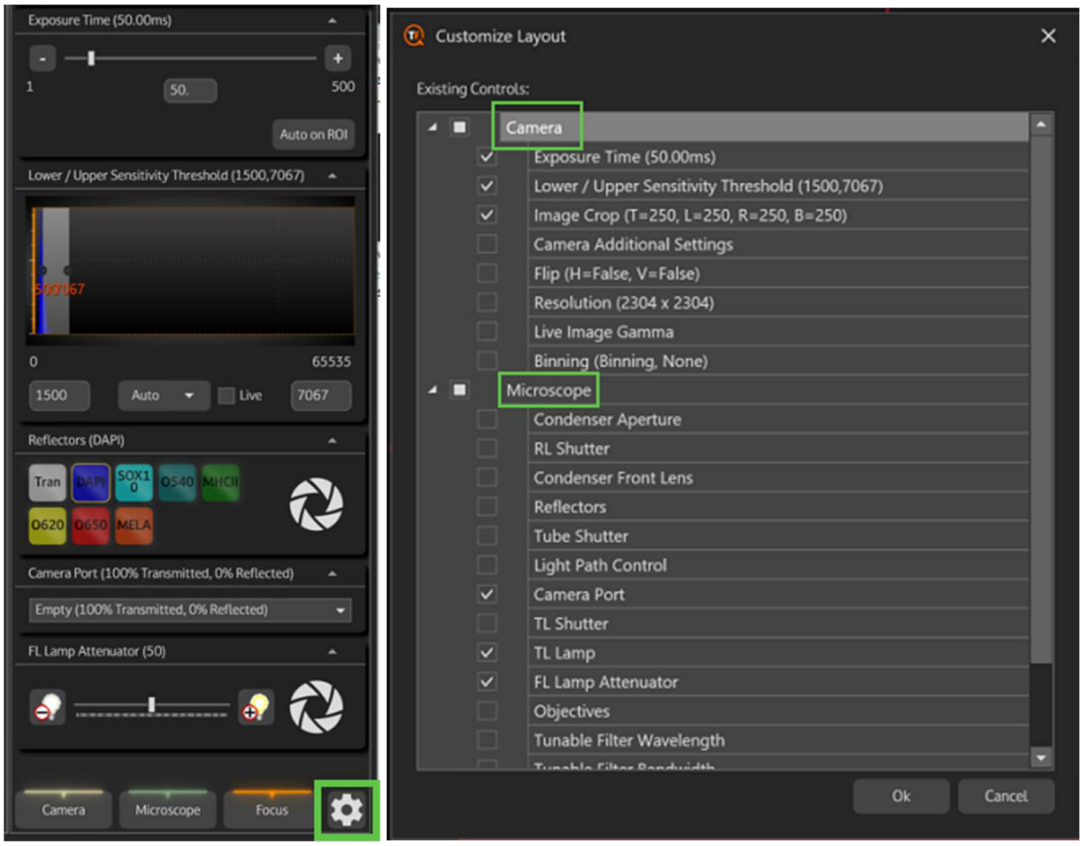


Save: press this button to save acquisition channels settings.

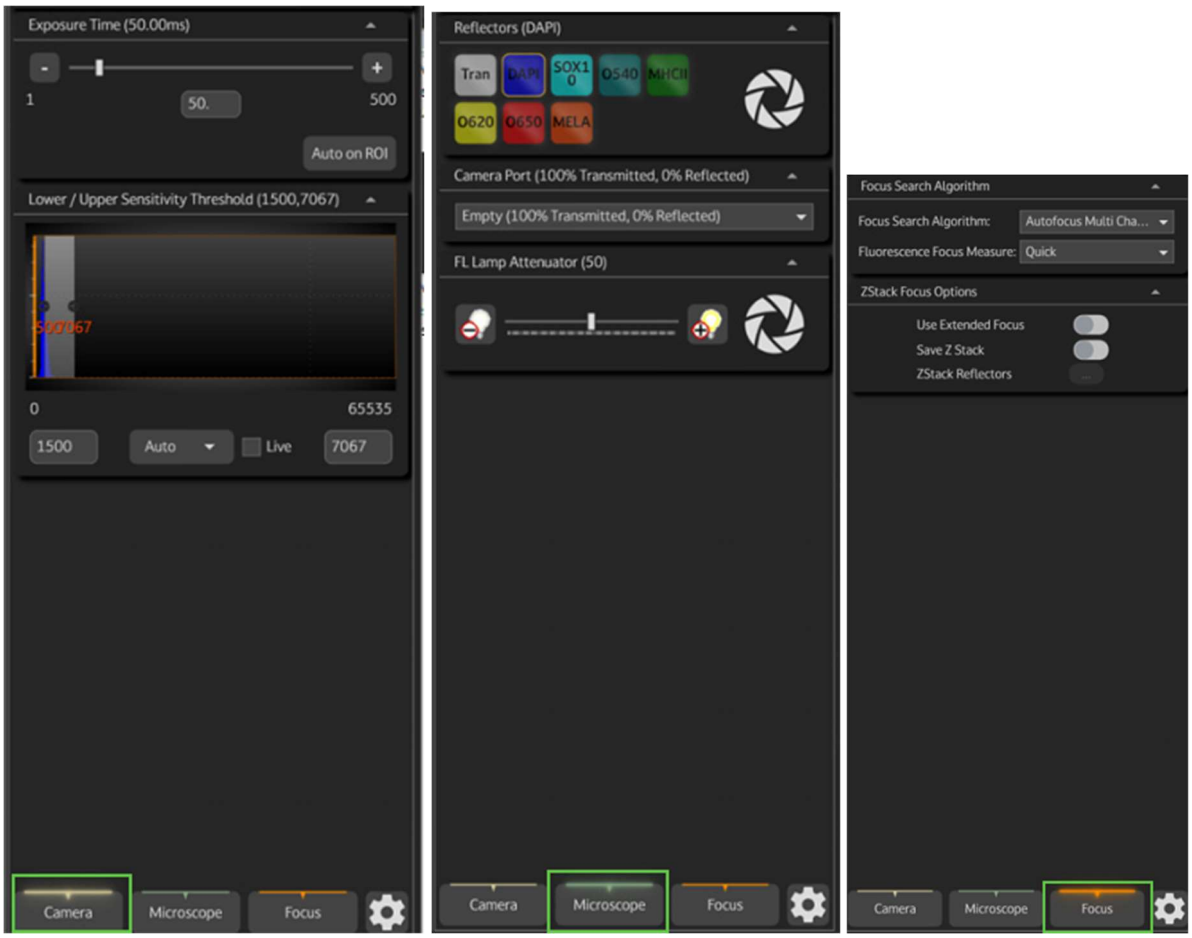
Controls Layout

By default, specific controls populate this section, depending on the experiment type (BF, FL, MS, Confocal). However, you can add or remove controls using **Customize Layout** window.

You can visualize the controls for camera, microscope or focus separately, by selecting only desired tab.



You can visualize the controls for camera, microscope or focus separately, by selecting only desired tab.



Focus Channel (for FL Experiments)

Focus Channel is the channel for which **TissueFAXS** will perform the autofocus in fluorescence experiments.

The default for **Focus Channel** in **TissueFAXS** fluorescence experiments is DAPI. If DAPI is absent from the experiment, **TissueFAXS** will automatically set **Focus Channel** to the first channel listed in the **Acquisition Channel** section.

By default, focus is done on 16-bit images, but it can be configured to run also on 8bit.

Second channel focus brings the possibility of focusing on a second channel after the tissue was found by focusing on the first channel.

The workflow of the second channel focus requires the following steps:

- focus on the first channel;

- from the position found on the first channel, perform a full focus on the second channel in an interval centred in that position and with specific stages for the second channel;

- the position found will be used to acquire all channels using the channel offsets.

Channels

- For Brightfield Experiment: only **Transmission** is available;
- For Fluorescence Experiment: choose the desired reflectors (including transmission).

Notes:

- After choosing the reflectors, the camera settings for each reflector must be readjusted.

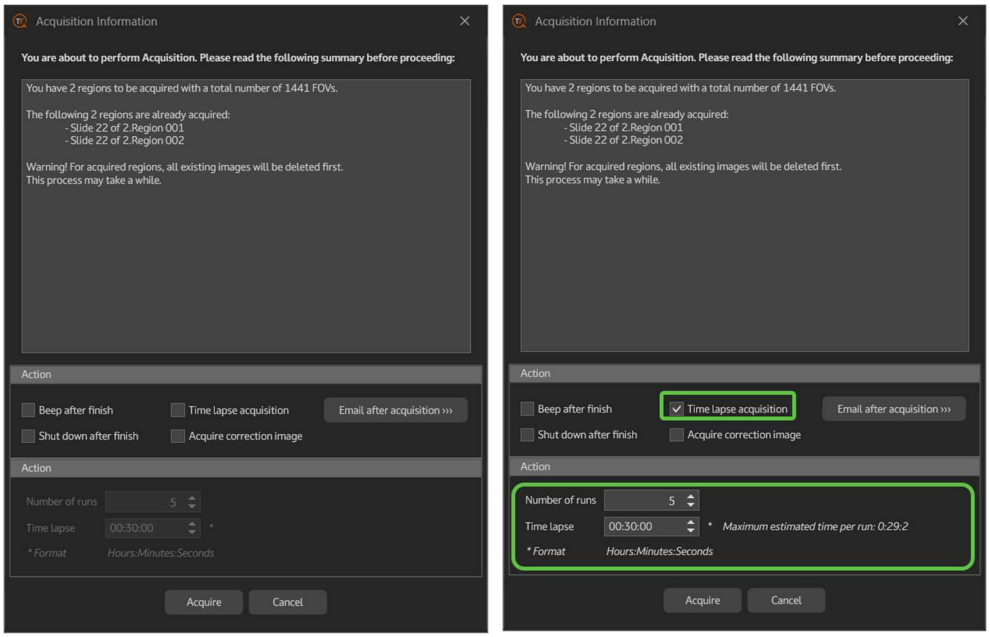
- The name of the channel is editable.

4.3.3. Standard and Time Lapse Acquisition

Standard Acquisition and Time Lapse Acquisition

A region can be acquired in **two modes**: **Standard** (normal) or **Time Lapse**.

You can specify the acquisition type in the **Acquisition Information** dialog that is displayed before the actual acquisition process is started.

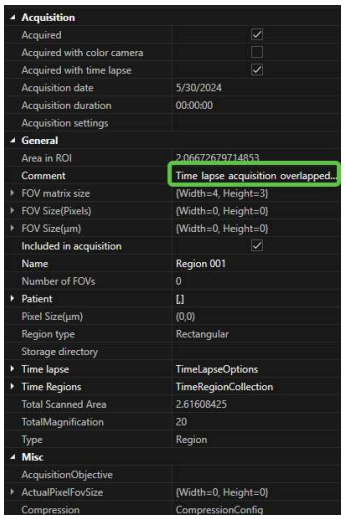


To enable **Standard acquisition** mode, the **Time Lapse acquisition** checkbox must not be checked.

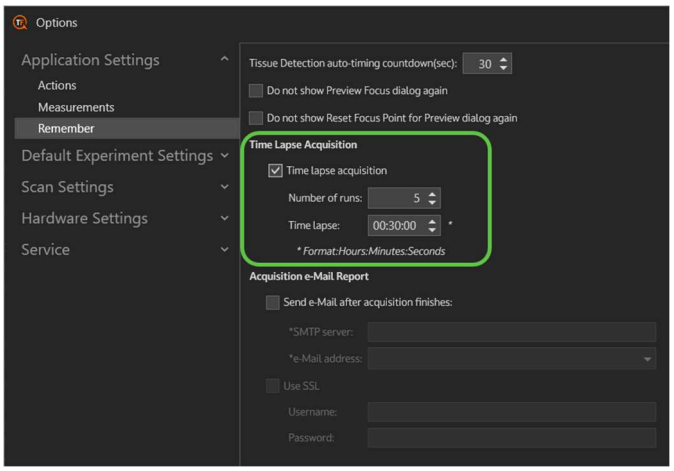
Check the box to enable **Time Lapse Acquisition**. The user should also set the values for the following parameters:

- *Number of runs*: how many times the acquisition will run;
- *Time lapse*: the interval between runs. The time lapse will be specified like this: *days. hours: minutes: seconds*.

For every region acquired with short time lapse, this information will be displayed in the **Region properties** section from the **Experiment Editor**.



Note: If you frequently use Time Lapse acquisition, you can set this option as default from **Tools** → **Options** → **Application Options** → **Remember** (along with the desired parameters values).



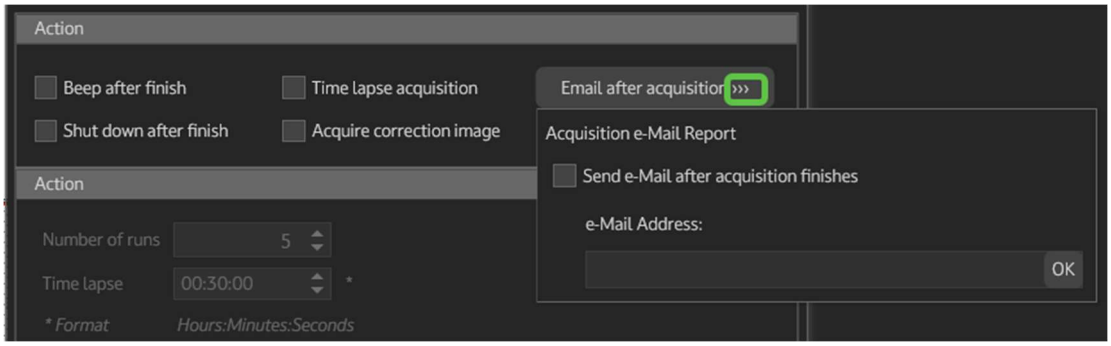
Time lapse acquisition consists of multiple acquisitions for a region or a set of regions. The multiple acquisitions will be performed after a certain time interval specified by the user.

The **Time lapse** feature is ideal for observing cell cultures.

Note: Time Lapse acquisition mode is not available for TMA samples. Therefore, the Acquisition Information dialog that appears before acquiring TMAs will display a notification that the Time Lapse acquisition option is disabled (see image below).

Acquisition e-Mail Report

Within the **Acquisition Information** panel, press the arrow button in order to access the **Acquisition e-Mail Report** dialog. Please check the **Send e-Mail after Acquisition Finishes** checkbox, then fill in the desired e-Mail address and press **OK**. **TissueFAXS** will remember this information for further use. An email notification is sent at the end of the acquisition process.

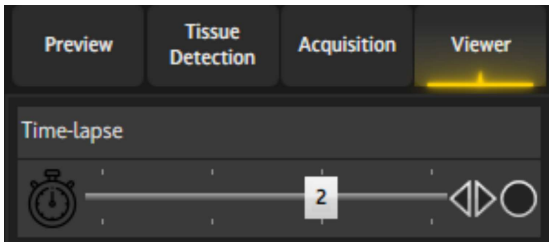


How to view

By default, when double clicking on a region in **Experiment Editor**, that region will open in **Time Lapse** mode (if time lapse images have been acquired) or in **Standard Mode** (if time lapse images have not been acquired).

It is important to note that not all operations normally available on a region are available on a set of **Time Lapse** images:

- Adding/Removing FOVs option is not available;
- Reacquisition of specified FOVs is not available;
- Resize of the region is not available;
- There is only one correction image for all Time Lapse images belonging to the same region;
- All the categories/subregions will be the same for all Time Lapse images belonging to the same region.



Assuming a region has both **Standard** and **Time Lapse** images, the user can switch between these two modes by using two buttons: **Standard** ↔ **Time Lapse**.

The user can choose to see the region at a certain moment by moving the cursor.

All time lapse images can be deleted by pressing the **Remove** button.

Notes:

- Time Lapse acquisition is not available for TMA samples.

- During the Time lapse acquisition process, the user can see the progress of the acquisition in the status bar of the application.

4.3.4. Multispectral Acquisition

Multispectral feature is only available on Multispectral systems.

To create **multispectral regions**, first you need to create a multispectral experiment, as shown in **Chapter** Generating New Experiments.

For multispectral experiments, there are two **types of regions** that can be acquired:

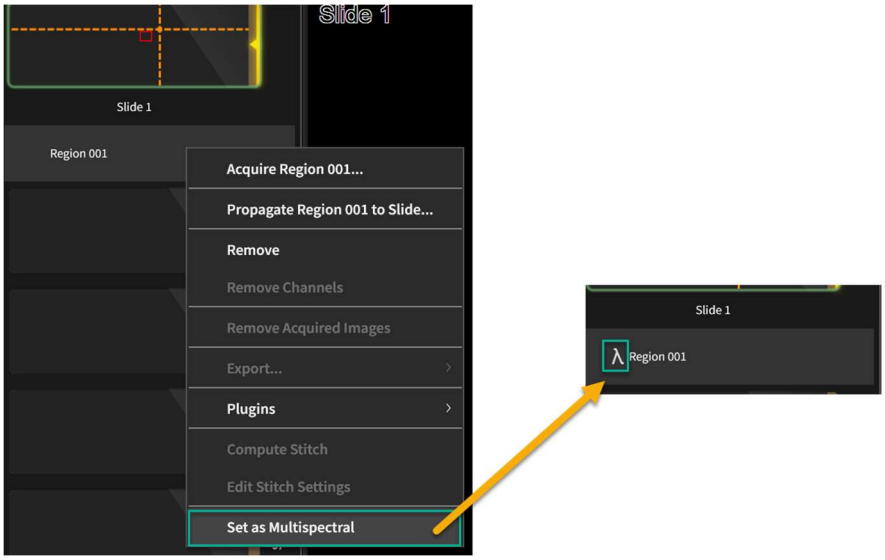
- Quick/normal regions (scanned as FL regions using only the peak wavelengths)
- Multispectral regions

To **create** multispectral regions:

1. Press **Multispectral Mode** button from **Tissue Detection** toolbar. All the regions that will be created after will be multispectral.

Note: When unchecking this button, the regions will be Quick Multispectral.

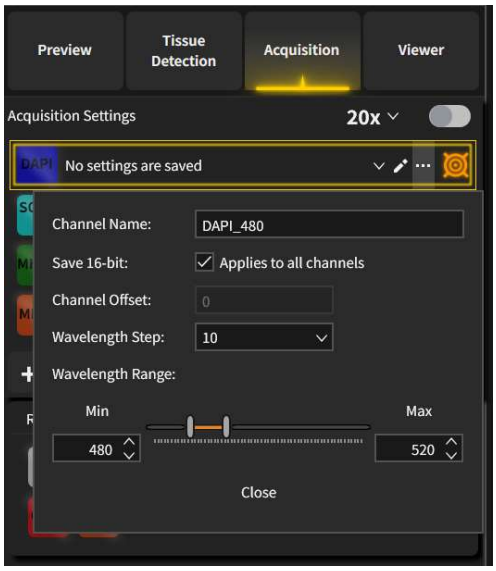
2. Using the contextual menu of the regions listed in the **Experiment Editor**. Once created, these regions will be marked as multispectral.



Another way of creating a new multispectral region is to choose from **Region Viewer** an already acquired normal FL region.

Go to **Image Viewer** toolbar -> **Subregions** -> **Show Multispectral Regions**. Now draw on the FL region the smaller areas that will be reacquired in multispectral mode. The new regions will be created as multispectral.

Multispectral acquisition **controls** per channel:



- **Channel name;**
- **Save 16-bit;**
- **Channels offset;**
- **Wavelength step;**

- **Wavelength range.**

4.3.5. Flags (Reacquisition)

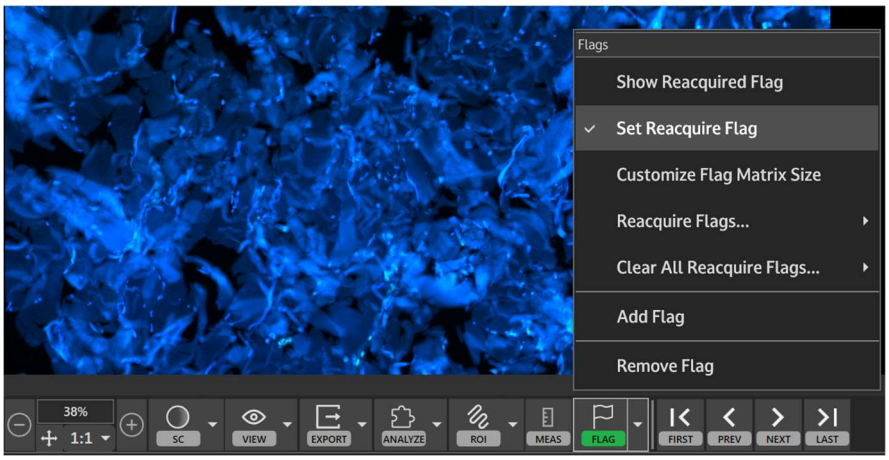
In **TissueFAXS**, the **Flag** feature is used for more purposes:

- In the reacquisition of FOVs, when the initial acquisition process has generated unclear or unsatisfying results.
- For modifying the shape of the region by adding or removing FOVs.

Reacquire Flags

To view and manage the flags, enable the **Set Reacquire Flag** option from the **FLAG** button's contextual menu.

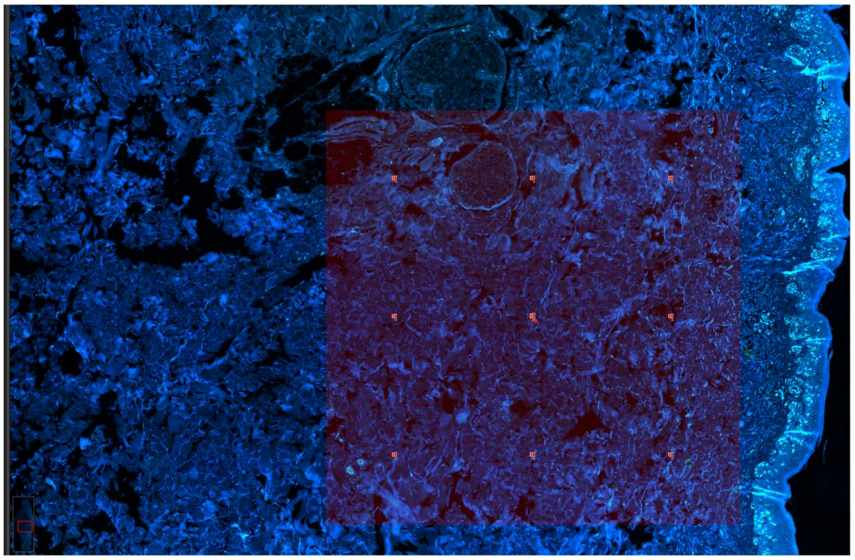
Note: **Reacquire Flags** feature is not compatible with the Time-Lapse acquisition and is therefore only available for Standard acquisition mode.



There are two options for adding reacquisition flags:

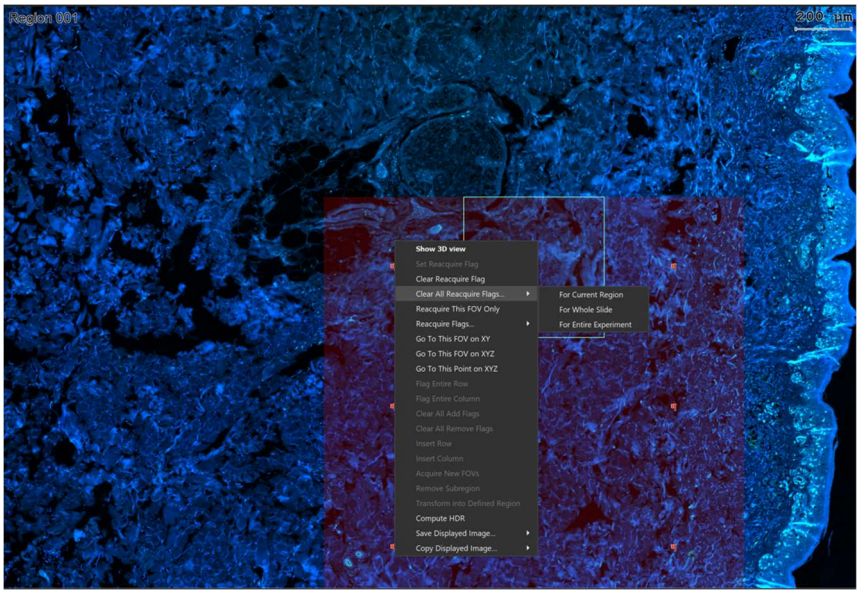
- Left-clicking directly on the FOV chosen for focus;
- Pressing "**Set Reacquire Flag**" from the contextual menu that appears after right-clicking on the FOV chosen for focus.

For both methods for adding reacquisition flags, the selected FOV will be set as a focus point and all its neighboring FOVs will be selected for reacquisition as well. The size of the neighboring area is the size specified by the "**Customize Flag Matrix Size**" option.



There are three options for removing reacquisition flags:

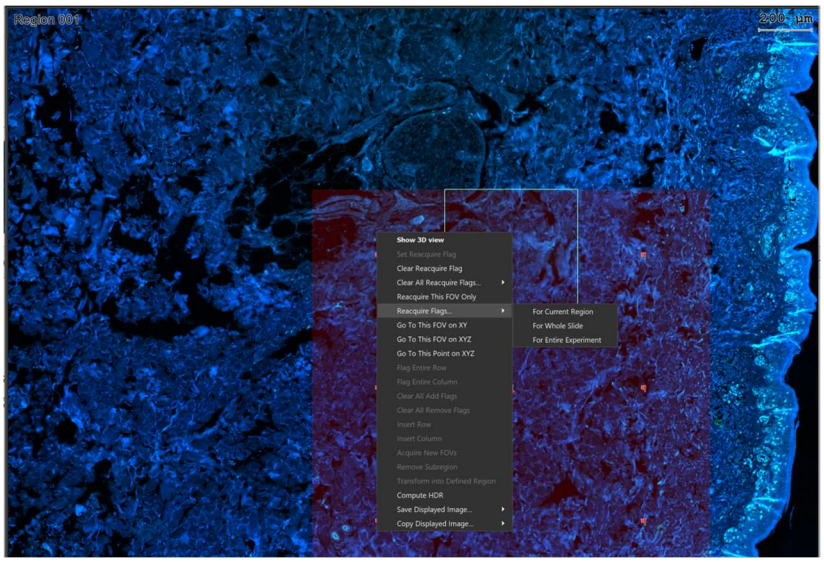
- Directly left-clicking on the FOV;
- Pressing "**Clear Reacquire Flag**" from the contextual menu that appears when right-clicking.
- Pressing "**Clear All Reacquire Flags**"**…** from the contextual menu that appears when right-clicking and then choosing one of the three available options: **For Current Region**, **For Whole Slide**, or **For Entire Experiment**.



Note: For the first two remove flags options, by clicking on the focus flag, all neighboring flags will be removed (including the focus flag itself). However, by clicking on a neighboring (non-focus) flag, only this flag will disappear.

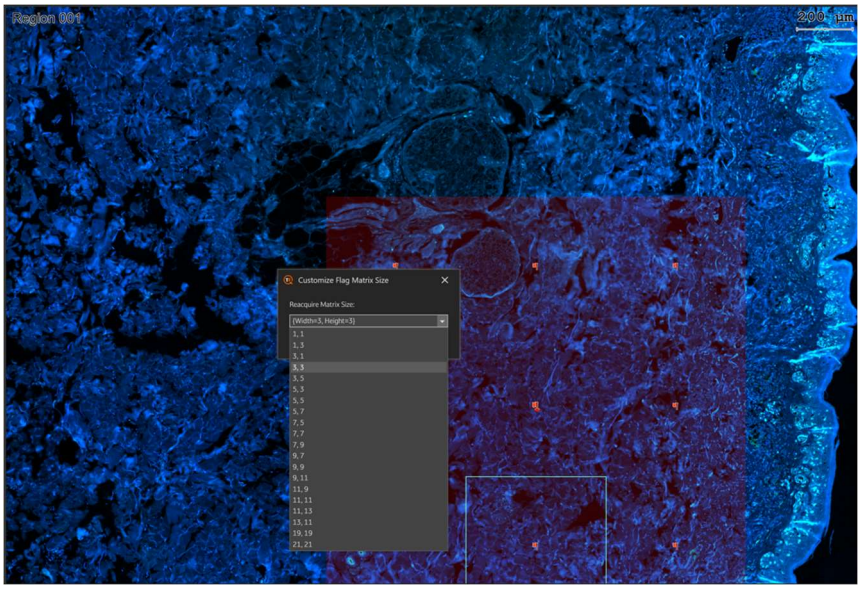
Reacquire FOVs

- If the **Reacquire This FOV Only** option is selected, only the current selected FOV will be acquired.
- If the **Reacquire Flags** option is selected, there are three choices: **For Current Region**, **For Whole Slide**, and **For Entire Experiment**.



Customize Flag Matrix Size

Flag matrix size can be modified for setting reacquisition flags. Choose a given value's configuration from the dropdown menu, then press **Ok**.

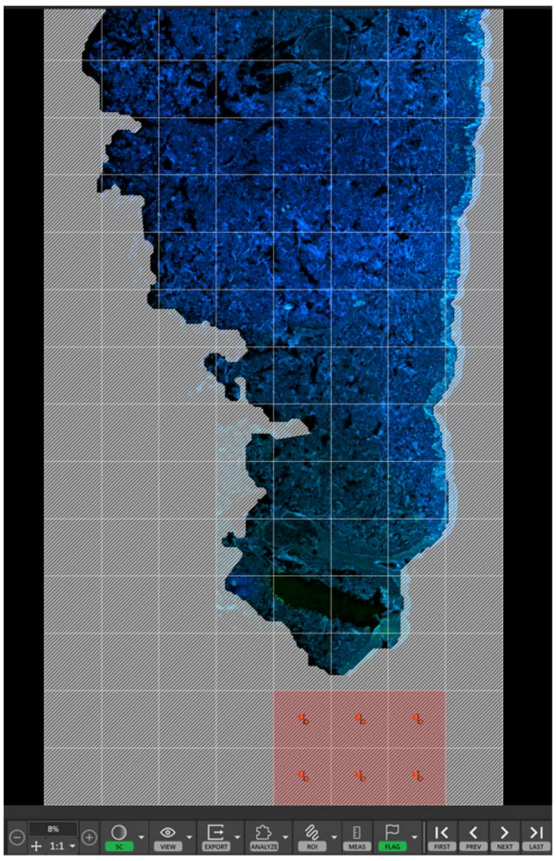


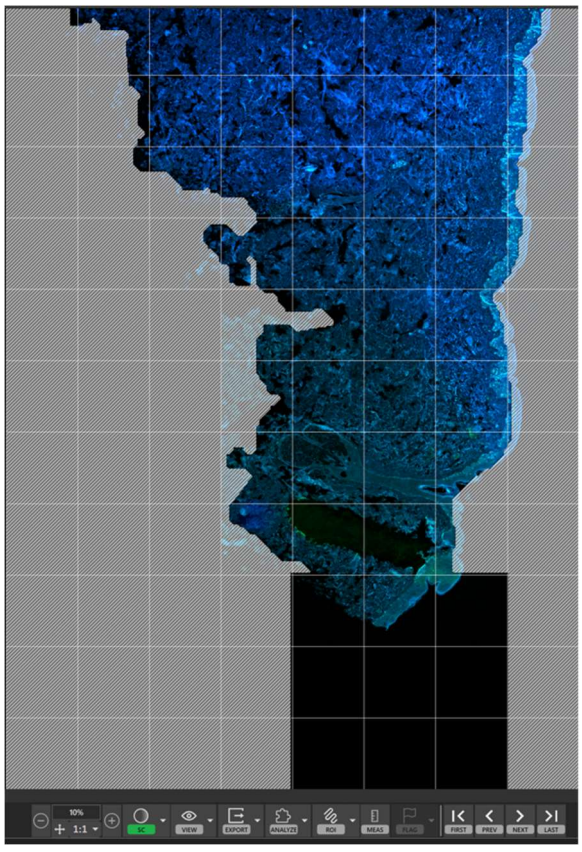
Add Flags

Add Flag feature allows you to enlarge the region size by adding new FOVs at the edge of the existing area.

There are multiple ways of adding FOVs:

- Click on the first/last row, to select **Insert row before/after** from the image contextual menu;
- Click on the first/last column, to select **Insert column before/after** from the image contextual menu;
- Enable the **Add Flag** option from the **FLAG** button's contextual menu, then click on an empty position outside the desired region shape (next to already existing FOVs). To remove the added flag, click again on the same empty position. After finishing this operation, uncheck the **Add Flag** button;
- Another option is to click **Flag to add entire row/column** from the image contextual menu that appears by right-clicking on the newly inserted row/column. To reverse this operation, choose **Unflag entire row** from the contextual menu;





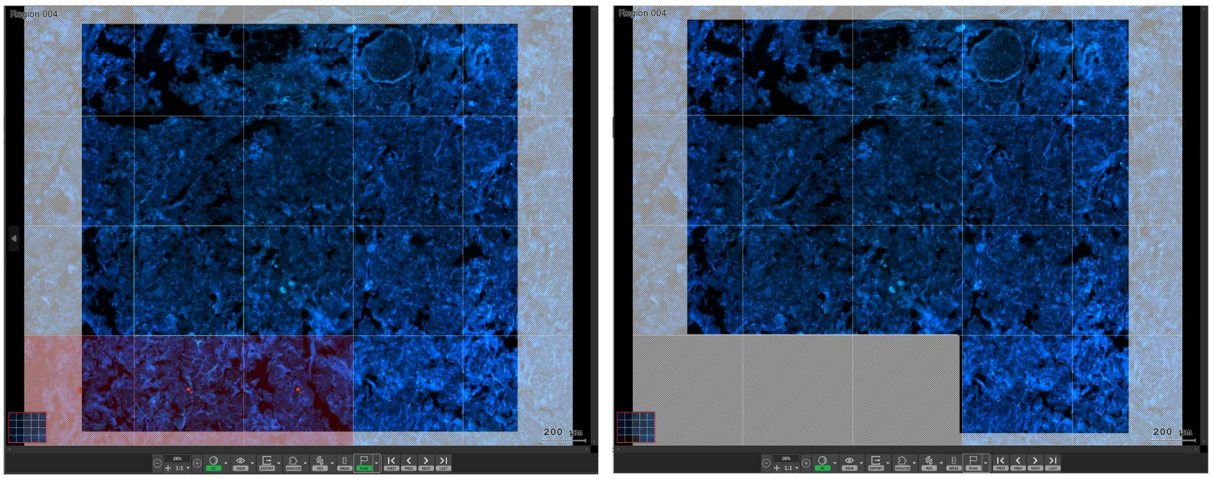
To remove all added flags, click "**Clear Add Flags**" from the **FLAG** button's contextual menu or choose "**Clear All Add Flags**" from the image contextual menu.

When you finished adding flags press the **Apply** option from the **FLAG** button's contextual menu.
Pressing the **Apply** button will effectively modify the region shape by adding/removing FOVs (will remove image files too).
Pressing the **Undo** button will discard all changes (insert rows/columns, **Add/Remove Flags**).

Remove Flags

Remove Flag feature allows you to shrink the region size by removing FOVs. A use-case for this would be when there are FOVs with no tissue (usually around tissue border).
There are multiple ways of removing FOVs:

- Enable the **Remove Flag** option from the **FLAG** button's contextual menu, then click on a border FOV that is not needed. To uncheck the added flag, click again on the same FOV.



- Another option is to click **Flag to remove entire row/column** from the image contextual menu that appears by right-clicking on the border row/column. To reverse this operation, choose **Unflag entire row** from the contextual menu.
- To remove all added flags, click **Clear Remove Flags** from the **FLAG** button's contextual menu or choose **Clear All Remove Flags** from the image contextual menu.

When you finished adding "**remove flags**" press the **Apply** option from the **FLAG** button's contextual menu.
Pressing the **Apply** button will effectively modify the region shape by adding/removing FOVs (will remove image files too).

Pressing the **Undo** button will discard all changes (insert rows/columns, **Add/Remove Flags**).

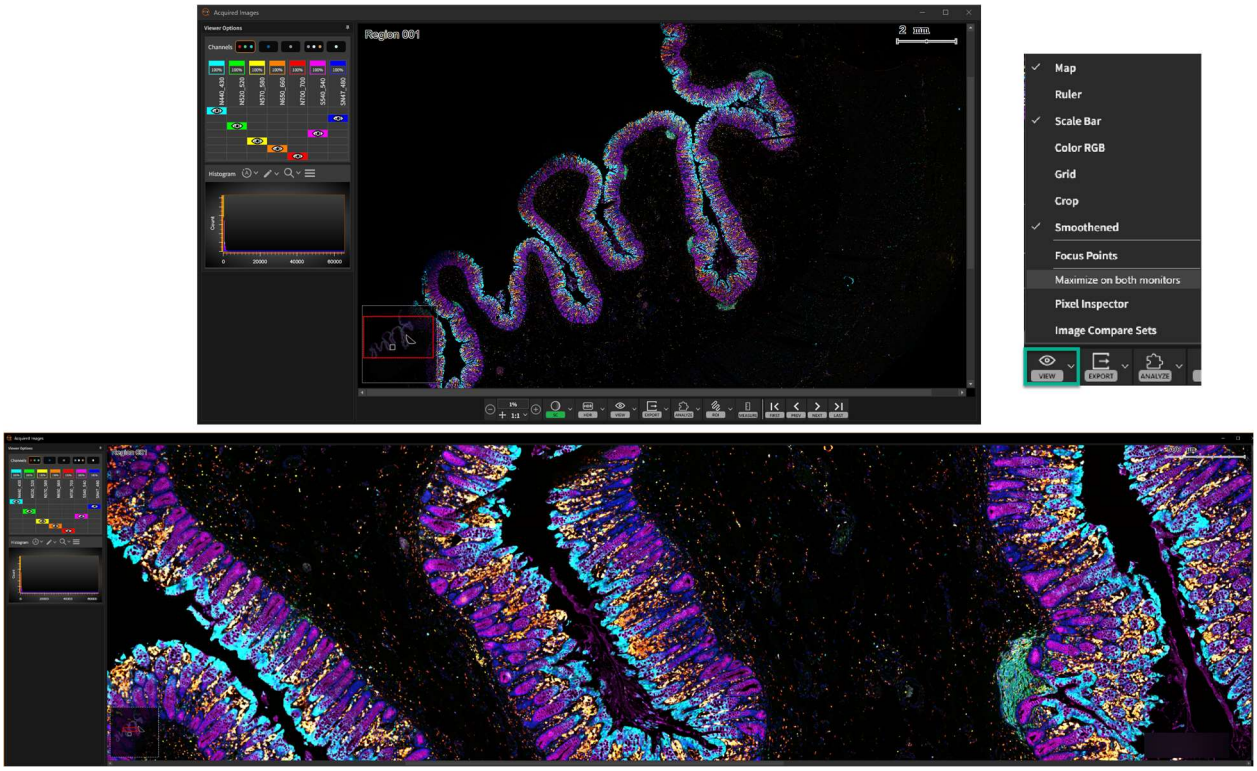
Acquire New FOVs

This option from the contextual menu will acquire all newly added FOVs.

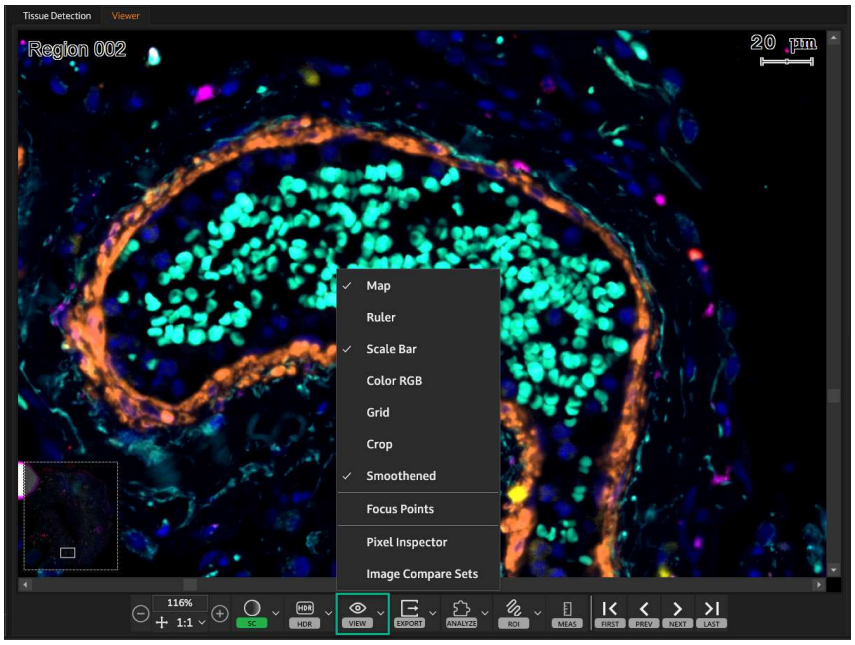
4.4. Images and Viewing Options

The image visualization tool in TissueFAXS is called **region viewer**.

A double click on the **Viewer** tab opens the viewer in a separate window, where **Maximize on both monitors** option can be found in the **View** menu (if the system has two monitors).



Double click on any acquired region to open it in region viewer.



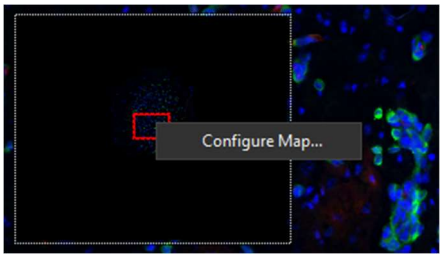
By pressing **View** button, a set of visualization tools will become available.

1. Map

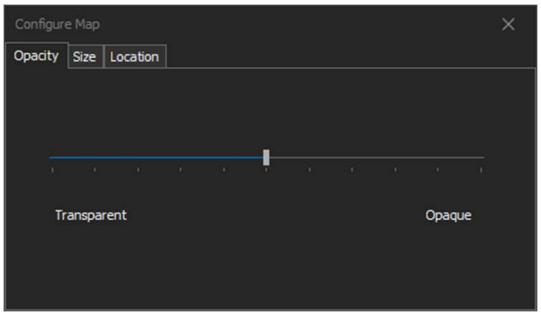
The map displays the entire sample. The part of the sample within the red frame is shown in the Region Viewer window.

The map has a set of parameters: size, opacity, location. These parameters are saved by the application.

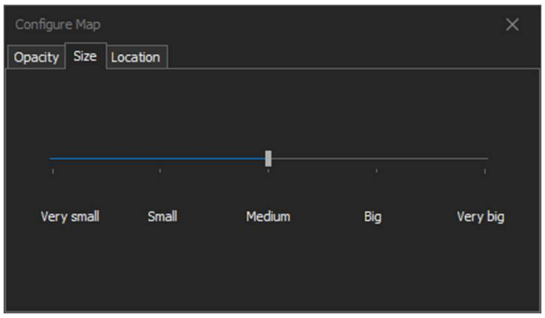
To change the default settings, right click on the map and choose **Configure Map…** from the displayed menu.



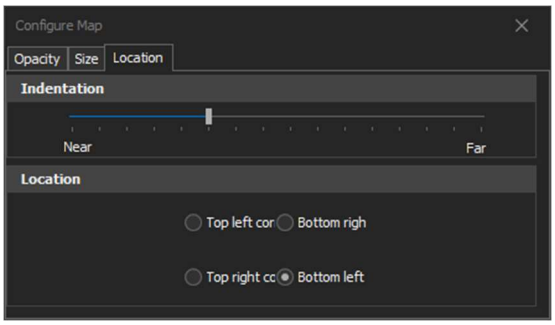
- **Opacity** - allows changing the degree of transparency for the map by using a slider.



- **Size** - allows changing the size of the map. By default, the value of this parameter is "**Medium**".



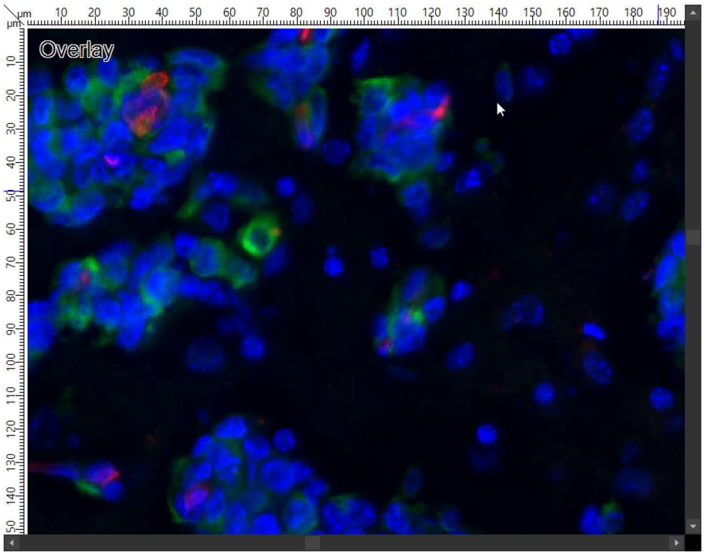
- **Location** - allows changing the corner of the Region Viewer in which the map should be displayed (top left, top right, bottom left, bottom right) and the indentation from the closest border. By default, the map is displayed in the bottom left corner.



2. Ruler

The **Ruler** shows the physical dimensions of a current viewing size.

A physical dimension is expressed in metrical units. The **Ruler** values are computed using the experiment's FOV size.

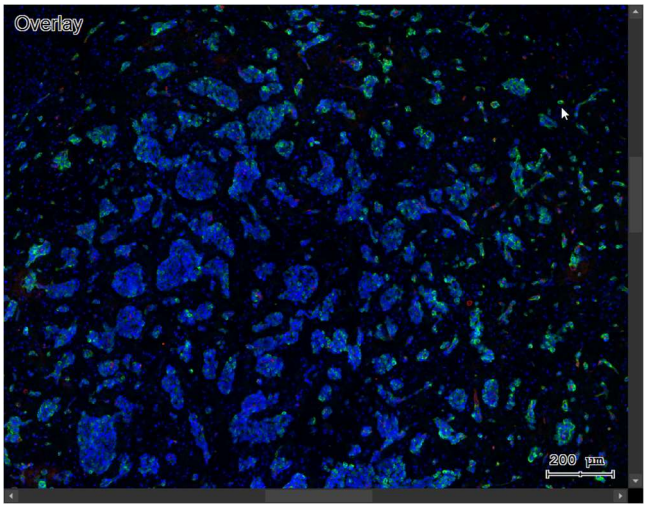


Notes:

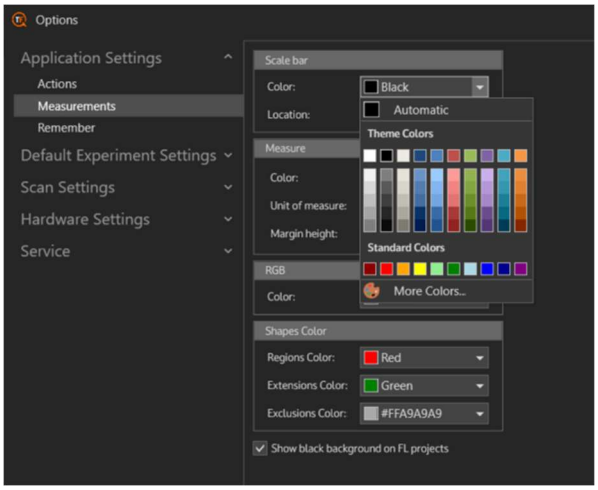
- Depending on the zoom level, the **Ruler** will display different divisions.
- If the user has pressed the **Ruler** button, **StrataQuest** will remember it next time it runs.

3. Scale Bar

The **Scale** Bar is a graphical element which shows a sample scale graphically.



To change the color of the **Scale Bar** and its location on the viewer, go to **TissueFAXS -> Tools -> Options:**

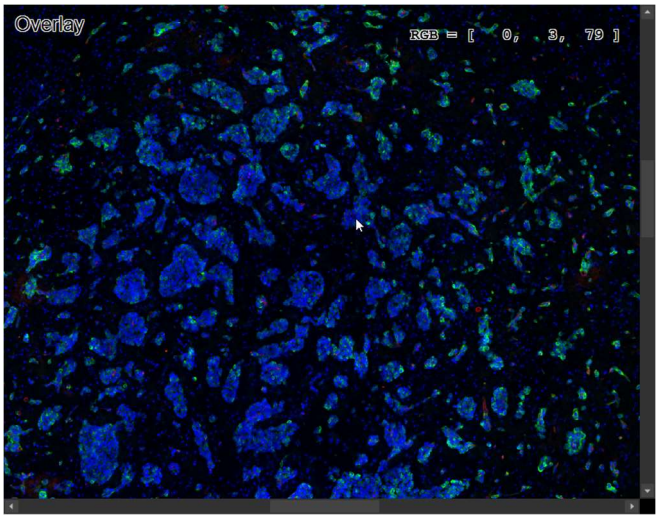


Notes:

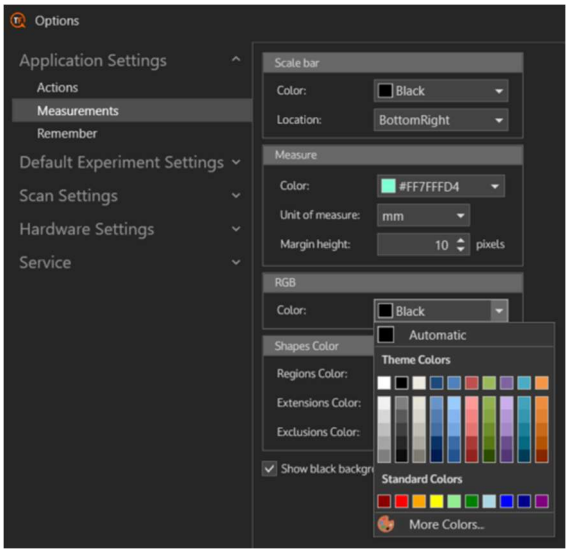
- If the user has pressed the **Scale bar** button, **TissueFAXS** will remember it next time it runs.
- If the user changes the FOV size in the **Project Properties** the values displayed by the scale bar will also change.

4. Color RGB

The **Show Color RGB** button shows the color at the current position of the mouse.



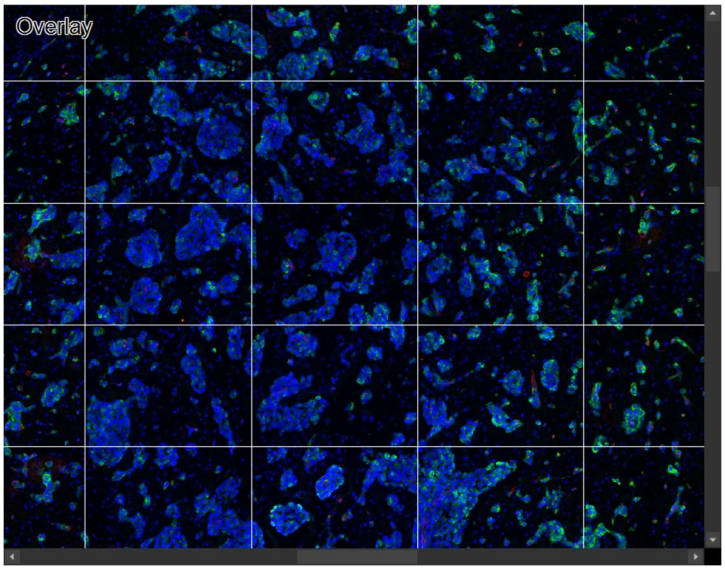
To change the color of the **RGB** and its location on the viewer, go to **TissueFAXS -> Tools -> Options**:



Note: If the user has pressed the **Show Color RGB** button, **TissueFAXS** will remember it next time it runs.

5. Grid

Show grid button shows/hides gridlines between FOVs.

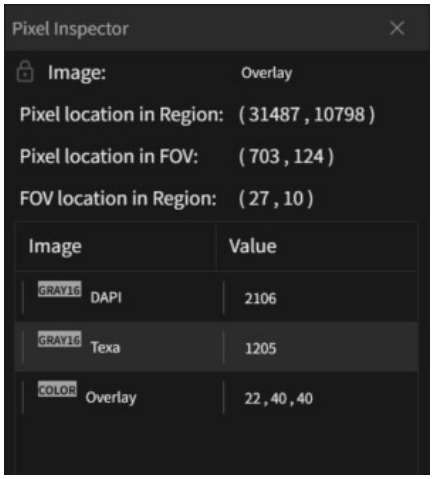


6. Crop: displays the shape of the region in the viewer.

7. Pixel Inspector

Pixel Inspector is a tool that allows you to visualize information about a pixel selected within the tissue.

When **Pixel Inspector** dialog opens, you will have to go on the region and select the desired pixel using the color picker.



Once the selection is done, you will be able to see the following data:

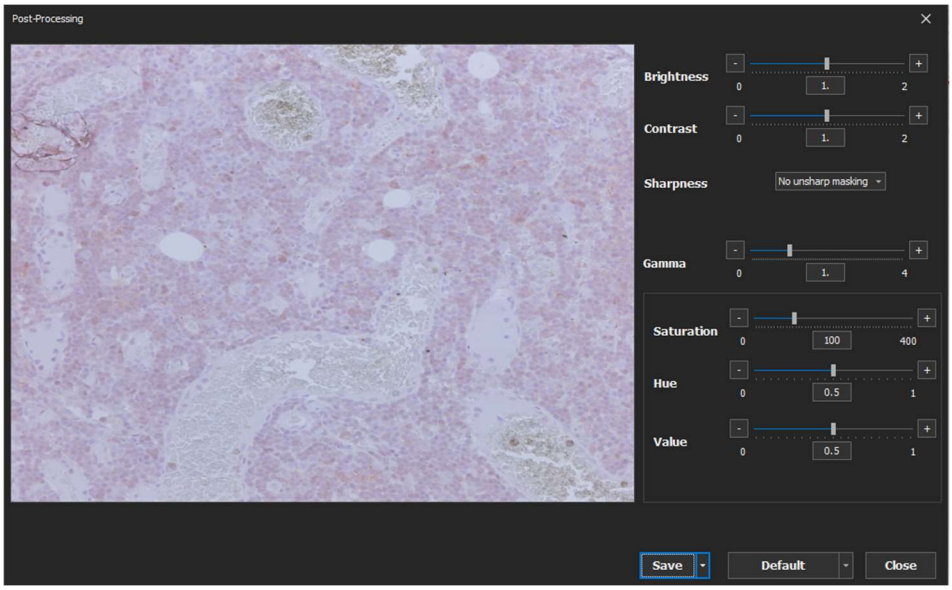
- Pixel location in region
- Pixel location in FOV
- FOV location in region
- Data regarding the channels

To close pixel inspector, close the window or uncheck the menu entry.

4.4.1. Post-Processing (BF Only)

For BF experiments, **TissueFAXS** helps you manually enhance the quality of acquired images by using a post-processing feature. It is located in **region viewer toolbar**.

Go on the displayed image, right click it and select **Configure Post-Processing** from the contextual menu that appears. **Post-Processing** window will open, displaying a selected FOV.



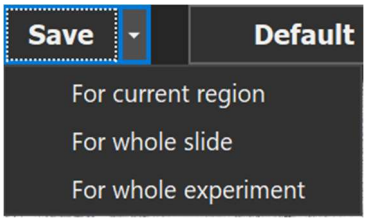
Here, images can be adjusted by using these image processing **parameters**:

- Brightness
- Contrast
- Sharpness
- Gamma
- Saturation
- Hue
- Value

After modifying the parameters above, there are two **reset** options (available by pressing the **Default** button):

- **Reset to default values**: original values of parameters will be restored;
- **Default from region**: default values of current region will be restored.

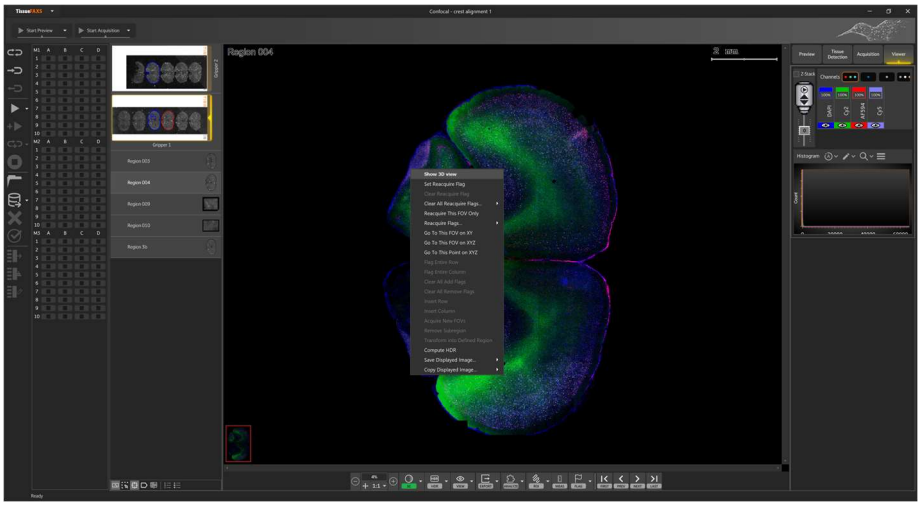
When done, choose one of the **Save** options: for current region, for whole slide, or for whole experiment.

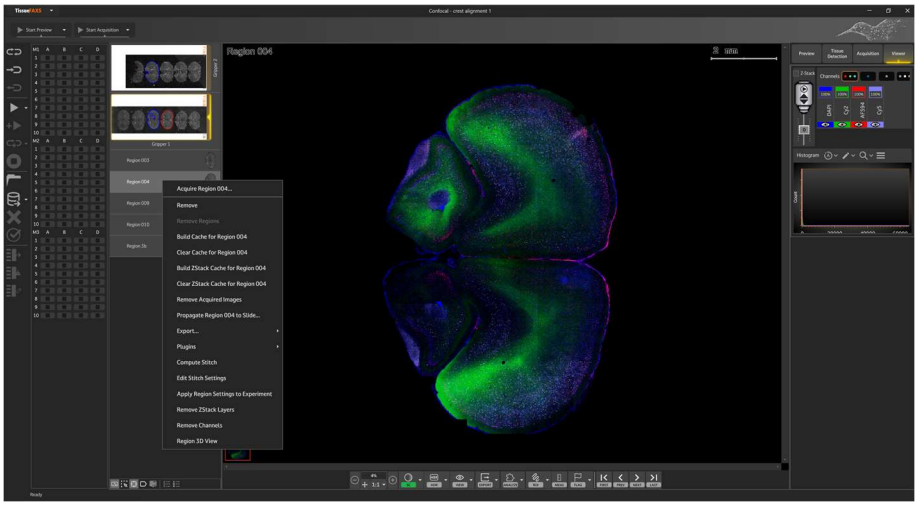


4.4.2. 3D Viewer (FL Only)

A region can be visualized in **3D Viewer** if it has been acquired with Z-stack.

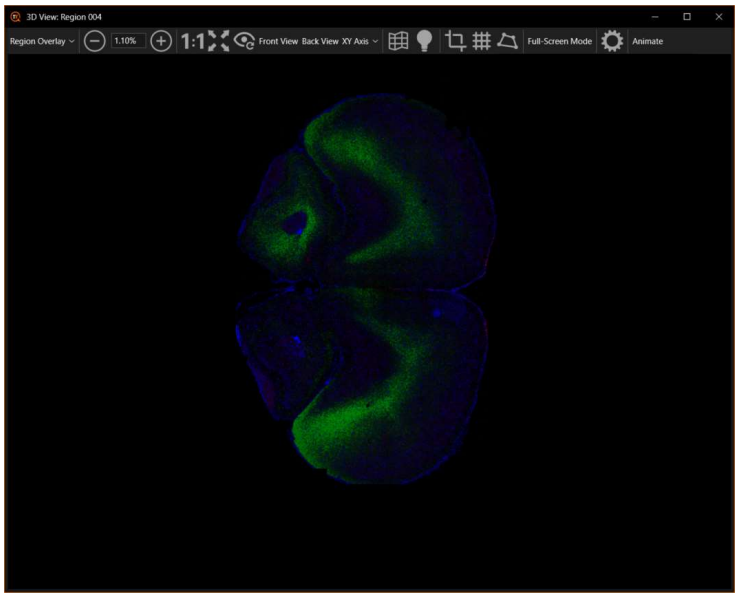
To access the **3D Viewer** feature, right click on a region and select **Region 3D View** from the contextual menu.

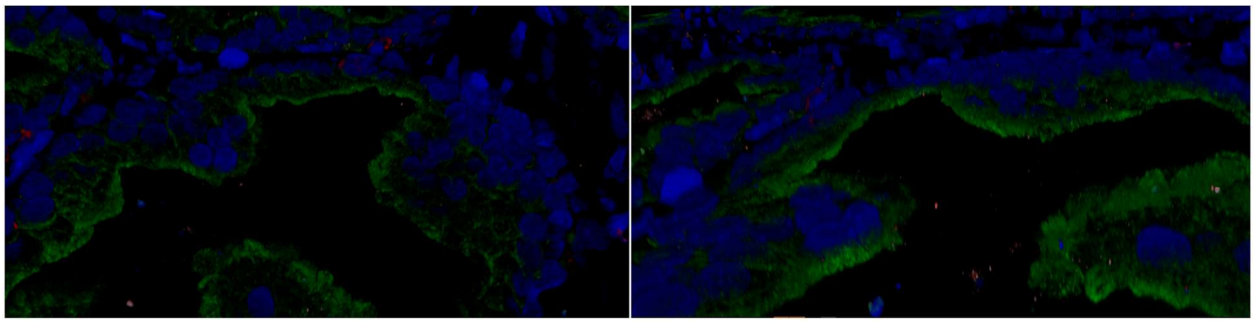




Note: Before accessing the 3D Viewer feature, the **Z Stack cache** needs to be built. This can be done by right clicking on a region and, from the contextual menu, selecting **Build Z Stack Cache**.

The selected region will open in full 3D Viewer mode:



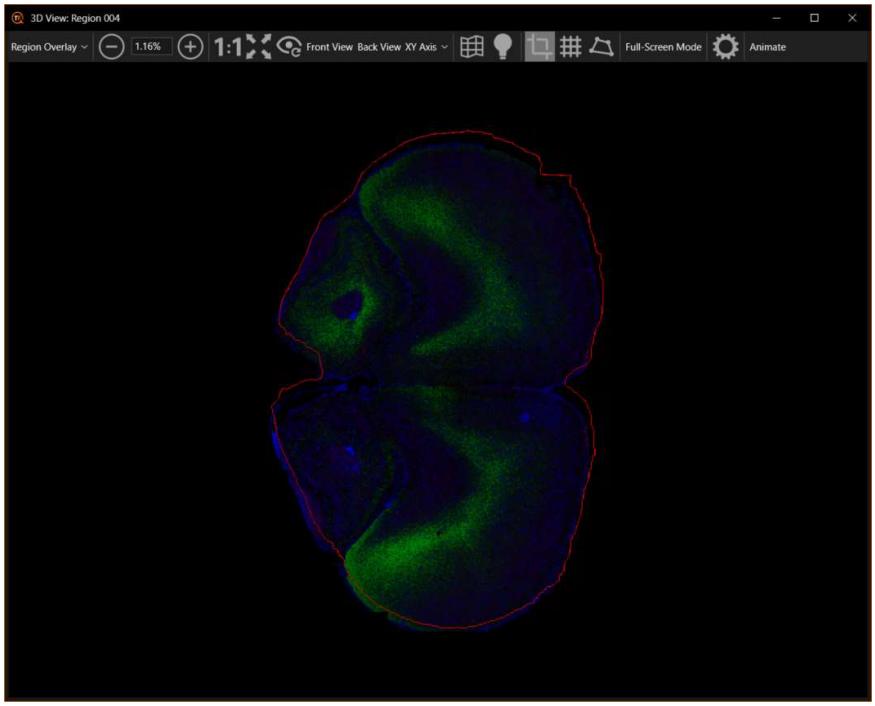


Full screen mode displays image in full screen mode.

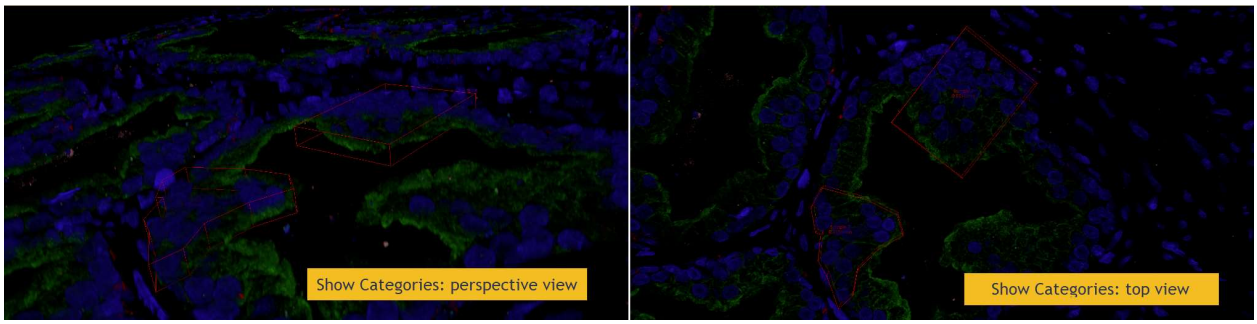
3D Viewer Toolbar

- **Region Overlay**: overlay feature in 3D Viewer helps selecting the channels to be displayed and adjust their values (intensity, color, range). See also Chapter Viewer Options;
- **Zoom in/Zoom Out;**
- **Original View;**
- **Best Fit View;**
- **Reset to Original View**: resets image to its original view, in other words the image will look like being freshly opened in 3D Viewer;

- **View Type: Front/Back/XY Axis**: choose to see the front/back of the opened image and select the rotation axis;
- **Map**: shows map; also see Chapter Images and Viewing Options;
- **Shading Correction**: activates shading correction; also see Chapter Illumination/Shading Correction;
- **Show Crop**: shows region's contour;



- **Show Grid**: shows the grid that separates the FOVs composing the image; also see Chapter Images and Viewing Options;
- **Show Categories**: shows categories (if any) in 3D mode. For more details see Chapter Categories;

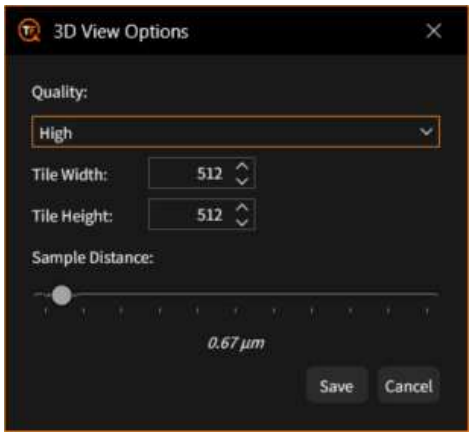


To handle 3D categories in the viewer:

- Select a region/category by using the mouse to click over the edge of the shape. To make multiple selections at once, hold down the CTRL key and click the edges of the shapes to be selected.

- You can highlight a region/category by hovering the mouse over the edge of the shape.

- **Full Screen Mode**: enters full screen mode. To exit, press Esc key;
- **Settings**: the advanced settings for 3D Viewer help you obtain an optimal view, getting the most of the graphical performance of your computer;



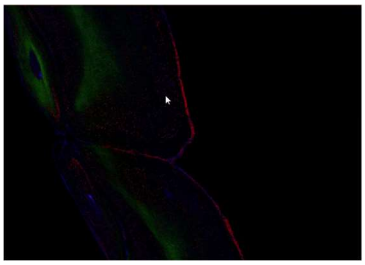
- Set the **quality** of the 3D image. Lower quality will generate a fast rendering, higher quality will generate a slower rendering but a better image. Select Highest or High if you have a performant graphic card, if not – select Medium or Low;

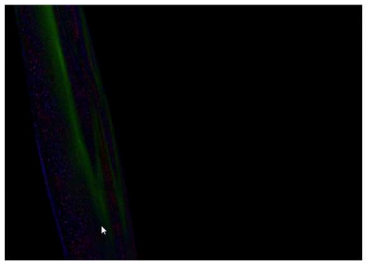
- Set **tile** width and height values;

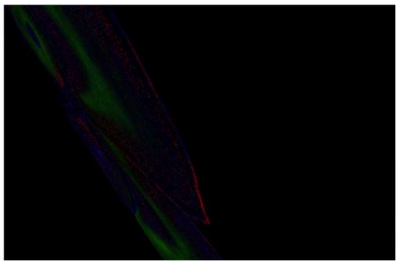
- Set **Sample Distance**: it represents the step made by a raycasting ray in the volume. The more points you have, the more detailed volume you will get. For optimal quality, 1 point per voxel is recommended (one sample per voxel). For low-quality graphic cards, you can increase the sample distance: some quality loss will occur in the image, but the overall performance will improve.

- **Animate:** TissueFAXS 3D Viewer allows creating and storing animation sequences.

- To create an animation, press **Add**. Enter values for X, Y and Z planes, and also a duration for the animation (in seconds). Press **Ok** to create the animation.







- To edit an animation, press **Edit**.

- To delete an animation, press **Remove.** To delete all animations from the list, press **Remove All**.

- Press **Loop** to run the animations continuously.

- Press **Play** to run once.

- Press **Stop** to end current rendering.

- **Animation View** is a graphical overview of the “playlist” of existing animations. You can select at what time the animation starts and ends. You can also have a good visual understanding of how many animations you have and also their duration.

Operations Shortcuts

The following shortcuts are available for more dynamic interactions with an image in the 3D viewer:

- Mouse Right Click → Rotates camera around the focal point;

- Mouse Left Click → Zooms in on a selection;

- Shift + Mouse Left Click → Zooms in on a selection (the center is the view on the selection);

- Ctrl + Mouse Left Click → Zooms in on a selection (the center is the view on the selection);

- Mouse Wheel → Zoom in and out;

- Shift + Right Click → Zoom in and out;

- Mouse Wheel Click → Pans the region;

- R key → reset to original view;

- B key → view best fit;

- F key → Fly to point (animation that zooms in to mouse pointer, for single channel regions);

Note: Given a position x, and a movement of the camera's current focal point to x, the movement is animated over the number of frames specified.

- Esc → exit full screen.

4.4.3. Illumination/Shading Correction

1. Brightfield Illumination Correction

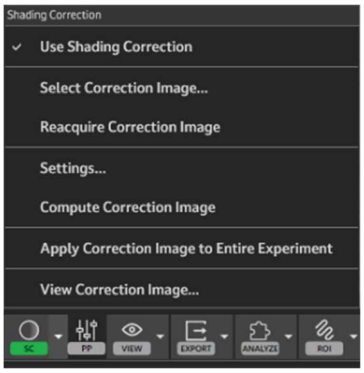
Occasionally, some shades may appear on acquired images. They can be caused by imperfections of any component of the light path, specks/impurities on the camera/objective.

TissueFAXS provides a way to remediate shading complications in brightfield experiments by using the **Illumination Correction** function.

The **Illumination Correction** menu can be accessed by pressing the **Illumination Correction** button from the **Region Viewer** control.

A *correction image* is *required* to apply illumination correction.

The **correction image** is an image computed to store information about the shades in the light path. By applying this image to a certain region, the shades will be removed and the images will be uniformly illuminated.



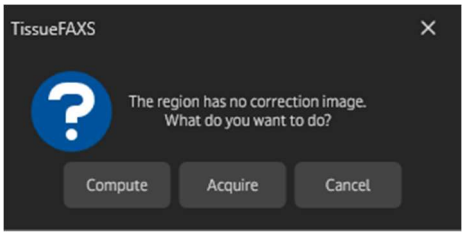
The **Illumination Correction menu** contains the following options:

- **Illumination correction**: choose this option to automatically apply the correction image to the region. If there is no correction image available, a message box will appear offering three possibilities:

- Compute a correction image, using the already acquired images. This is for when a correction image is not available and reacquiring the correction image with the same settings/hardware as the region is no longer possible.

- Acquire a correction image (equivalent to **Reacquire correction image** from the **Illumination correction** menu);

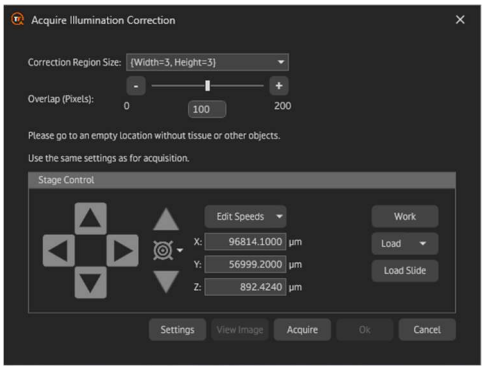
- Cancel the whole process of illumination correction.



- **Select correction image**: Choosing this option will display a dialog with a list of regions (containing correction image), from which a correction image can be selected. The region highlighted in red the currently opened region.

Select the image and apply the illumination correction.

- **Reacquire correction image**: to correct the illumination for an acquired region using the following panel:



Correction region size: Specify the width and height, expressed in FOVs, of the area used to compute the correction image. Generally, a large area size is preferred, but this is not mandatory.

Overlap: the overlap parameter helps control the size of the overlapping area shared by neighboring FOVs that form the correction region. The slide bar can adjust the number of pixels that can be shared (overlapped) by two FOVs. Usually, increasing the value results a better correction image and, thus, a better illuminated region.

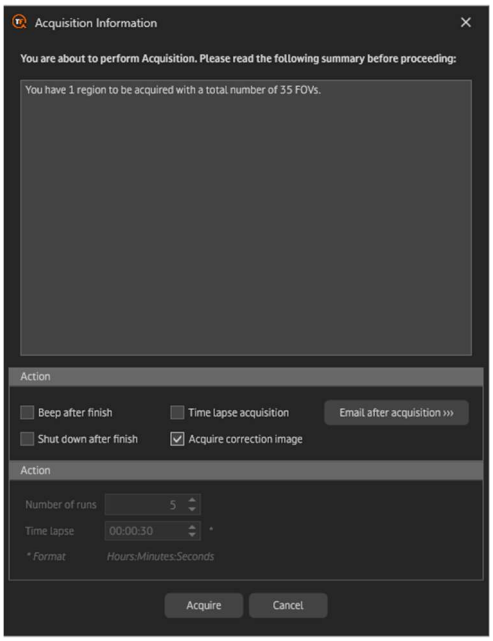
Press the **OK** button to save the correction image or the **Cancel** button to cancel the acquisition process.

- **Apply this correction image to the entire experiment**: the correction image of the current region from the region viewer will be applied to all regions of an opened experiment.

Notes:

- Each time, before starting the acquisition process, an **Acquisition Information** dialog will be displayed where the Acquire correction image option can be checked in order to obtain a correction image for the respective region. TissueFAXS will use the same acquisition settings for the region and for the correction image.

- The correction image must be acquired on an area with no tissue. Also, slide edges must be avoided.



The Shading correction images acquisition dialog will appear (see above the *Reacquire correction image* paragraph).

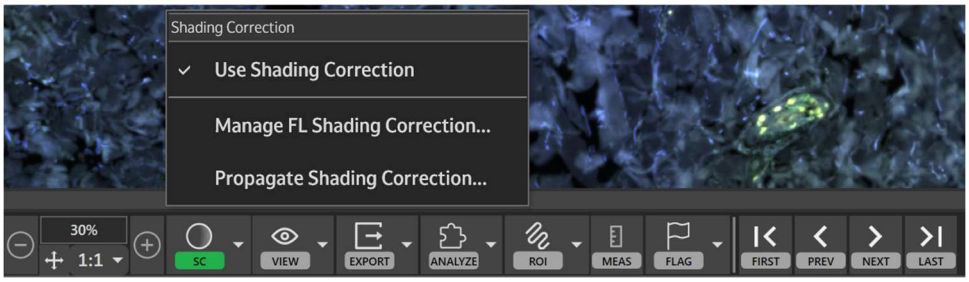
Note: If images are acquired frequently, this option can be set as default from **Tools → Options → Application Options →Remember.**

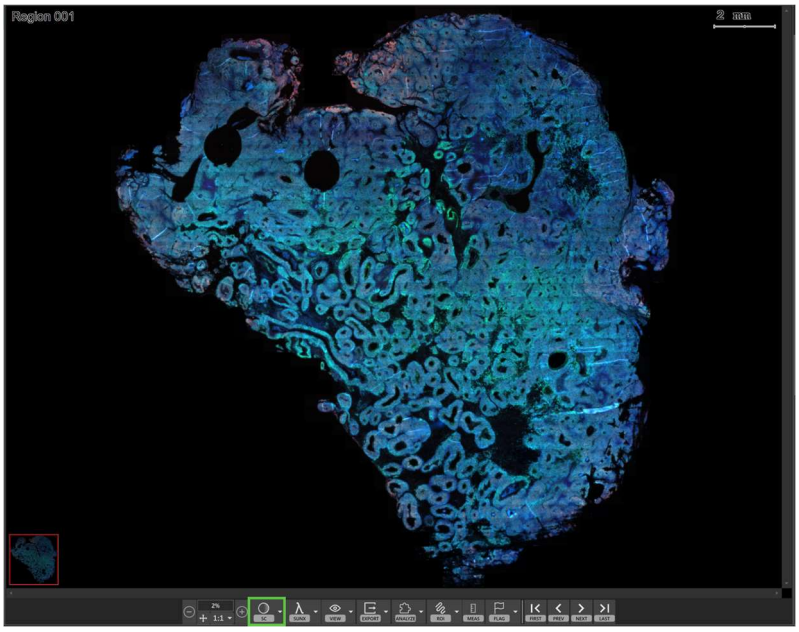
2. Fluorescence Shading Correction

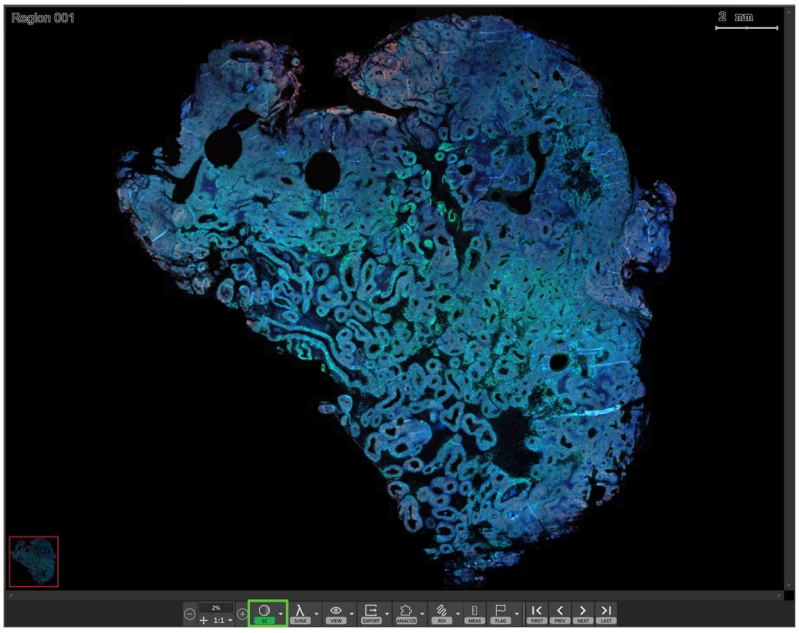
Occasionally, some shades may appear on the acquired images. They can be caused by imperfections of any component of the light path (e.g., FL Lamp, filters, etc). That is, specks/impurities on the camera/objective.

TissueFAXS can correct these shading problems in fluorescence experiments by using the **FL Shading Correction** function.

The **FL Shading Correction** menu can be accessed by pressing the **FL Shading Correction** button from the **Region Viewer/Acquired Images** control.







A *correction image* is necessary to apply shading correction.

The **correction image** is an image generated to store information about the shades in the light path. By applying this image to a certain region, the shades will be removed and the images will be uniformly illuminated.

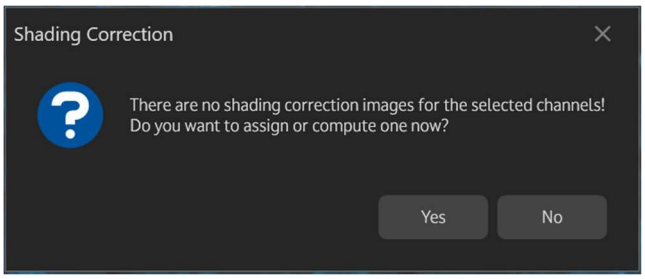
The shading reference image for Z-stacks has to be computed for each Z level.

The shading reference is specific for each channel, including confocal channels.

Note:

- The shading reference corrects shading that comes from the light path - *it cannot correct optical aberrations* that come from the sample itself. In some samples, there are "shading effects," which come from the tissue, different preparation, or fixation methods. These effects might appear in some areas while not visible in other areas or in other samples. This is not considered shading. Such effects are optical aberrations that have their origin in different optical properties of the sample. They will not be corrected by the shading correction operation.

If no correction image is present, the following message will pop out:



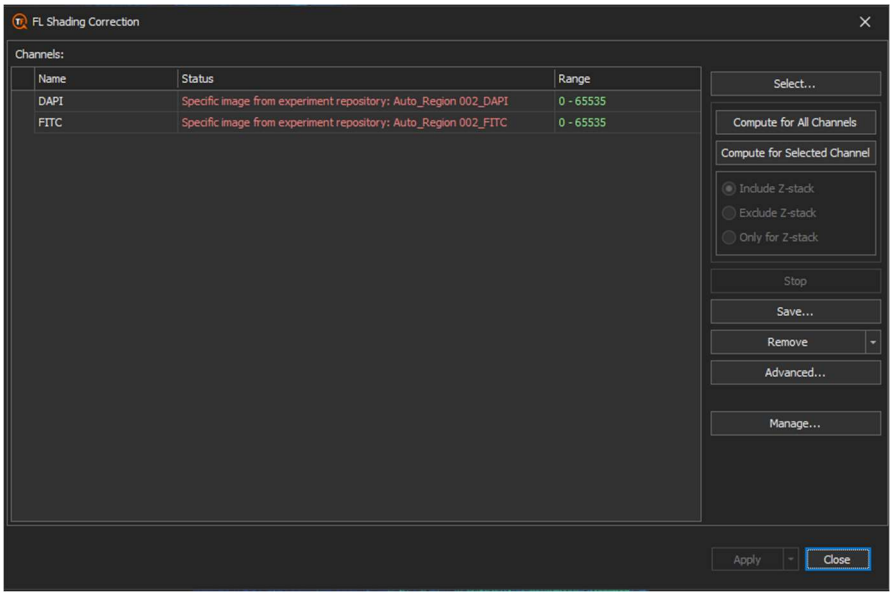
To compute a correction image, press **Yes** in the above message or choose **Illumination Correction** to access the **FL Shading Correction** panel.

Managing FL Correction Images

TissueFAXS provides a management panel for existing correction images.

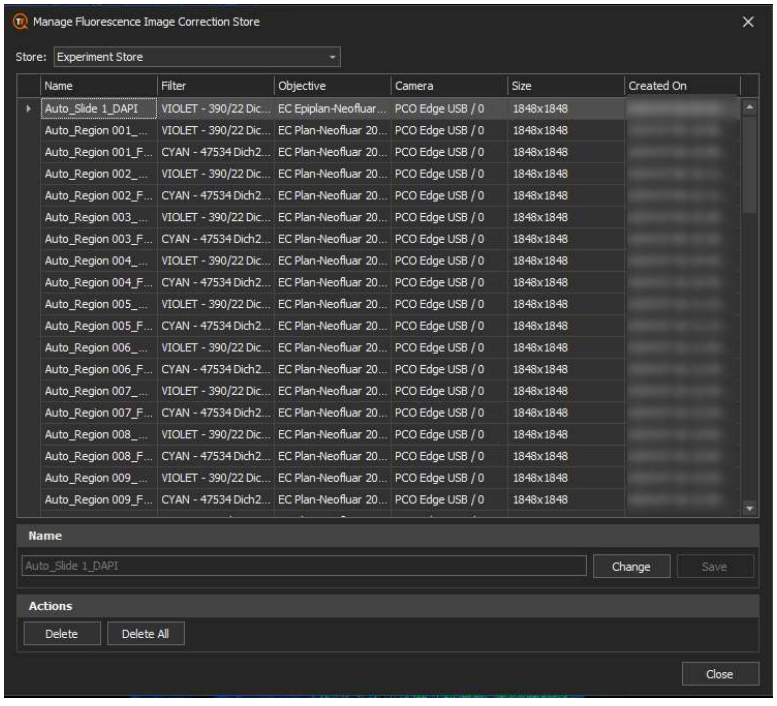
To manage the correction images, choose the **Manage FL Shading Correction** option and then press the **Manage…** button. The **FL Shading Correction** panel will open.

The name, status, status for Z-stack, and intensity range will be displayed for each channel.

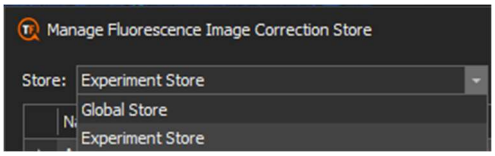


Note: The correction image will be affected if overexposed areas are present when computing correction image. The **Flag** feature can be used in order to select which images to keep for computing shading correction.

Press the **Select** button to open **Manage Fluorescence Image Correction Store**.



First, select the type of store where the correction images are located: a global store or experiment store. The **global store** will make the images available to all the experiments, while the **experiment store** will only make the images available for the current experiment.



Compute for All Channels: computes correction image for all channels;

Compute for Selected Channel: computes correction image only for a selected channel;

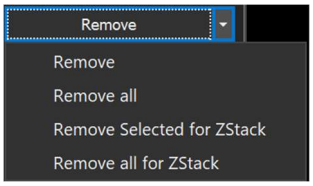
Include Z-Stack: computes a correction image for all the images that include Z-Stack;

Exclude Z-Stack: computes a correction image for all the images that exclude Z-Stack;

Only for Z-Stack: computes a correction image only for Z-Stack images.

You can **remove** the images as follows:

- **Remove**: will remove selected correction image;
- **Remove all**: will remove all the existing correction images;
- **Remove Selected for ZStack**: will remove selected image only for ZStack;
- **Remove all for ZStack**: will remove all the correction images only for ZStack.



In the end, the user must select where to **apply** the correction image(s):

-To the current **region**;

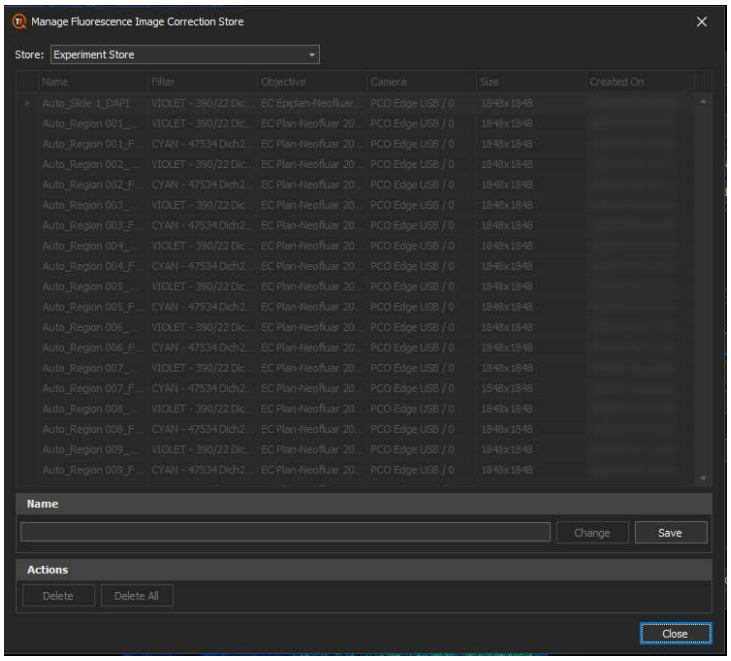
- To the current **slide**;

- To the current **experiment**.

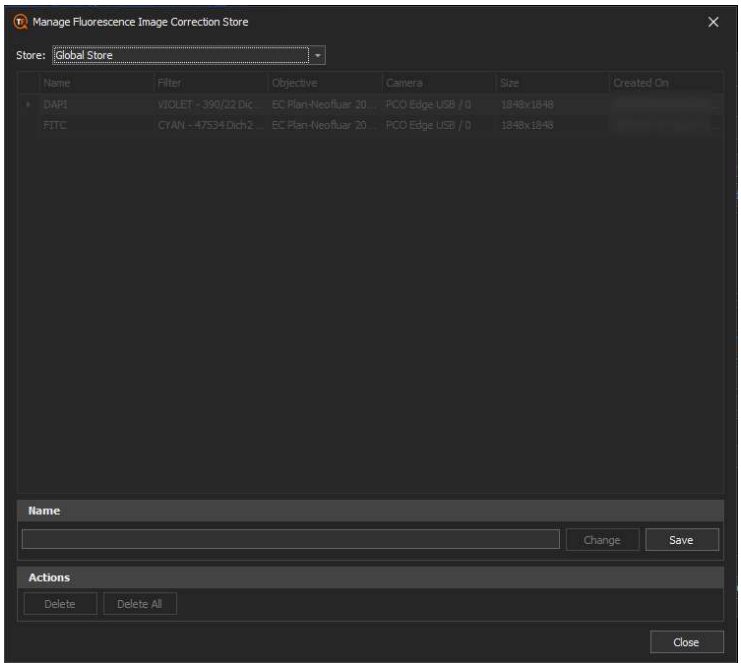
Stop: will halt the process of computing correction images for large regions.

Save: there are two ways of saving correction images:

- In **Experiment store**: the correction images saved here will be used only for the current experiment;
- In **Global**: the correction images saved here can be used in any other experiment.



If you need to make changes to the correction images store, press **Manage**. The **Correction Image Store Manager** will open.



Press the **Change** button to edit the names of a correction image, then press **Save**.

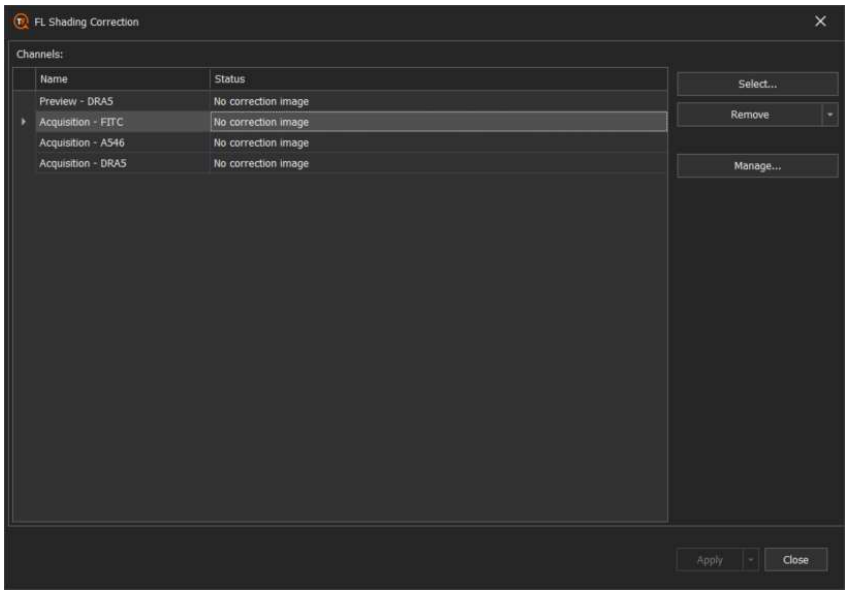
The selected correction image can be deleted by pressing **Delete**. If pressing **Delete All**, then all the correction images from the respective storage will be deleted.

A correction image can be computed for:

- All existing channels;
- Only for selected channels;
- If the project was acquired with Z stack, the correction images can be computed for selected channels for Z stack slices (each slice will have its own correction images).

FL Experiment Shading Settings

In the **FL Experiment Shading Settings** from the **TissueFAXS** main menu-> **Experiment**, you can select the images you want to use for the experiment before preview/acquisition. The correction images selected here will be used during the acquisition process.

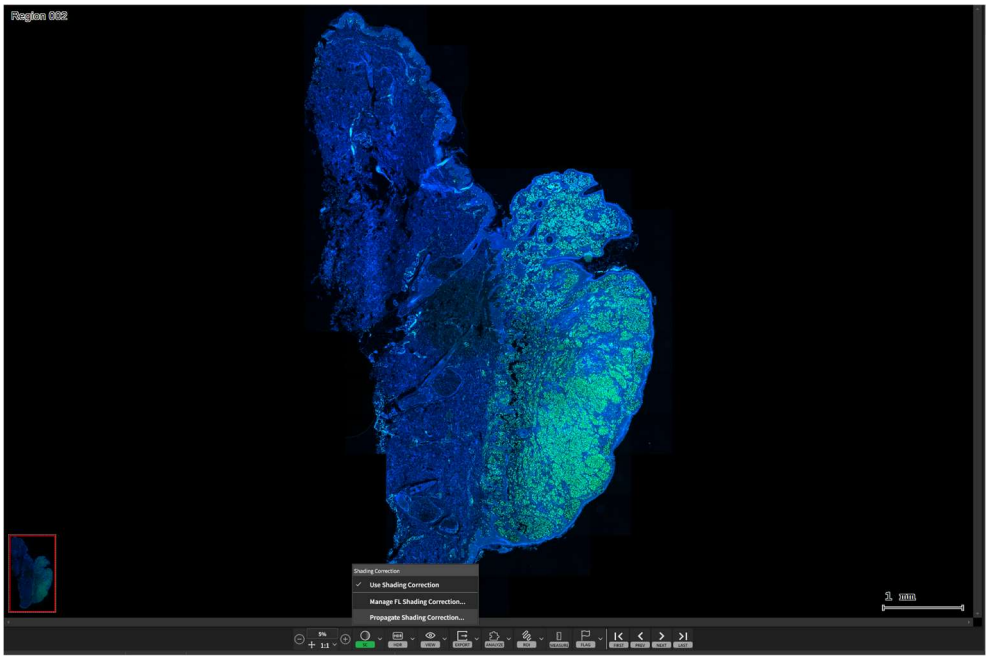


Press the **Select** button to open a store and pick a correction image for the desired preview/acquisition channel.

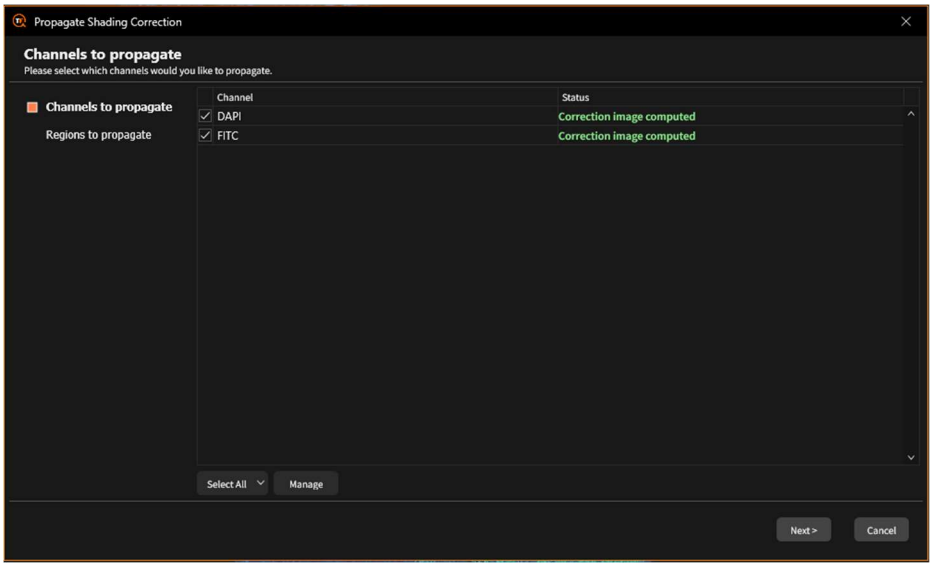
Propagate Shading Correction

A shading reference image can be propagated only to user-specified channels.

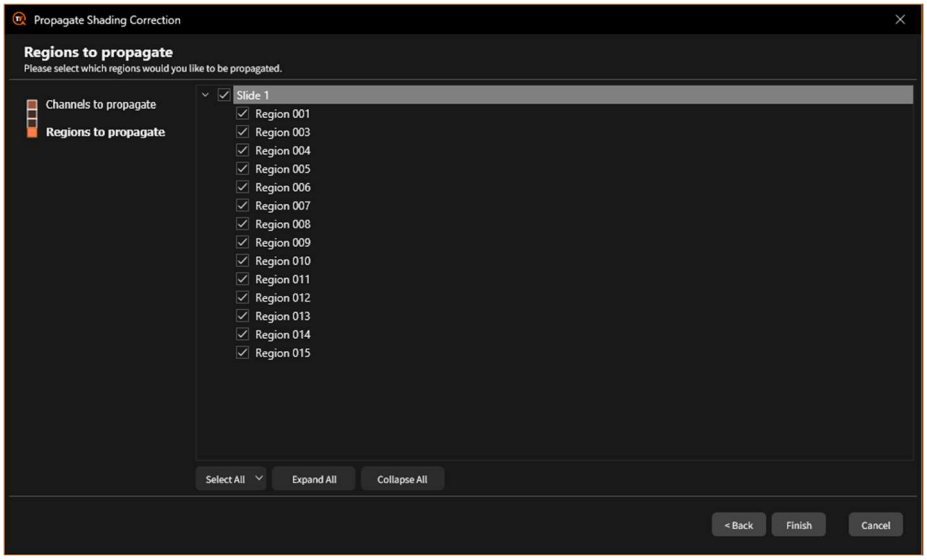
This option is located at: **Region viewer** -> **Shading correction** -> **Propagate Shading Correction**.



Channels to propagate:



Regions to propagate:

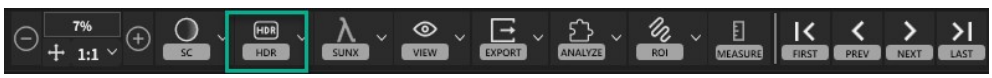


4.4.4. HDR Visualization (FL Only)

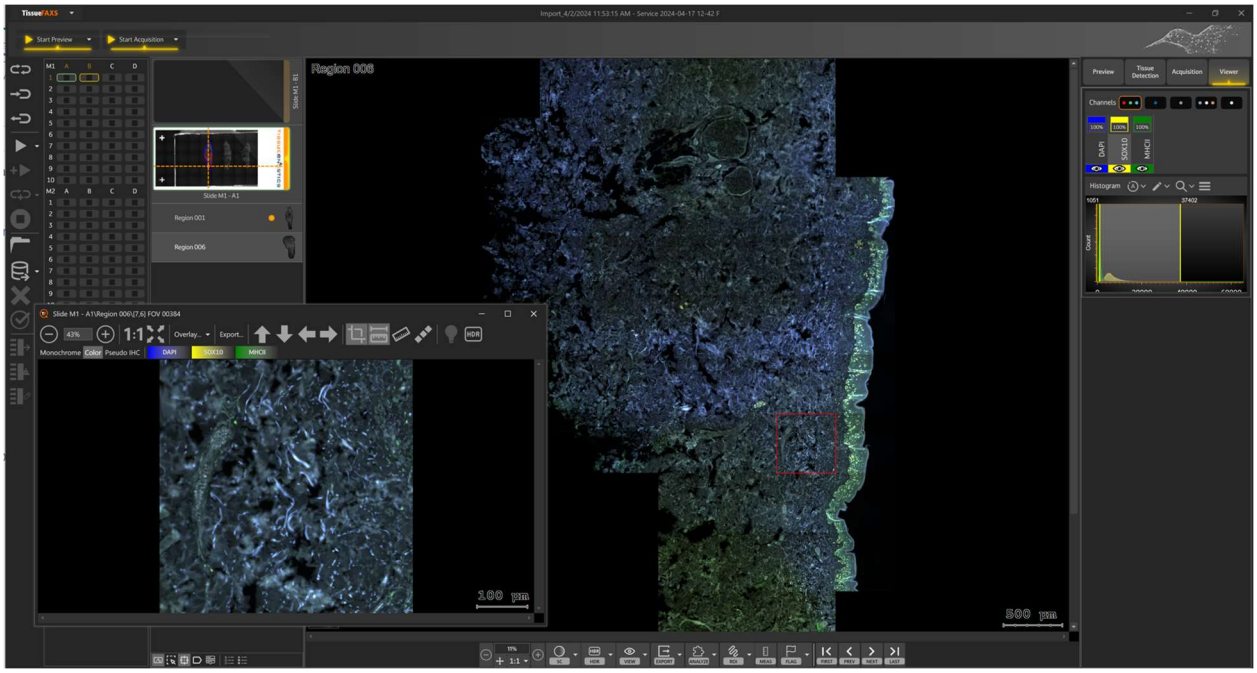
With TissueF**AXS,** regions can be visualized as **HDR** (High Dynamic Range) images.

In other words, a non-linear display can be used to improve the dynamic range of the images, enhancing details in shadows (areas with weak signal) and highlights (areas with strong signal).

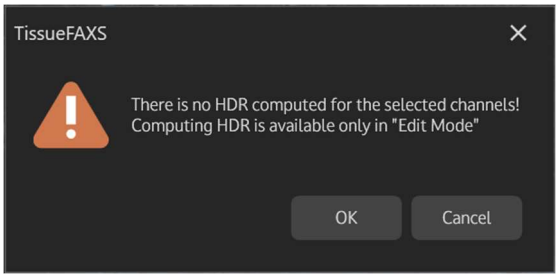
Use the **HDR** button from the image viewer toolbar to activate **HDR** visualization mode.

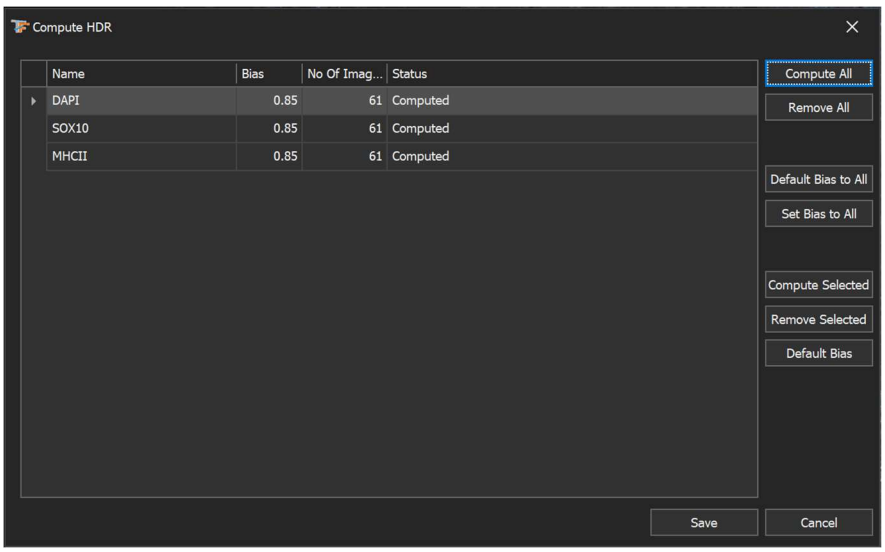


The **HDR** button is available for region viewer and FOV viewer.



If a region is missing the **HDR** image, a message prompt will ask whether to compute it and for which channels.

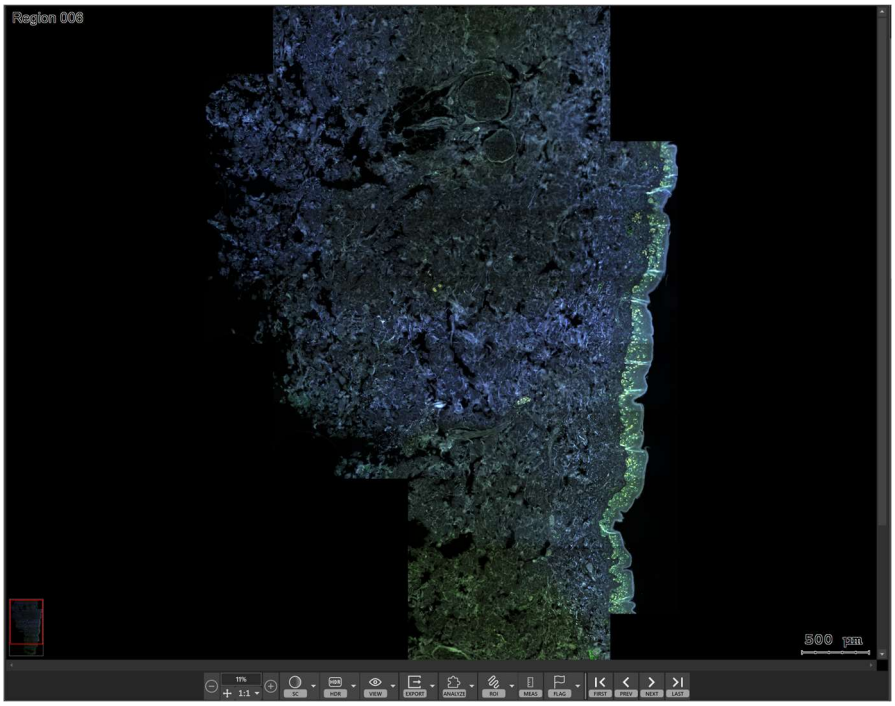


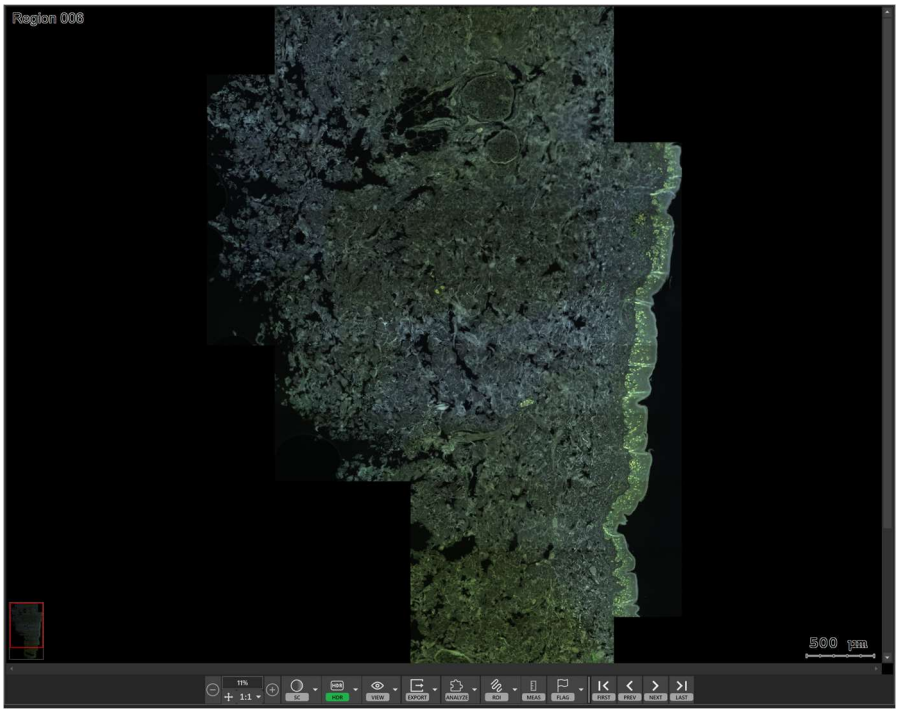


HDR Operations

In the **Compute HDR** window, the following operations are available:

- **Compute Selected/Compute All** HDR images. In order to generate HDR images, information from the images composing the sample must be collected. This operation needs to be completed only once for each channel. It is also possible to compute it for all channels by choosing Compute All.
- **Remove Selected/Remove All**: HDR images can be deleted for some channels (select channels + **Remove**) or all channels (**Remove All**).





Bias operations

The Bias parameter can be used to control the overall intensity increase in the generated image.

A lower value will generate a bigger amplification for lower intensities. If the HDR image tends to be over-saturated, the Bias value should be increased.

- **Default Bias to All**: apply default bias value to all channels;
- **Set Bias to All**: set same bias value to all channels;
- **Default Bias**: choose the default bias value.

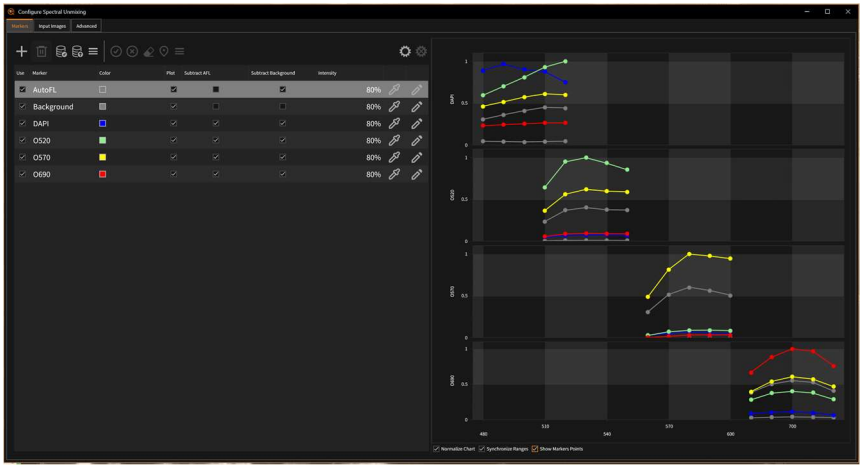
4.4.5. Spectral Unmixing

Spectral Unmixing feature is only available for FL Multispectral Experiments only.

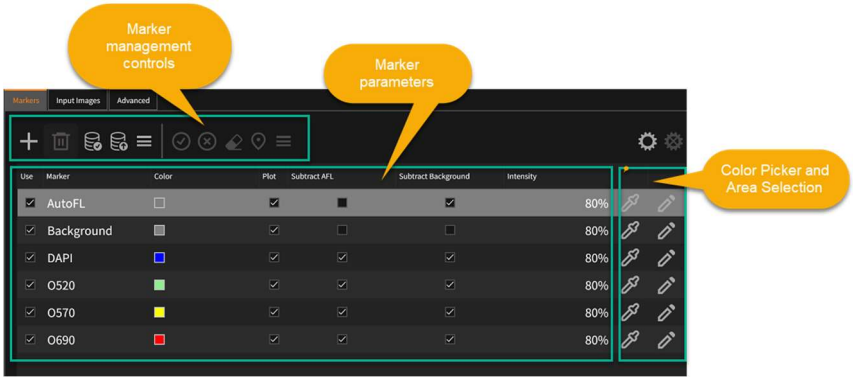
To configure the **Spectral Unmixing** settings, press the **Configure Spectral Unmixing** button from the region viewer:

Configure Spectral Unmixing dialog will open.

It has three sections: **Markers**, **Input Images**, and **Advanced**.



Reference Marker Definition



Markers management

To manage markers, use the following controls:

- **Add Markers**: adds a new marker to the list of markers.
- **Remove Marker**: deletes a specified marker from the list of markers.
- **Remove All Markers**: deletes all markers from the list of markers.
- **View Values**: displays the numeric values for all markers present in the list of markers.
- **Save Markers to Spectral Database**: saves the reference values of a specified marker into the database.

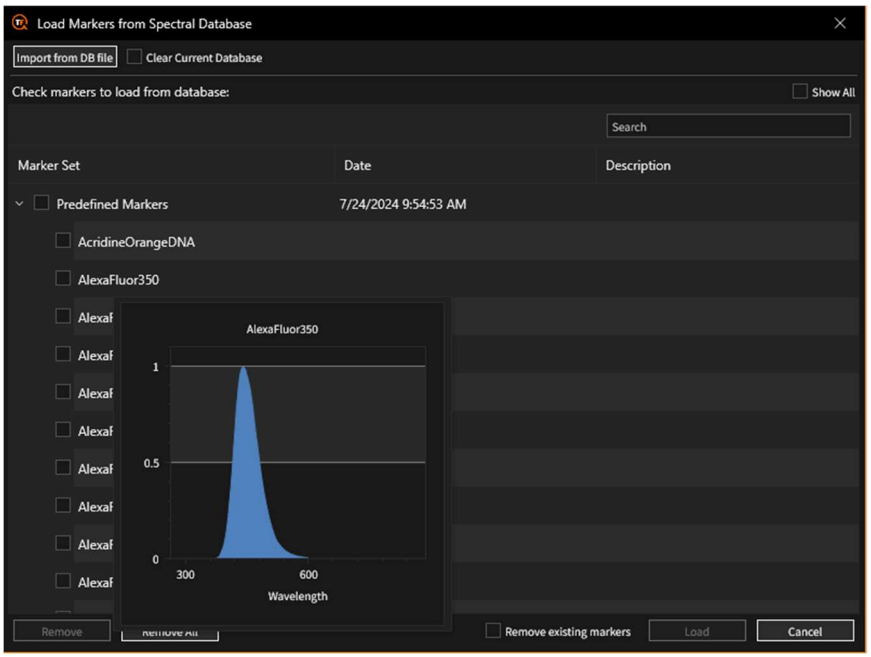
The following parameters can be set for each marker:

- **Marker name:** defines the marker name.
- **Color:** defines the marker color.
- **Plot:** enables plot display.
- **Subtract AFL:** this parameter works like a flag, which specifies that the auto FL component will be subtracted from the marker.

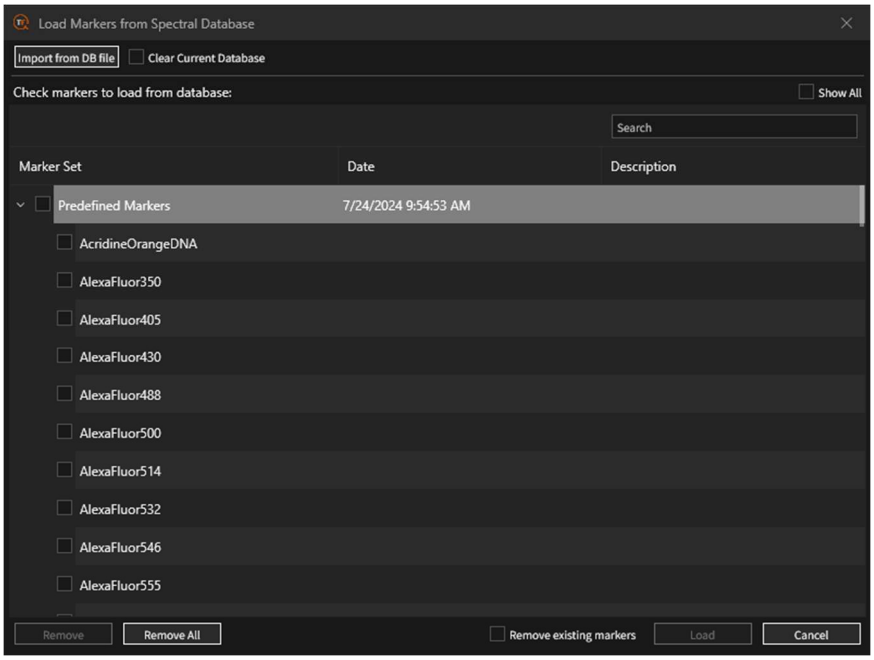
Load from Spectral Database

Previously saved markers or predefined standard markers are available for loading from the spectral database for usage. If using the **Import from DB File** option, markers will be imported from another database (for example StrataQuest database). Before importing from another database, the current database can be removed by using **Clear Current Database**.

Hoovering the mouse on the marker's name will open a small window showing the marker's spectrum.



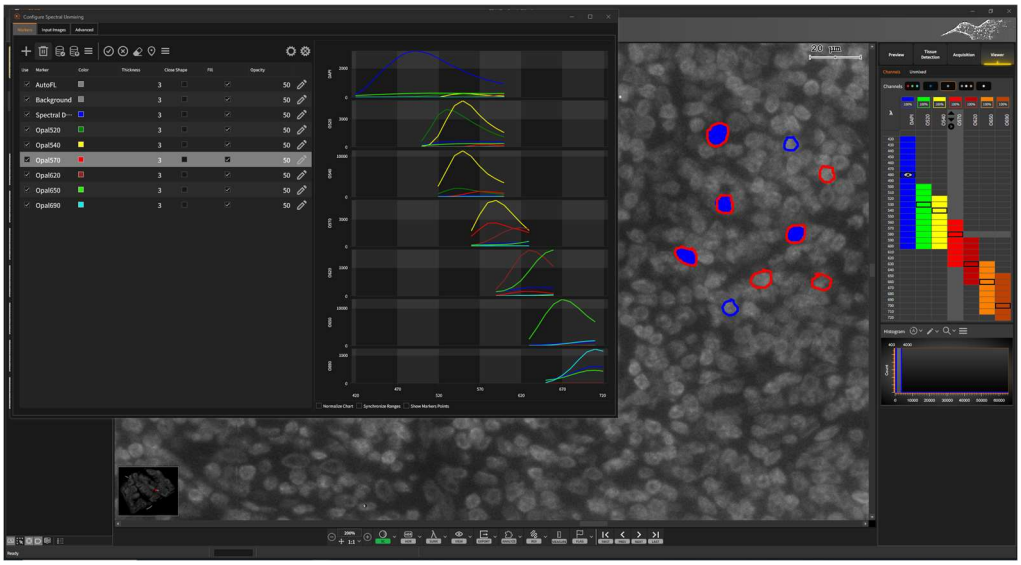
There's a list of the most used predefined standard markers. The search option on the top right side of the window makes the selection easier.



Once the selection is made, the loading process is finalized by pressing the **Load** button.

Color Picking and Area Selection

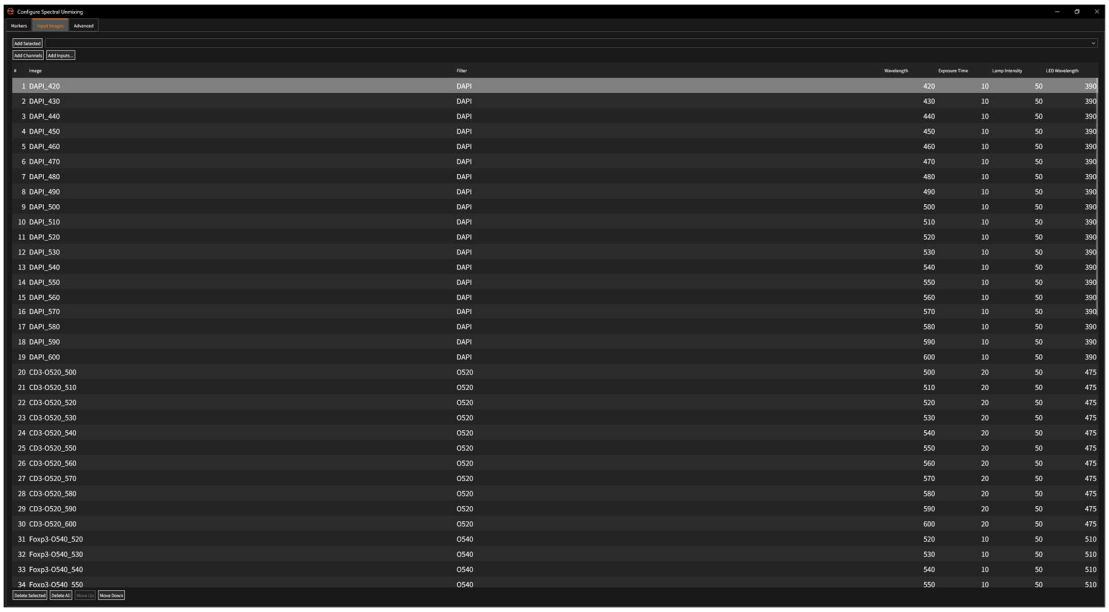
For a proper definition of the reference spectrum of a specified marker, it is recommended to use a single marker stained sample. Otherwise, it is possible to select an unwanted mixture of two or more markers, which will generate the result (of that particular mixture selected spectrum) to be a mixture also.



- **Color picking** – the user selects a single pair of coordinates (x, y). These coordinates are used to collect all the reference values of the marker, from the images corresponding to the wavelengths defined within the Lambda stack.
- **Area selection** – the user draws a mask using a brush, meaning a collection of coordinates (x, y). All (x, y) positions indicated by the drawn mask will be used to generate the reference values of the marker, from the images corresponding to the wavelengths defined within the Lambda stack.

Input Images

Input images can be selected in this tab. Input images (or the Lambda stack) represent a list with all images used in the unmixing process.



Input Operations

The following **operations** are available to manage the input images list when defining an input:

- **Add Selected**: selects an image to add to the Spectral Unmixing input images list
- **Add Channels**: adds all original wavelengths for each channel to the input
- **Add Inputs**: adds more images to your input at the same time (batch)
- **Remove Selected**: removes selected images
- **Remove All**: removes all images
- **Move Up** / **Move Down**: controls change the position of the selected image (+1, -1)

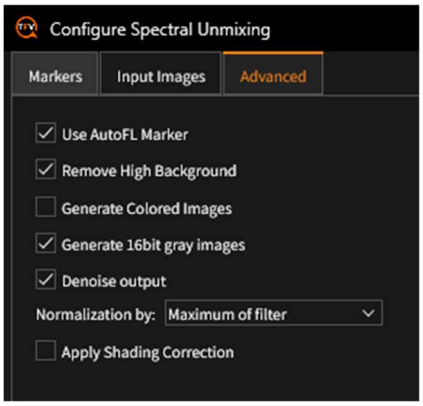
Note: All available input images are gray images acquired with 8 bit or 16bit.

Advanced

The **Advanced** tab contains the following settings:

- **Use AutoFL Marker:** enables auto fluorescence. It behaves like a marker in the unmixing process.
- **Remove High Background**: offers the ability to define the background. Usually, in 16-bit images, the background is never 0-value. Any value above 0 is considered signal and will be decomposed into the defined marker's components.

- **Generate Colored Images**: generates colored images for each unmixed marker. The color used is the one associated with each marker.
- **Generate 16bit gray images**: The grayscale images generated for each unmixed marker will be on 16-bits.
- **Denoise output**: A small filter is applied on the unmixed images to remove noise.
- **Normalization by**: specifies the method used to normalize the reference marker's values - maximum on filter or maximum on marker. Normalization is only for the plot.
- **Fluorescence mode**: switches between fluorescent / brightfield mode.
- **Apply Shading Correction**: enables shading correction on the input image.



After finishing the configuration for Spectral Unmixing, press **Analyze** button.

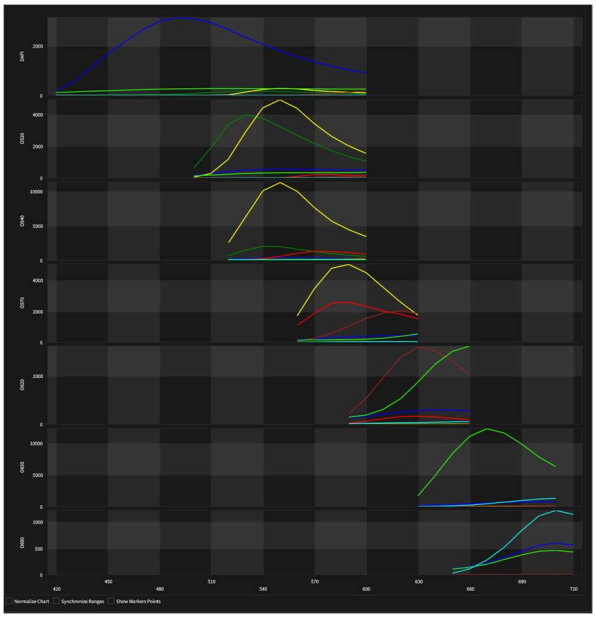
Based on the input, a set of images will be generated: the images for all individual channels used as input and a mixed image with all the channels superposed. To stop the run process, press **Clear Spectral Unmixing**.

Plot

The plot can be visualized after applying the selection for color picker/area selection.

The following settings are available:

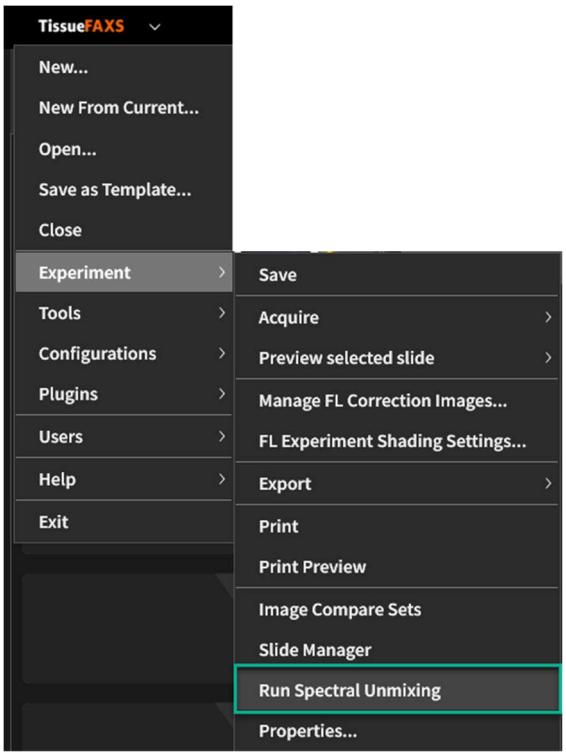
- **Normalize Chart**: if selected, normalized values will be used in the plot, using the marker values. If not selected, the raw values will be used.
- **Synchronize Ranges**: if selected, all the plots will have the same range on y axis.
- **Show Marker points**: displays the values on the plots as points.



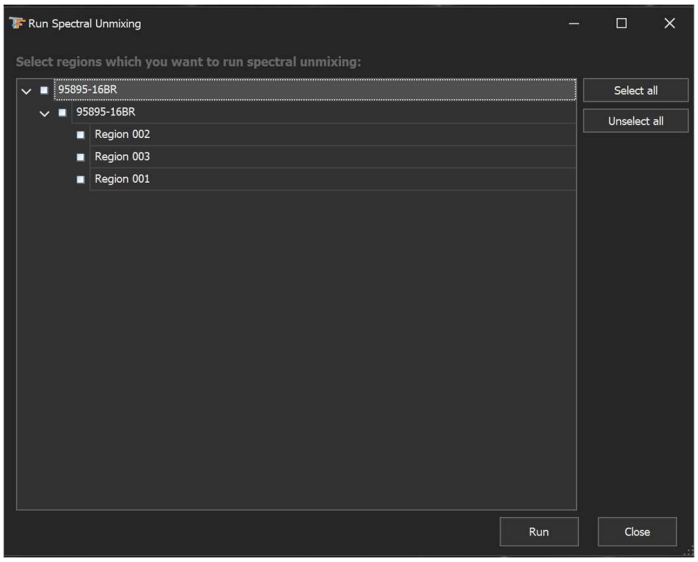
Propagating and running spectral unmixing

To run **Spectral Unmixing,** there are more options:

1. Run **Spectral Unmixing** from main toolbar



If pressing the **Run spectral unmixing** button from main toolbar, the **Run Spectral Unmixing** panel will open:



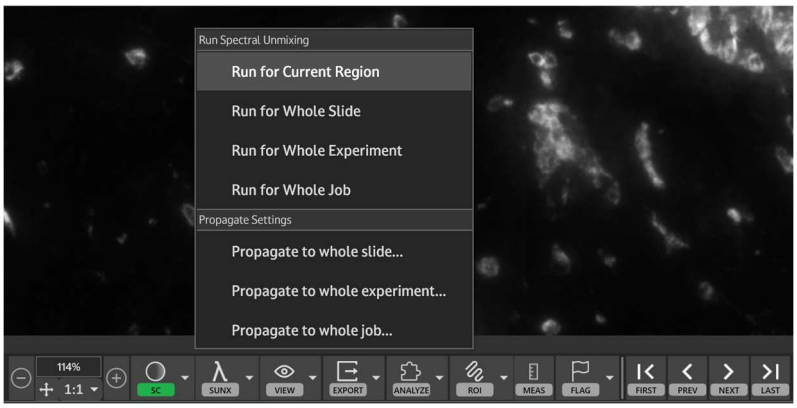
Items for running spectral unmixing can be selected:

- by manually selecting the sample and the regions;
- by using the **Select All** option in order to run spectral unmixing for all the listed items.

When the selection is done, press **Run**.

2. Run **Spectral Unmixing** from Region Viewer

If pressing the arrow near the **Configure spectral unmixing** button from the region viewer, two options will appear:

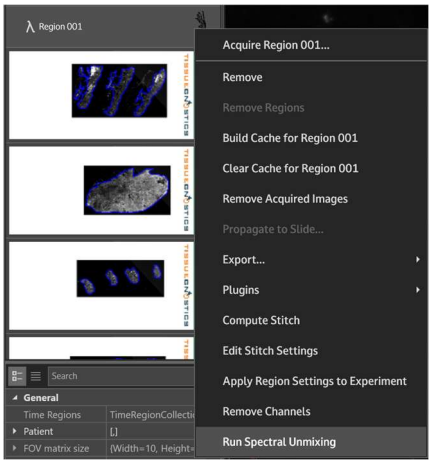


- **Run for current region**: runs spectral unmixing for the current region.
- **Run for whole slide**: all the current settings for spectral unmixing will be propagated to the whole selected slide.

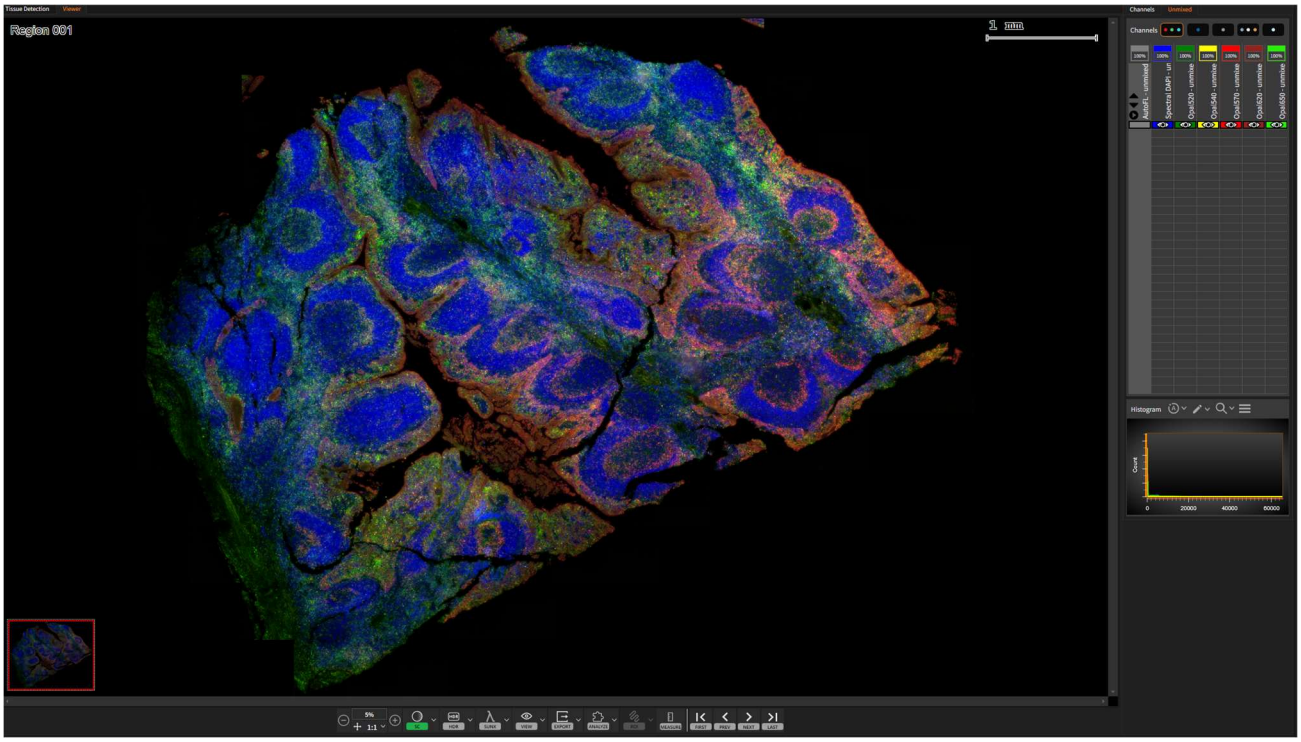
- **Run for whole experiment**: all the current settings for spectral unmixing will be propagated to the entire experiment.
- **Run for whole job**: all the current settings for spectral unmixing will be propagated to the entire experiment.

3. Run **Spectral Unmixing** using the contextual menu

Running spectral unmixing can also be accessed from the **contextual menu** of a region or a slide, as shown in the images below:



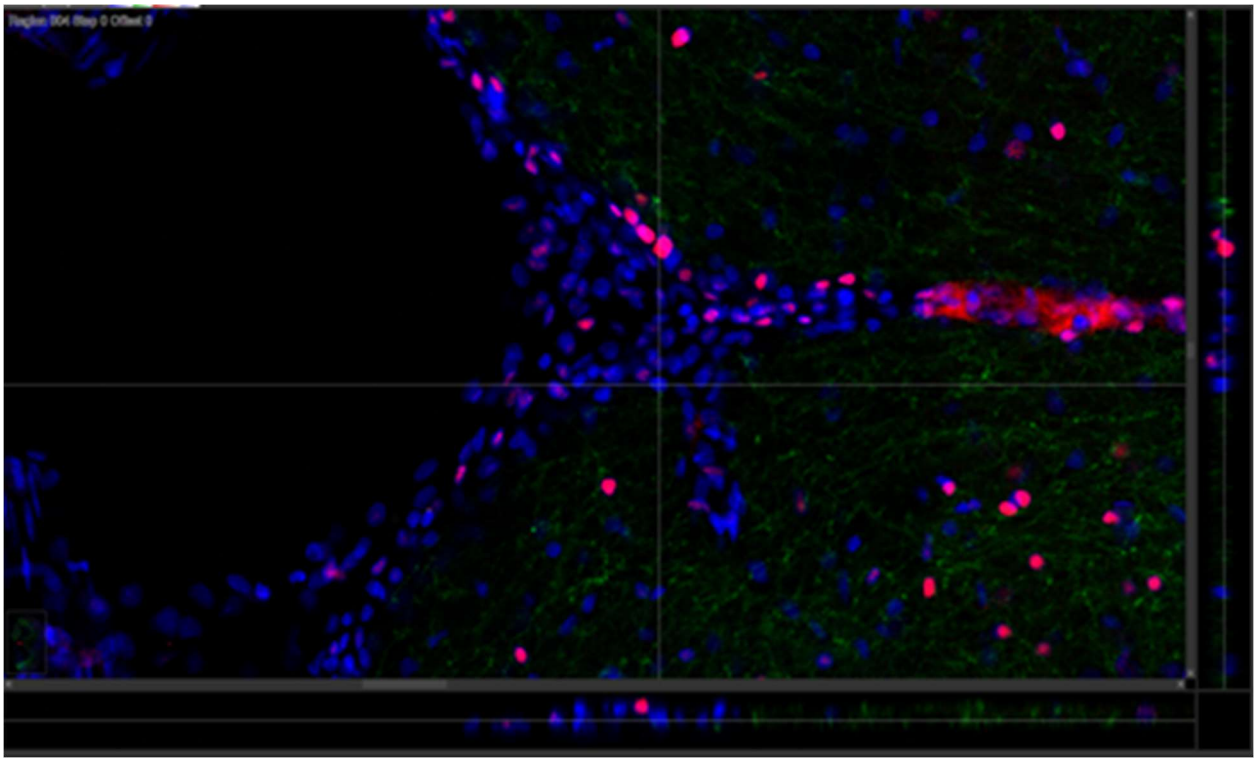
After running the algorithm, the unmixed images will become available in the toolbar as buttons.



4.4.6. Z-Stack Virtual 3D View

Z-Stack virtual 3D view provides a virtual look at the sample along the Y axis on right and the X axis on the bottom (at the selected position).

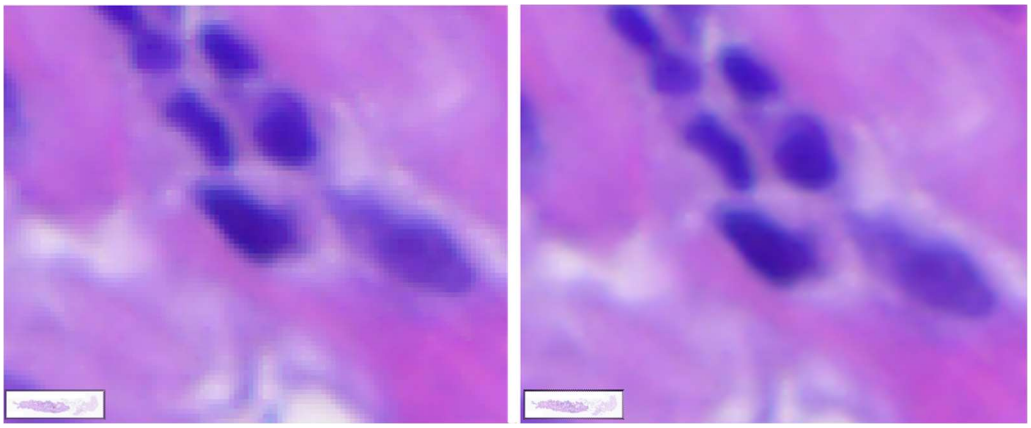
Scrolling through Z stack provides only a look along Z axis.



4.4.7. Smooth Image Effect

Smoothen Image button, found in the main viewer toolbar, is enabled by default in **TissueFAXS**.

It will smoothen the image from the viewer for a better general visualization.

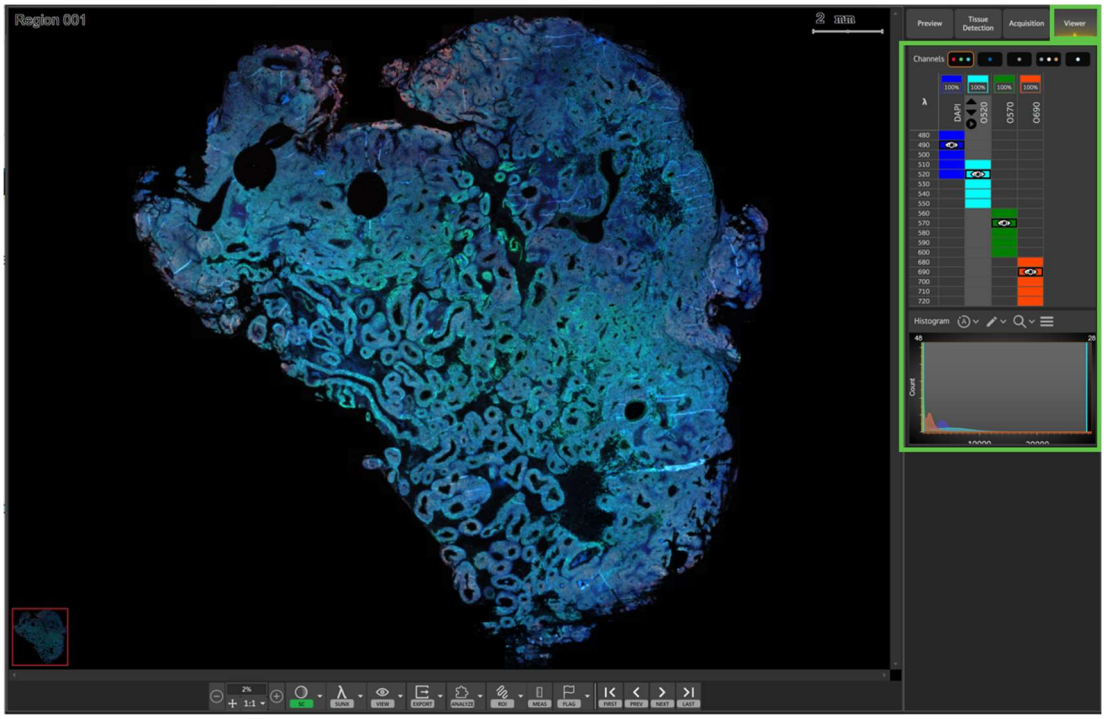


It can be disabled at any time.

4.4.8. Viewer Options

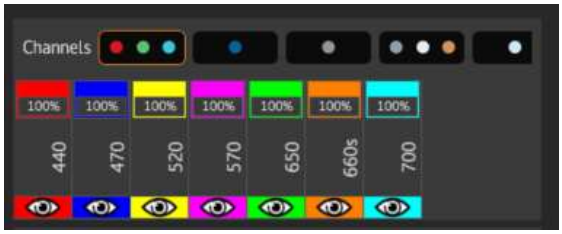
ROI Overlay

This feature allows choosing which channels to view in the acquired image. Here, adjustments can be made to the color, light intensity, and dynamic range (only for channels acquired with 16bit) for each channel.



Channels

To visualize the channels there are more **display types:**



- Show multiple color channels
- Show single color channel
- Show single gray channel
- Show multiple Pseudo IHC channels
- Show single Pseudo IHC channel

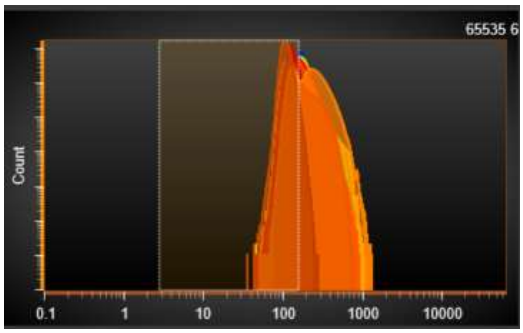
The **View button** () helps navigating the channels and configuring the visualization:

- Press the eye button for each channel you want to visualize on the sample. Once the button is pressed for a channel, that channel becomes visible in the region viewer. For single channel displays, only one button is available.
- You can toggle between channels by using the eye button.

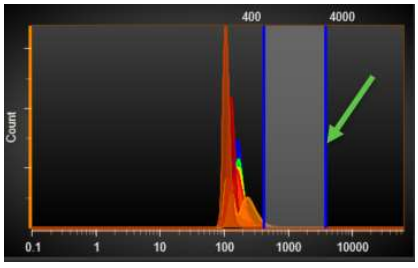
Histogram

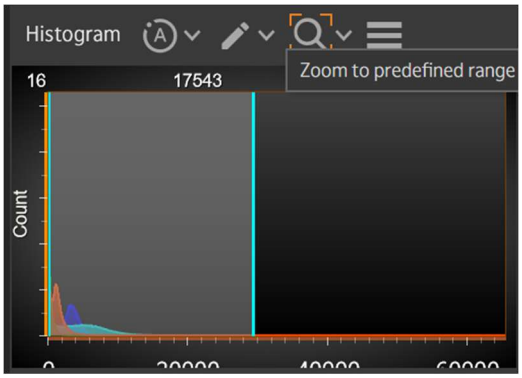
The **histogram** (for FL projects) shows a graphical representation of the displayed channels of the signal. By scrolling with the mouse, you can zoom in or out the histogram. To visualize the histogram at its original fit, double click on it.

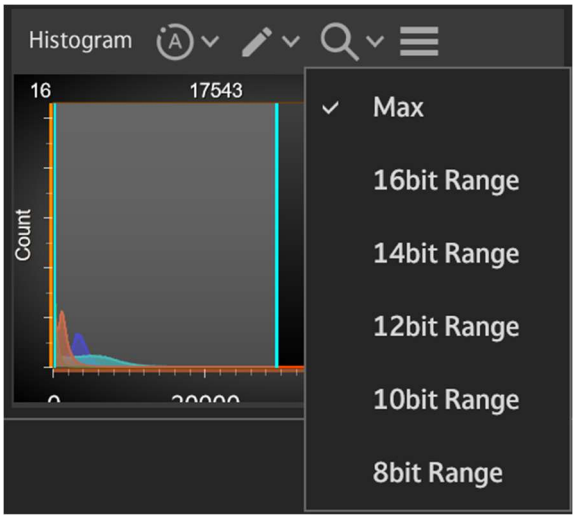
- Double clicking on the histogram will reset the zoom range to the entire range.
- **Auto** and **Default** deal with the 16bit range. They operate on the current visible channels.
- It is possible to adjust grid visibility and logarithmic scales.
- Right click and move the mouse to define a zoom rectangle.

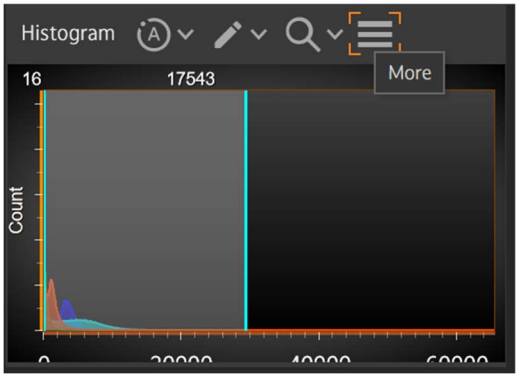


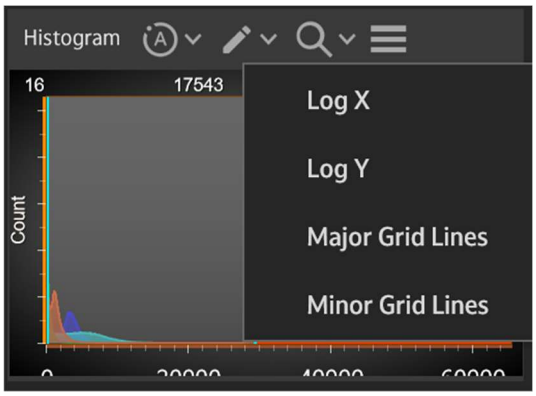
- Selected channel shows the 16bit range that can be adjusted with drag and drop.





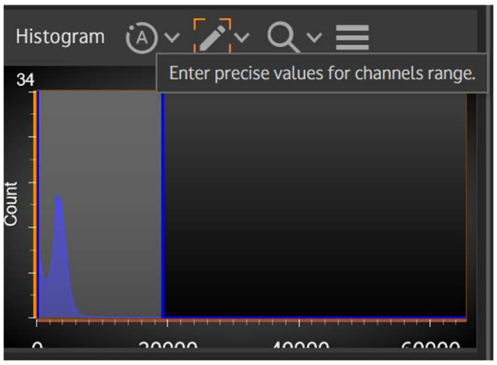


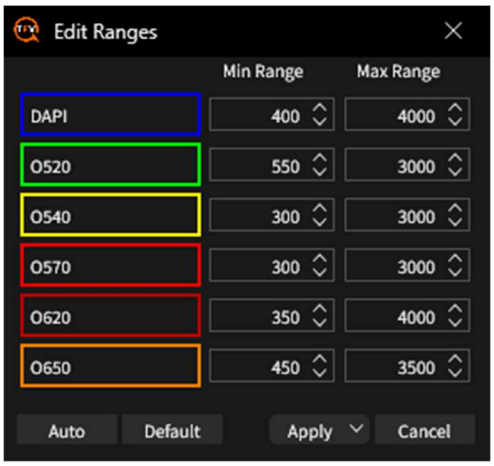




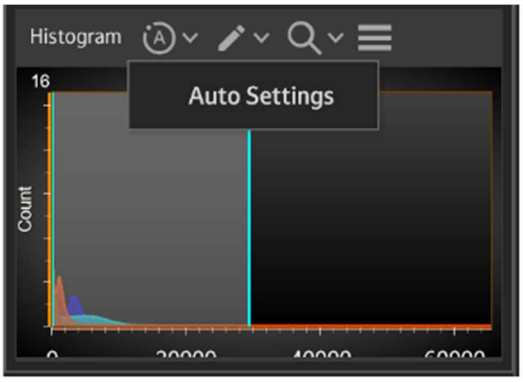
Dynamic Range

The **Dynamic range** can also be set manually, by manually entering values, as shown below.



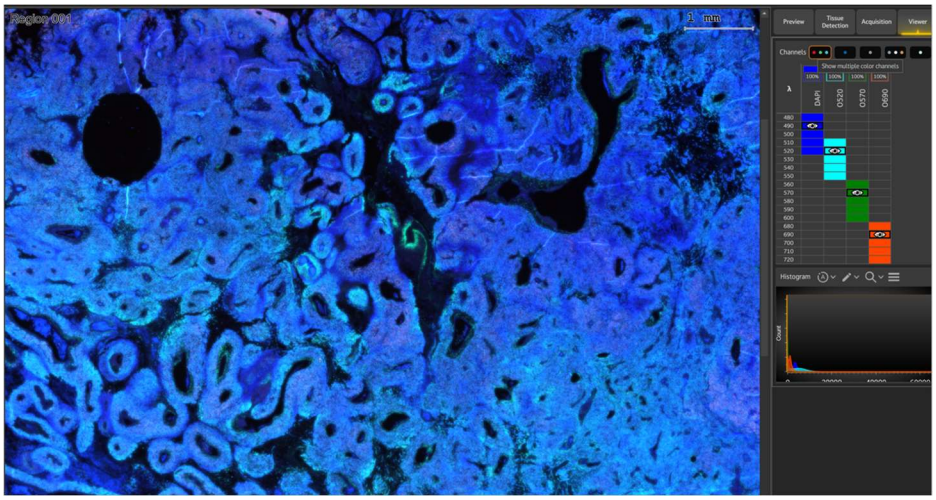


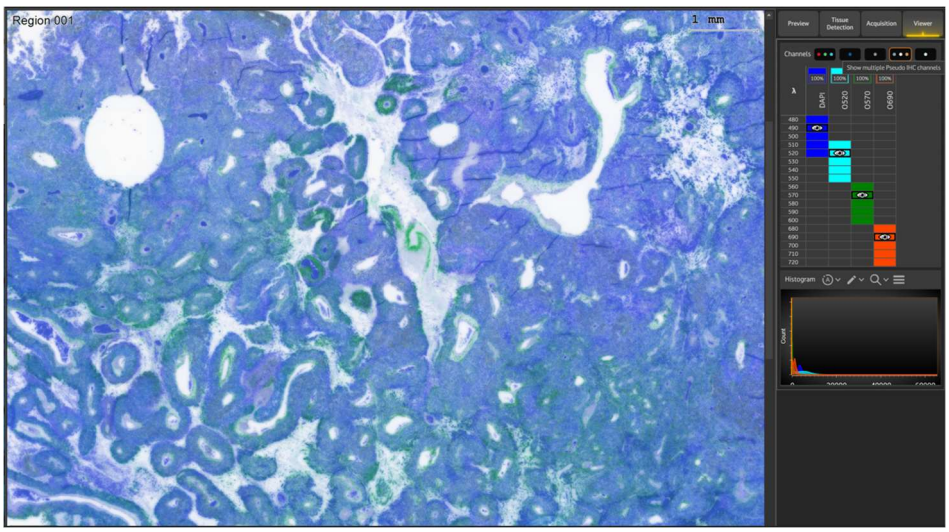
Pressing **Auto** will automatically compute the proper dynamic range settings.



Pseudo IHC Mode

The Pseudo IHC view mode takes a monochrome image and converts it to a 24bpp color IHC-like image. In other words, a user can visualize individual channel fluorescent images as converted in brightfield images. The purpose of this conversion is an easier visual evaluation of morphological details.

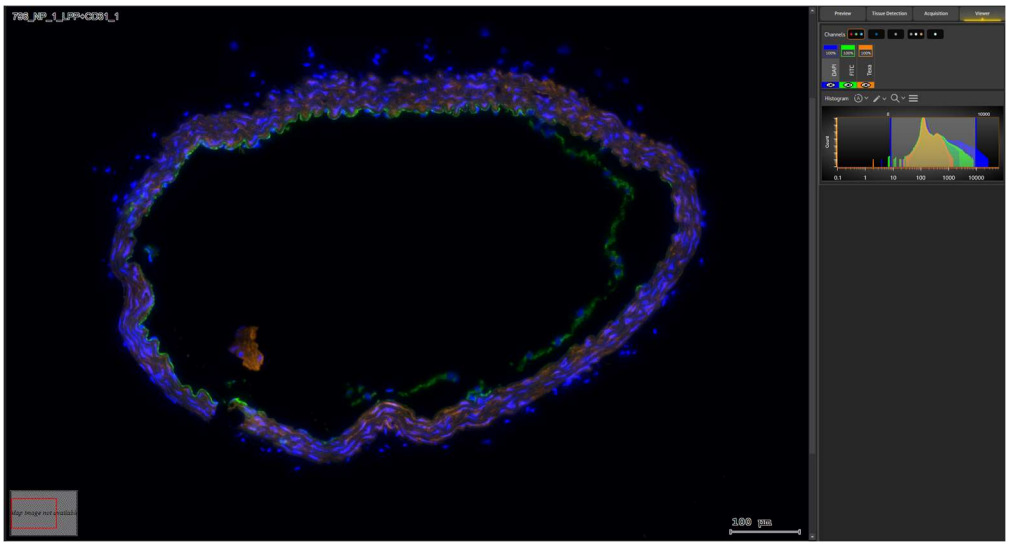


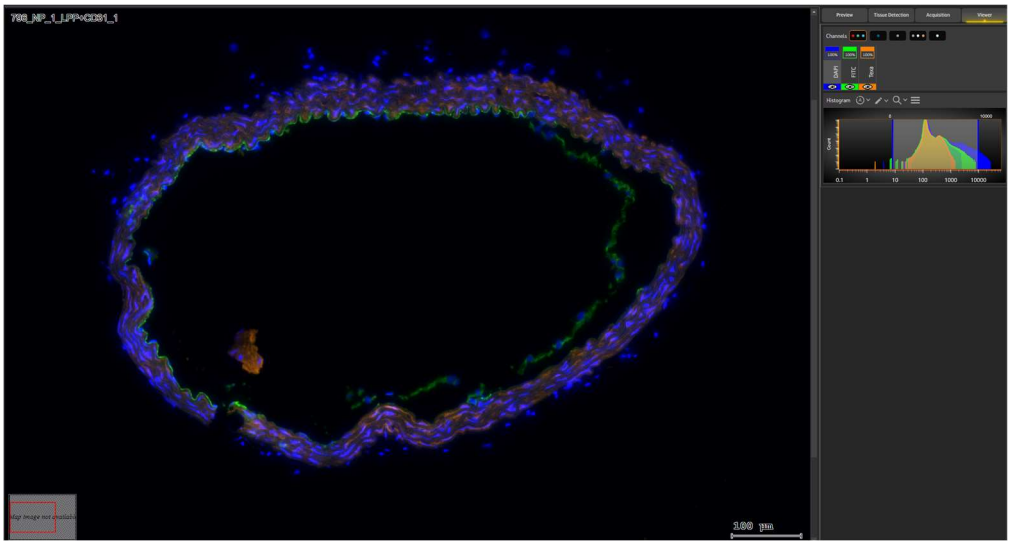


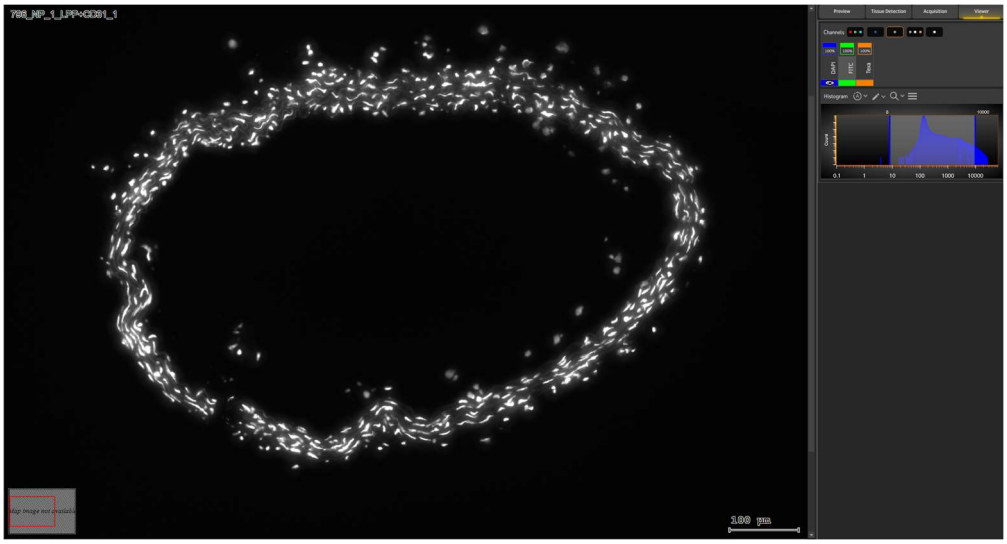
Easy visualization for overlay images

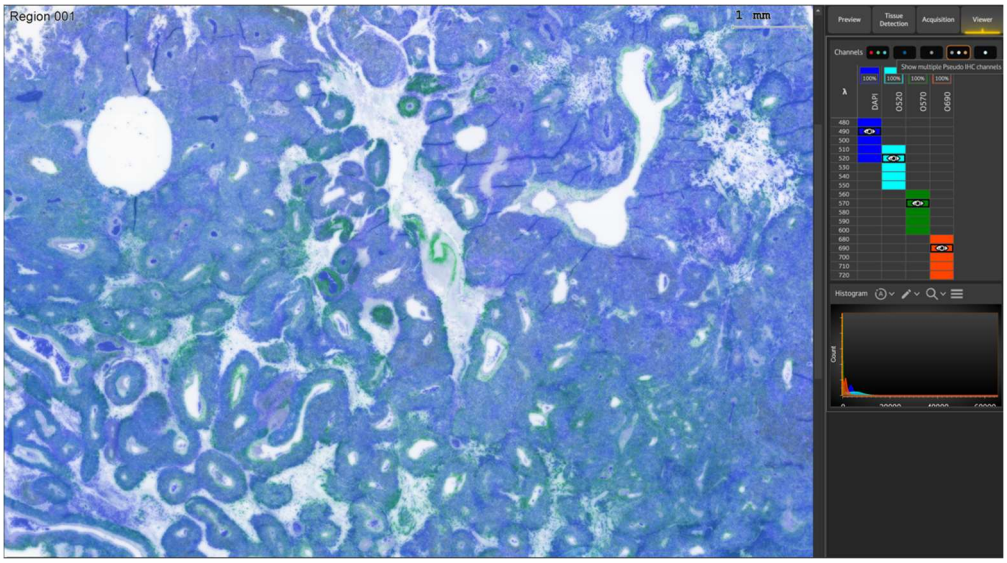
For fluorescence projects, the **Region Viewer** displays visualization buttons (the eye shaped button on each channel) for an easy selection of the channels.

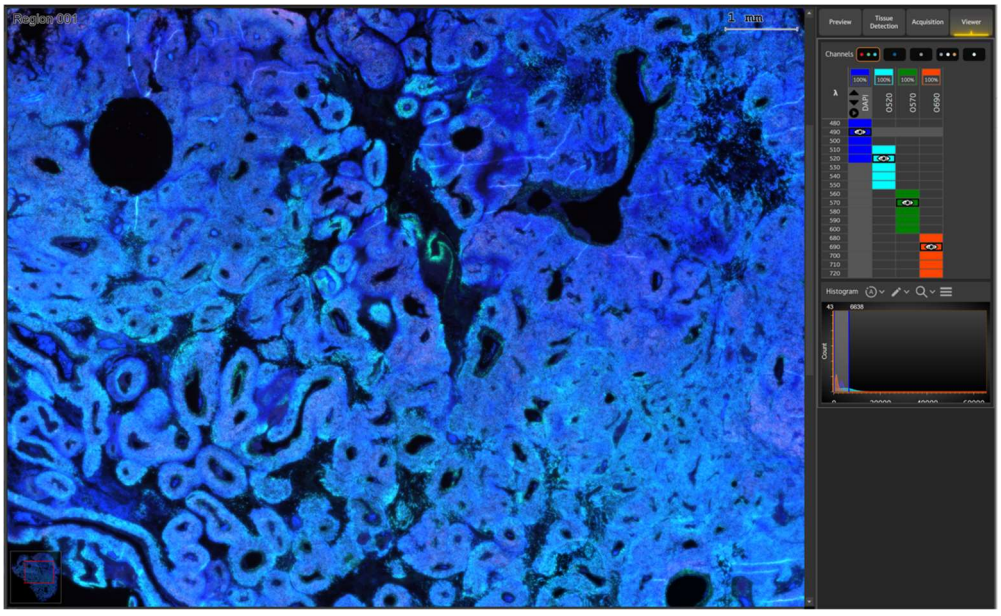
Below, you can see fluorescence and multispectral images. Please notice the differences between the channel selection.

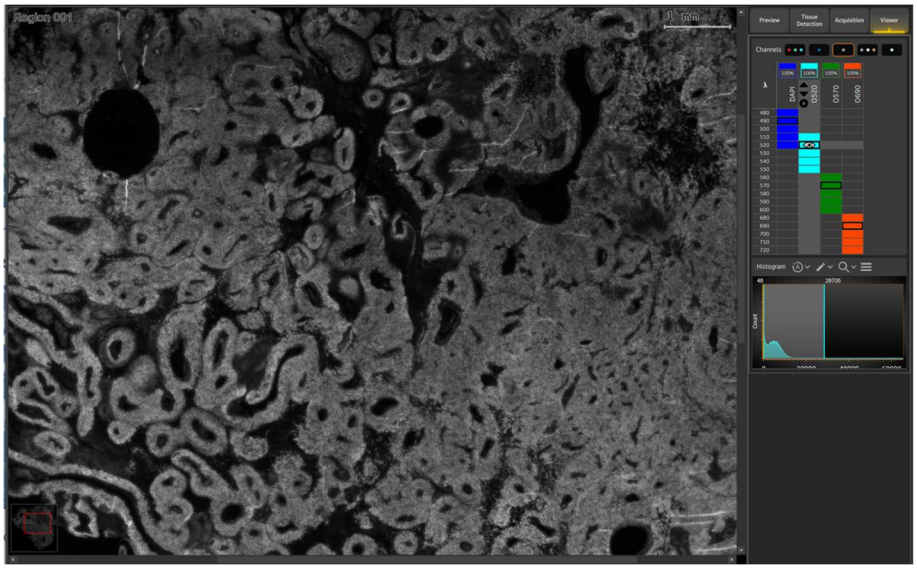





100 µm


Region 001
1 mm


Region 001
1 mm



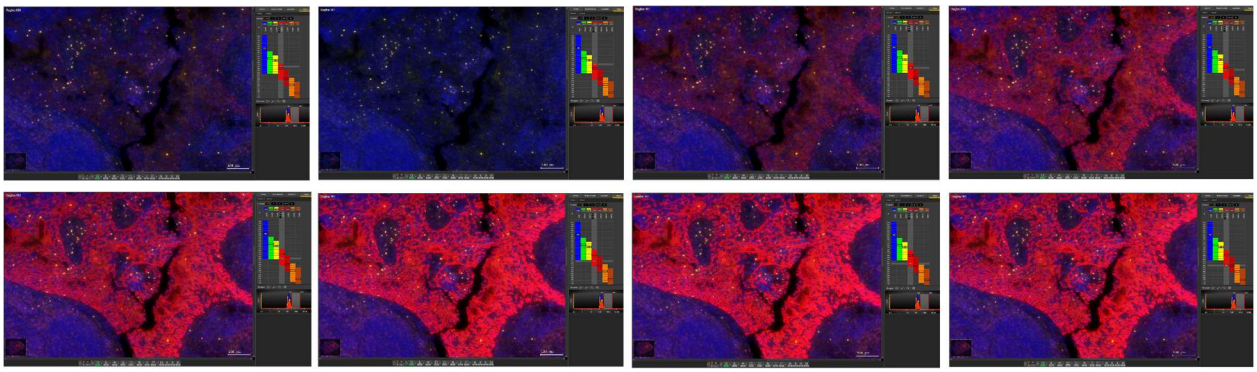
In **multispectral** experiments, acquired images display a specific feature: *wavelength selection channel*.

The **Eye button** controls the wavelength displayed for the selected channel.

Show Peak Wavelength: the image selected with a black rectangle will display the wavelength value giving the best signal for the selected channel.

The image that will be displayed in the **Region Viewer/Acquired Images** is an overlay (if **Color Mode** is selected) between the channels checked in the **Overlay** section for each selected wavelength.

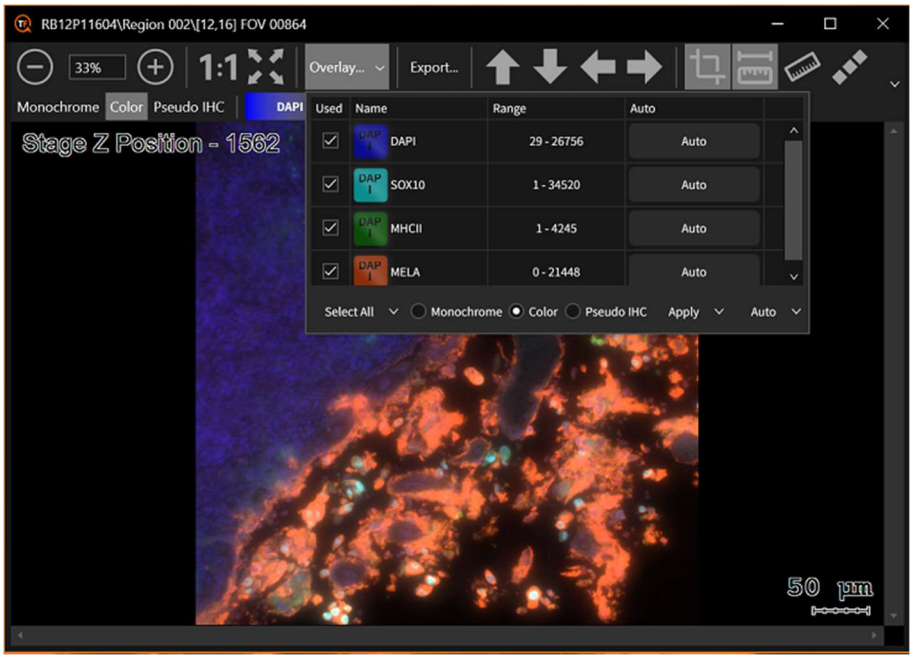
In the example below you can see how the overlay image changes depending on the selected **Lambda** stack image (in this example the step is set to value “10”) for a single reflector.



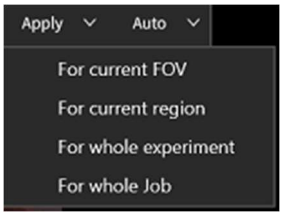
Note: In monochrome mode, only one channel will be selected.

Field of View Overlay

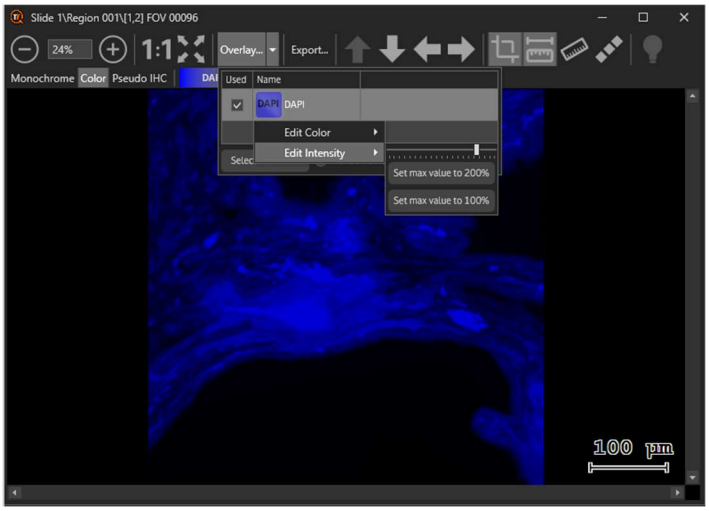
The **field of view (FOV) Overlay** displays a panel for adjusting channels intensities and colors.

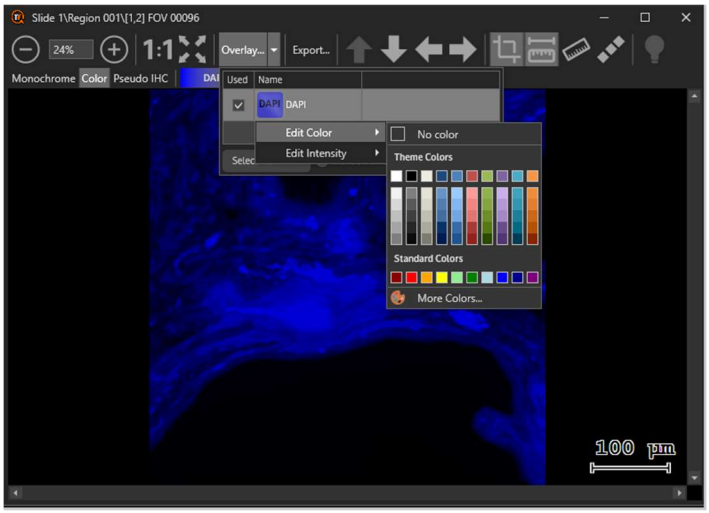


Apply button has multiple options:



The user can also adjust the **intensity** and color for each channel by typing the desired value in the corresponding field.

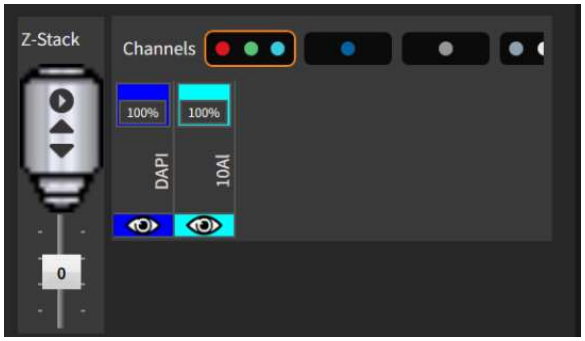




Any modification done in the **Overlay** window can be seen in real time.

Z-Stack display control

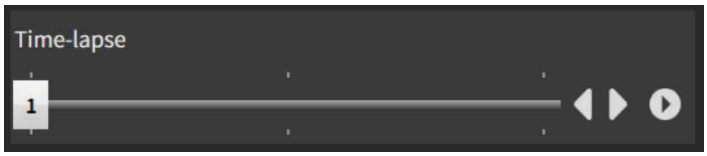
You can scroll through the Z-stack and “play” slices from a Z-stack.



Time Lapse Control

TissueFAXS time lapses are visible as individual time frames. Time lapses are available for both BF and FL projects.

You can scroll through the time points and “play” the images from the recorded time points.

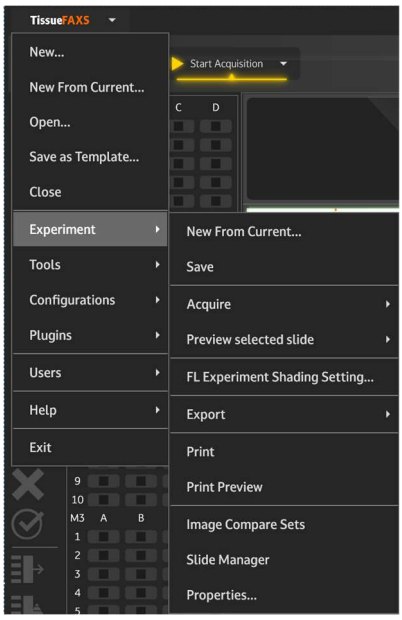


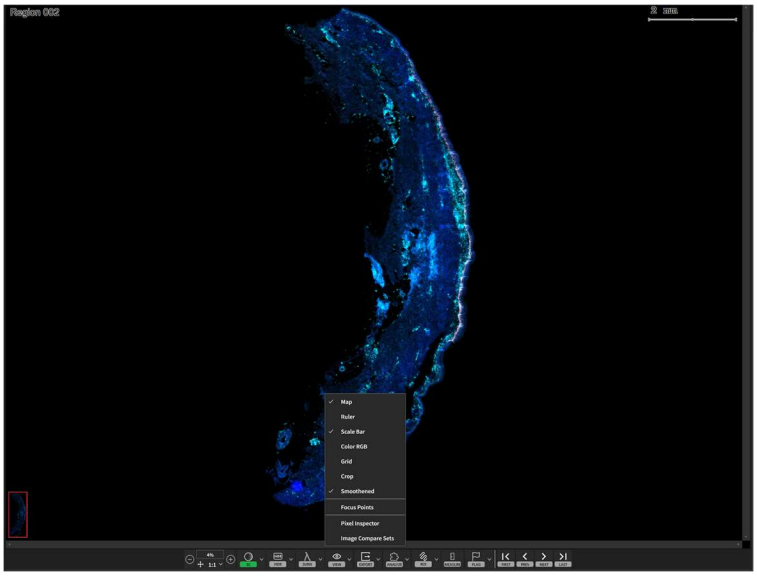
4.4.9. Images Compare Sets

Image Compare Sets is a visualization mode that is used to see two or more similar tissues at the same time. It has a synchronization feature, so that operations like zoom and scroll are performed in parallel.

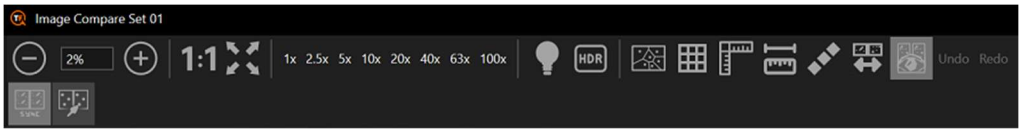
It allows visual side by side comparison of multiple samples, ROIs or groups, through an automatic synchronized display.

This option can be found in **Main Menu -> Experiment -> Image Compare Sets**:

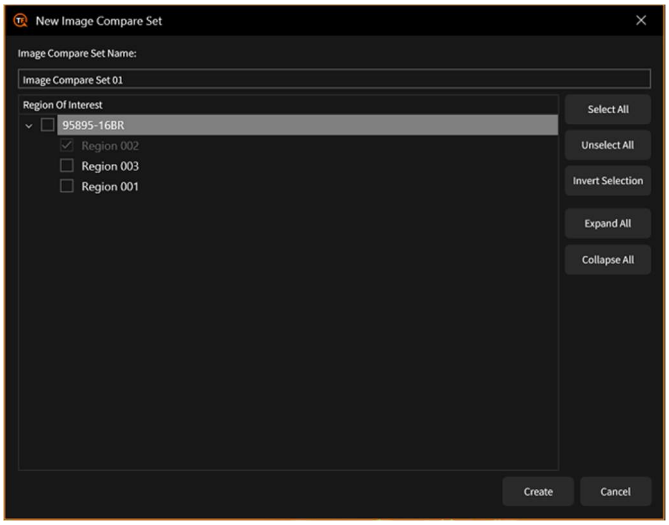


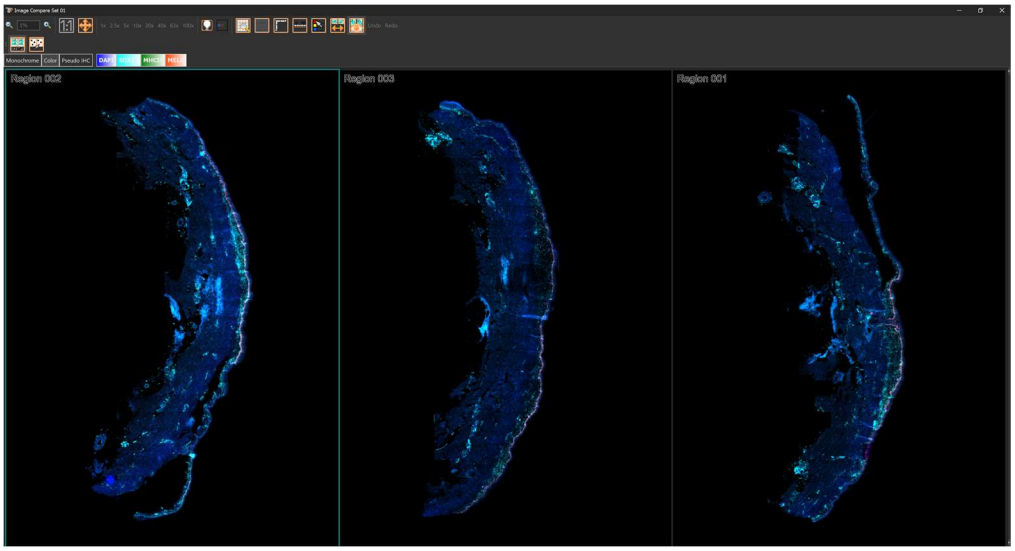


When accessing the **Image Compare Sets** feature from the region viewer, the default reference region is now the currently opened region.



The reference region can be selected in the **New Image Compare Set** dialog. The region is marked in gray in the input regions list.



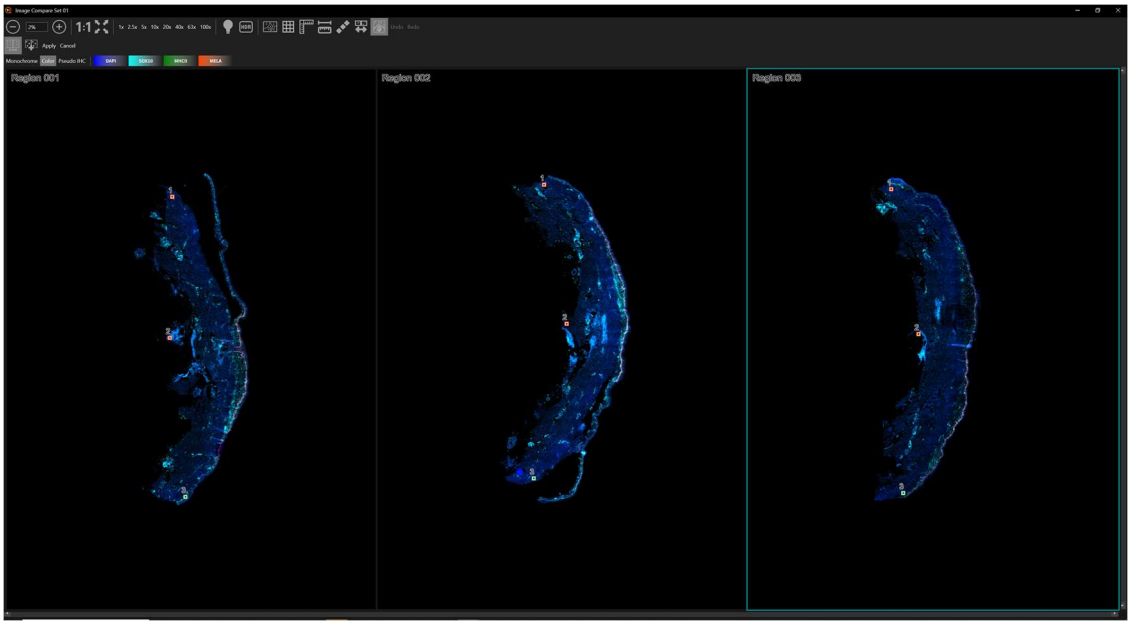


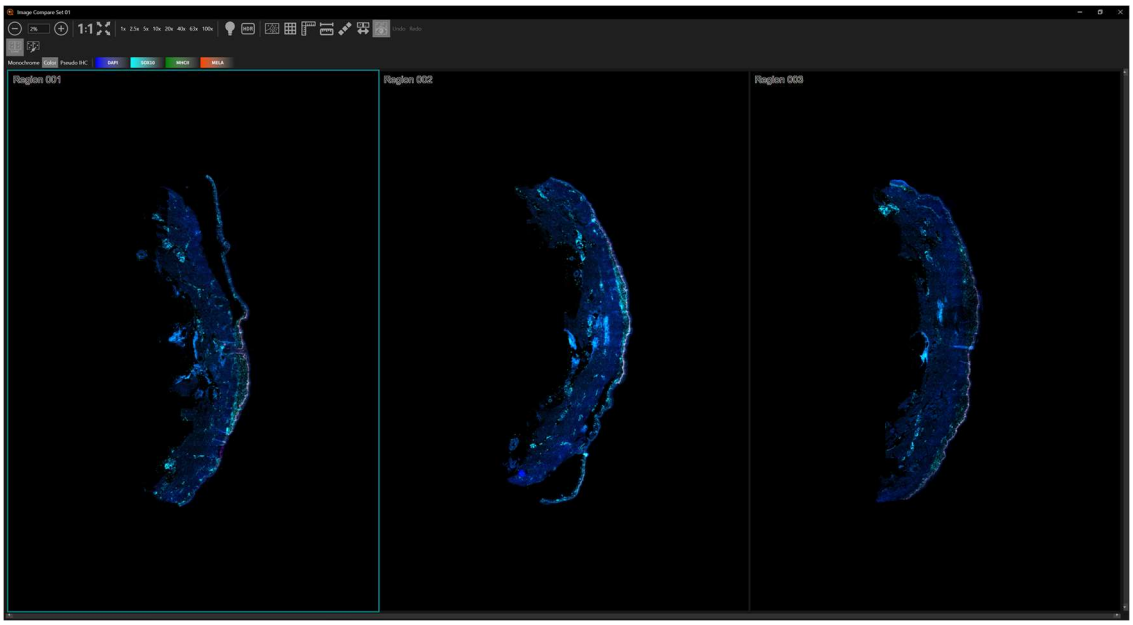
- **Maximize on both monitors**: simulates maximizing a window on both monitors.
- **Show registration toolbar**: shows/hides a toolbar used to synchronize multiple similar tissues.
- **Synchronize samples**: enables/disables the synchronization mode:

When the button is pressed, the synchronize mode is activated and all the items in the viewer share a common scrollbar.

- **Adjust registration points**: similar tissue synchronization is realized based on a set of corresponding points from the tissues. The option allows effectively editing these points.

After pressing the button, **Apply** and **Cancel** options will appear in the toolbar. Also, three default registration points will appear on the samples.





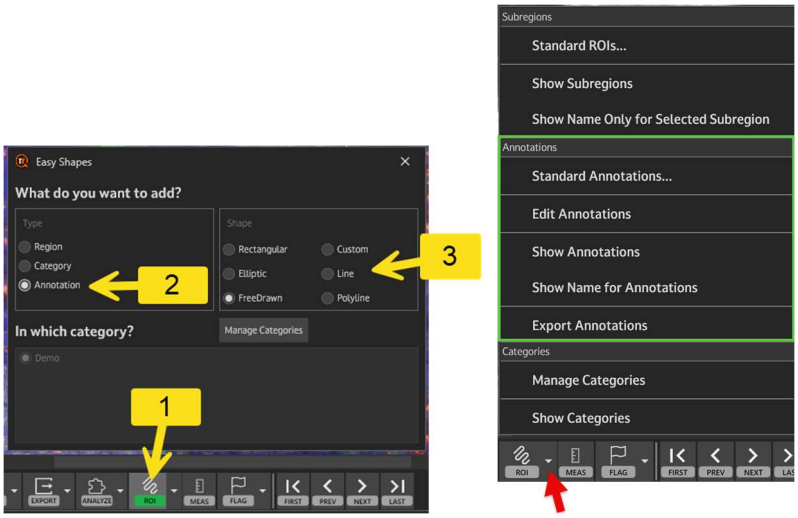
4.4.10. Annotations

An annotation is a tissue section that can be defined within a region. The annotation cannot be acquired, but it has a set of metadata (notes, area, etc.).

Create Annotations

To create an annotation, press **ROI** button from **Region Viewer Toolbar**, then, in the **Easy Shapes** panel, select **Annotation**.

Then, select a shape for the annotation you will draw.



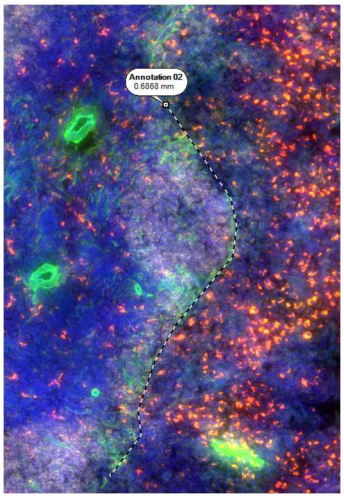
Now, add desired shape on the viewer using the mouse, as with any normal region (see **Chapter** Generic ROIs).

To be able to see all added annotations, check the **Show Annotation** option from the **ROI** button menu.

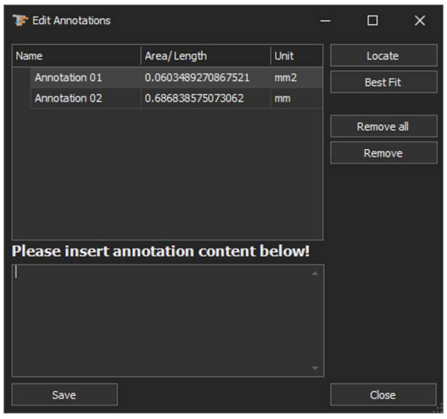
PolyLine Measurements

This feature allows drawing a line shape in order to measure a certain area.

Press the **PolyLine** button and draw the desired shape on the sample.



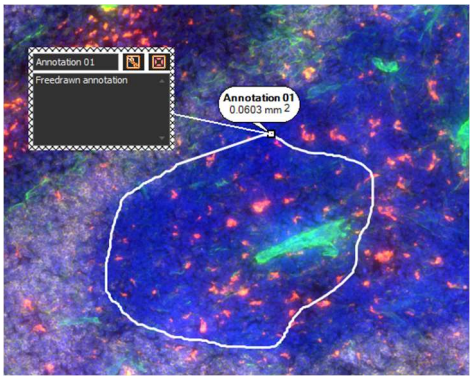
Manage Annotations



- **Locate**: selected annotation will be located on the sample and displayed at the current size of the sample.
- **Best Fit:** selected annotation will be located on the sample and displayed at the current size of the region viewer.
- **Remove**: removes selected annotation.
- **Remove all**: removes all existing annotations.
- **Please insert annotation content below**: write some info for the respective annotation.

Edit annotation directly on the sample

An annotation can also be edited directly on the image viewer: it can be renamed, its content can be changed, or it can be removed. To edit an annotation, press the little black framed square on the contour of the annotation. When pressed, this little square displays an edit box, which enables the following actions:



- Edit the name of the annotation: write the desired name in the upper-left corner of the box and press the **Enter** key to save the changes;
- Write a comment in the space below the name. Existing comments can also be erased (changes to the notes are saved automatically);
- Delete the annotation by pressing the button;
- Close the edit box by pressing the button.

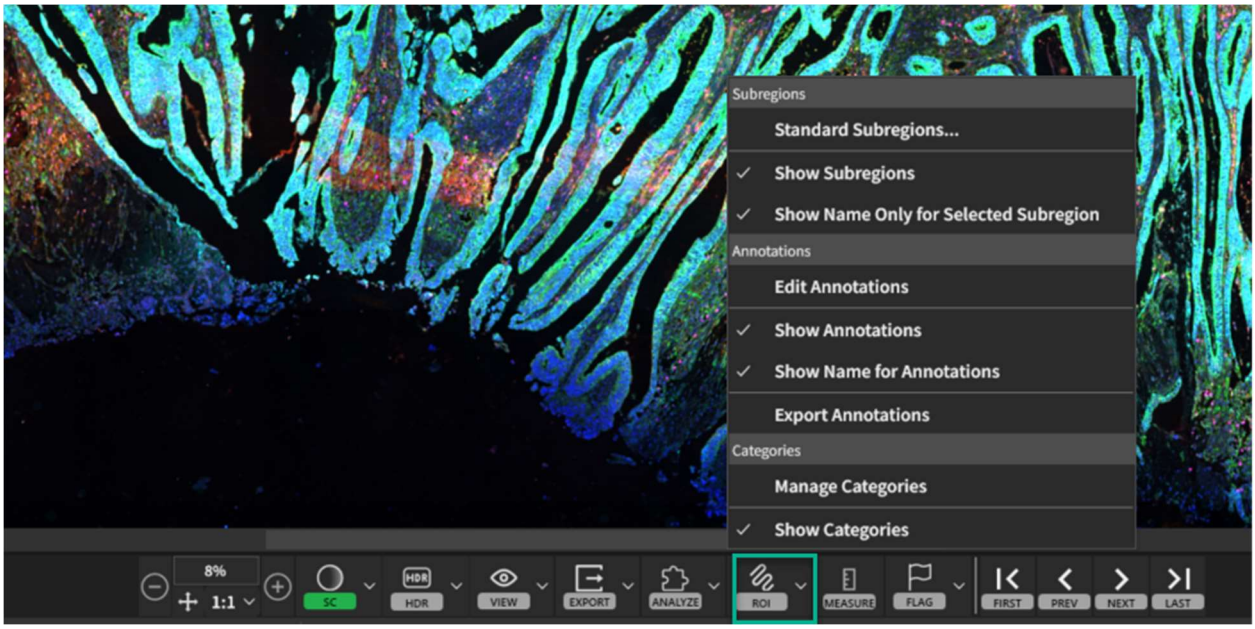
Export Annotations (please see **Chapter Export and Printing**).

4.4.11. Subregions

Subregions

Subregions are normal regions defined within the Region Viewer, having the same properties as the regions from the Slide Preview.
They can be seen in the experiment editor and can be acquired just like normal regions. The advantage of the subregions is to ensure higher flexibility and more thorough inspection of a more detailed area of tissue.

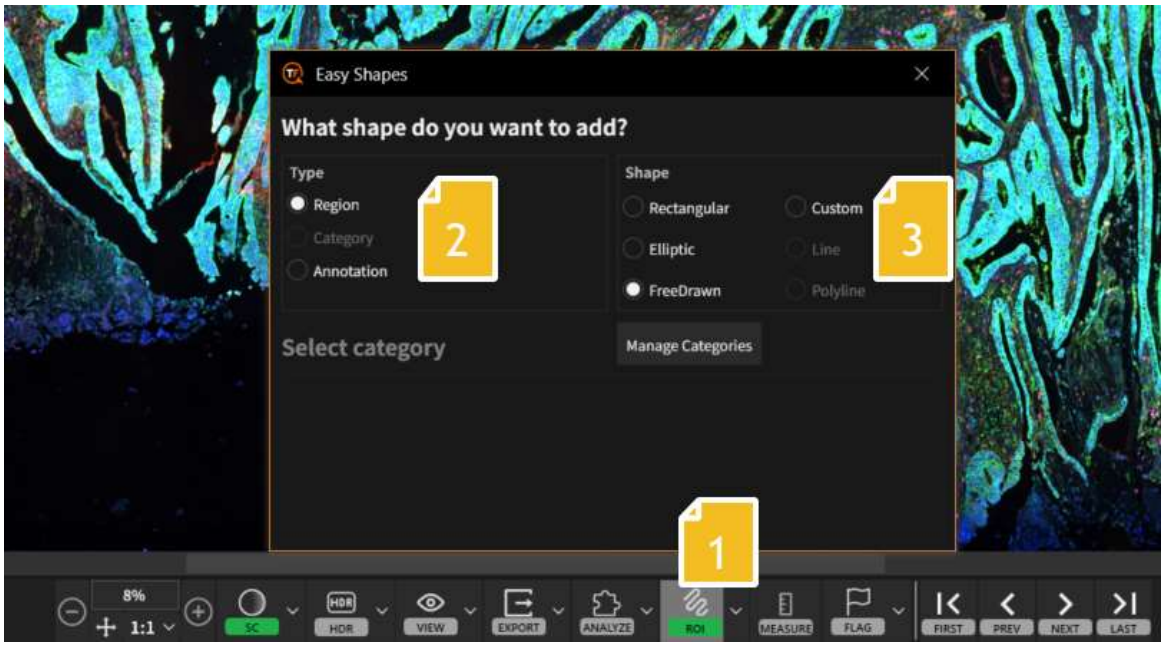
Another type of use for this feature would be that, on a 20x image, to be able to identify relevant sections to be scanned with different settings: higher magnification, Z-stack, other channels etc.



To add subregions, go to **Region Viewer Toolbar → ROI → Standard Subregions → Easy Shapes.**

In the **Easy Shapes** panel, select **Region**.

Then, select a shape for the region you will draw.



Now, add desired shape on the viewer using the mouse, as with any normal region (see **Chapter Generic ROIs**).

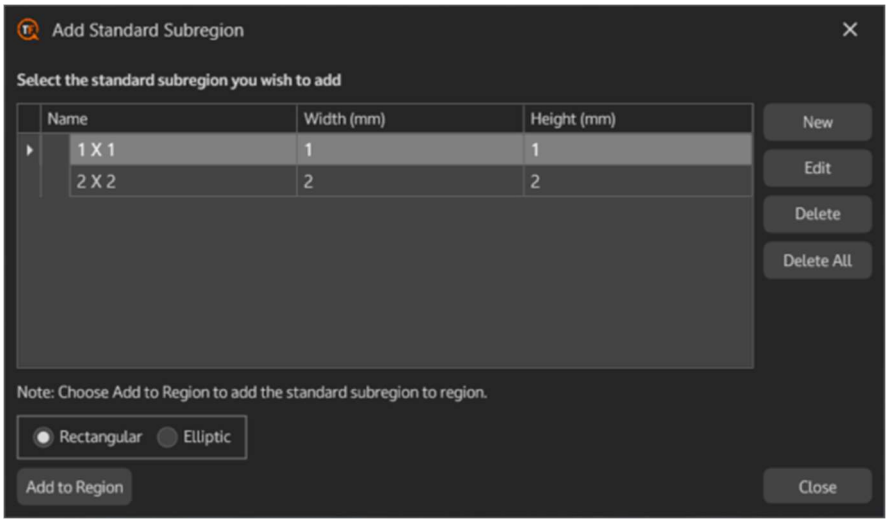
To be able to see all added regions, check the **Show Subregions** option from the **ROI** button menu.

Note: For Multispectral regions, this option from the contextual menu is called **Show Multispectral Regions**.

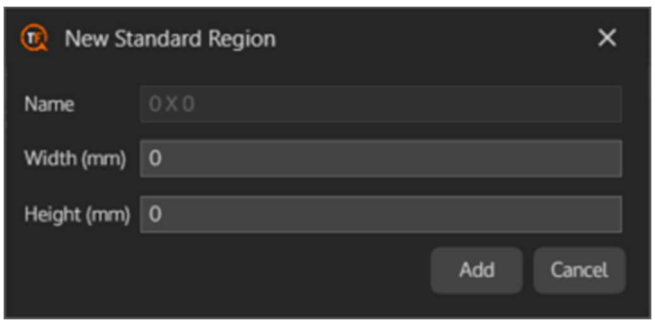
Standard Subregions

Standard Subregions allows creating identical subregions with predefined size and shape (for example rectangular regions with 1x1 mm).

To create standard subregions, go to **Region Viewer Toolbar → ROI → Standard Subregions → Easy Shapes**. **Add Standard Subregion** dialog will open.



First, choose the desired type of shape: rectangular or elliptic.



Next, press **New** button to set the height and the width of the new ROI, then press **Add** in order to include the new subregion in the list.

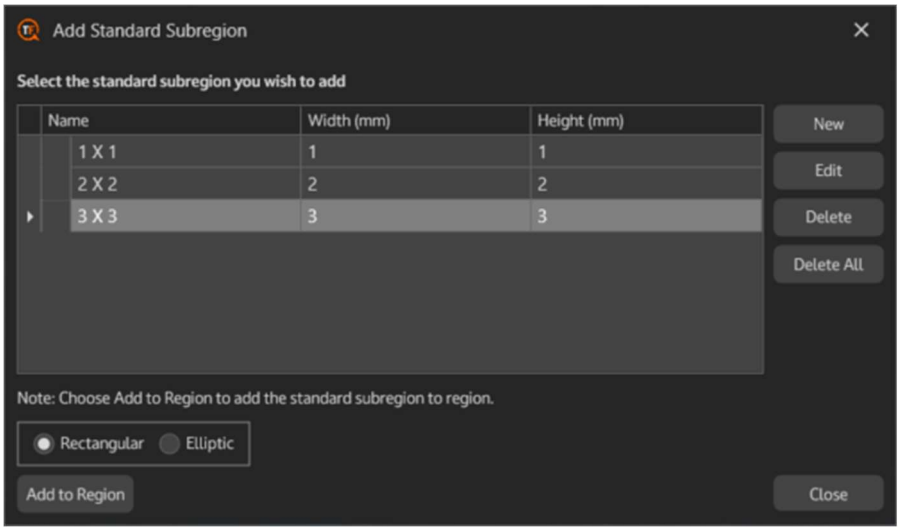
Notes:

- For the elliptic regions, the values from the Width and Height fields refer to the diameters of the ellipse (radius value x 2).

- The last used standard region is always remembered. It is selected automatically when opening the **Add Standard ROI** dialog.

To start adding the standard subregion on the region, press **Add to Region** button. Once clicked, the shape of the subregion (rectangle or ellipse) follows the mouse, to help center the region on the desired area.

The name of the standard subregions will include its size.



- **Edit** - modifies the properties of a ROI from the list.
- **Delete** - deletes a selected ROI from the list.
- **Delete All** - deletes all ROIs from the list.

4.4.12. Categories

Sometimes, you may want to emphasize certain small areas on a region that could contain high-interest research information. These areas can be exported for analysis or they can be used as a highlight tool.

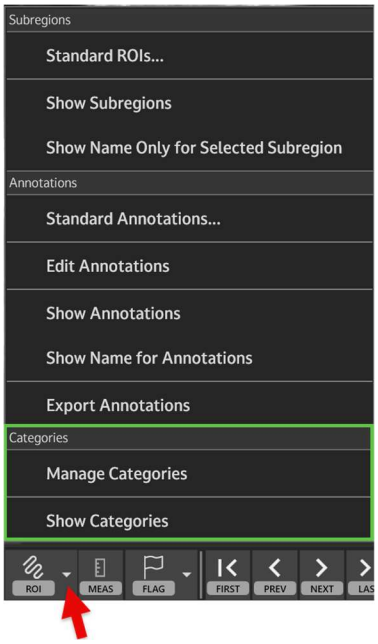
For instance, a tissue may contain both tumor areas and normal adjacent tissue (non-tumor) areas. For each type of area (i.e., tumor and non-tumor), a **category** can be created to highlight that particular area on the image.

Using **categories** can assist in performing a more precise analysis.

The advantage of categories is to ensure higher flexibility and more thorough analysis of a specific area of tissue.

Add Categories

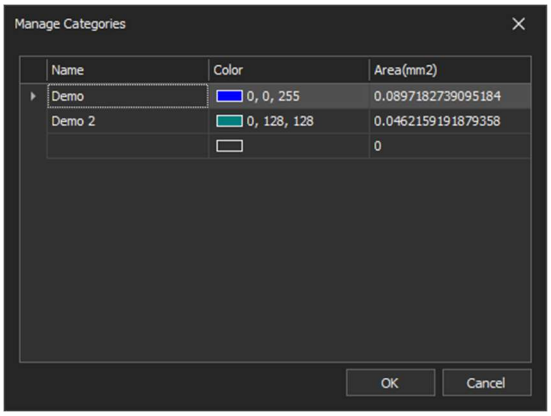
To add categories, go to **Region Viewer Toolbar** → **ROI** → **Easy Shapes** → **Manage Categories…** or **Region Viewer Toolbar → ROI → Manage Categories…** A dialog will appear (containing any previously added categories), where new categories can be added. Each one requires a name and an assigned a color. The list of categories is per project, not per region.



To draw categories, press **ROI** button from **Region Viewer Toolbar**, then, in the **Easy Shapes** panel, select **Category**.

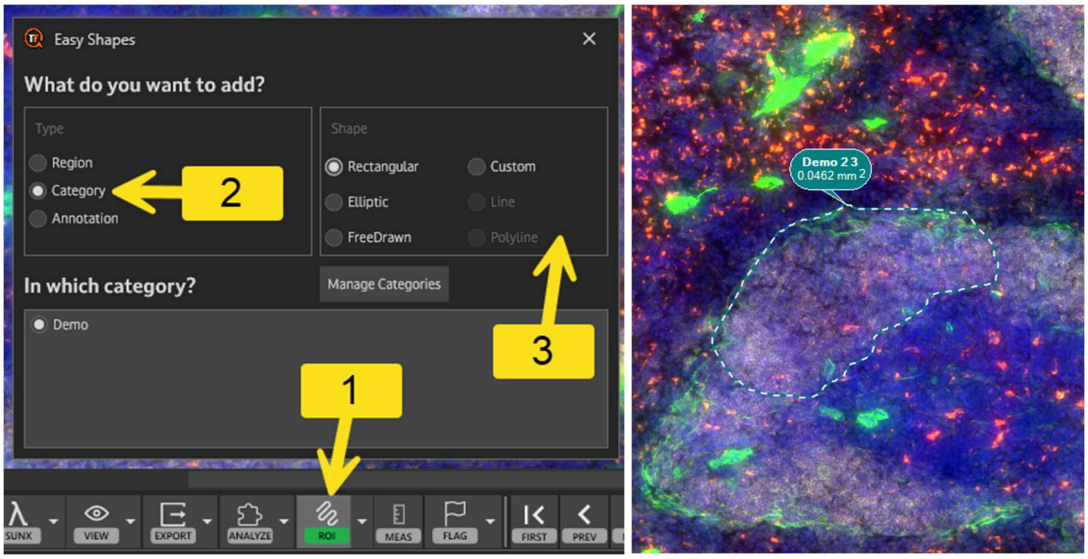
Manage Categories button and the category list will be enabled.

At this point, you can add categories, remove or edit them.



From existing list, select a category where the new shapes will be added.

Then, choose a shape to be included in the category.



Now, add desired shape on the viewer using the mouse, as with any normal region (to learn how to draw regions, see Chapter Generic ROIs).

To be able to see all added categories, check the **Show Categories** option from the **ROI** button menu.

To **remove** categories: go to **Region Viewer Toolbar → ROI→ Easy Shapes→ Manage Categories…,** select the category to be removed, then press the **Delete** key on the keyboard.

Export: Categories can also be used when exporting images. See **Chapter** Export and Printing for further details.

Transforming regions from category shapes into subregions for reacquisition

If you need to reacquire the tissue area from a category, it is possible to transform it into a **Subregion** and then perform reacquisition.

In the region viewer, right click on the border of a category and from the contextual menu choose the **Transform into Subregion** option. The category will transform into a **Subregion**, disappear from the region viewer, and be visible in the experiment editor (so it can be reacquired).

Notes:

- When exporting the **Region Overview**, there is an option to also export the categories.
- When exporting the **FOV images, there is an option to only export images belonging to the respective category.**

4.5. Export and Printing

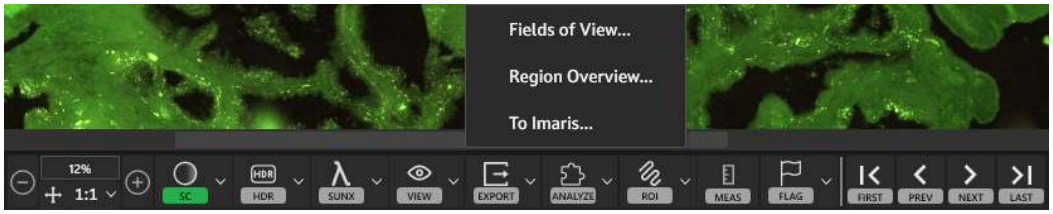
4.5.1. Export Options

TissueFAXS allows **exporting** acquired images as a complete region overlay or image by image.

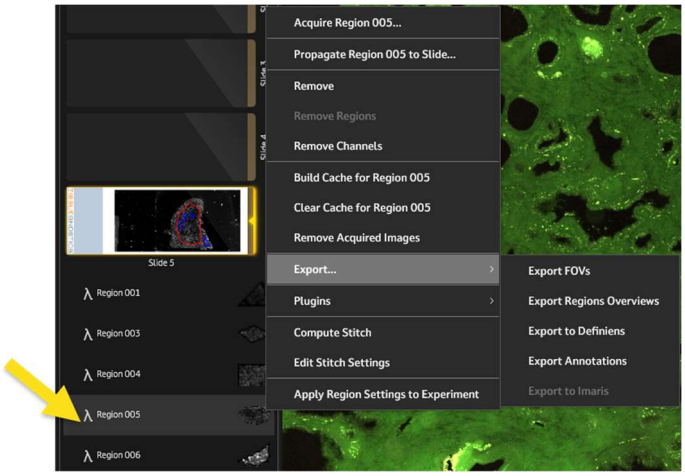
In certain cases, *csv* files can also be exported.

Export options are found in more places, for an easier access:

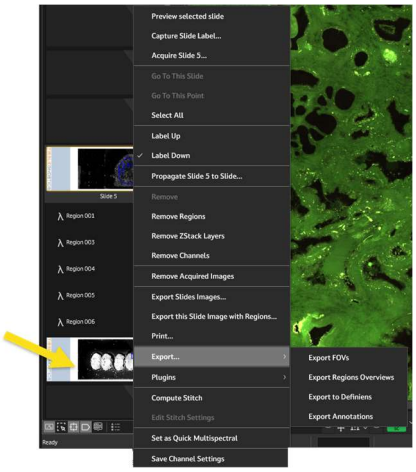
- Image viewer toolbar, by pressing **Export** button.



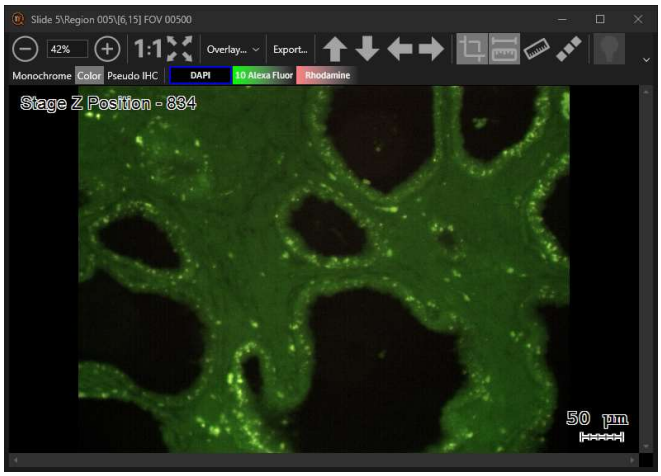
- Contextual menu of a region



- Contextual menu of a slide



- Field of View contextual menu



4.5.1.1. Export Region Overview

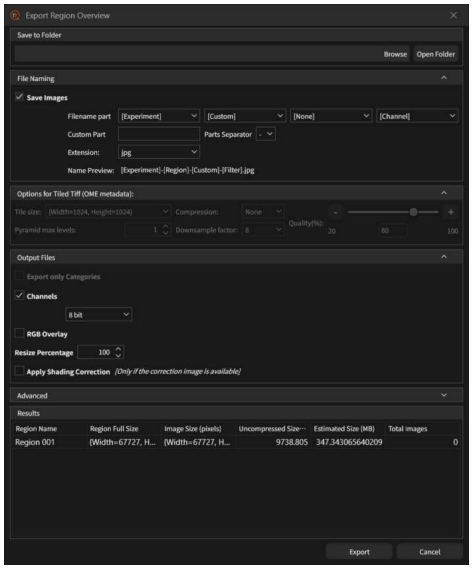
This option allows exporting an entire scanned region as a stitched global image. More image formats are available for export.

To export just parts of the region, use categories feature to mark the areas to be exported and choose the Export only overviews from categories option.

It can be accessed from**:**

- **TissueFAXS main menu → Experiment → Export → Export Region Overviews** for entire experiment;
- **Region Viewer/Acquired Images → Export → Region Overview** for current region;
- **Slide Browser → Slide or Region Contextual Menu** for selected items;
- **Export Job button**

The following dialog will appear:



Save to folder

- Pick a folder to save export results. It is also possible to open that folder to see its contents.

File naming

- Select how the files to be exported are going to be named.
- Some of the parts are to be chosen from the dropdowns, some of them are customizable.

- You also have to select the parts separator character and the extension type. *Tiled Tiff (OME metadata)* is available for exporting larger images

Options for Tiled Tiff

If choosing *Tiled Tiff* as the extension, the items from the **Options for Tiled Tiff** (OME metadata) will be enabled:

- **Tile size**: is the dimension of an image (tile) composing the tiff (there are three predefined options);
- **Compression**: there are two options – **None** and ***jpeg;***
- **Pyramid max levels;**
- **Quality**: the quality of the compression can be adjusted using the slider.

Tiled Tiff containing **OME-TIFF** (OME stands for Open Microscopy Environment) metadata embedded in exported TIFF images provide the following details:

- Objective information
- Acquisition type
- Pixel size
- Channel information
- Z-stack information

Notes:

Restrictions regarding the images to be exported:

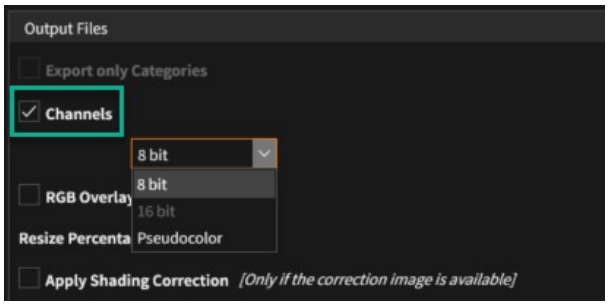
- For all export formats (excluding Tiled Tiff) the image size must not exceed 80000000 pixels.

- For Tiled Tiff (OME metadata) the uncompressed size must not exceed 2000 Mb.

Output Files

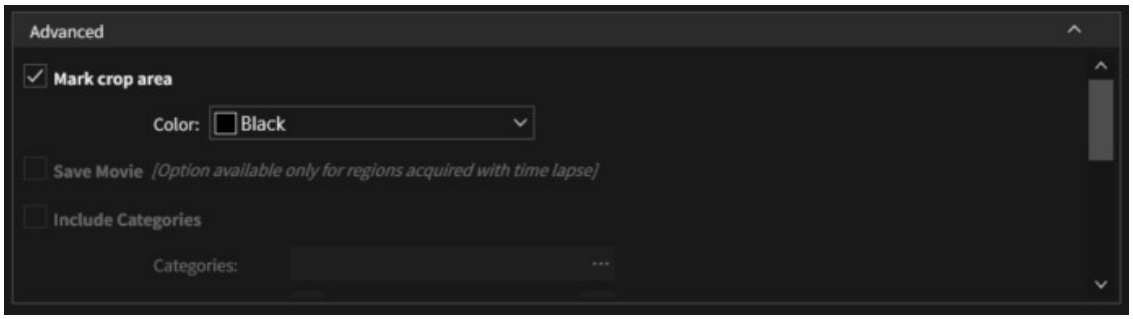
The following options are available for multi-channel experiments like fluorescence or multi-spectral experiments:

- **Channels**: include the images for each channel as acquired.
- **Export 8-bit, 16bit or 8-bit pseudocolor**



- **Export channels as single Tiff files**: when Export Region Channels is checked, all channels will be exported within the same Tiff as Tiff pages.
- **RGB Overlay**: the super-imposed image of all channels is exported into an RGB color image.
- **Resize Percentage**
- **Apply Shading Correction** (only if the correction image is available).

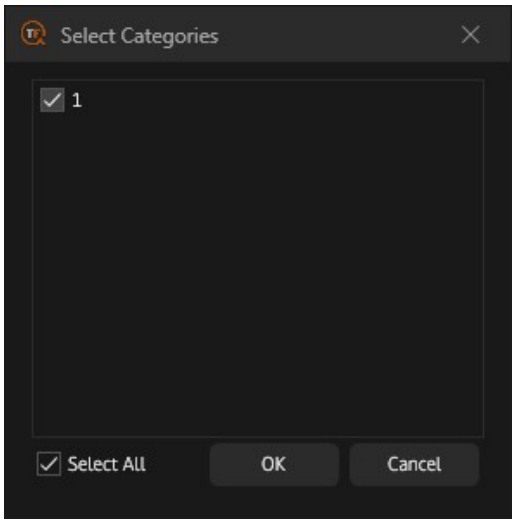
Advanced



- **Mark crop area**: the color of the region crop area on the exported images is customizable;
- **Save movie**: only for regions acquired with time-lapse;
- **Include categories**: will include categories (if any) in the exported images. The opacity of the categories is customizable.
- **Scale Bar**: will include the scale bar in the exported images; you can choose it color and its location;
- **Dots per Inch**: set a resolution value for the exported images.

Export Overviews from Categories

To export categories, first check **Export only overviews from categories**. Press the (**...**) button to select the types of categories that will be exported. The following dialog will appear, listing the available categories:



The lower part of the **Export Region Overview** dialog will now show a section that includes all the overviews that will be exported. The name and image size will be displayed for each overview.

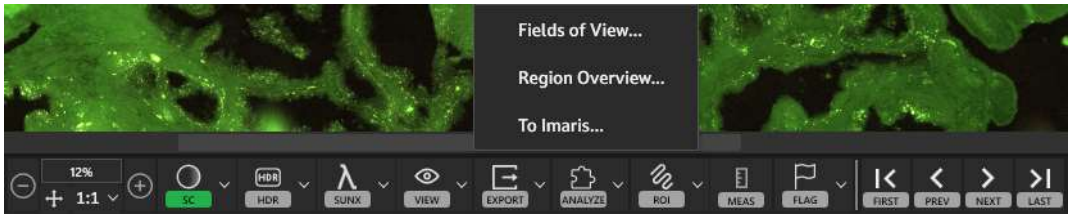
Results

The **Results** section lists the regions to be exported and their details: name, size, number of images.

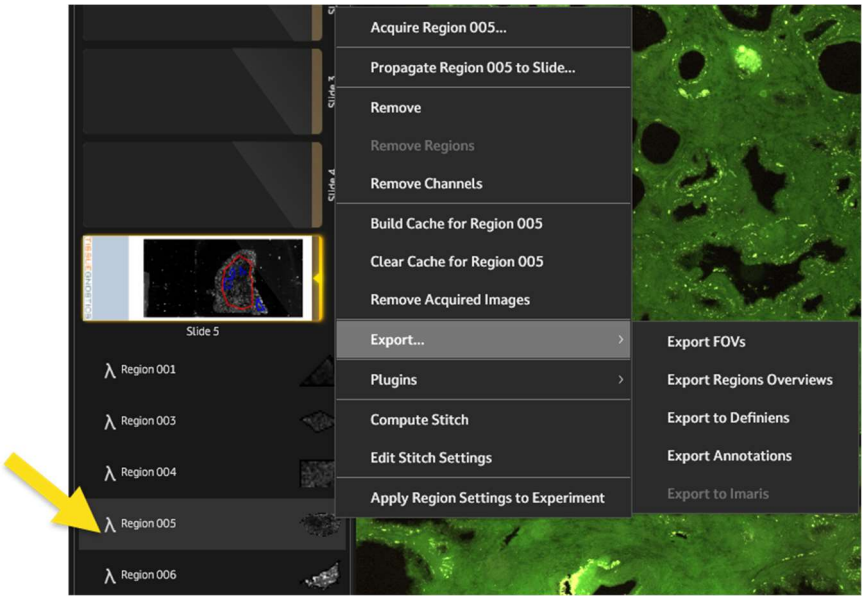
4.5.1.2. Export FOVs Images

This option can be accessed in two ways:

- **Region Viewer/Acquired Images → Export → Fields of View**;



- **Experiment Editor → Region Context Menu**.

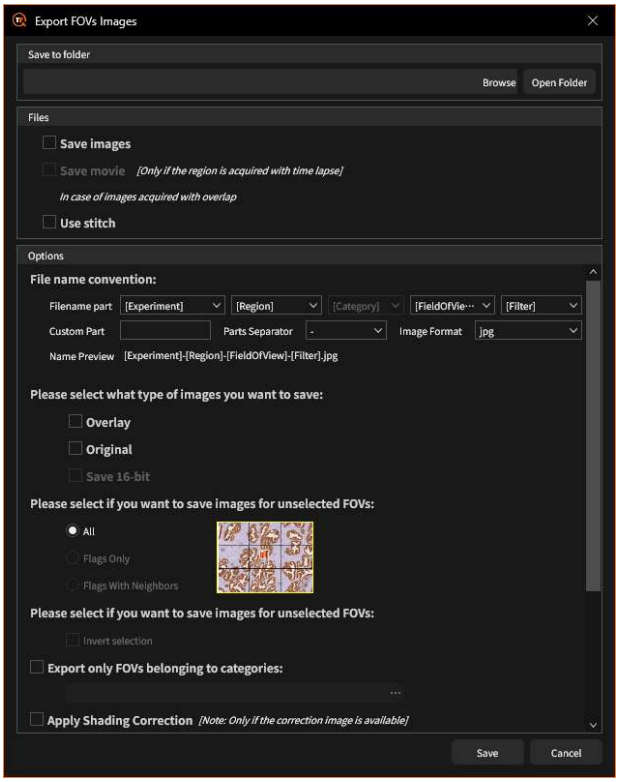


Notes:

- Export more regions or FOVs simultaneously by selecting multiple items from the Experiment Editor tree.

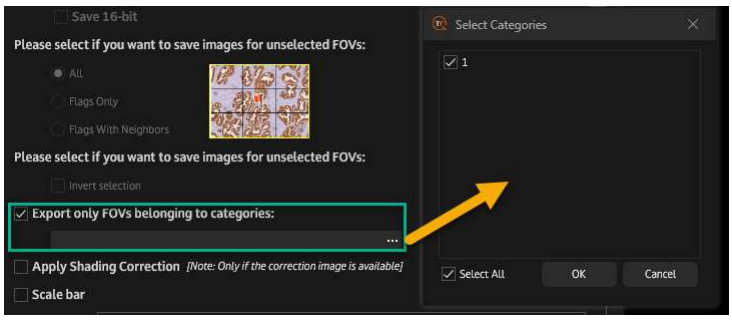
- To export all the regions or FOVs, select experiment item from Experiment Editor tree.

In both cases, the following dialog will appear:



TissueFAXS enables the precise configuration of an export, allowing a user to select the exact contents of interest. In the dialog displayed above, a user can adjust the following **settings**:

- Select the **storage folder**;
- Select **files** to be exported:
- Images
- Movies (option available only if the region was acquired with time lapse)
- Use stitch
- Select the **file name convention** (Name Parts, Custom Part, Parts, Image Format, or Name Preview);
- Select the **type of images** to save:
- **Overlay –** The FOV images will be composed from all the channels as currently specified in Region Viewer/Acquired Images.
- **Original** – The FOV images will be exported separately for each channel as they were acquired.
- **Save 16 bit**;
- Select **what images will be saved**:
- **All**;
- **Flags Only** and **Flags with Neighbors** (these options are only available if at least one flag set).
- **Invert selection** – Only the FOVs not marked with a flag or neighbors of the flagged FOVs (applies if **Flags with Neighbors** is selected) will be exported;
- **Export only FOVs belonging to categories**: in this case, only the FOVs that belong to the selected categories are exported, by pressing the browse button (...).



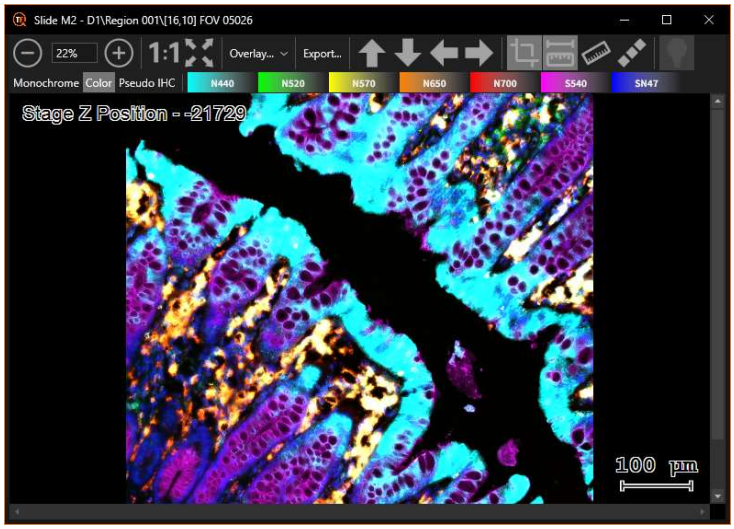
Note:

In the selection list, only categories that contain tissue areas on their overview image will be listed.

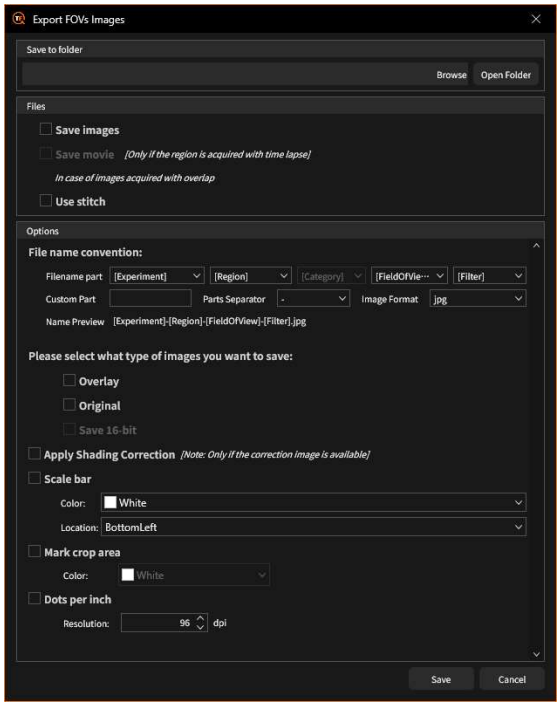
- **Mark Crop Area**: it marks the contour of the region for FOVs near the region border; its color can also be selected;
- **Scale bar;**
- **Apply Illumination Correction**: this option is enabled only for **Brightfield** experiments. The correction image will be applied to exported images if the correction image is available;
- **Apply Shading Correction**: this option is only enabled for **Fluorescence** experiments. The correction image will be applied to exported images if the correction image is available.

4.5.1.3. Export One FOV Image

This option can be accessed by pressing the **Export** button from the One Image Viewer toolbar.



The following dialog will appear:



- Select the **storage folder**;
- Select **files** to be exported:

- Images

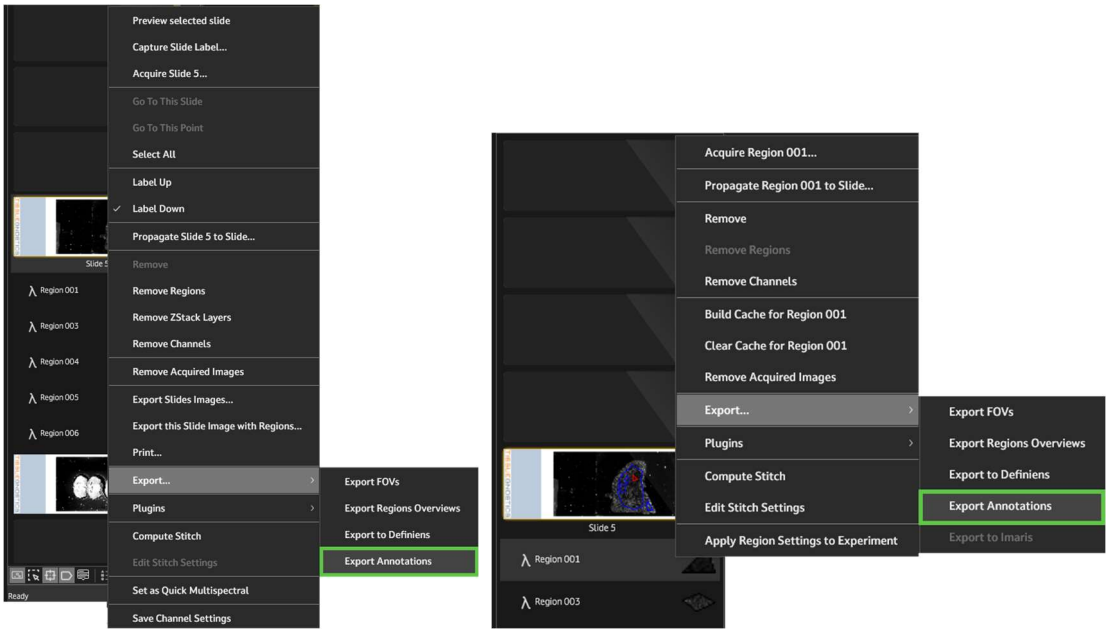
- Movies (option available only if the region was acquired with time lapse)

- Use **stitch**
- Select the **file name convention** (Name Parts, Custom Part, Parts, Image Format, and Name Preview);
- Select the **type of images** you want to save:
- **Overlay**: the image as it appears in the One FOV Viewer.
- **Region Channels**: the images for each channel as acquired.
- **Save 16bit**: a user can also export 16bit images if acquisition was made with 16bit. Only channel images can be a 16-bit type. For the fluorescence regions, a user can export the region overlay, as well as images from each channel separately, with the option of exporting them as 16bit.
- **Mark Crop Area**: marks the contour of the shape for each FOV; its color can be selected by the user;

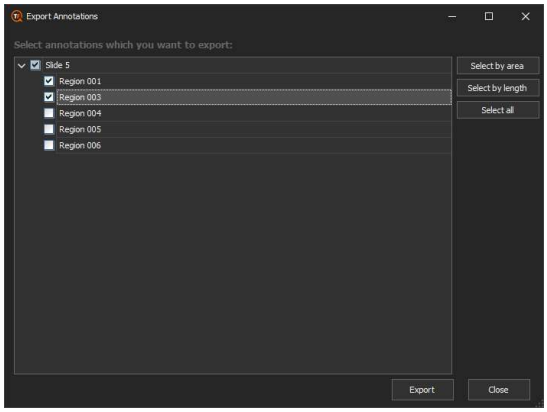
- **Scale bar**;
- **Apply Illumination Correction**: is enabled only for **Brightfield** experiments. The correction image will be applied to exported images if the correction image is available.
- **Apply Shading Correction**: is enabled only for **Fluorescence** experiments. The correction image will be applied to exported images if the correction image is available.

4.5.1.4. Export Annotations

To export annotations, go to the contextual menu of a region/slide, then choose **Export**... -> **Export Annotations.**



The following dialog will appear:



The first step to export is to select the desired item(s) from the list.

Items can also be selected by using the buttons on the right side of the dialog:

- Select by area;
- Select by length;
- Select all;

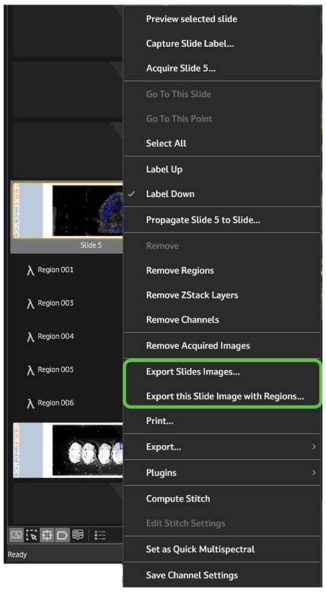
In the browser that opens, press the **Export** button and choose the location folder. Specify the name for the *csv* file to be exported. Finally, press the **Save** button to finish the export.

The exported *csv* file will contain the following data:

- Experiment name;
- Slide name;
- Region name;
- Annotation name;
- Area/Length;
- Unit measure;
- Description (if any).

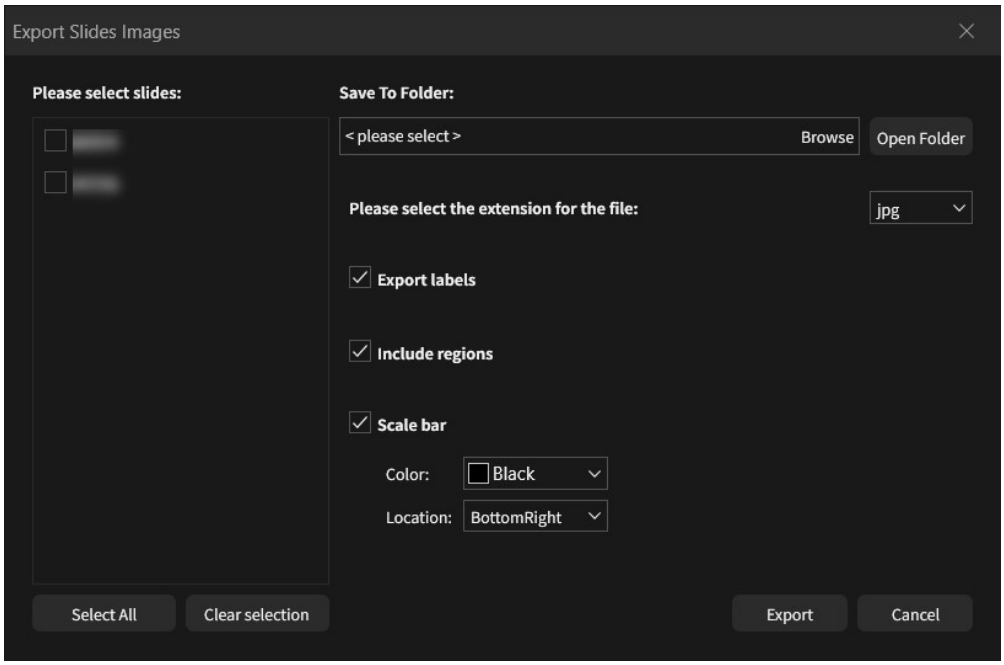
4.5.1.5. Export Current Slide Image

TissueFAXS **slides** images can be exported by going to each slide's contextual menu.



Export slides images

This option will export more slides at once, but first you have to select the slides for export in the dialog shown below.



- **Select the storage folder;**
- **Select the file extension;**
- **Export labels**: also exports the label of the slide;
- **Include regions** checkbox –choose whether or not the export image should contain the region shapes;
- **Scale bar;**
- **Location**: select location for scale bar.

Export this slide image with regions

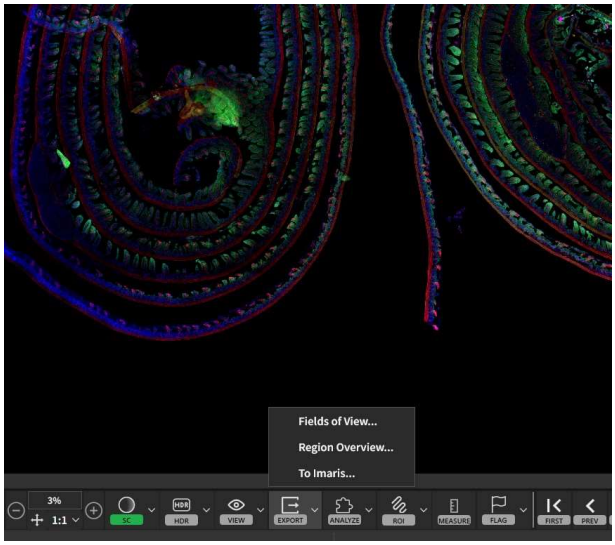
This option will export selected slide's image, including its regions, if any.

4.5.1.6. Export to Imaris

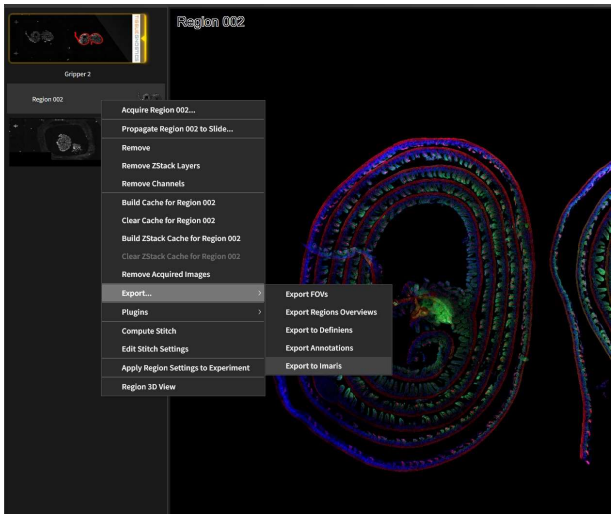
You can export regions acquired with Z-stack in *.*ims* format, which can be used in **Imaris** software to reconstruct 3D.

This export feature can be accessed from:

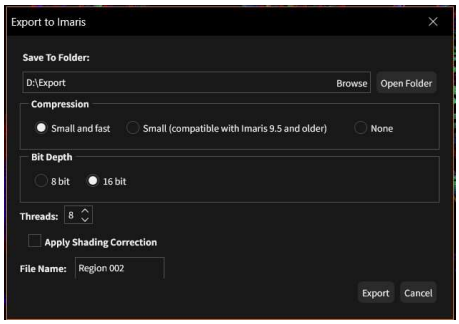
- **Image viewer -> Export**



- **Region's dropdown menu -> Export**

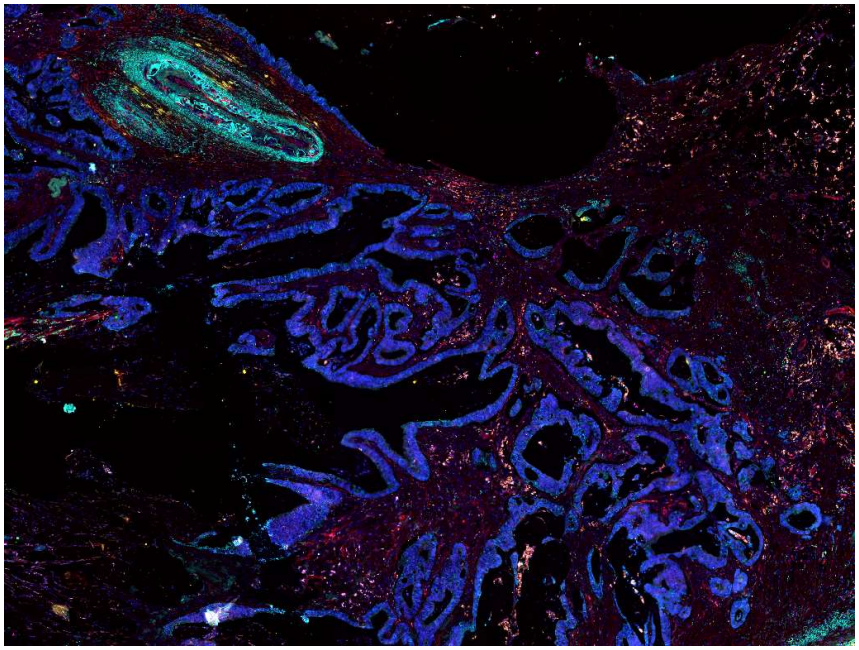


Export to Imaris window will open:



Before exporting, you can make these **settings**:

- Choose a storage folder;
- Select compression type;
- Select Bit Depth;
- Select number of threads;
- Apply shading correction;
- Choose a name for the exported file.

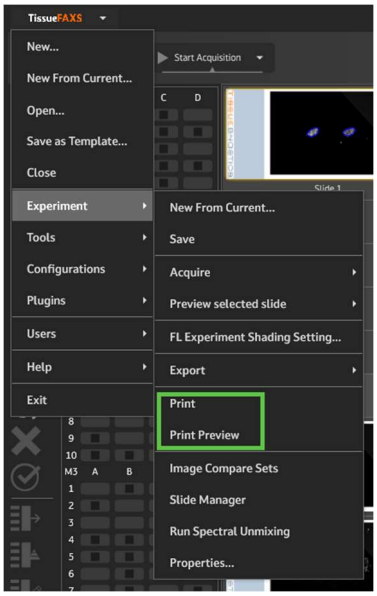


4.5.2. Print Experiment

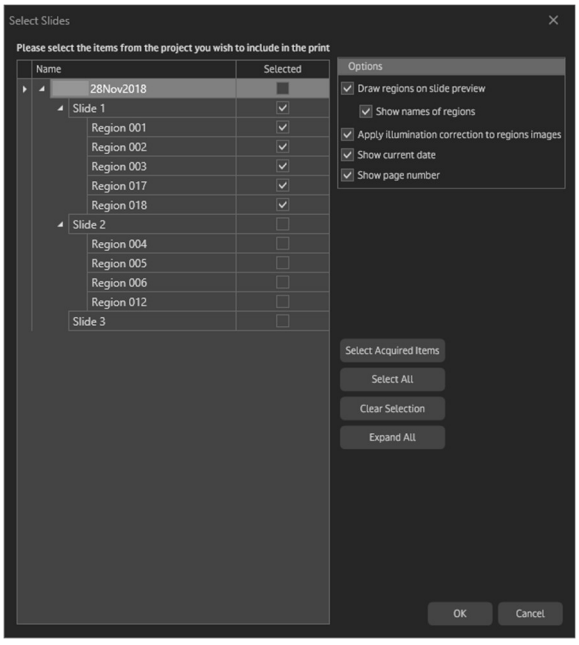
At some point it will be useful to print information from a **TissueFAXS** project, especially for the fact that Although the software does not a function to simply print an entire project, a user can decide which information to include in a report and how the data will be displayed.

1. Print Options

To access the **Print** option and the **Print Preview** option, go to **TissueFAXS main menu** → **Experiment**.



Printing is only possible for an opened experiment. When the dialog appears, select the items (acquired items, all items, etc.).



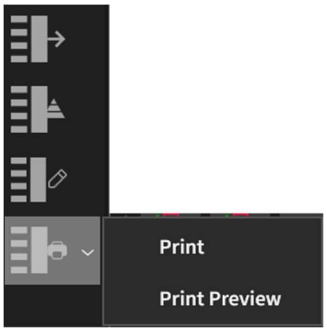
The default selection for this dialog is the selected slide in the slide viewer. It also offers a set of **options**:

- Draw regions on slide preview: The preview image may also contain region shapes;
- Show names of regions: is available only if the first option is selected;
- Apply illumination correction to regions: is available only for **Brightfield** experiments, the correction image will be applied to exported images if the correction image is available;

- Show current date: the print date is visible on each page of the report;
- Show page number: the page number is visible on each page of the report.

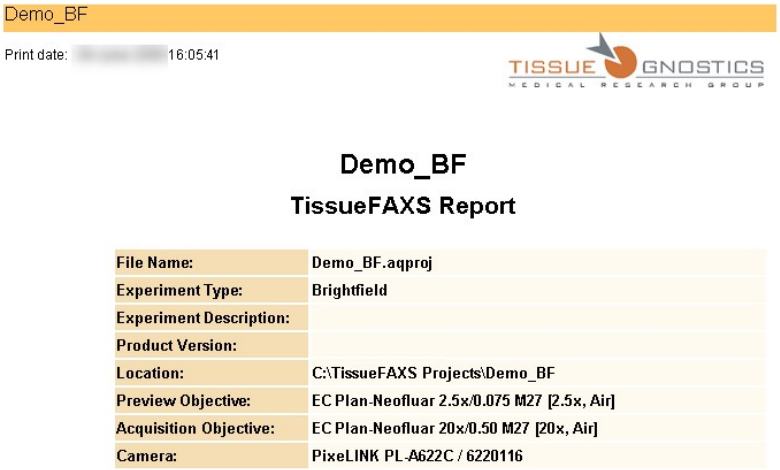
Print Job

It is possible to print an entire job (with its acquired slides and regions), by using the **Print Job** button from the Jobs toolbar.



2. Printing Items

- Selecting the **Print** option will bring up a dialog where the printer can be chosen.
- Selecting the **Print Preview** option will generate a preview report.



The **preview report** contains the following:

- Experiment Name;
- File Name;
- Experiment Type;

- Experiment Description;
- Product Version: the **TissueFAXS** version used to print this info;
- Location: the location of the experiment;
- Preview Objective: the objective lens used for the preview operation;
- Acquisition Objective: the objective lens used for acquisition;
- Camera: the camera used for this experiment;
- Each Slide selected in the list:

- Slide Name;

- Slide Image;

- Content Type;

- Comments: the comments referring to this slide;

- Objective: the objective lens used for preview.

- Slide Preview Channels: is a table that contains the channel list for the current slide and some properties for each channel in the list:

- Checked: this flag indicates if the current channel is used for overlay;

- Name: the channel name;

- Intensity: the channel intensity;

- Color: the channel color.

- Region list for each generic slide:

- Region Name;

- Region Image;

- Comments: the comments referring to current region;

- Acquired: this flag indicates if current region is acquired or not. Two possible values are present: **Yes** or **No**;

- Path: the path for current region files;

- Objective: the objective used for acquire current region;

- Rows: the number of rows for region;

- Columns: the number of columns for region;

- FOV's Count-: the number of FOVs items;

- Patient Name: the patient name;

- Patient Reference number: the individual reference number;

- Time Lapse (if acquired with time lapse);

- Number of Runs (if acquired with time lapse);

- Time between Runs (if acquired with time lapse).

- Regions Channels: is a table that contains the channels list for current region and some properties for each channel in the list:

- Checked: this flag indicates if the current channel is used for overlay;

- Name: the channel name;

- Intensity: the channel intensity;

- Color: the channel color.

- TMA Blocks list for each TMA slide:

- TMA Block Name;

- TMA Block Image;

- Comments: the comments referring to current TMA block;

- Acquired: this flag indicates if current TMA block is acquired or not. Two possible values are present: **Yes** or **No**;

- Objective: the objective lens used for acquisition of the current region;

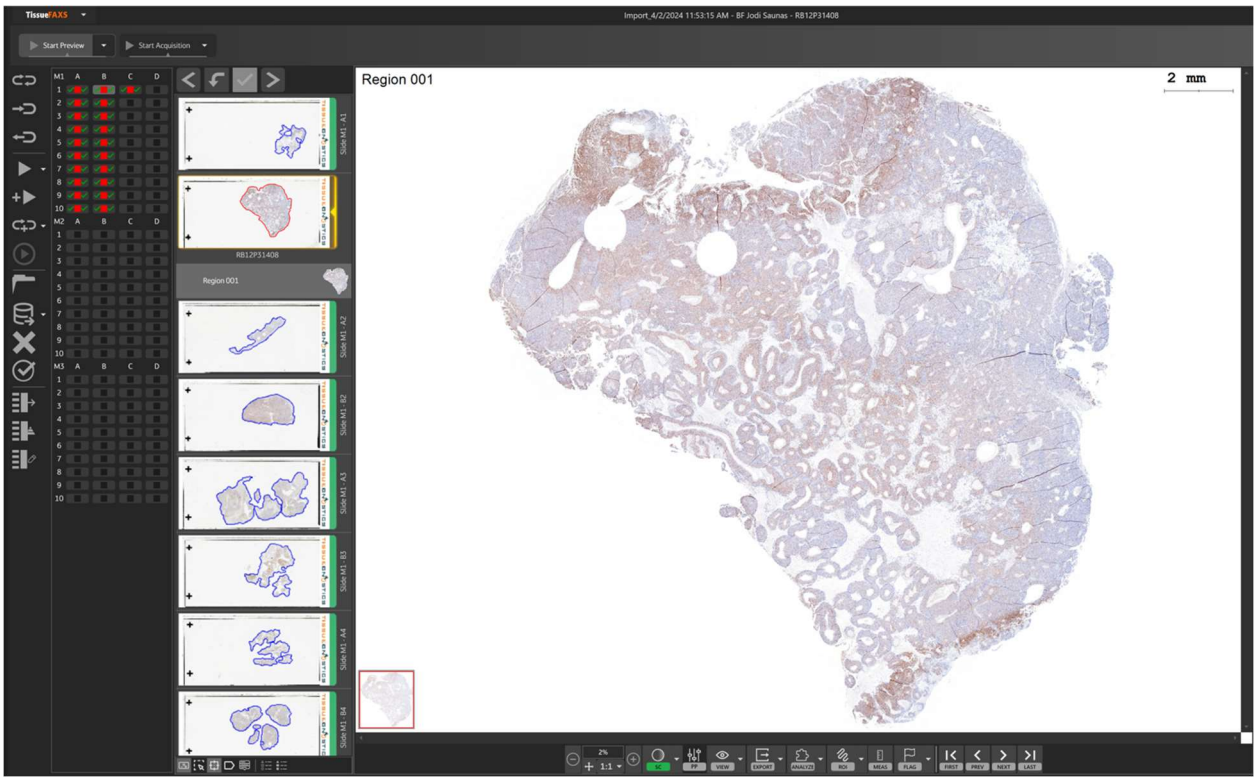
- Rows: the number of rows for the current region;

- Columns: the number of columns for the current region.

- TMA Spots Count-: the number of spot items.

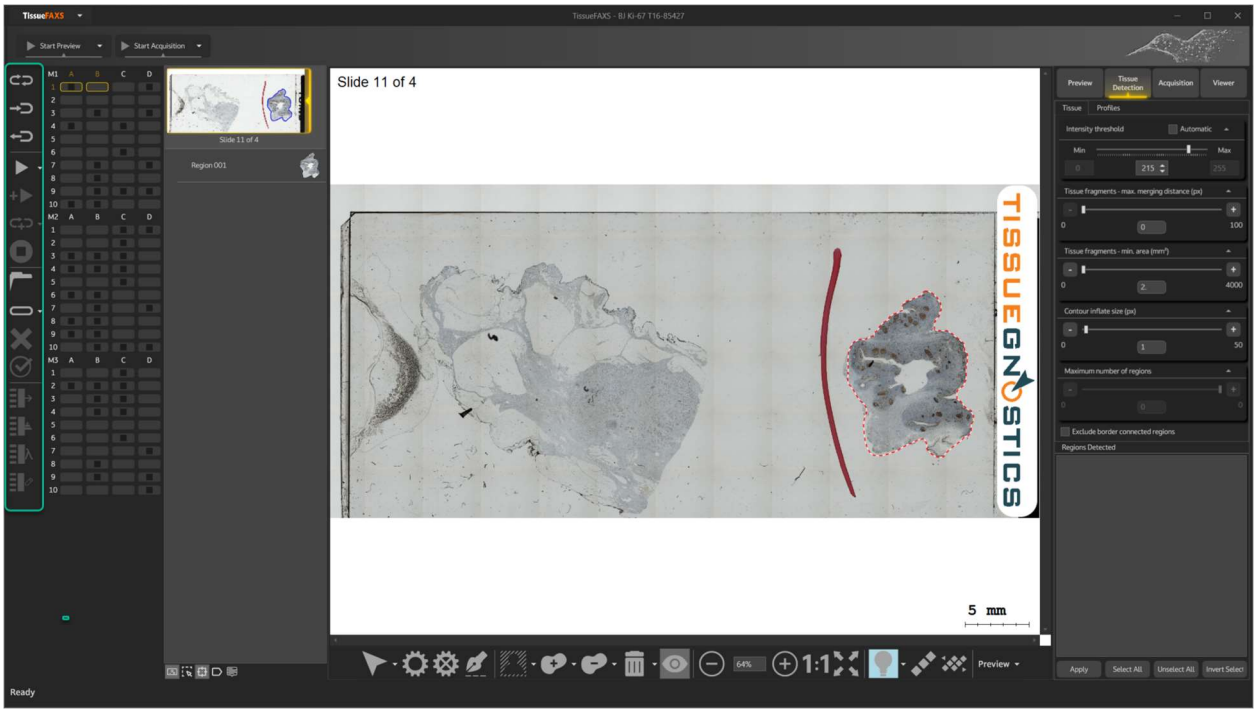
5. Job Workflow

TissueFAXS is the ideal tool for digitalizing many batches of slides grouped into what we call ***JOBS***. It comes with a dedicated automated workflow for **slide job processing** and, for optimal results, also allows the user to manually intervene during the process.



5.1. Job Overview

Job Toolbar includes the following operations:



Slide Loader operations

-**Reload Cassettes/Magazines**: moves up the cassettes/magazines to allow the user to change the contained slides;

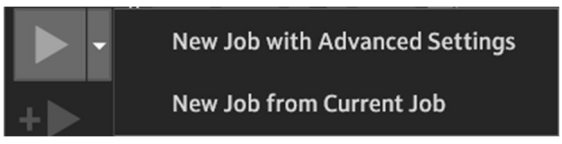
-**Load Slide**: loads slide from the loader cassettes/magazines into the gripper for further analysis;

-**Unload Slides**: moves slides present in the gripper back to the loader;

Note: The term cassettes is used for TissueFAXS 200 and the term magazines for TissueFAXS SL (120).

Job Acquisition

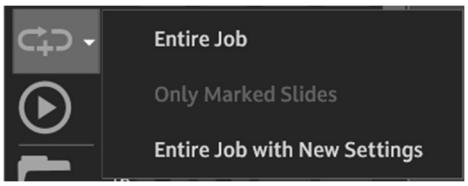
-**New Job**: When using the **New Job from Current Job** option, all physically available slides will be included in the job and the scanning of the job will be done using one template. When using the **New Job with Advanced Settings** option, the scanning of the job allows using more templates.



-**Add Slides to Job**: adds extra slides to your job.

-**Reacquire Job**:

- **Entire Job**: reacquires entire job.
- **Only Marked Slides**: reacquires slides previously selected for reacquisition.
- **Entire Job with New settings**: **TissueFAXS SL** supports restarting jobs with validated previews using different acquisition settings. This is useful if there is a need to change acquisition settings after cancelling job acquisition or checking acquisition results in **Validate Acquisition** phase.



-**Continue Job**: stops/continues the current job.

Job Operations

-**Open Job**: enables browsing for a job stored on a drive;

Note: If the job comes from another computer or another installation, the application will automatically save it on the server.

- **Open Database Job**: open jobs available in a database.

-**Close Job**: closes job;

-**Validate Job**: opens a validation window according to the current state of the job. Use the provided options to validate either the preview or the scan results.

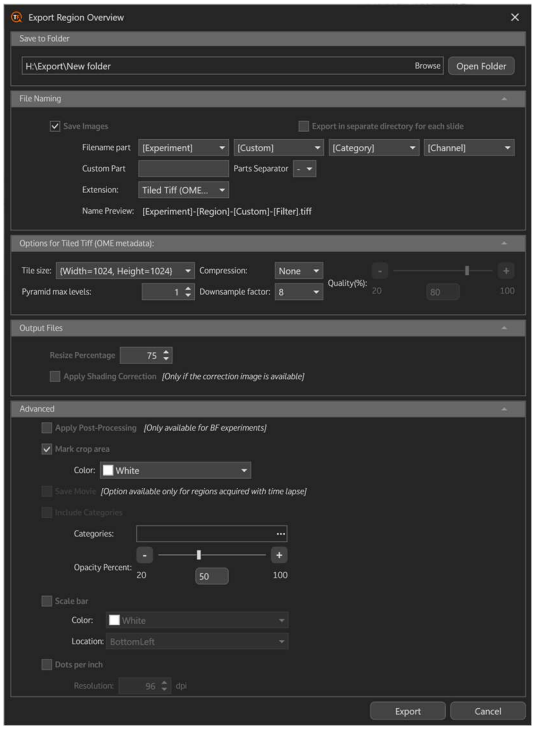
Notes:

- If TissueFAXS is currently acquiring the previews of the job, the user will be able to asynchronously start the Preview Validation process.

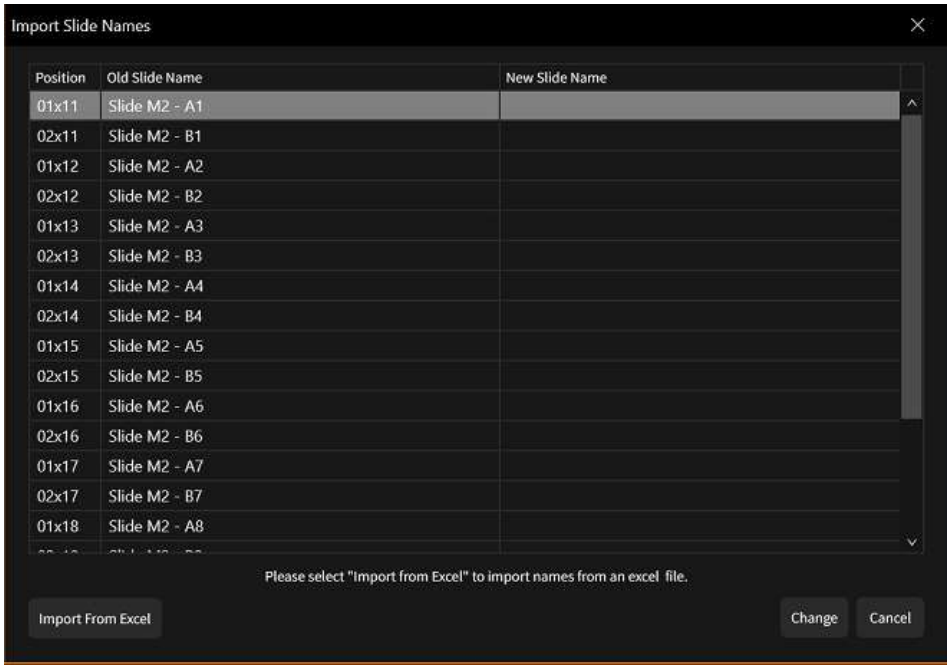
- If TissueFAXS is currently acquiring the slides of the job, the user will be able to asynchronously start Acquired Images and Regions Validation (Scan Validation).

- If TissueFAXS has completed scanning a job, the user can still access the scan validation in order to add new regions for reacquisition or flag previously acquired images.

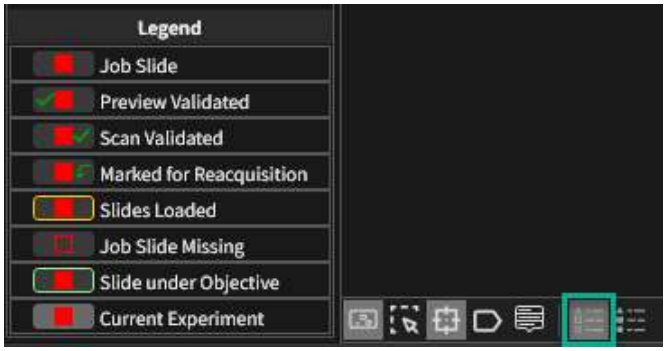
- **Export Job**: exports region overviews for all the experiments in the job. See Export chapter.



- **Slides Names**: Slides can be renamed manually, one by one, by typing the new names in the **New Slide Name** fields. Names can also be imported from an excel file by pressing **Import from Excel** and browsing for the excel file containing the names. Press **Change** button to save the name changes.



- **Build cache for Job**
- **Legend**: helps you know the status for each slide;



- Job Slide: is the slide included in acquisition;

- Preview Validated: is the slide with validated preview;

- Scan Validated: is the slide with validated scan;

- Marked for Reacquisition: marks which slide will be included in reacquisition;

- Slide in Gripper: is the slide currently in the gripper;

- Job Slide Missing: is the slide previously acquired but has been removed and now is physically missing from loader;

- Slide under Objective: is the slide currently in the gripper and under objective.

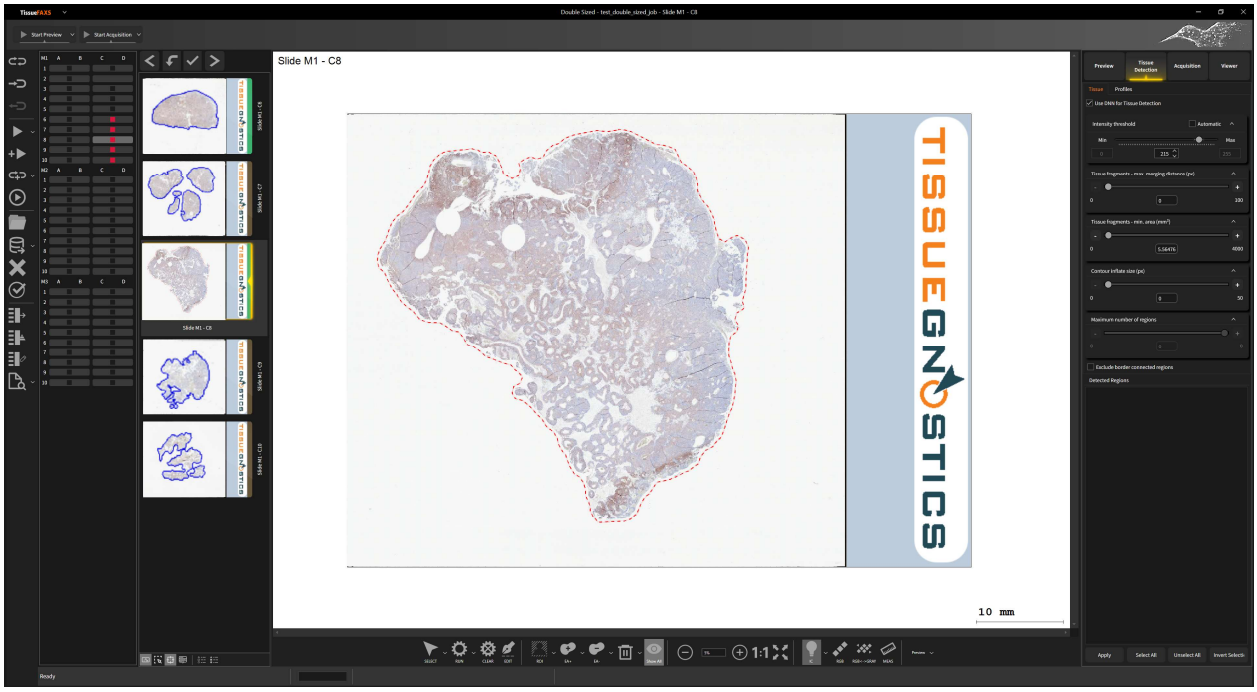
- Current Experiment: marks the currently opened experiment.

Slide Loader Display Control

This section displays all the slide loader positions. The empty positions look clear; the occupied positions are shown as tissue slides.

Scanning Double sized Slides

TissueFAXS allows scanning normal slides (1x3 inches) but also double size slides (2x3 inches).



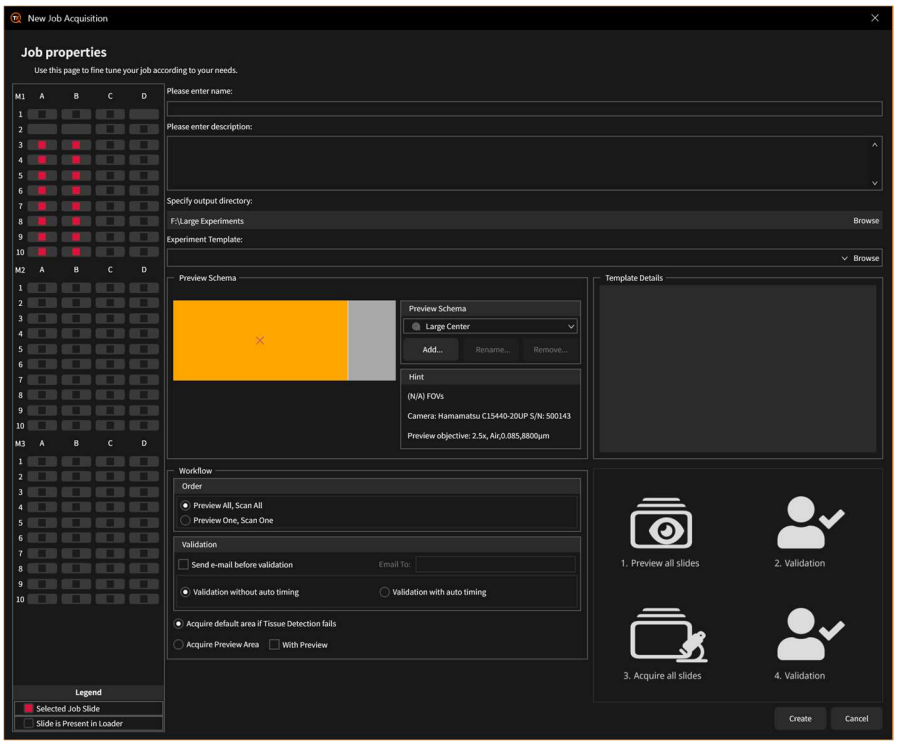
Notes:

- Normal slides cannot be scanned in the same configuration as the double size slides!
- The detection of clip type (which contains normal or double-sized slides) is only performed when loaded from the magazine. Thus, scanning in a job will skip the different clip types.

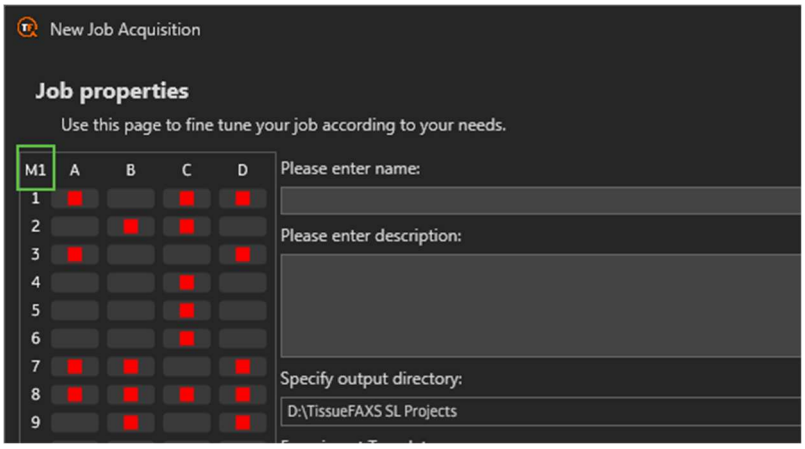
5.2. Workflow

Creating new jobs

To begin, press the **New Job** button to open the **New Job Acquisition** page. Use this page to perform fine tuning on the job.



Note: Pressing the name of the **M1** Magazine from the job list acts as a **Select/Unselect All Slides** feature.



- Enter a job name
- Enter a job description (optional)
- Specify a storage folder
- Choose a template for the experiment. Once chosen, its details can be viewed in the Template Details section

- Set a preview schema
- Set workflow preferences:

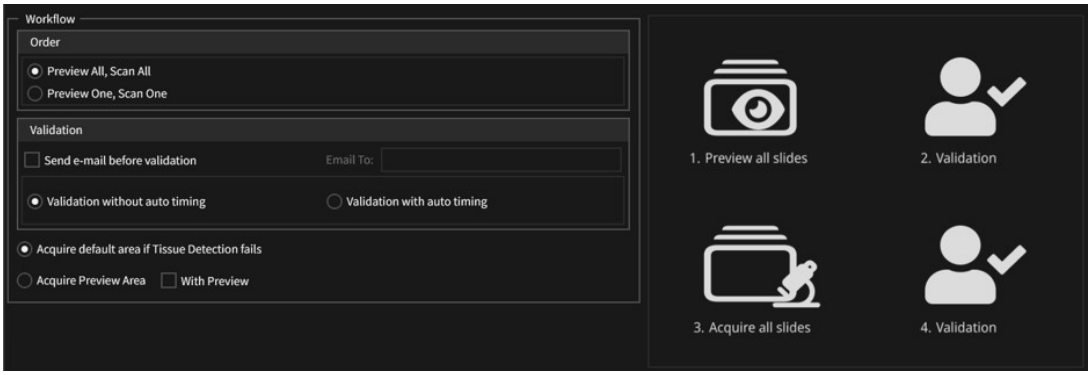
1. Preview All, Scan all

If choosing this option, the workflow is: **Preview all slides → Validate Preview → Acquire all slides → Validate Acquisition**.

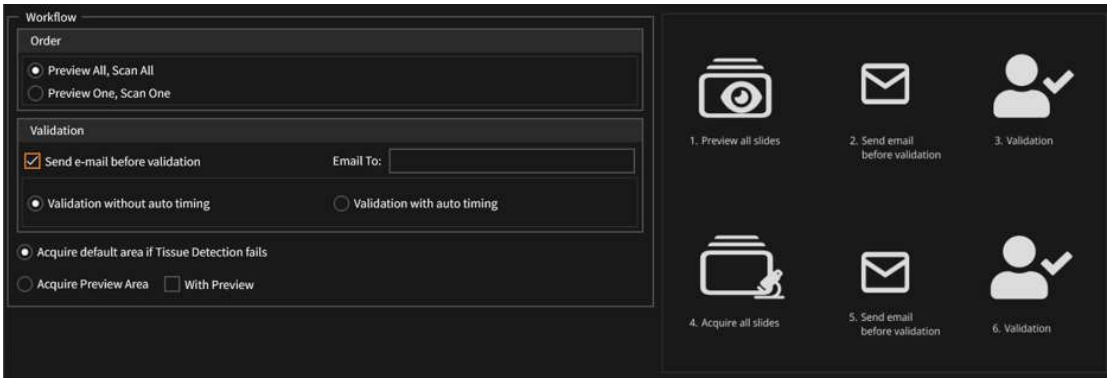
Note:

-The user doesn't have to wait for all slides to be previewed in order to start Preview Validation. The validation process can begin at any time, asynchronously.

-The user doesn't have to wait for all slides to be previewed in order to start Scan Validation. The validation process can begin at any time, asynchronously.

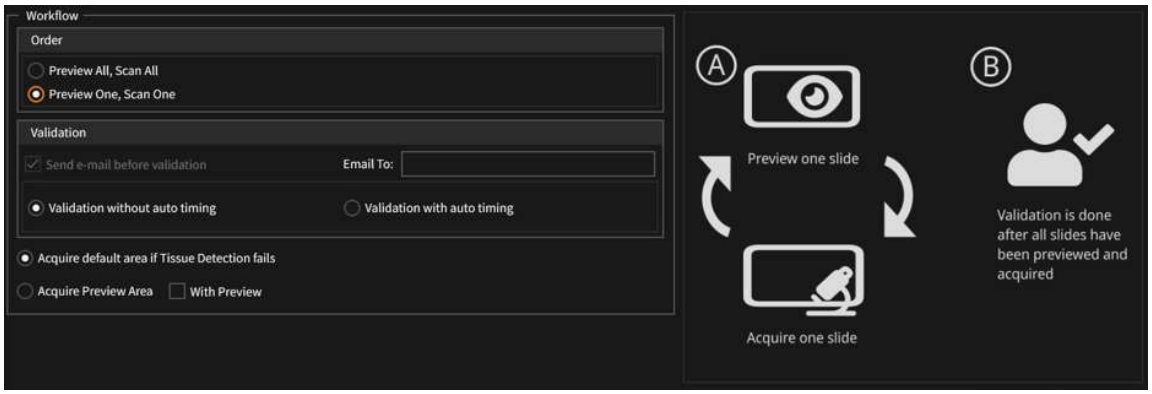


Choosing **Preview All, Scan All** with **Send e-mail before validation** checked, the workflow is: **Preview all slides → Send e-mail before validation → Preview Validation → Acquire all slides → Send e-mail before validation → Scan Validation.**



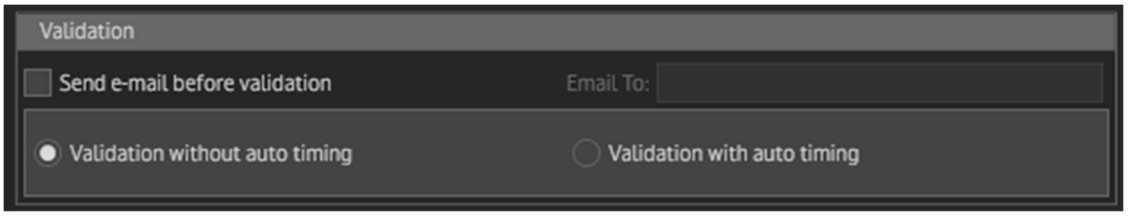
2. Preview One, Scan One

If this is chosen, **Preview Validation** will be skipped and the regions will be automatically detected and scanned. The workflow will be: **Preview one slide → Automatic Tissue Detection → Acquire one slide**. The user will be able to validate scanned results after **all** slides have been acquired. The scan validation dialog is the same as Preview All, Scan All.



Validation

To receive an email when the acquisition process reaches the validation step, check **Send e-mail before validation.**



Now the user has **two validation options**: with or without auto timing.

- Validation with auto timing

If no action is taken within a previously determined amount of time, **TissueFAXS** will automatically detect regions and proceed to acquisition.

- Validation without auto timing

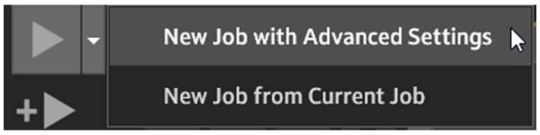
Acquire default area if Tissue Detection fails: after preview is done, if tissue detection fails, TissueFAXS SL will acquire the preview area.

Acquire preview area: TissueFAXS will directly acquire the preview area, with or without preview (check/uncheck **With Preview**).

Configurable Scan

When using the **Configurable Scan** option, more templates can be used in the scanning of the job.

Press **New Job with Advanced Settings** to open the **Job Acquisition Wizard:**



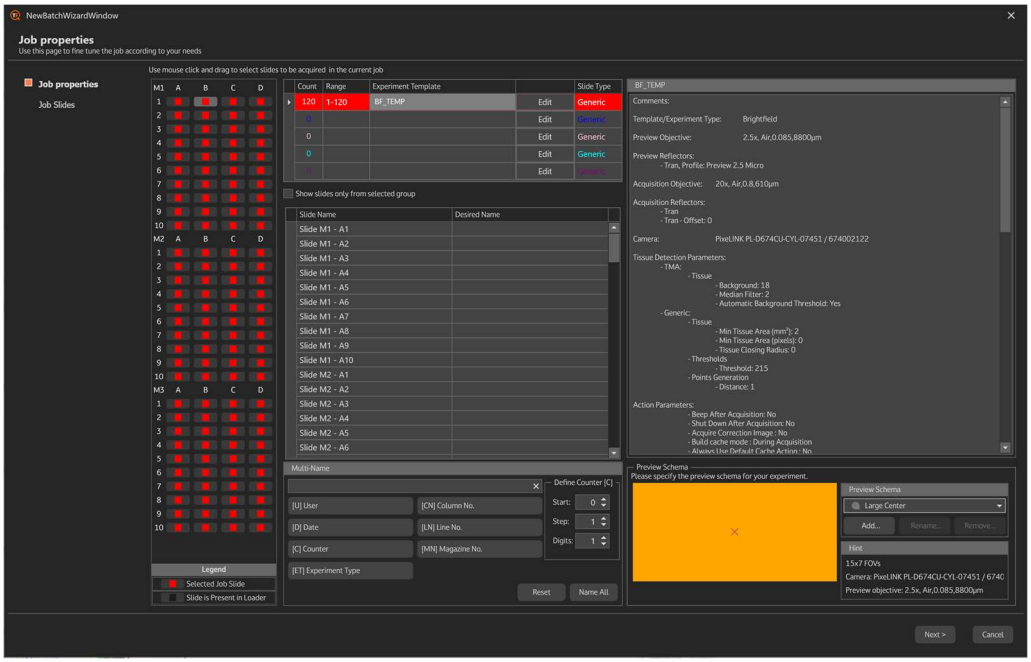
In the first step of the Wizard, **Job Slides**, a user can configure the slides that will be included in acquisition.

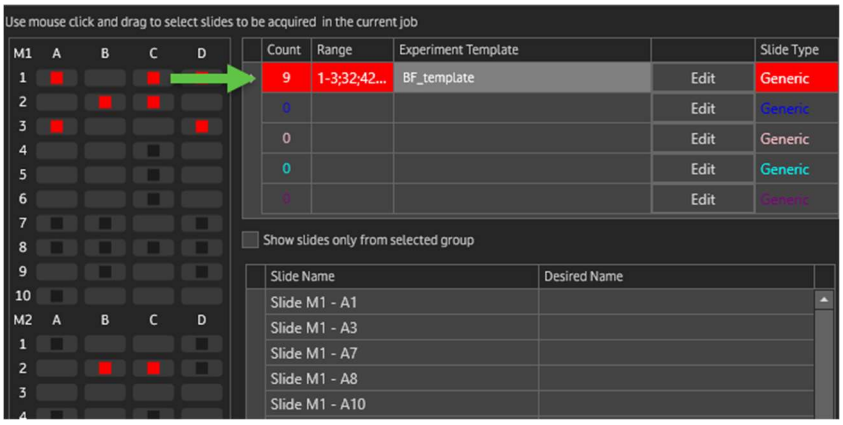
Groups

First, each slide must be assigned to a group. The slides of a group share the same template.

Using the mouse, select a slide or a range of slides and assign an experiment template.

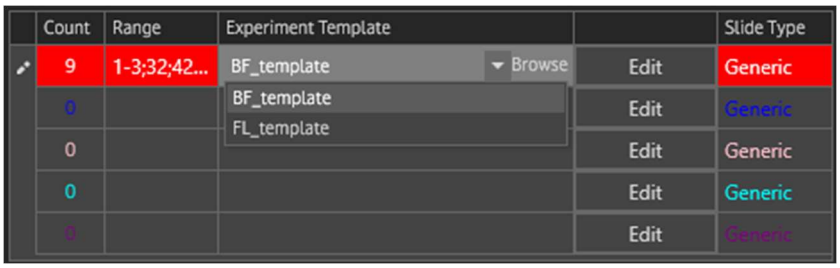
To select all slides, press in the upper left corner of the window.



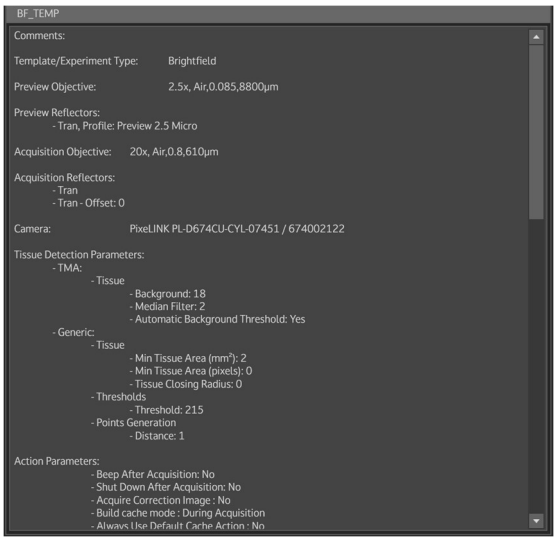


After selecting the desired slide(s), browse the folders and choose a default experiment **template**. An existing experiment can also be chosen instead of a template.

Note: By default, all templates available in the default location will be made available in the drop-down menu.

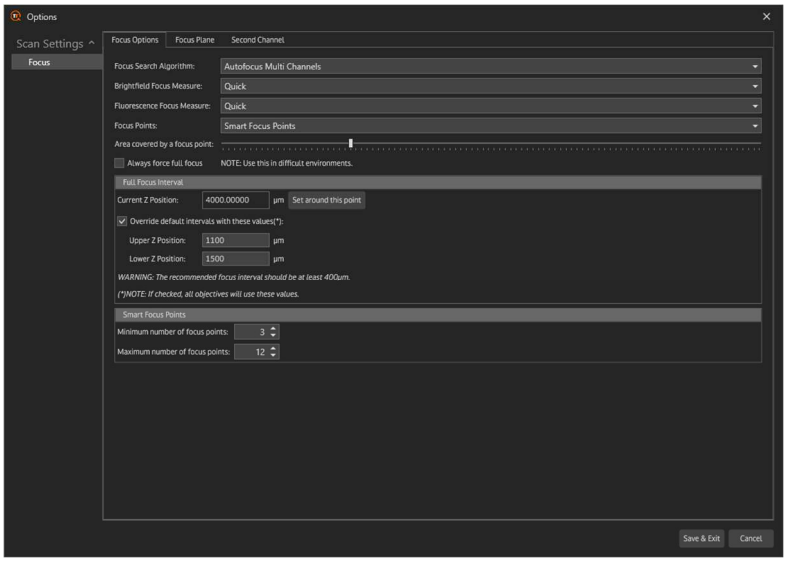


Once settings for the slides have been selected, the template details can be visualized in a dedicated section.



Edit button allows changing settings for the focus operation, before or during job acquisition.

For more details regarding focus, see Chapter Focus Settings



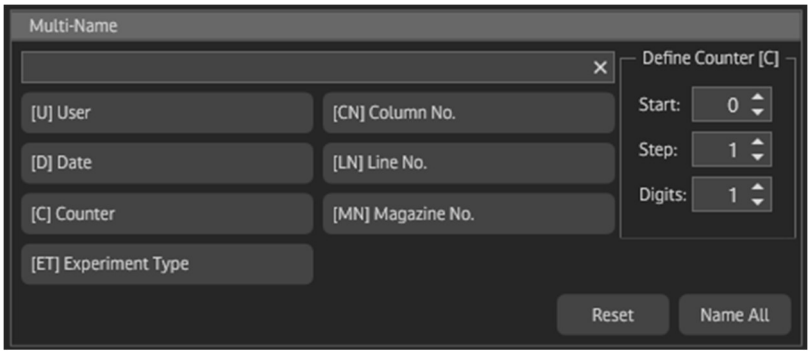
You can also change the **slide type**: generic or TMA.

Due to the large number of slides forming a job, **TissueFAXS** also provides a **Multi-Rename** tool.

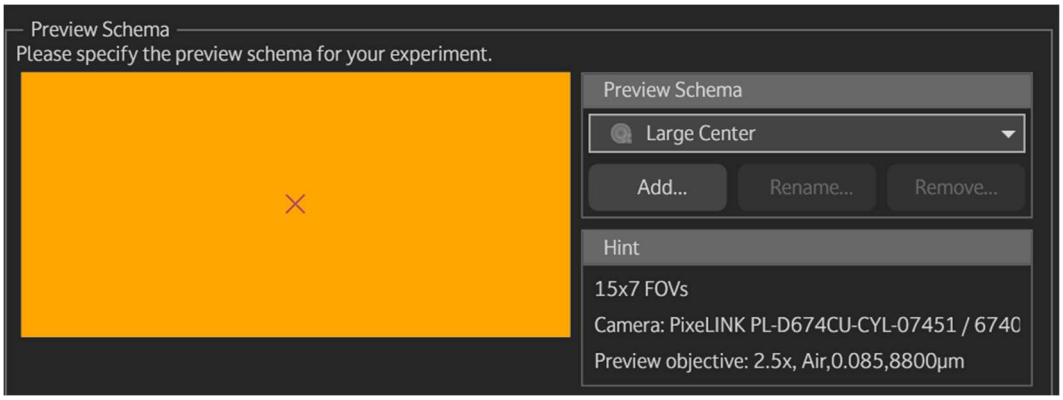
In the **Multi-Rename** field, pre-defined criteria can be selected: user, date, counter, cassette number, line number or slide type, magazine number, or experiment type. Text can also be entered manually.

A configurable counter is available.

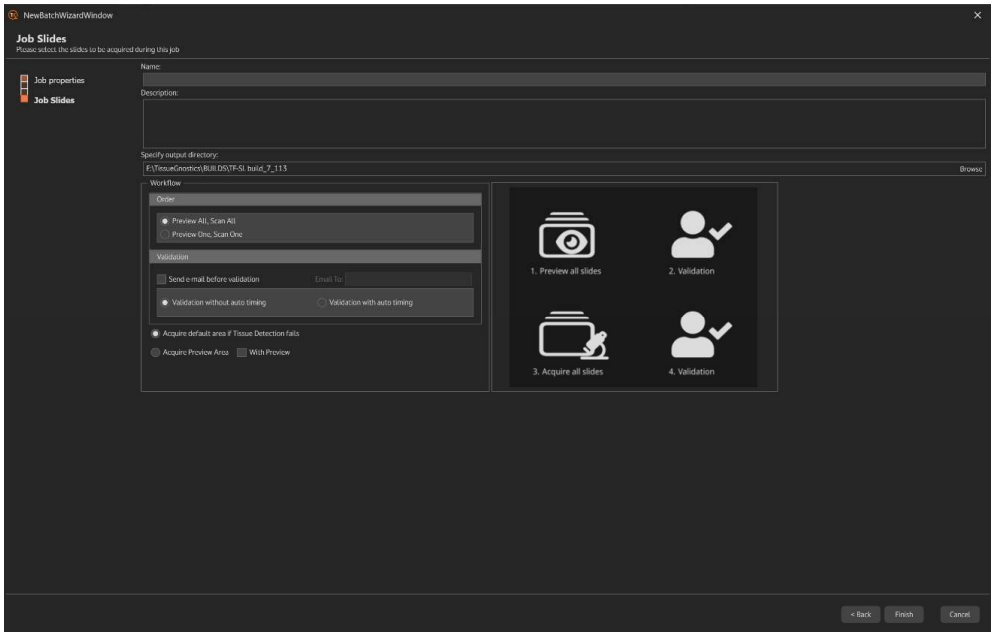
There is also a **Reset** function and a **Name All** function.



The preview schema inherited from the template can be modified.



When finished with the settings, press **Next** to access the second step of the wizard, which looks exactly like the **Scan All** page above.

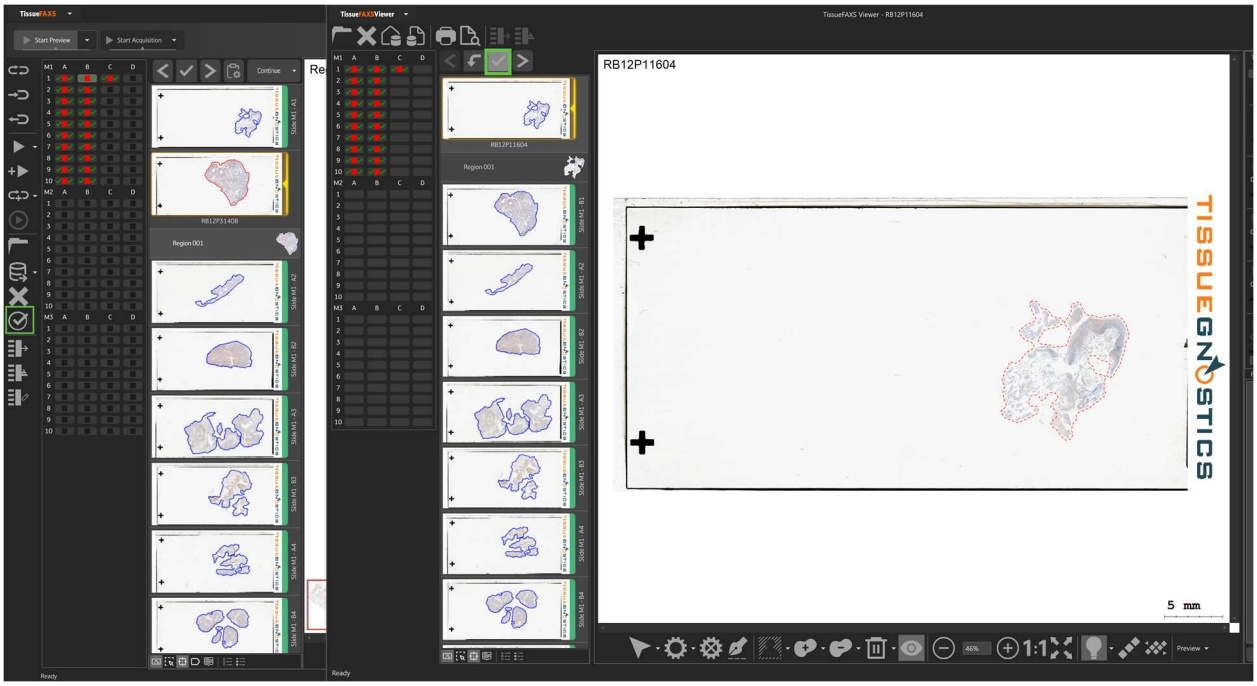


Finally, press **Finish** to start the job preview and acquisition.

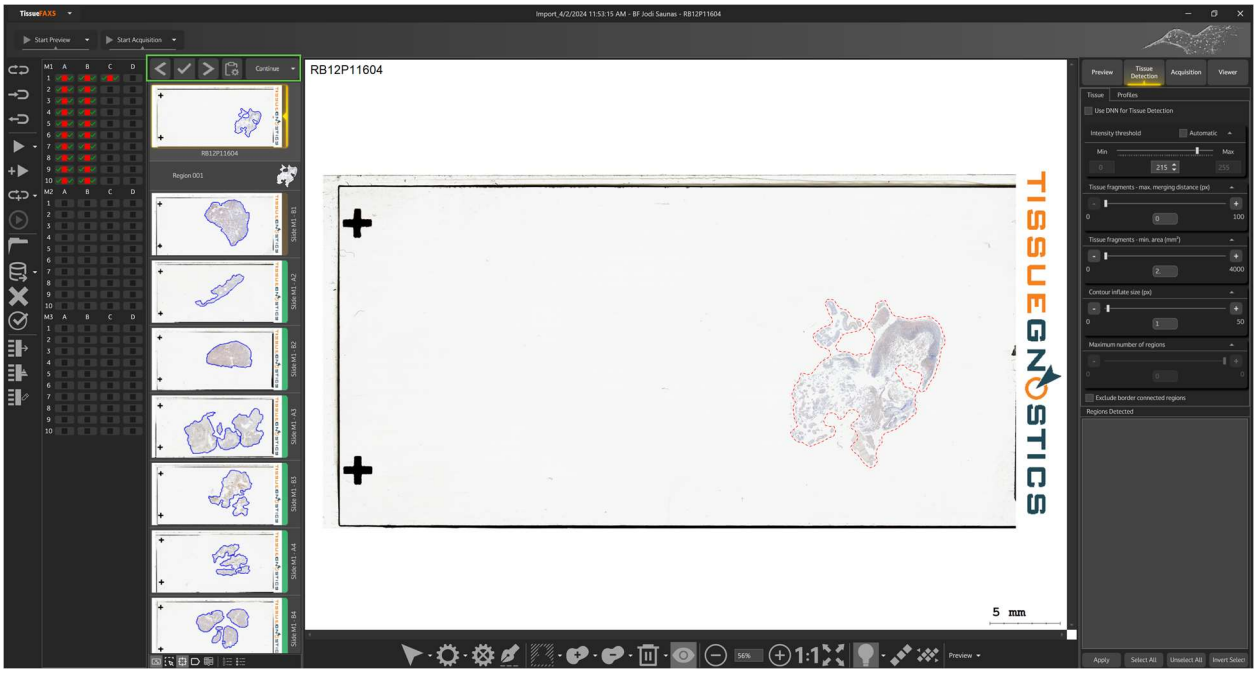
Validate Preview

You can automatically open the job for validation in **TissueFAXS Viewer** by using **Validate** button.

During the preview acquisition, you can define regions (by automatic tissue detection or manually) and validate each slide by using TissueFAXS Viewer.



After **TissueFAXS** ends the preview (only for the Preview All/ Scan all workflow), you can start validating your slides.

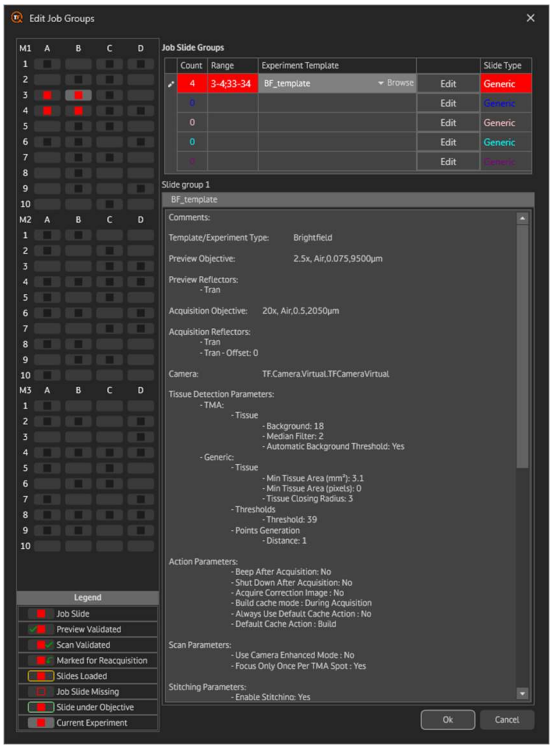


You can navigate through slides and validate them using the validation controls: previous, next and validate slide.

Note: When all slides are validated, acquisition process will start.

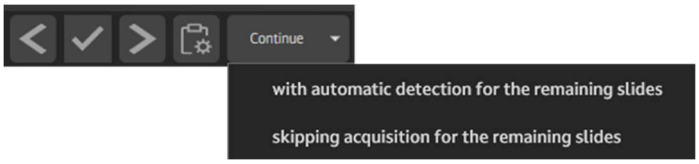
At this point, if necessary, manual tissue detection can be done. For details on how Tissue Detection works, see Chapter Tissue Detection.

- Adjust Tissue Detection parameters;
- Choose Tissue Detection Templates;
- Detected Regions: listed detected regions can be found here. Right click on this section or on a detected region for additional options: **Rename Region**, **Naming Order** and **Order by;**
- If right clicking on a drawn region, the region can be set as Multispectral by choosing from the context menu **Set as Multispectral**.
- **Edit Job Groups** button allows changing focus options once more, if necessary. For more details, please see above in this chapter.



Continue Job

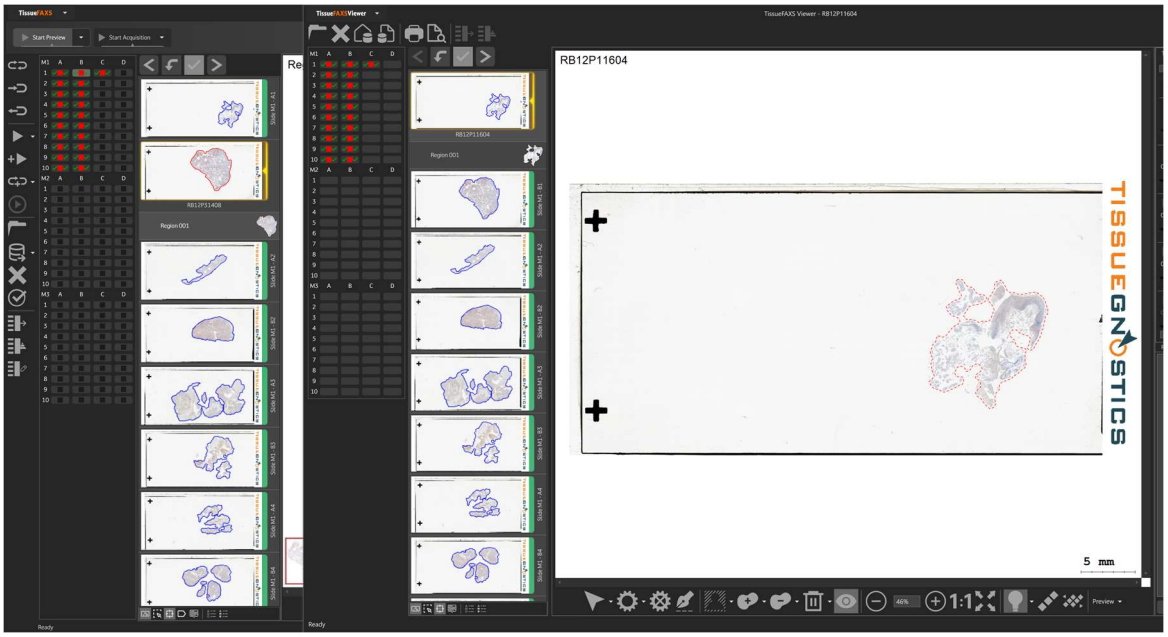
When the slide settings are complete, press the **Continue Job** button to start the preview operation.

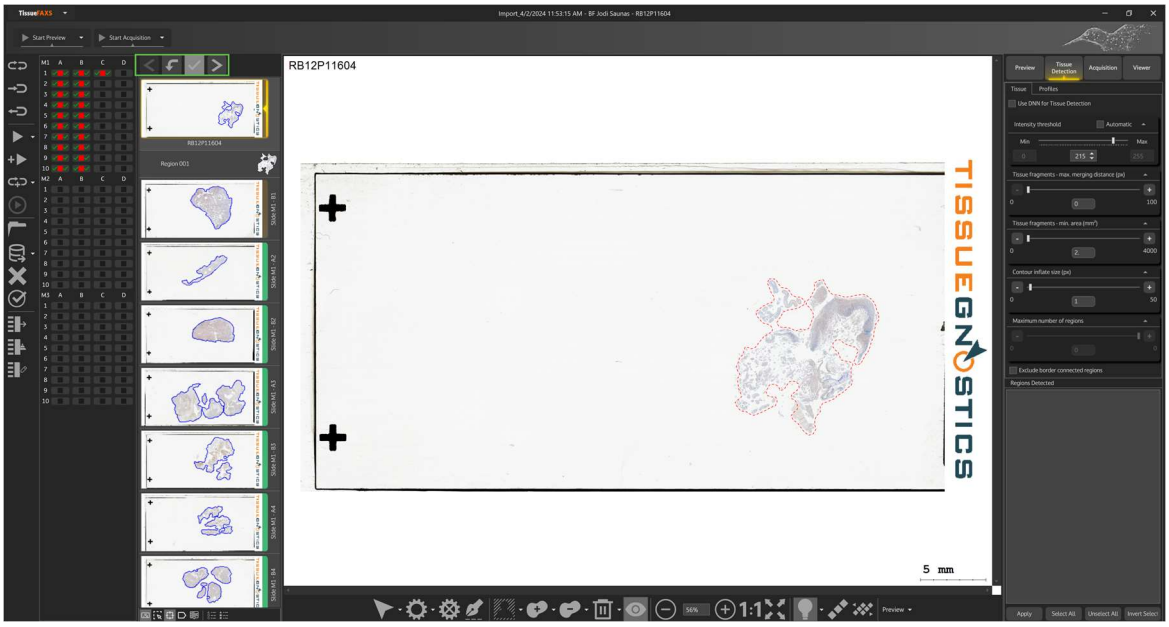


- **Continue job with automatic detection for the remaining slides**: skips validation of remaining slides, performs automatic tissue detection, and starts acquisition.
- **Continue job and skipping acquisition for the remaining slides**: skips remaining slides and automatic detection, then starts acquisition.

Validate Acquisition

As for preview, you can validate the regions from TissueFAXS, after the job acquisition is done, or during job acquisition, by using TissueFAXS Viewer.





Reacquisition

You can reacquire items in more ways:

- From the **Slide Validation** toolbar within the **Tissue Detection** panel - **Mark/unmark for reacquire** button: includes/excludes slides in reacquisition.

Notes:

- Mark a slide for reacquisition to acquire new regions defined on the preview of the slide or to reacquire FOVs marked on the existing regions.

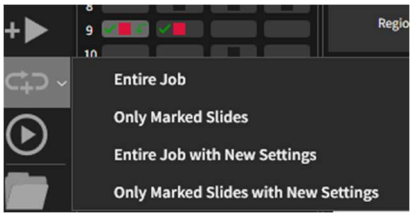
- You can also mark a slide for reacquisition if you erase acquired images from the scanned regions.

- Slides that will be marked for reacquisition without new added regions or flagged FOVs will not trigger any reacquisition. If the user defines new regions or flags existing FOVs, there will be an option to mark the slide for reacquisition.

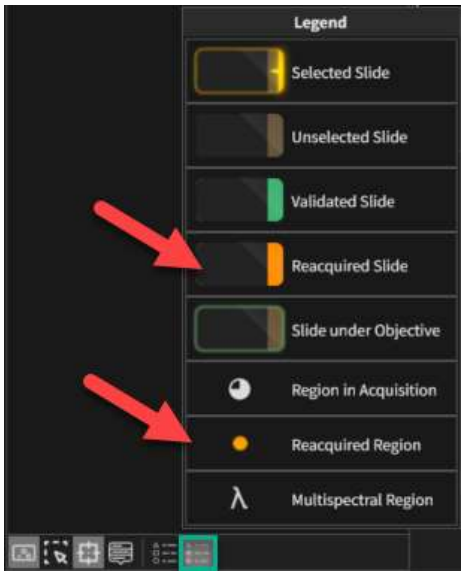
- By using **Flags** feature: see **Chapter** Flags (Reacquisition).

- By using **Reacquire Job** button from the **Jobs** section:

- **Entire Job**: reacquires entire job.
- **Only Marked Slides**: reacquires slides previously selected for reacquisition.
- **Only Marked Slides with New Settings**: reacquires slides previously selected for reacquisition using different acquisition settings.
- **Entire Job with New settings:** TissueFAXS SL supports restarting jobs with validated previews using different acquisition settings. This is useful if there is a need to change acquisition settings after cancelling job acquisition or checking acquisition results in Validate Acquisition phase.



The reacquired items have a specific graphical marking within the experiment editor, as shown in the **Legend** below:



Toolbar

Validation **toolbar** assists when navigating between slides and validating them or marking them for reacquisition:

The default **buttons** are:

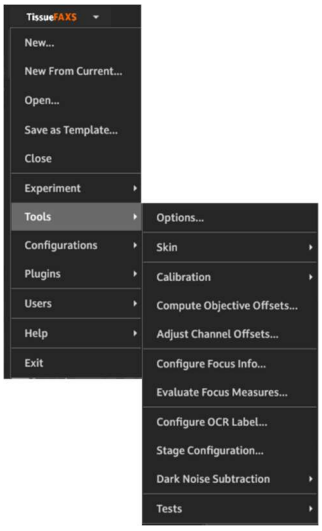
- **Go to previous slide**: press to go to a previous slide;
- **Mark/unmark for reacquire** (see section above);
- **Validate slide**: to validate a selected slide;

Note: For viewing region acquisition results, you can open that region in Region viewer (see Chapter Images and Viewing Options).

- **Go to next slide**: press this button to go to the next slide.

6. TissueFAXS Core Settings

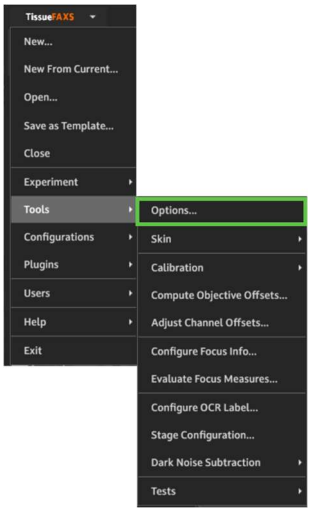
The **core settings** panel is useful in managing the main settings of the application. Here, by accessing the listed items, you can visualize the available settings and modify them.



6.1. Options

Access **Options** by going to **TissueFAXS Main Menu -> Tools -> Options**.

Th items listed among **Options** may vary depending on the type of user logged to the application.

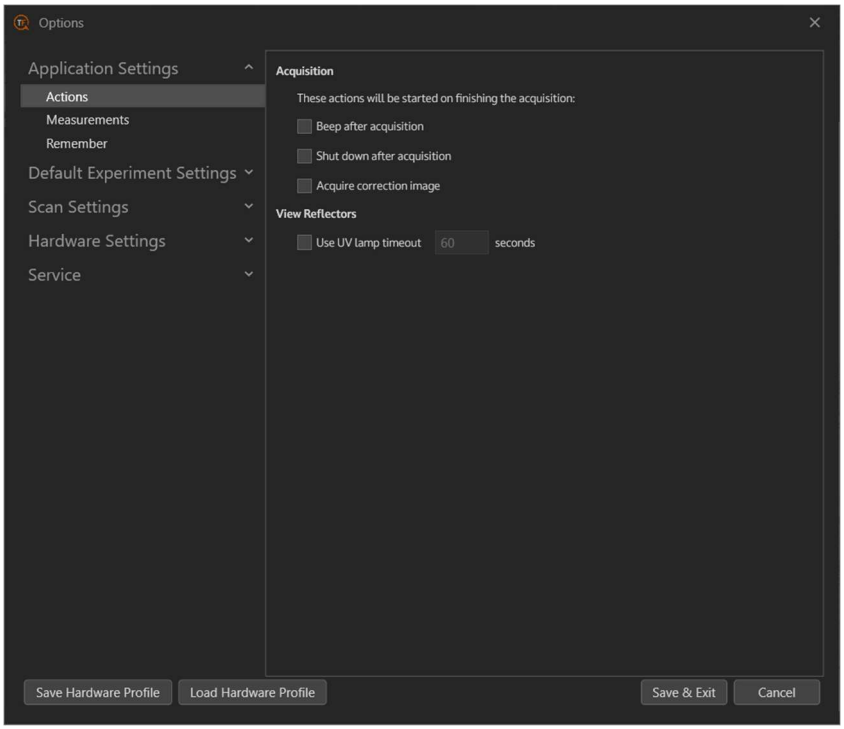


6.1.1. Application Settings

This section contains TissueFAXS generic settings.

6.1.1.1. Actions

Actions



Acquisition

After finishing the acquisition, three options can be set:

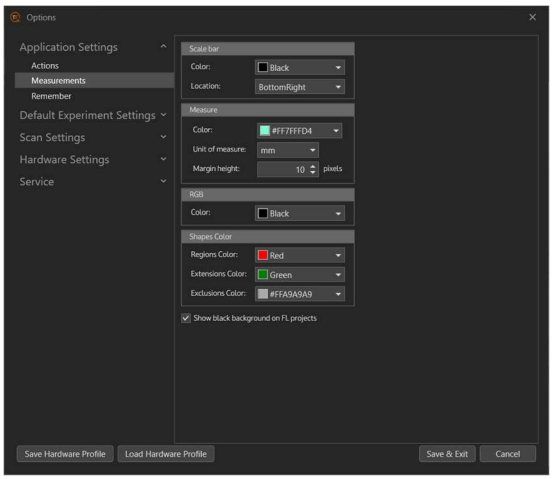
- **Beep after acquisition**: **TissueFAXS** will indicate the completion of the acquisition process with repeated beep sounds;
- **Shut down after acquisition**: The computer will automatically shut down after acquisition.
- **Acquire correction image**: a correction image is acquired before the acquisition process.

View Reflectors

Use View UV lamp timeout: when viewing a FL channel, there's the risk of leaving the FL lamp open and bleaching the tissue. By checking this option, the lamp will automatically close after a user-defined interval.

6.1.1.2. Measurements

Measurements

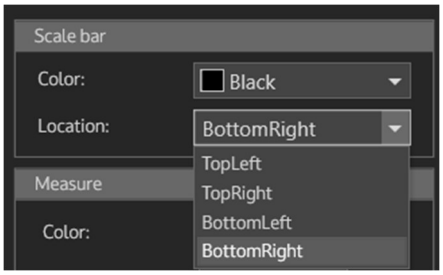


Scale bar

The **Scale bar** is represented by a segment that indicates the scale of the image.

It has two adjustable attributes:

- The **color**;
- The **location** list with four values:



Note: The default values are Black (for color) and Bottom Right (for location).

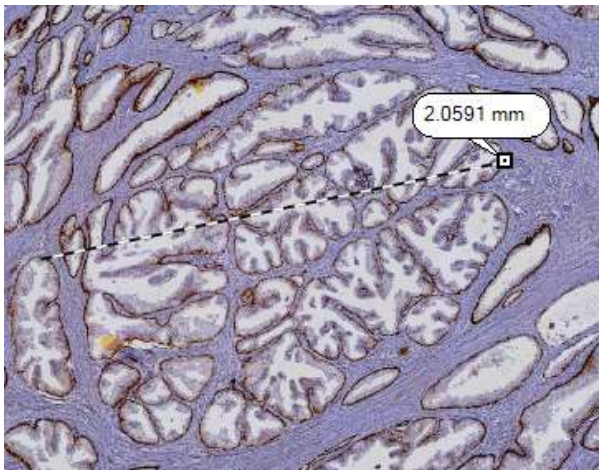
The scale bar can be found on the user interface in more places:

- Live Image;
- Slide Preview;
- Region Viewer;
- One Image Viewer;
- Exported Images.

Measure

This function is used to measure the distance between two points (on the sample) specified by the user (by clicking on the start point, then on the end point).

The distance and the unit of measure are displayed on the measured image.



Note: There are two adjustable attributes: the color and the measure unit. The default color is **aquamarine** and the default measure unit is **millimeter**.

This function is available on:

- Live Image;
- Slide Preview;
- Region Viewer;
- One Image Viewer.

RGB

When using RGB, you can select the color used to display information about **RGB** or **Grey value** for a pixel on the camera live image.

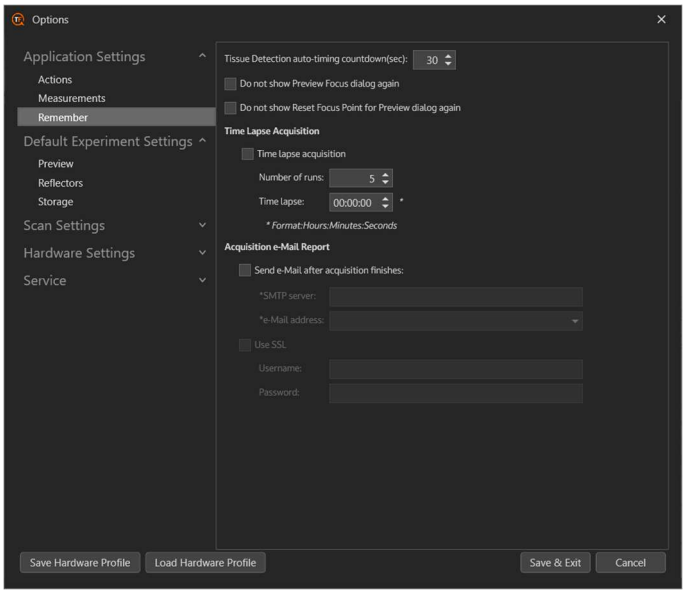
Shapes Color

- Assigns individual colors for the user-defined regions, for the extension areas and the excluded areas.

- Shows black background on FL projects: check this to see the background of fluorescence projects in black.

6.1.1.3. Remember

Remember



This dialog can be used to specify default values for some of the most commonly used options of the application.

- Uncheck **Do not show preview focus dialog again** if you want that dialog to be displayed before the preview operation; otherwise, check this option;
- Uncheck **Do not show reset focus point for preview dialog again** if you want that dialog to be displayed when you reset the preview focus points; otherwise, check this option.
- **Do not show slide renaming dialog again**: This option (checked by default) will prevent the slide renaming dialog from appearing each time you create a new experiment.
- **Time Lapse Acquisition**: Check this option in order to set by default multiple acquisitions for a region or group of regions. Please set desired values for the following parameters:

- *Number of runs*: how many times the acquisition will run;

- *Time lapse*: the interval between runs.

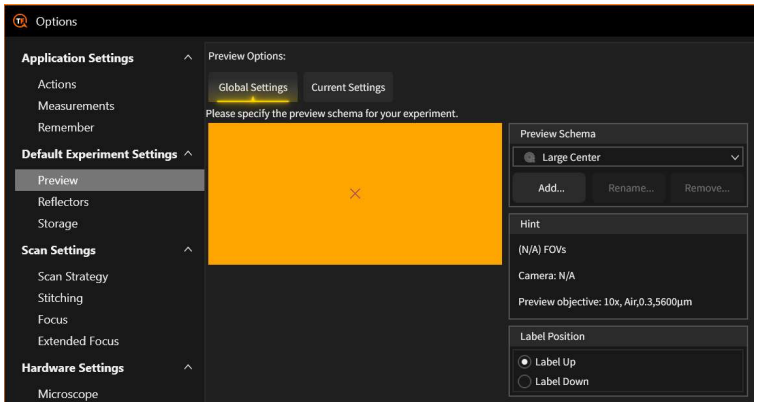
- **Acquisition e-Mail Report**: this feature allows the user to be notified by email when the acquisition process ends. This is especially useful when acquiring very large areas of tissue with many FOVs. In order to enable this option, check the **Send e-Mail after acquisition finishes** checkbox. You must also fill in the information regarding the **SMTP server** and the **e-Mail address**. Once you fill in this information, **TissueFAXS** will remember it for further use.

6.1.2. Default Experiment Settings

This section contains essential experiment settings.

6.1.2.1. Preview

Preview



In the preview dialog you can manage the stored preview schemes. The adjustable orange area allows the user to define the default preview region on the slide(s).

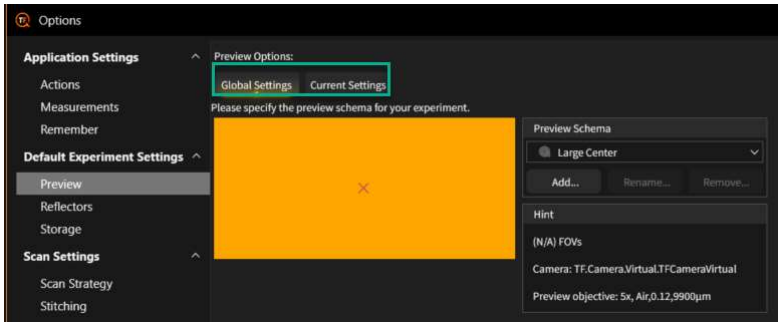
- By default, more **preview schemes** (**All Slide**, **Large Center**, **Normal Center**, **Small Center, TG-Standard**) are present, which cannot be deleted or modified. These schemes are grey. You can add, rename or delete additional schemes from this panel.
- Create new preview area on slides: press the **Add…** button to create a new preview schema according to the size and the location of the sample you are currently using. After giving a name to the newly created preview area, you can modify its size (resize using the mouse cursor on any edge of the shape) or move it (by using the mouse cursor to drag & drop the area to the desired location).
- Set the default preview area on slides by selecting the desired entry from the **Preview Schema** combo box.
- **Set Focus Point**: You can manually set the focus point on the preview schema by right clicking on the preview schema and selecting the desired point.
- Select the label position from the **Label Position** section (default label position is **down**).

Notes:

- The options mentioned here will only affect newly created projects. Please remember the label position is not stored in the preview schema.

- Changes in this panel will affect future experiments. If you want to change the preview area of a current experiment, use the **Preview Options** drop-down from the **Experiment Options** section (in the same dialog).

Preview Options



You can load one of the following **Preview Options**:

- **Global Settings**: the default settings applicable to all future experiments.
- **Current Settings**: the settings made for the current experiment.

6.1.2.2. Reflector settings

Reflector Settings

Fluorescence images are usually grayscale images (from black to white over a certain number of gray values), the intensity of the gray values representing a certain marker staining intensity. In order to acquire a marker, a corresponding reflector (filter block) must be used. When using multiple markers, in order to get a better overview for the entire region, **TissueFAXS** allows you overlaying images acquired with different reflectors. You can choose for each reflector (color channel) the optimal color (Look-Up Table) that will represent the corresponding marker in the final image.

Notes:

Later on, we will use the following terms:

- A *Region Overlay* is the image created by superimposing images of the same region acquired with different reflectors.

- A *channel* is the image of a region acquired with a certain reflector.

You can change the names of the reflectors by going to **Tools → Options → Reflectors**.

The colors of the reflectors will only be used as default for new experiments. Each region can have its own color configuration after acquisition.

You can edit these default colors according to your needs in the dialog below:

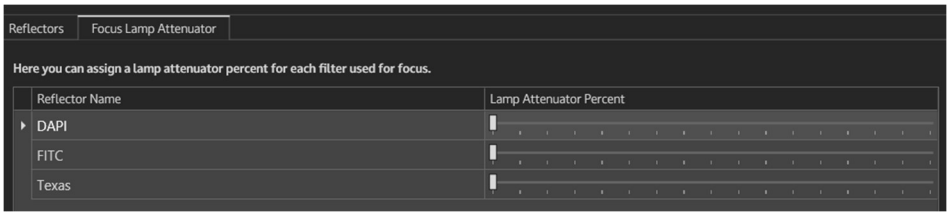
- **Use inverted image for Transmission channel in fluorescence experiments**: choose how the transmission image will appear in the overlay image - the normal image or the inverted image of the Transmission channel (which means a ‘‘fluorescence-like’’ appearance).

- **Use white in preview overlay of fluorescence slide**: for fluorescence experiments, if only one channel is present in the preview settings, the default color for that channel is white. If the preview is made on a nuclear marker like DAPI and is displayed in dark blue, the resulting preview image will be difficult to see against the black background. White, however, gives a stronger contrast.
- Finally, you can choose how the **name** of the reflector will be displayed in the application: **Full name**, **Short name,** or **Abbreviation**.

Focus Lamp Attenuator

This feature plays the role of an anti-bleaching adjustment during autofocus.

In this section you can assign a lamp attenuator percent for each filter used for focus, to avoid bleaching the tissue. The percent of attenuation is applied to the intensity value previously saved for the acquisition channel.



Note: It is necessary to test before scanning if the focus works on darker areas of the tissue with the selected lamp attenuation value.

6.1.2.3. Storage

Storage

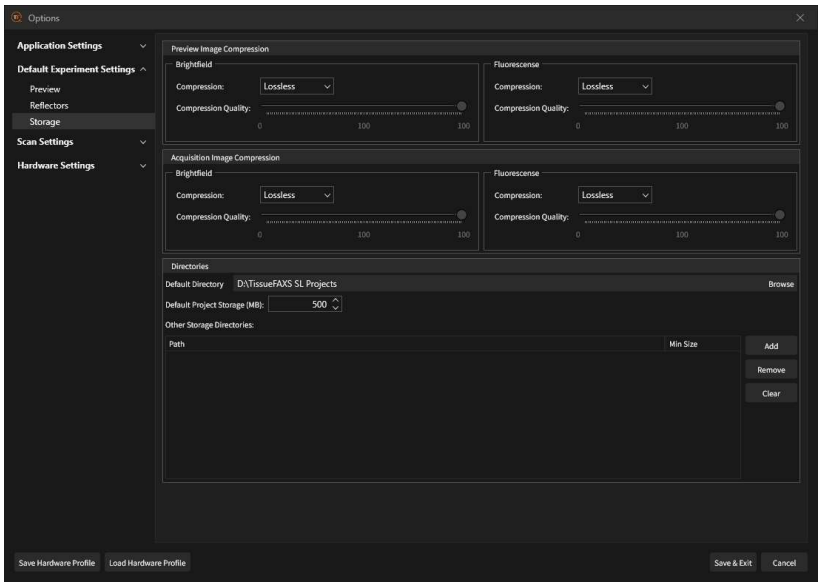


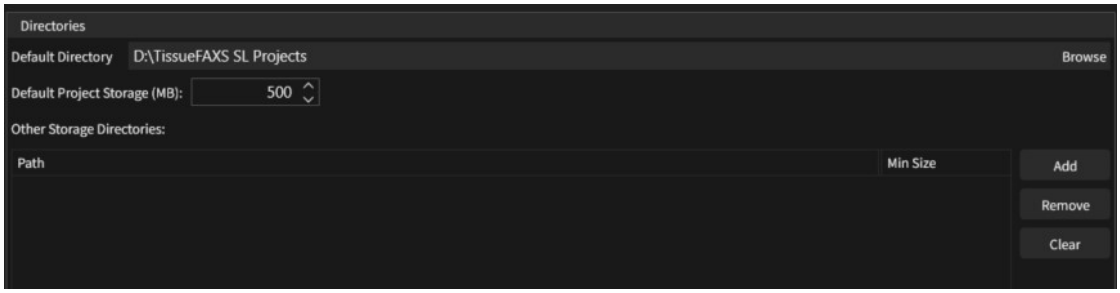
Image Compression

Can be configured for preview and acquisition images.

Compression **types**:

- **None**: no compression is applied. The files are uncompressed images which will occupy a lot of space;
- **Lossy**: offers the best compression ratio, but the quality of the images will be decreased. use Compression quality slider to adjust the compression percent;
- **Lossless**: images will be compressed without quality loss. This ensures the best quality/size ratio.

In the **Directories** section you can define details regarding the storage directories.



- **Default project storage** (in MB): if the storage space is under this amount, TissueFAXS will use the next secondary storage, which has more space than its set minimum size.

- **Other Storage Directories**: you can add in the list more storage directories, remove them or clear the whole list. They are used in case the default storage has less storage capacity than the Default Project Storage.

Note:

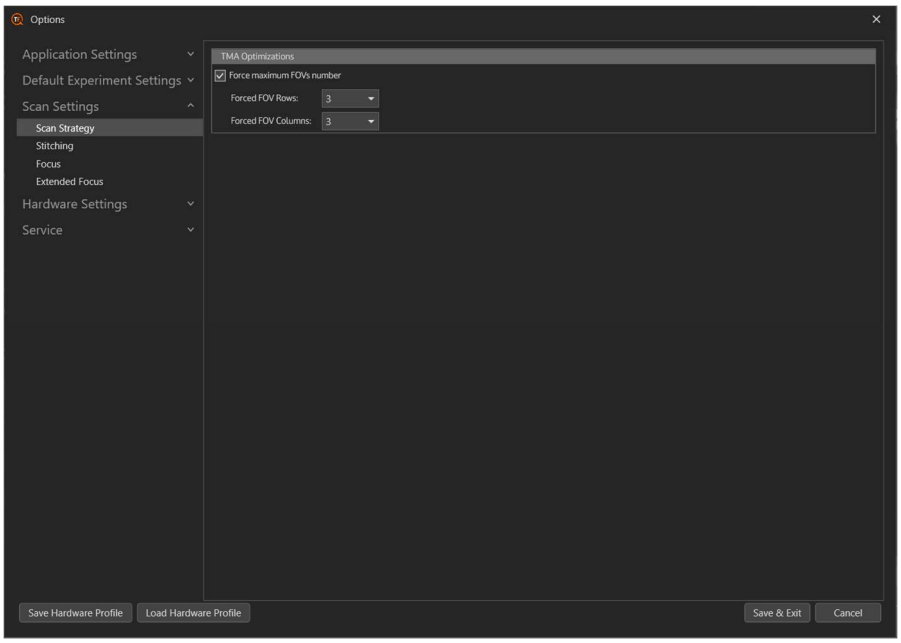
Important: Please avoid storing projects on Desktop, My Documents, or in any other folder located on the system drive (usually C). If the drive is nearly at its full capacity, the system might behave erratically or even stop working if the drive completely runs out of space. Usually, systems delivered by TissueGnostics have a dedicated storage medium for projects. It is advisable to use it to increase TissueGnostics products performance and avoid issues with low disk space. Also, in the event the system needs to be reinstalled (virus infection, system files being deleted, etc.), you might lose data located on the system drive.

6.1.3. Scan Settings

This section contains essential scan settings.

6.1.3.1. Scan Strategy

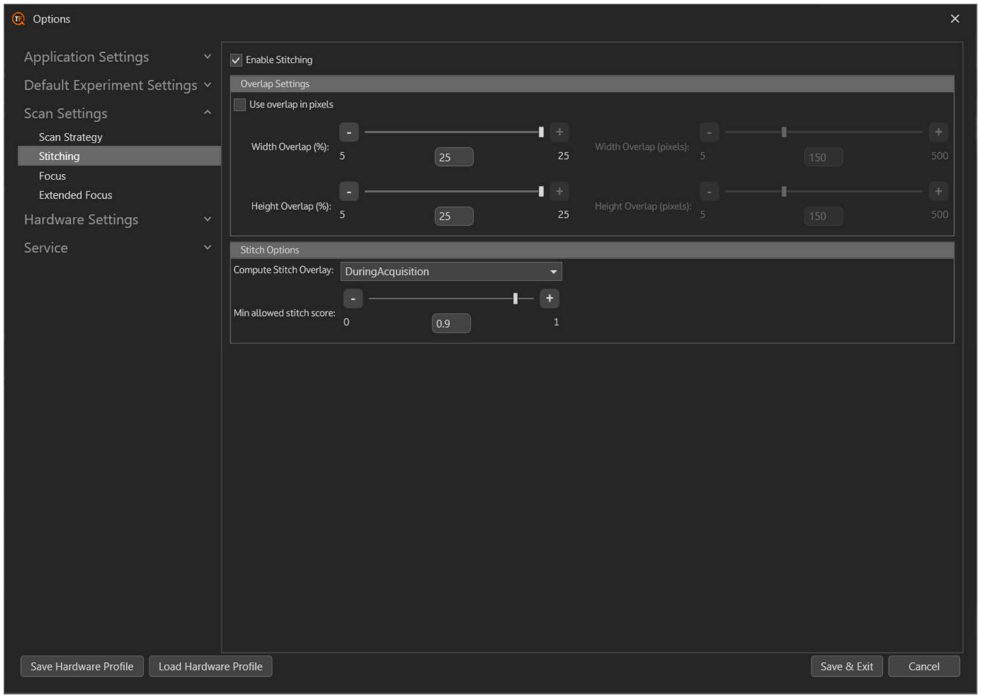
TMA Optimizations



Well Plates and TMA Optimizations: This is a feature that allows the user to specify the maximum number of FOVs that will be acquired for Well Plate regions and TMA Spots. You must check the option and then chose one of the predefined values for FOV Rows and FOV columns.

6.1.3.2. Stitching

Stitching



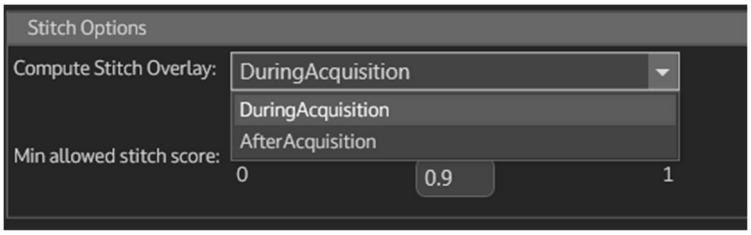
Stitching feature allows seeing high quality pictures without any inconveniences caused by stage errors, camera calibration or other issues that might interfere with proper image display/acquisition.

To enable this feature, you must mark the **Enable Stitching** checkbox.

Overlap settings

- **Overlap settings**: the external and internal stitching will work properly if the regions are acquired with an overlap that hardware errors. A value of at least 15% is recommended. Avoid acquiring regions with large empty areas when using stitching.
- **Width/Height Overlap (in percentage and in pixels)**: these are used to specify the percentage or amount of pixels of width/height that will overlap neighboring FOVs. The overlap can also be set in pixels.

Stitch options



Compute stitch mode:

- **During Acquisition**: this option will slow down the acquisition process; the user will be able to see the acquisition results in real time.
- **After Acquisition**: this option will call the stitch after each region has been acquired.

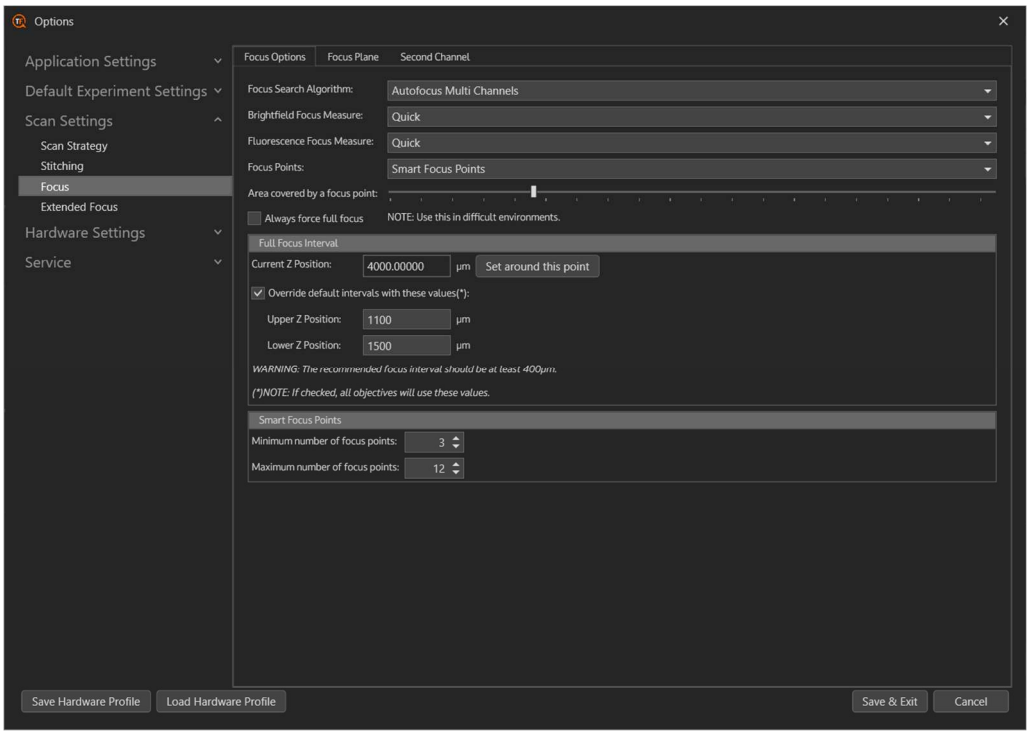
Min allowed stitch score: decrease this value only if you are acquiring regions with a large empty area. Otherwise, it should be set to the default value.

Note: Stitching will not be able to compensate for major hardware errors (defective stages) or major errors in configuration (FOV sizes, rotated cameras, etc.)!

6.1.3.3. Focus Settings

In this section, you can adjust focus-related parameters, which organized in two tabs: **Focus Options** and **Enhanced Focus**.

Focus Options tab



1. Focus Search Algorithm

- **Autofocus Multi Channel**: this is based on customizable focus measure algorithms - predefined and user custom defined, which can be separately set for BF and FL (more details later in this chapter).

  Predefined Focus Measure Types for BF and FL:

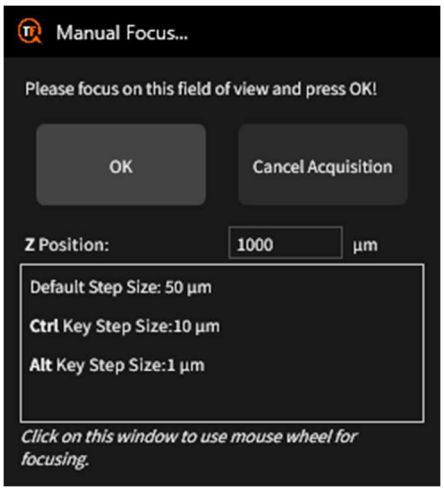
  - **Quick:** faster focus;
  - **Balanced:** best compromise between focus speed and accuracy.
  - **Quality:** best focus, but takes a little longer.

Note: The Full Focus will use all the focus stages defined in a focus category and the Quick Focus will use the focus measure defined within the last step.

- **Autofocus (Legacy)**: when using this type of focus, **TissueFAXS** will automatically detect the best focus position based on the parameters described below. The **Autofocus** method consists of two steps. In step one, using a fast method, **TissueFAXS** tries to determine a short interval where the focus is best. In step two, using a high precision method (specified by the **Fine Focus Measure** parameter), **TissueFAXS** determines the best focus position from the fine focus interval determined in step one. Generally, to determine the fine focus interval, **TissueFAXS** uses the **Rough Focus Measure** and scans the entire

interval specified in the "**Focus interval**" section. This approach is called "**Full Focus.**" However, to improve the speed of the focus, if a previous focus position was found, **TissueFAXS** will directly use the **Fine Focus** on a small interval around the last focus position. This approach is called "**Quick Focus.**"

- **Manual**: when using this type of focus, the user will be prompted to manually focus the image when necessary. For **Manual Focus** method, the window allows the focus position to be set by manually entering the Z position or by using the mouse wheel in the same way as in live image. This is very useful for manual focusing on systems where the microscope Z wheel is hard to reach.



- **Current Position:** no type of focus will be performed and the stage will maintain the same focus position for all FOVs during acquisition.

Notes:

- If the **Quick Focus** method fails, a **Full Focus** will be automatically performed.

- If focus plane is used, then **Full Focus** will be used for each focus point.

The following parameters are for autofocus only and have no effect on the **Current position** or **Manual Focus**.

2. Always force full focus

The last focus position is ignored and the focus is always searched in the full interval. This method will take more time, but the obtained results will be better for difficult samples and environment (e.g. samples with many tissue folds).

3. Focus Points

- **Fixed Focus Points**: the focus is performed on the FOV from the center of the block determined by the **Focus Strategy Neighbors**. **Fixed Focus Point** is most frequently used in the acquisition process, when your tissue sample is compact (without holes, for example);
- **Floating Focus Points**: the focus is performed in a point that may not be the center of the block. **Floating Focus Points** are especially used for biopsies or when your regions to be acquired are not compact (e.g. they include holes). The method identifies the location of the tissue within one block of FOVs and selects the most inner one. This location may be between several FOVs and not necessarily identical to one of the FOVs.

Notes:

- **Focus Strategy Neighbors** can be adjusted according to the flatness of your tissue section, as the focus directly depends on the subsidence of the tissue. If the tissue section would have been perfectly flat, a single focus for its entire surface would have been enough. But more often, each tissue section has its own relief and the focus must be performed repeatedly. The higher the magnification, more the relief will influence the focus.

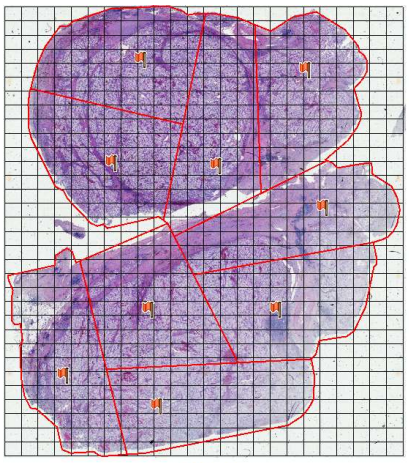
- **Focus Strategy Neighbors** helps you save time when performing the focus, by avoiding unnecessary focusing. The regions to be acquired will be split in blocks of Fields of View. The **Focus Strategy Neighbors** parameter defines how many FOVs will share the same focus estimation. In the actual example, 3x3 is selected, in which case **TissueFAXS** will focus once for every block of 3X3 fields of view. (The location of the focus point within the block is based on the Focus Points parameter). After focusing in one point, **TissueFAXS** will scan all neighboring FOVs using the same focus position.

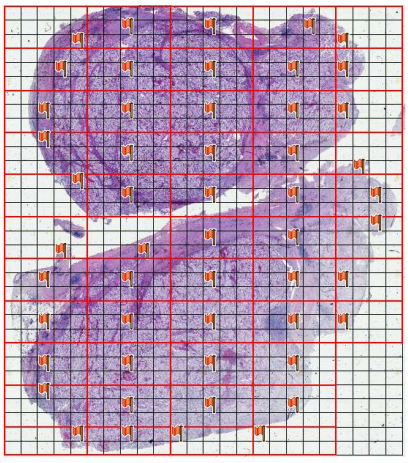
- **Smart Focus Points**: a number of focus points is determined by the user selected area covered by a focus point. The number of focus points is limited by a minimal and a maximal given value (minimum 4 and maximum 12), depending on the area of the region. This option is available when Compute focus plane is enabled and it optimizes the number of focus points used for a plane. This focus points distribution strategy uses the preview image to find the best positions in order to avoid low contrast areas (holes which are usually outliers) that are outside of the tissue plane.

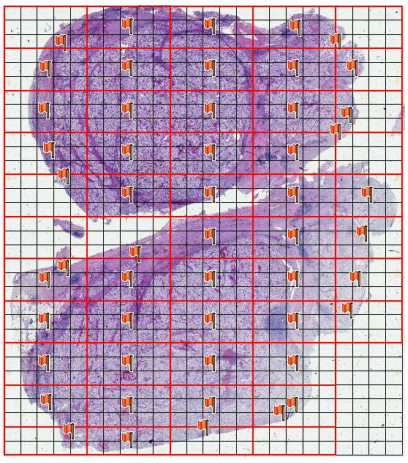
You must select the minimum and the maximum number of focus points.

Note: The number of focus points of the region is calculated based on the area covered by a focus point.

- **Fixed Focus Points by FOV Score (experimental)**: the focus is calculated based on the FOV score. If the FOV score value is below a certain threshold, this method helps to avoid focusing on areas without tissue.


SMART FOCUS POINTS


FIXED FOCUS POINTS


FLOATING FOCUS POINTS

Override Focus Interval

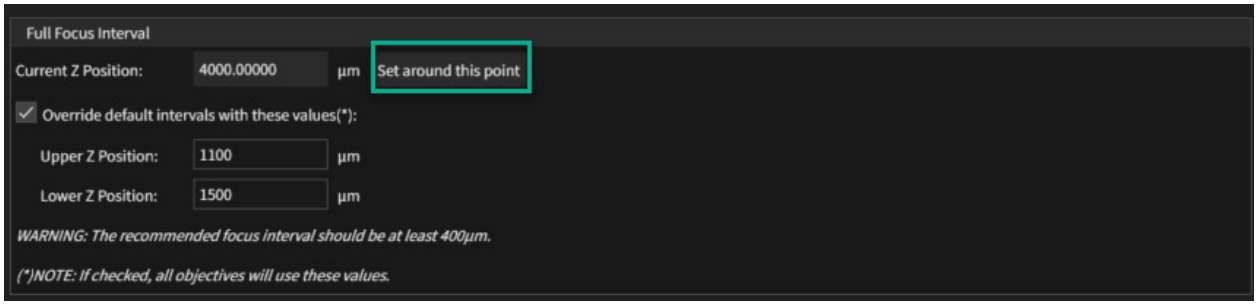
For each focus measure, there is a configured focus interval (i.e. for BF Quick 20x you can have a different focus interval than for Bf quick 2.5 etc.).

If you use this override option, previous focus interval settings will be ignored.

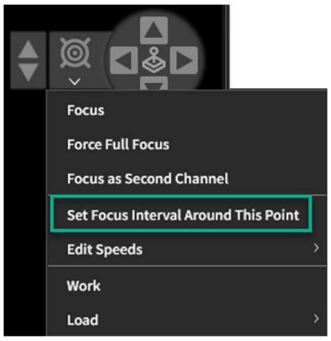
To determine the proper focus interval, we recommend manually focusing with the objective of interest. You can then check the current Z-position in the camera window and add 250 µm above and below this position for the upper and lower Z-positions of your interval. The current Z

position should be read from the **TissueFAXS** software (in Camera window) and not from any display of the microscope. Larger focus intervals will make the autofocus slow and may cause the microscope to focus on objects laying on the cover slip.

The **Set around this point** is meant to automatically set the Z interval within +/- 200 around the current Z position (the position where the tissue is in focus), to restrain the focus interval and to reduce the duration of the focus process.



This option can also be accessed from the Live Image Focus section.



Notes:

- If you want to use the specified focus interval for all objectives, check **Override default intervals with these values**.

- If you want to use the default focus interval only, uncheck **Override default intervals with these values**.

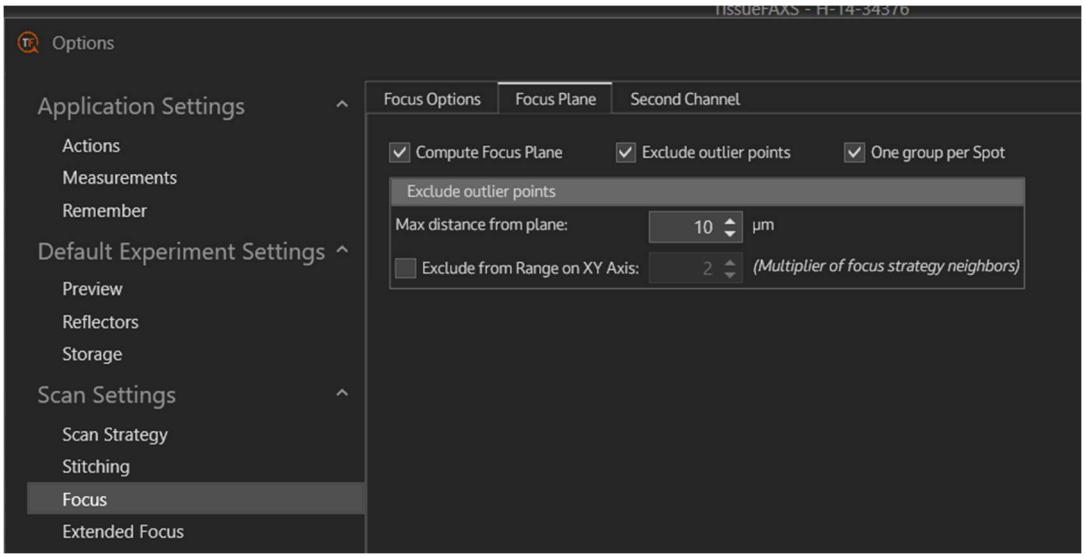
- If checking **16 bit**, the focus will be computed on 16 bit images.

- *Remember to be very careful when making focus related changes, as wrong settings could cause damage to your sample and your objectives.*

- For all the items described in the **Focus** section, some **tool tips** are available. This is a short description that appears when the cursor is over an item.

Focus Plane tab

The focus plane method focuses on the tissue at the beginning of the scan. A focus plane is computed based on the resulting focus points.



The multi-plane focus calculates an “equilibration surface” over the entire tissue. Each FOV might have a slightly different focus compared to its neighbors but big differences between one focus group and a neighboring focus group will be avoided. This multi-plane focus can also detect outliers and will skip them (not take them into consideration for the calculation of the equilibration plane) if their distance from the average is above a certain threshold.

The multi-plane focus is still based on the “focus group” strategy, i.e. the autofocus is done in a matrix of 3x3, or 5x5, or 7x7 FOV – as always, BUT all focus points are acquired BEFORE the actual scanning starts. As previously mentioned, each FOV might have a slightly different focus. Focal changes across a sample will lead to a continuous change of the focus position from FOV to FOV rather than sudden larger changes.

Compute Focus Plane: computes focus plane if checked; otherwise the standard focus group strategy will be used.

Exclude outlier points: if checking this option, after all focus points are computed, the ones that exceed the established limits will be excluded when computing the focus plane;

One group per TMA spot: because a TMA spot is quite small, a single focus action is usually sufficient to reduce the acquisition time. However, when working with a larger TMA spot, you can uncheck this option to ensure that the entire tissue will be in focus.

Exclude outlier points

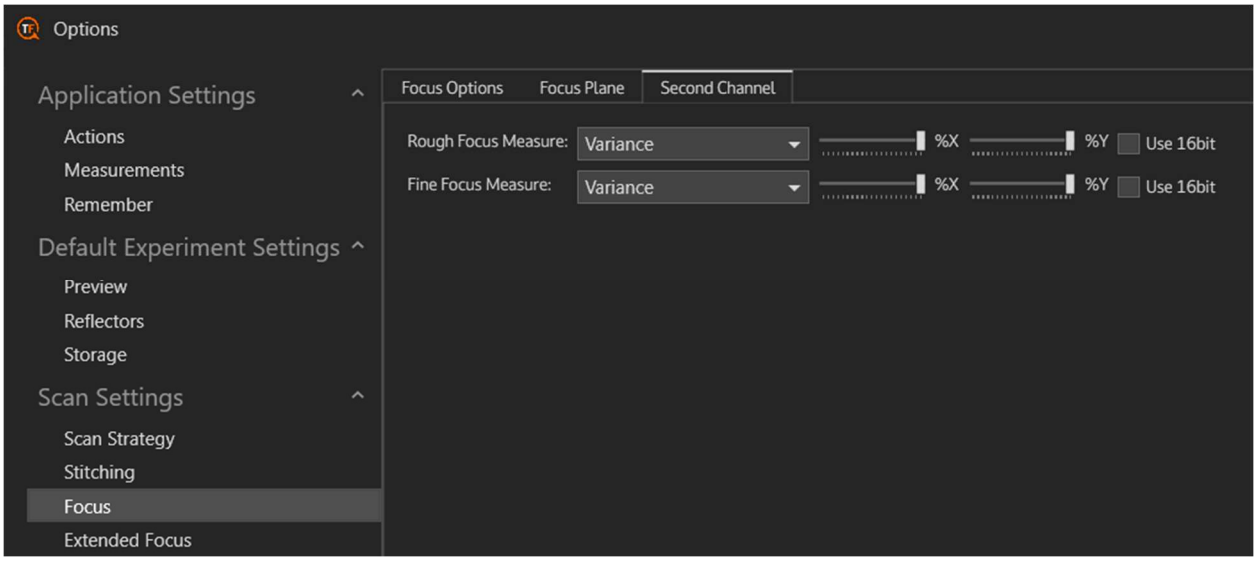
- **Max distance from plane**: focus points that exceed the maximum distance from the average focal plane will be considered outliers and excluded from computing the focus plane;
- **Exclude from range on XY axis:** if checking this option, each focus point will be checked as outlier based on all neighboring focus points situated in a specified range on XY.

Second Channel tab

The second channel focus gives the possibility of scanning on a second channel.

The regular channel specific offsets are not effective on biological structures which are in a different layer of the sample. For example, when the focus is performed on DAPI, the second focus channel allows performing another fine focus on the TexasRed channel, so that structures appearing in that channel are in full focus.

The workflow of the second channel focus implies the following steps:
- focus on first channel as usual
- from the position found on first channel, do a full focus on second channel in an interval centered in that position and with specific stages for the second channel
- the position found will be used to acquire all channels using the channel offsets



The following settings are available:

- **Rough Focus Measure**: choose the desired method (Variance, HP, HP Fine, Average, Average Complement) and then set the percent for both X and Y axis.
- **Fine Focus Measure**: choose the desired method (Variance, HP, HP Fine, Average, Average Complement) and then set the percent for both X and Y axis.

6.1.3.4. Extended Focus

TissueFAXS software offers the option to enhance image quality for each scanned Field of View by capturing a stack of images below and above the focus position and creating a merged image.

The **Default** algorithm works for both Brightfield and Fluorescence acquisition and creates an image with better details blending the information from the image stack.

There are also three **alternative projection methods**: **SUM, MAX** (recommended for Fluorescence, especially Confocal type) and **MIN** (for Brightfield only). SUM means that each pixel in the final image is a sum of all the corresponding pixels from the image stack). MAX means that each pixel in the final image has the maximum intensity value of all corresponding pixels from the image stack. MIN is like MAX, but it takes the minimum intensity.

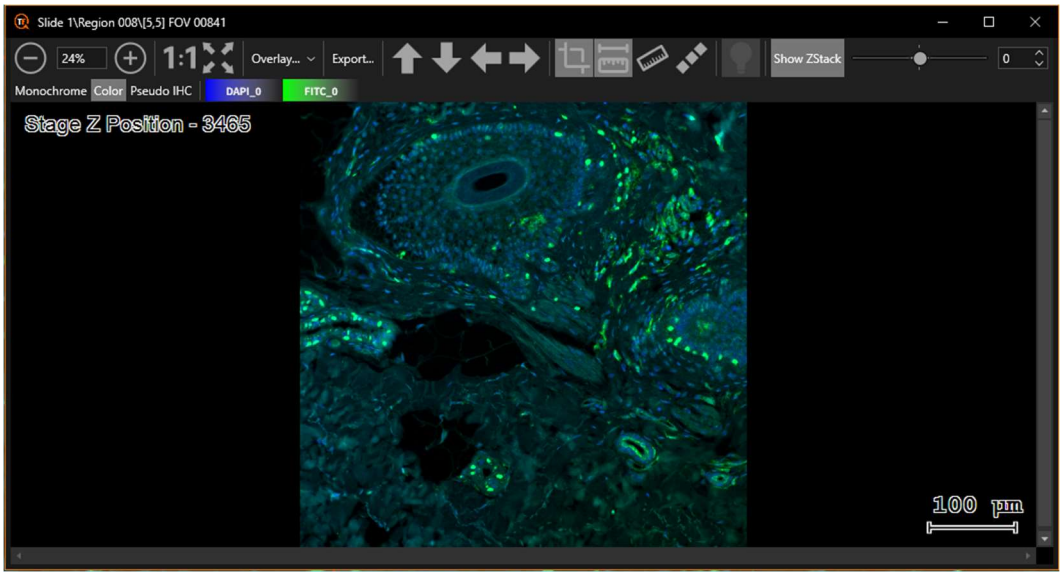
Z Stack

Z Stack represents a set of images that are acquired above and below the optimal focus position.

Check this option only if you want to visualize the region in 3D using TissueFAXS or some external software for 3D or image fusing. Enabling this option will require more space on the hard drive.

You can choose to use the Z stack for each individual reflector by marking the corresponding checkbox from the **Save Z Stack** column.

Note: To be able to use the Z stack for individual channels, you must have checked the Save Z Stack option below the reflectors' section.



You can navigate through a Z-stack by using the slider to look at different focal planes.

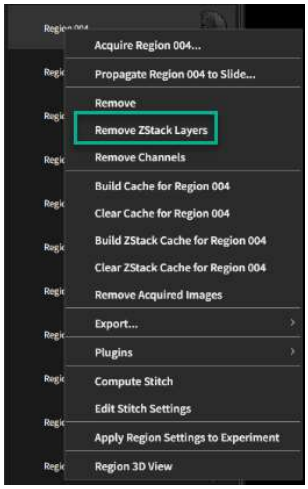
Notes:

- Computing the cache for an entire z-stack will be time consuming and require a lot of memory. Therefore, the computation of the cache for an entire z-stack is only done if the user chooses this operation.

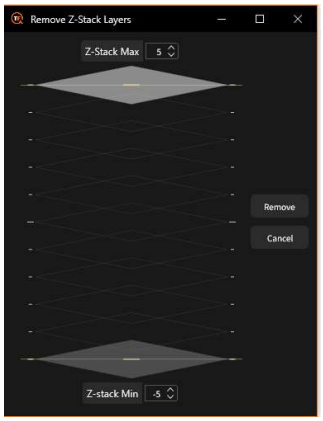
- You can remove z-stack layers by choosing the **Remove Z Stack Layers** option from the contextual menu of the region.

Deleting individual layers from a Z-Stack

By right clicking on a region acquired with ZStack, you can access the **Remove ZStack Layers** option.



In the **Remove ZStack Layers** dialog, you set the desired steps above and below to remove ZStack layers from your region.

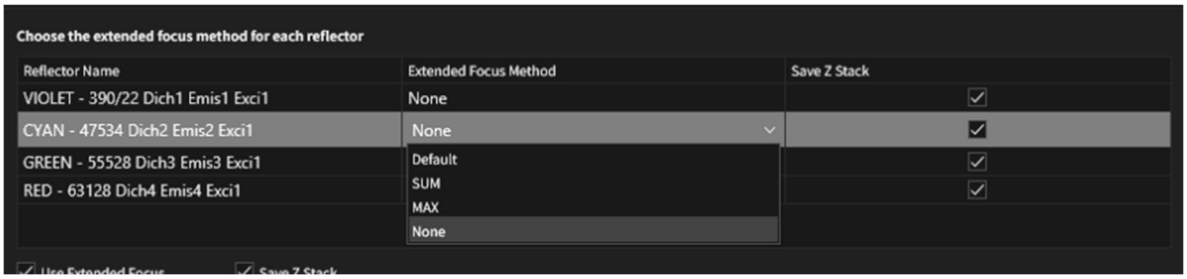


Use Extended Focus

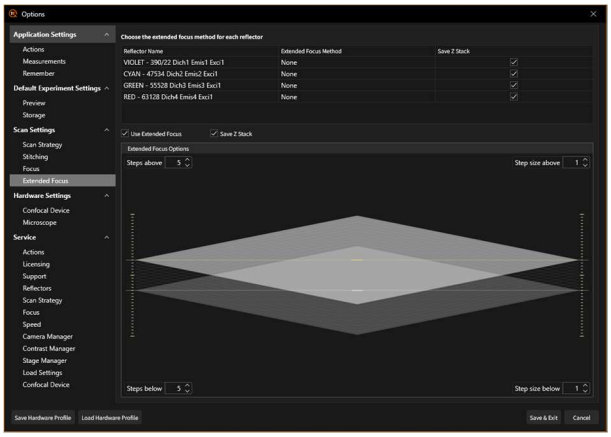
For each reflector present in the system, you can choose the **extended focus method** used to combine the Z Stack.

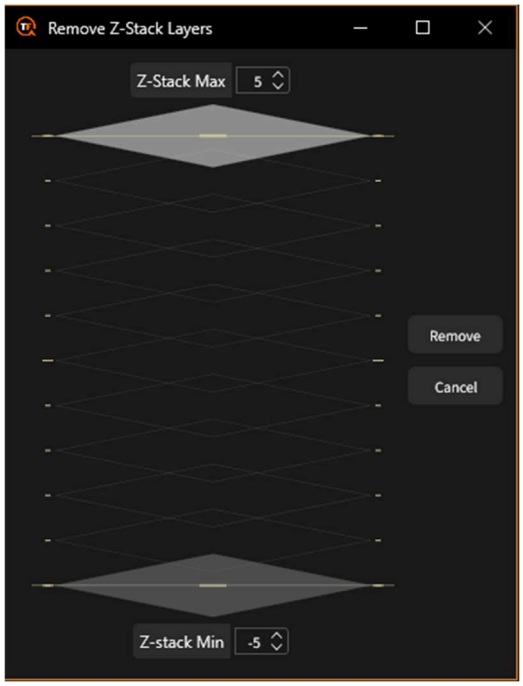
Check this option only when objects are present on multiple focus plans.

If you don't want to apply the extended focus to one or more reflectors, choose the **None** option from the reflector's dropdown list.



Extended focus options





You can specify the number of steps above or below the automatically computed focus position as well as the step size.

Notes:

- To be able to use the extended focus for individual channels, you must have checked the **Use Extended Focus** option below the reflectors' section.

- Using any of the two options (**Use Extended Focus**, **Save Z Stack**) will significantly increase the acquisition time.

6.1.4. Hardware Settings

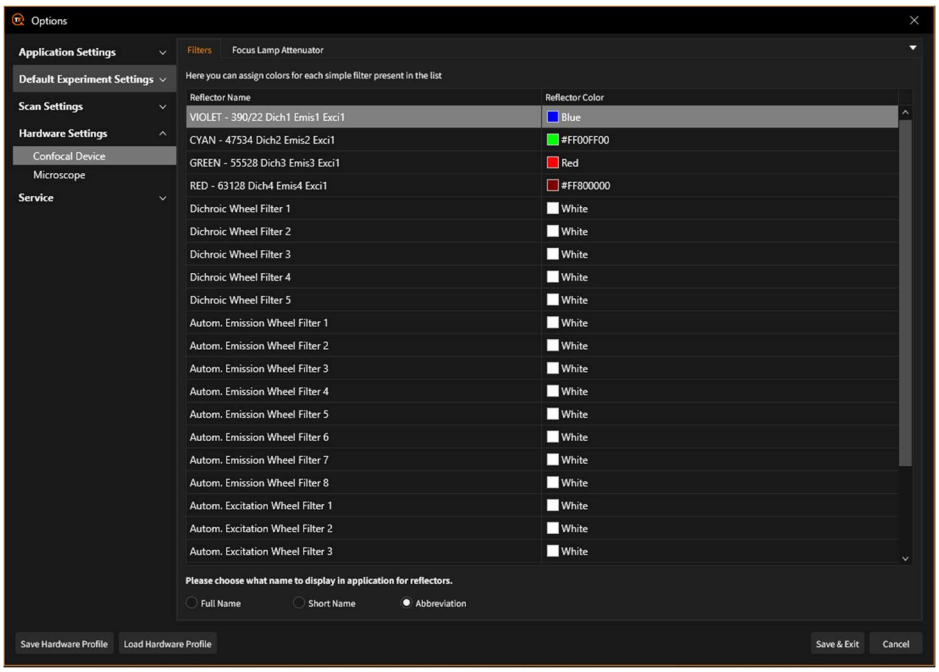
This section contains essential hardware settings.

6.1.4.1. Confocal Device

Filters

Colors can be assigned for each simple filter present in the list.

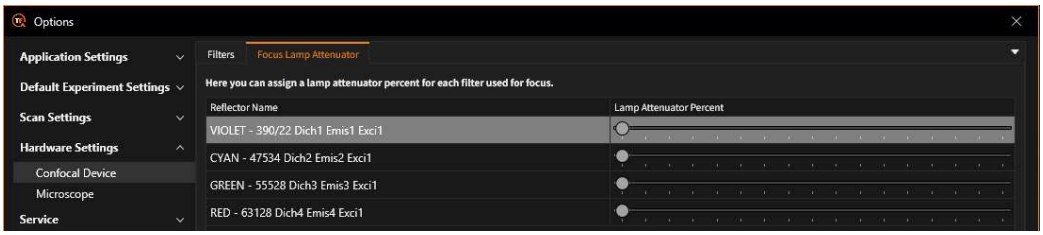
Names can also be set to display in the application for the reflectors.



Focus Lamp Attenuator

This feature plays the role of an anti-bleaching adjustment during autofocus.

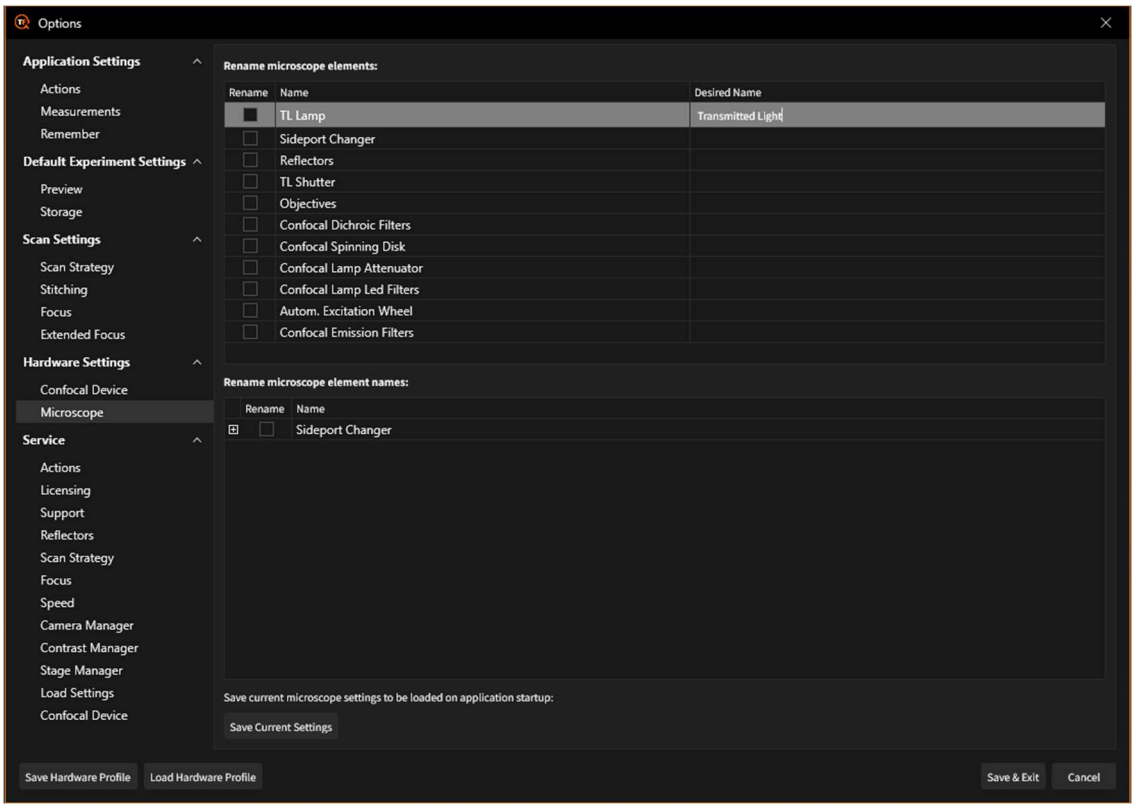
In this section, a lamp attenuator percent can be assigned for each filter used for focus to avoid bleaching the tissue. The percent of attenuation is applied to the intensity value previously saved for the acquisition channel.



6.1.4.2. Microscope

Rename microscope elements: select an item then type desired new name.

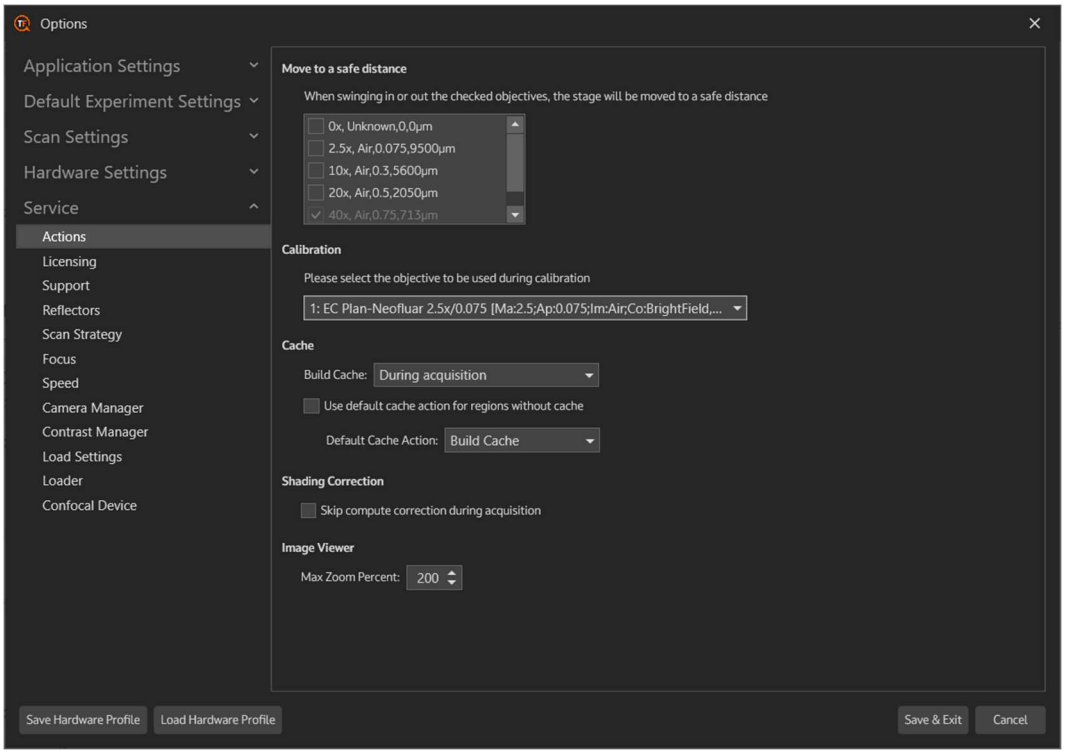
Also, the position of the elements can be configured.



6.1.5. Service

This section is only available if logged in as **Service**. It contains fine tuning settings for TissueFAXS and its hardware.

6.1.5.1. Service Actions



Safety distance

Safety distance means the Z-position to which the stage is lowered in order to ensure that no objective lens collides with the slides or any part of the stage.

When moving between slides or when switching between objectives, the stage will be moved to a safe distance.

Note: By default, for any objective with magnification higher than 20x, **TissueFAXS SL** will lower the stage to the safe distance. For the rest of the objectives, this behaviour may be customized in the dialog shown below. Usually, this behaviour is enabled for any longer objective or for objectives that come very close to the slide for focus. For inverted microscopes, the best action is to enable it for all objectives.

Calibration

This is a safety that should be accessed after installing **TissueFAXS SL** to choose a safe objective for calibration. This choice means that the application is helping to avoid possible damages of the objectives, stage or slides, caused by an inappropriate objective being used during the calibration procedure.

If an objective with a magnification higher than 20x is selected for calibration, **TissueFAXS SL** will send a warning message before performing the calibration.

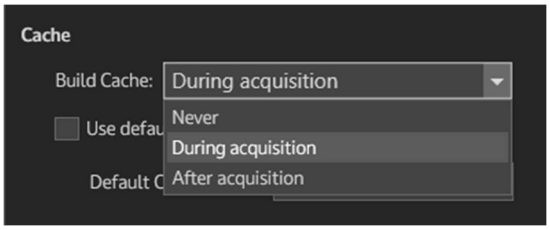
Cache

The image cache consists of scaled-down acquired images. It is used to speed up the display of large regions and decrease the memory requirements for this operation. If no cache is built, image viewing is available for large regions from a minimum zoom level and fits only a few FOVs displayed at the same time.

Note: Other operations like export and analysis are not affected in any way by the presence or lack of the cache.

Building the cache:

- The cache can be built by choosing one of the options from the Build Cache dropdown box (please see image bellow)



- **Never**: The cache will not be built during acquisition. This will slightly decrease acquisition time. The decrease will only be noticeable with regions larger than 1000FOVS.
- **During acquisition:** As each FOV is acquired it is processed and smaller versions are created. Larger regions might increase the total acquisition time. The GUI will respond slower if the region is opened.

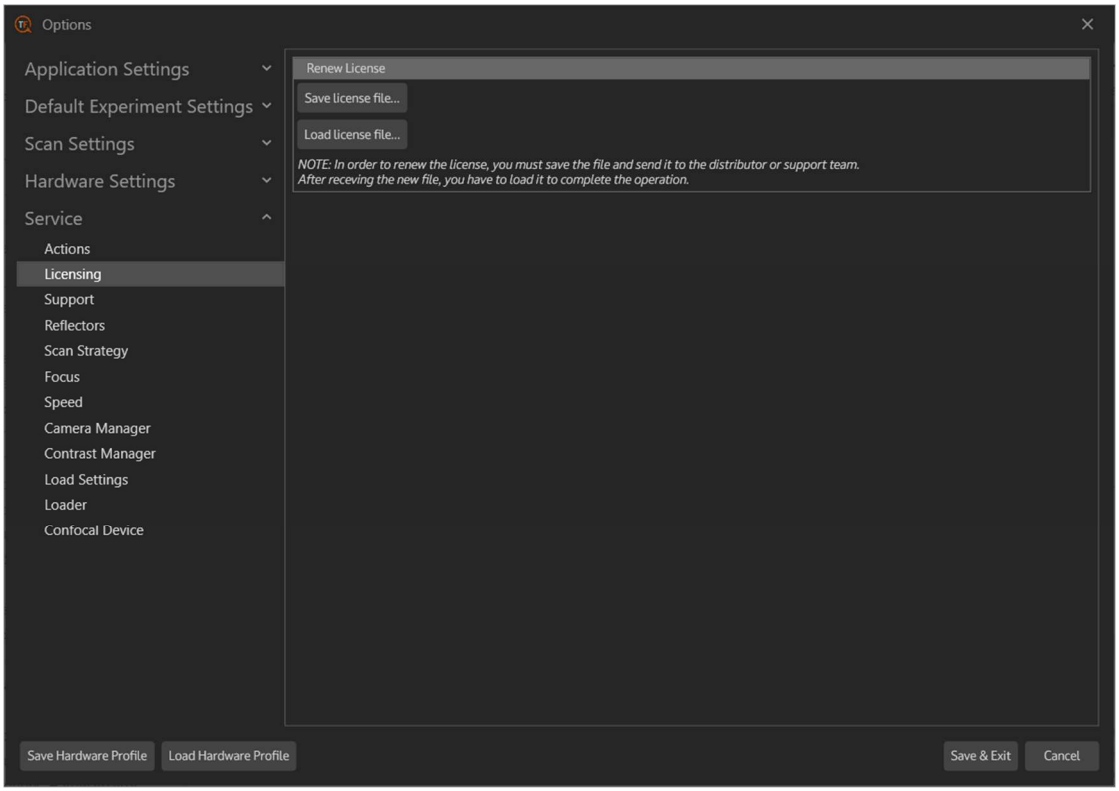
- **After acquisition:** if the cache is performed at the end of the acquisition process, a progress bar will inform the user about the status of the action. This will increase the acquisition time by 10 to 15 percent.
- The cache can also be built when opening a region, if **TissueFAXS** → **Tools** → **Options** → **Actions** → **Use default cache action for regions without cache** is checked and **Build Cache** is set for **Default Cache** actions**.**

Notes:

- If **Use default cache action for regions without cache** is not checked, **TissueFAXS SL** will ask if you wish to build it when opening a region without cache.

- **16-bit mode** available for building cache.

6.1.5.2. Licensing



The **TissueFAXS** license can be renewed in the **Licensing** dialog by following these steps:

- Use the **Save license file…** button in order to save the license file on your hard drive;
- send this file to your local TissueGnostics distributor to be renewed;

- use **Load license file…** to reload the new license file to the application.

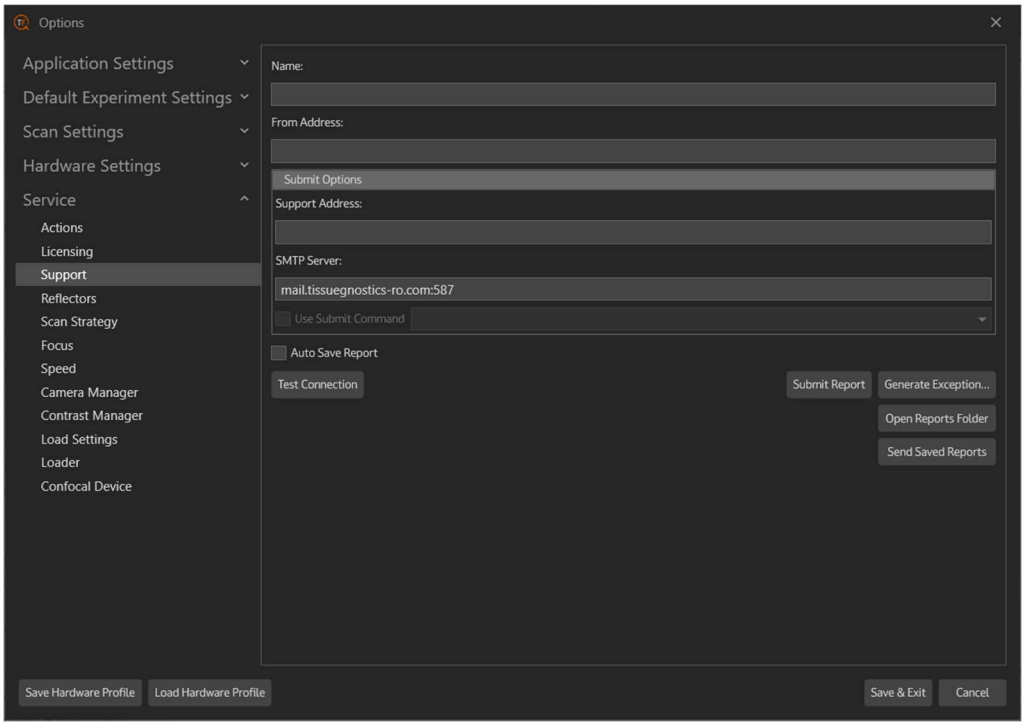
Notes:

- Please use the **Licensing** feature ONLY if you are instructed by TissueGnostics personnel or authorized distributors.

- **Licensing** feature is only available if logged in as service.

6.1.5.3. Support

As with any software application, occasional errors may be encountered during the use of **TissueFAXS**, The Support section was designed to help in situations requiring assistance and answers to TissueFAXS-related questions.



Enter the following **information for dynamic error reporting**:

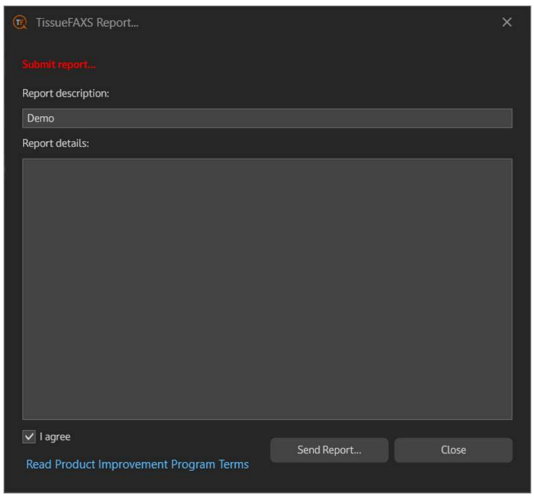
- The real name of the user who bought the software (e.g.: institution name);
- The email address that will be set as sender;
- The email address of Tissue Gnostics support;
- The available SMTP server/port to be used when sending the error report. (Contact your network administrator for details).

Use Submit Command: check this to access an external executable file that will send the files to a server.

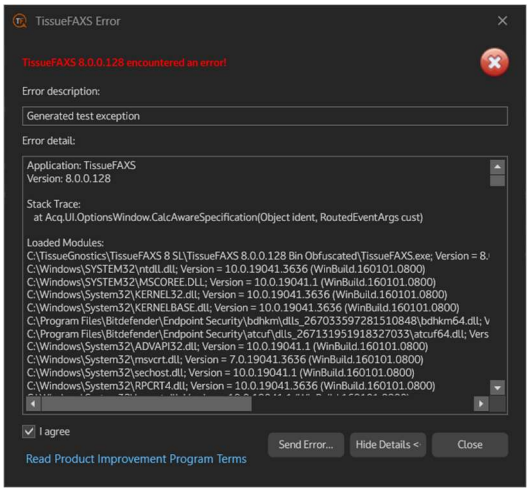
Auto Save Report: check this option to save the log files on the hard drive, whether or not the report is sent.

Test Connection is used to see if the connection to SMTP server works.

Submit Report will help send an email containing your questions or problems regarding **TissueFAXS**. Beside the information typed in the form, the email automatically includes the log files, the running processes, and the configuration files of the application. To effectively send the mail, press the **Send Report** button.



Generate exception is used to simulate an error to test if sending emails with the specified settings works.



- **Error description**: is a short description of the error.
- **Error detail**: is detailed data related to the error.

Open Reports Folder: if a report is not sent, it will be automatically stored in a local folder; press **Open Reports Folder** to open that folder.

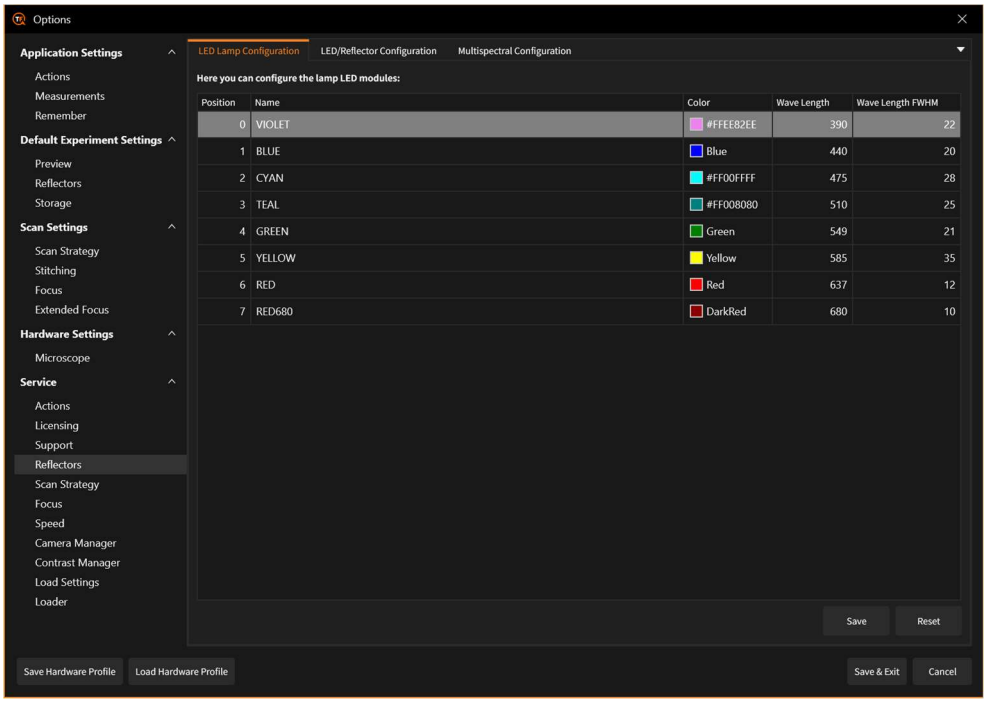
Send Saved Reports: press this button to send all unsent reports, if any. After effectively sending saved reports, they will be automatically deleted from the local folder where they were stored.

6.1.5.4. Service Reflectors

Fluorescence images are usually gray scale images (from black to white over a certain number of gray values), with the intensity of the gray values representing a certain marker staining intensity. A corresponding reflector (filter block) is used to acquire a marker. When using multiple markers, in order to get a better overview for the entire region, **TissueFAXS SL** allows overlaying images acquired with different reflectors to get a better overview for the entire region. For each reflector (color channel) the optimal color (Look-Up Table) can be selected to represent the corresponding marker in the final image.

LED Lamp Configuration

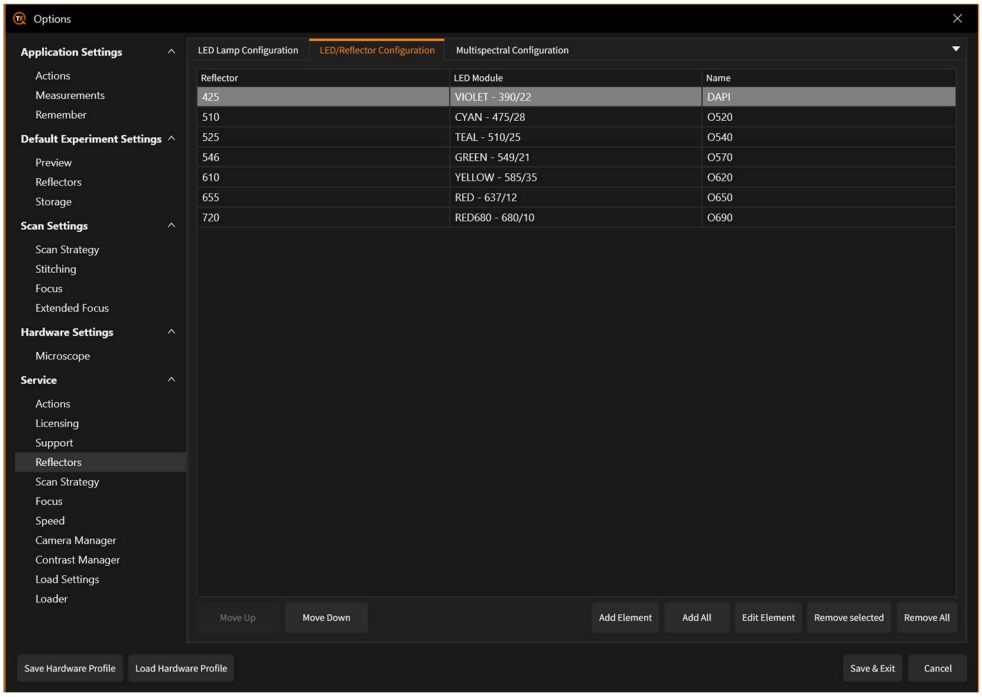
Here you can configure LED Lamp led modules: name, color, and wavelength.



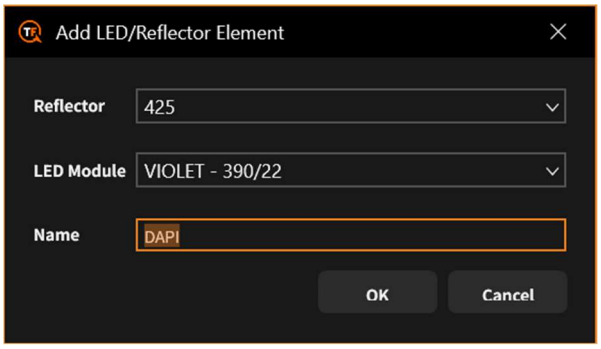
LED/Reflector Configuration

In this section you can manage the LED/Reflector configurations for your system.

For TissueFAXS systems with FL lamps with multiple configurable light sources, such as X-Cite XLED1 or Lumencor Spectra X and Spectra 3 lamps each independent LED has its own spectrum and must be matched to a reflector from the microscope turret. An LED is considered a single independent light source from the lamp.



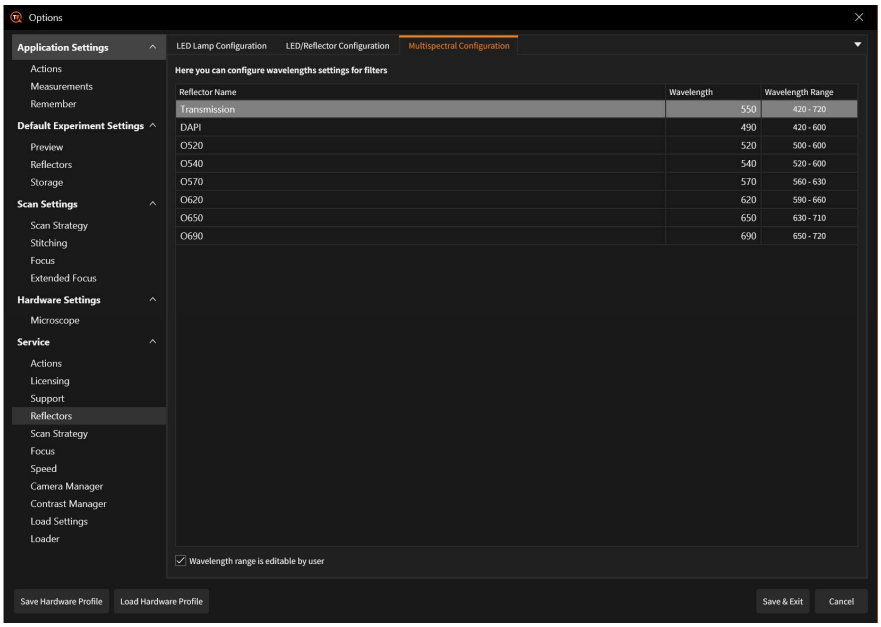
Press **Add Element** to create a new Led/Reflector element. Select a reflector, an LED module, choose a name for the new element, and then press **OK** to create.



Press **Remove selected** to remove selected element or press **Remove all** to remove all the elements from the list.

Multispectral Configuration (for Spectra systems only)

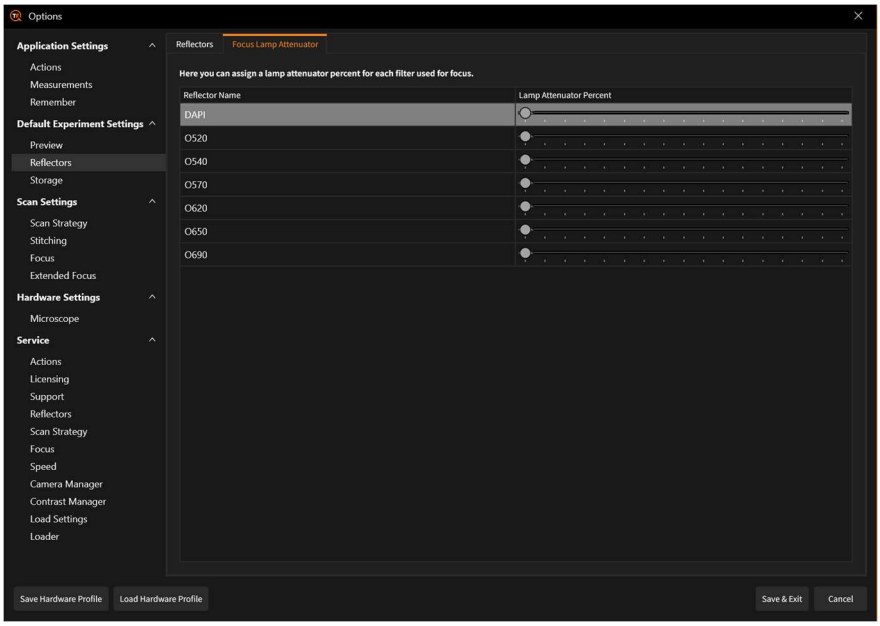
The Multispectral configurations for the system can be managed in this section.



Focus Lamp Attenuator

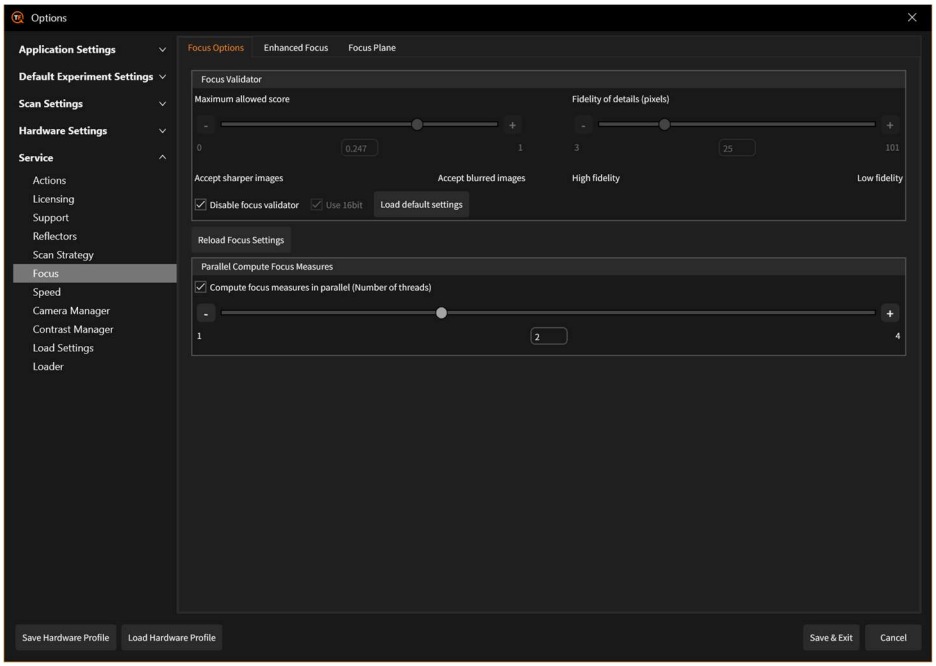
This feature plays the role of an anti-bleaching adjustment during autofocus.

In this section, a user can assign a lamp attenuator percent for each filter used for focus in order to avoid bleaching the tissue. The percent of attenuation is applied to the intensity value previously saved for the acquisition channel.



Note: First test to determine if there will be enough signal in images for an accurate focus.

6.1.5.5. Service Scan Strategy



Use software backlash correction: This feature helps to avoid certain errors that may occur when changing the moving direction of the stage (caused by physical characteristics of the stage) during normal **TissueFAXS** operation. It applies only to the XY axis of the stage. Using this feature to improve he stage precision may increase the acquisition time as much as 30%. The backlash correction should be used when the acquired images do not properly fit to each other. This option should only be used with stages with low precision.

Note: If all the images and camera alignment have a stitch problem, you should check the FOV calibration settings.

If software backlash is enabled you can configure on which axis needs to be applied and also on which direction: i.e. for X axis when movement is done from right to left if option “Right to Left” is checked or left to right if option is unchecked.

Acquisition Scan Strategy

Choose the type of scan strategy:

- Snake scan (will use the default scan strategy)

- Scan right to left
- Scan left to right

Filter Sort Mode

Select the filter sort mode:

- Normal: runs filters 1,2,3,4 then 4,3,2,1.
- Transmission optimized: normal sort but will select the closest transmission reflector.
- Best permutation: computes shortest path by checking all possible permutations.
- No sorting: will not sort the order of the filters, will run filters 1,2,3,4, then 1,2,3,4.
- Reverse no sorting: no sorting and reverse order 4,3,2,1 then 4,3,2,1.

Note: Important - Use only if instructed by TissueGnostics support team. Otherwise, leave unchecked.

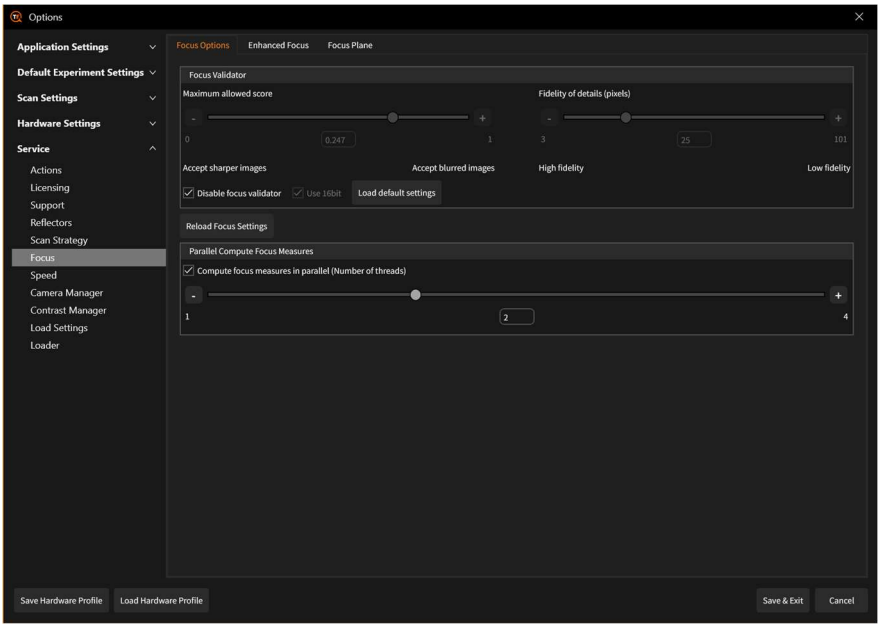
Use Enhanced Camera Mode: this feature, available on some cameras, will increase the data transfer speed.

Maximum size for the image saving queue: can increase or decrease the number of images enqueued for saving until the acquisition stops temporarily in order to allow image saving process. When the queue is full, in TissueFAXS status bar you will get the message: "Saving pending data". This usually happens on Z-Stack or multispectral acquisition.

Filter change delay: on some system, it is necessary to add a delay when filter changes from a position to another, as the filter turret may introduce vibrations and cause images with motion blur.

6.1.5.6. Service Focus

Focus Options



Focus Validator

The **Focus Validator** checks and confirms that the focus search algorithm has obtained a good quality image.

It is used to confirm that the **Quick Focus** method has worked correctly. If the quality score it provides is low, then a Full Focus search is initiated.

It is also used to confirm that the **Full Focus** method algorithm has obtained a sharp image. If this validation fails, the system will use the last known good focus position.

Normally, the default values for the validator options should be enough to provide a good acquisition.

- **Disable focus validator**: will disable any focus validation and, as a consequence, any images returned by the focus algorithm will be accepted as being in focus. It should only be used as a last resort when the sample has a very low contrast.
- **Maximum allowed score**: increases the tolerance of the focus validator. Setting low values means that only very good images will be accepted as being in focus. Setting high values means accepting lower quality images (e.g. blurred). Therefore, this parameter should be increased when using samples of a lower contrast.
- **Fidelity of details (pixels)**: size of relevant details. If set to a minimum (3), then the finest details will be searched and will make a difference in the validator score. If you set

to a higher value, then only rough elements will count in computing the score. However, this depends on the images being processed. Most of the time, the default value should provide good results.

- **Use 16 bit**.
- **Load default settings**, if pressed, will restore the default values for Maximum allowed score and Fidelity of details.

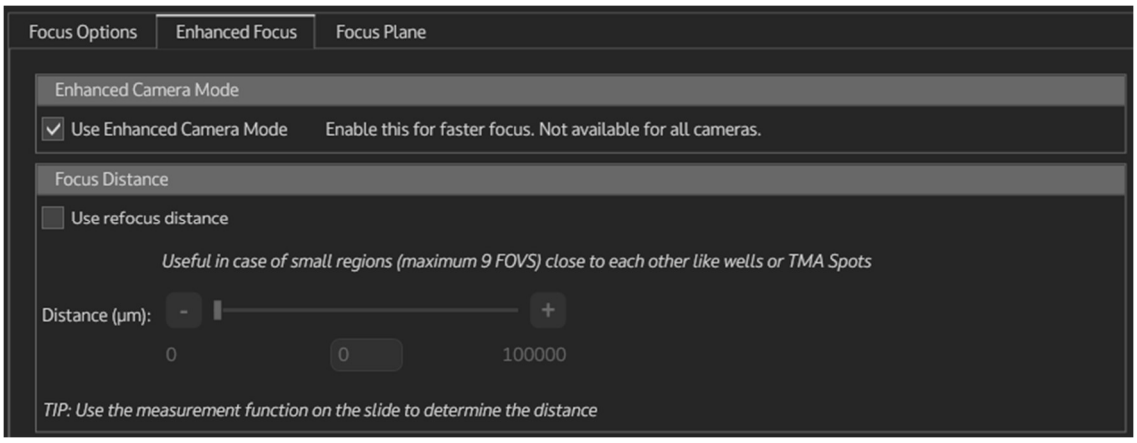
Parallel Compute Focus Measure

While focusing, captured images can be processed in parallel if **Compute focus measure in parallel** is checked.

The number of threads is configurable.

Note: This option can improve focus speed but it depends on the exposure time, camera speed and selected focus algorithm.

Enhanced Focus

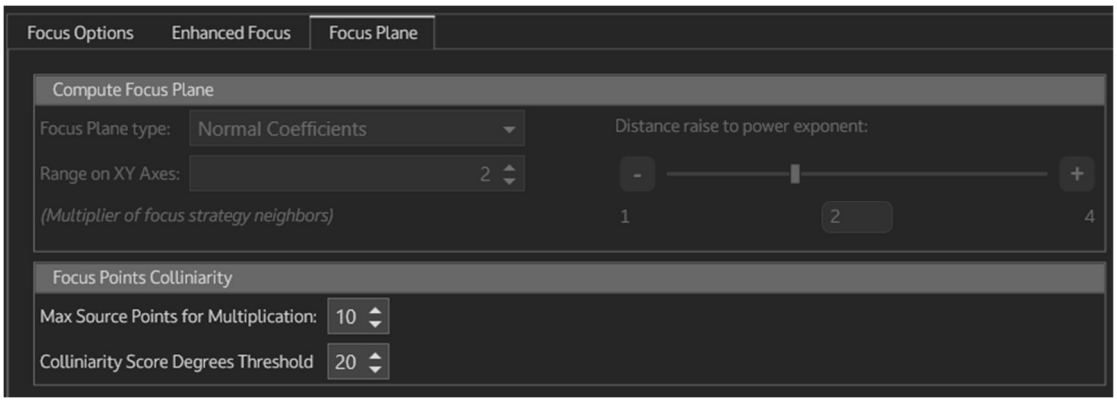


The options from **Enhanced Focus** tab will increase the speed of the focus. However, they are available only on certain cameras.

Use Enhanced Camera Mode: will increase data transfer speed.

Refocus distance: allows the focus position distance to be specified, decreasing the time needed to focus. This option must be used only for TMA or any other small regions that are close to one another. The distance parameter can be determined using the measure function on the slide preview.

Focus Plane



In the focus plane method, the focus on the tissue will be done at the beginning of the scan and a focus plane will be computed using the resulting focus points.

The multi-plane focus calculates an “equilibration surface” over the entire tissue meaning each FOV might have a slightly different focus compared to its neighbors. However, greater differences between one focus group and a neighboring focus group will be avoided. This multi-plane focus can also detect outliers and will skip them (not take them into consideration for the calculation of the equilibration plane) if their distance from the average is above a certain threshold. The multi-plane focus is still based on the “focus group” strategy, i.e. the autofocus is done in a matrix of 3x3, or 5x5, or 7x7 FOV – as always, However, all focus points are acquired BEFORE the actual scanning starts. Each FOV might have a slightly different focus. Focal changes across a sample will then lead to a continuous change of the focus position from FOV to FOV rather than sudden larger changes.

Focus Plane Type: you can select various focus plane types from the dropdown menu. Normal Coefficients represents the average equilibration plane and is the default option. The other types (Range Nearest Neighbors, Range Linear, Range Plane, Inverse Distance Weight, Range Inverse Distance Weight, and Delaunay Triangulation) are based on the two parameters in calculation the Z position for each inlier focus point.

Range on XY Axes is a value in microns which is the maximum radius from the XY position of the FOV of an area around it in which you find the focus points used for computing the Z value of the FOV (e.g., a mean of the Z from the focus points in the range will give the final Z value for a FOV).

Distance raise to power exponent designates what type of weight will have each focus point in computing the Z value for an FOV. As in the previous example, using the mean of Z's in computing the FOV's Z, with a weight of 1, the computation is linear. If a weight of 2 is used, the weight for each focus point Z will be distance^2 (distance from FOV to focus point raised to the power of 2). Closer focus points will have a higher weight than normal. The allowed values for this are from 1 to 5.

Focus points collinearity

Check if the generated focus points are colinear on their (x, y) positions; in this case, a focus plane cannot be properly generated, so this will be treated as a special case.

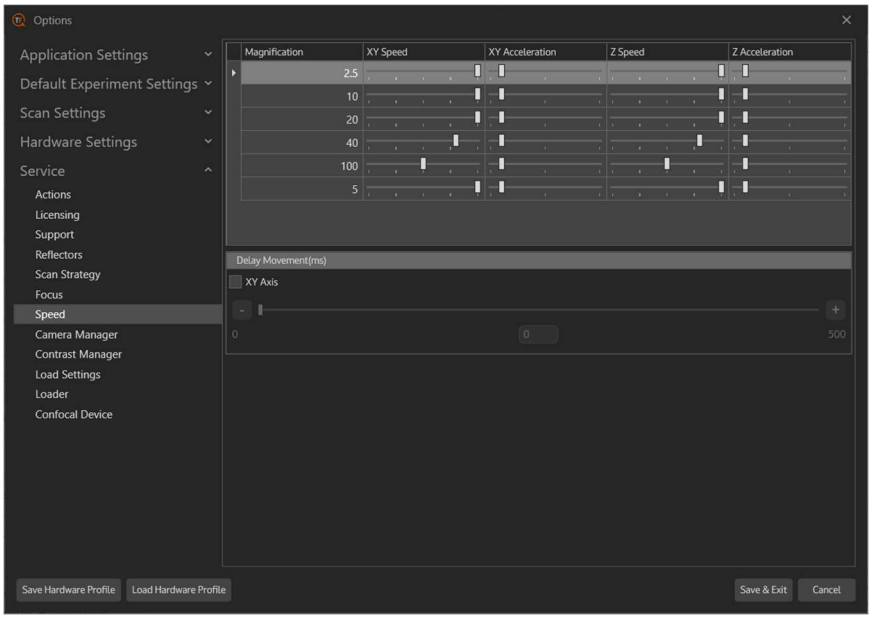
- **Max Source Points for Multiplication**: check if focus points are colinear only if the number of focus points is below this max value;
- **Collinearity Score Degrees Threshold**: a collinearity score for the focus points list is computed as the average angle between all points; this will be a value in degrees in the interval [0-90]; the lower the value is, the closer to colinear the focus points are, the higher the score is the better; a score below the parameter will flag the focus points list as a special case (colinear points).

6.1.5.7. Speed

The speed of stage movement during acquisition can be adjusted in the speed dialog. These settings do not affect the joystick speed settings. If working with well plates or cell cultures in general, consider reducing speed values (to avoid disturbing the content).

Also, high speed values can affect acquisition quality for 20x objectives or higher, because of the vibrations that might be generated.

The **Acceleration** parameter describes how fast the stage will try to reach the defined speed.



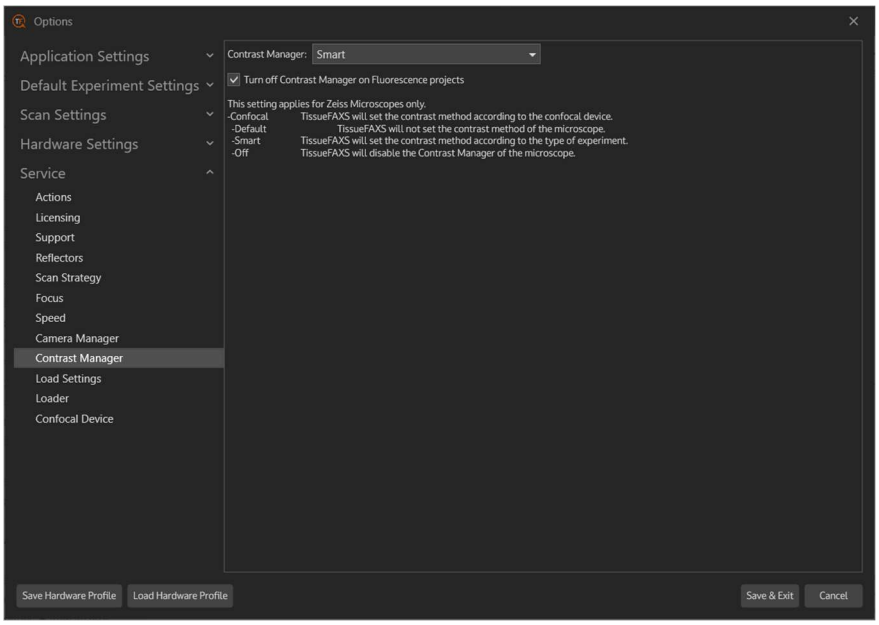
Delay Movements: a delay interval between a stage movement and the next image capturing can be set after each stage movement to avoid capturing motion blurs.

6.1.5.8. Camera Manager

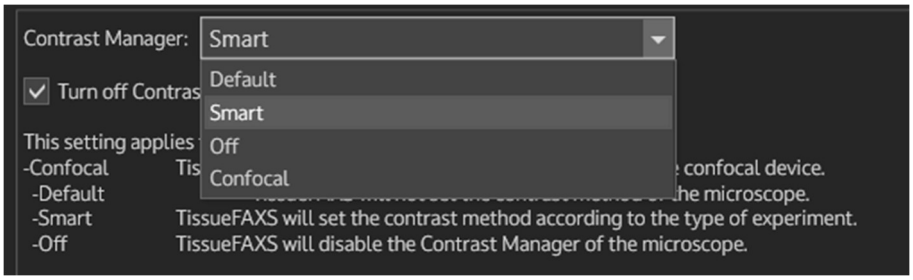
This option enables adding or removing a camera from the list of the cameras connected to the computer. It also helps with setting the current camera See **Chapter Cameras** for more details.

6.1.5.9. Contrast Manager

The **Contrast Manager** is a feature of the software that automatically adapts the contrast method of the microscope based on the type of experiment being conducted.



The **Contrast Manager** has three options available in the dropdown menus:



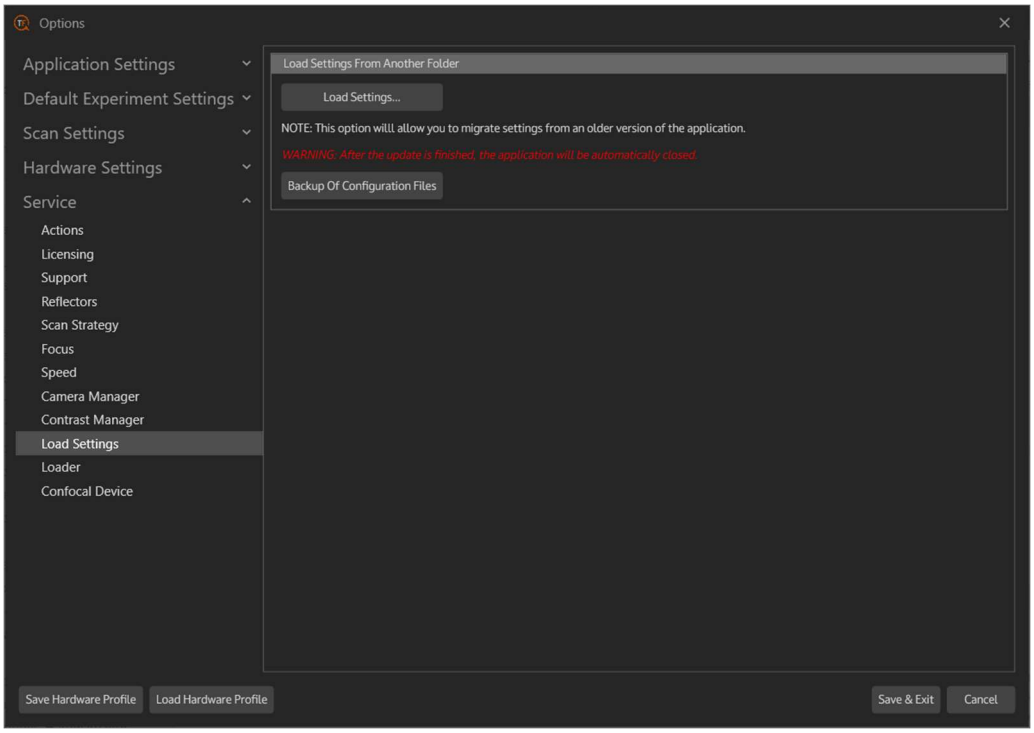
- **Confocal**: **TissueFAXS** will set the contrast method according to the confocal device.
- **Default**: **TissueFAXS** will not try to set the contrast method of the microscope. For optimal results, manually setting the contrast method on the microscope is recommended.
- **Smart: TissueFAXS** will set the contrast method according to the type of experiment. This is the recommended setting for the Contrast Manager.
- **Off**: **TissueFAXS** will disable the Contrast Manager of the microscope. Use this option if **Default** and **Smart** options do not return the desired results.
- **Turn off Contrast Manager on fluorescence projects**: disables the Contrast manager of the microscope only for fluorescence projects.

Notes:

- If the contrast method is not available for the microscope, it will revert to brightfield.

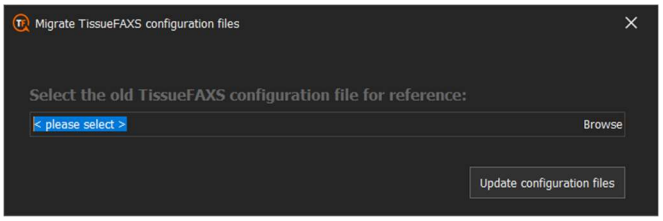
- For a detailed view over contrast manager and contrast method, please consult the microscope operating manual.

6.1.5.10. Load Settings

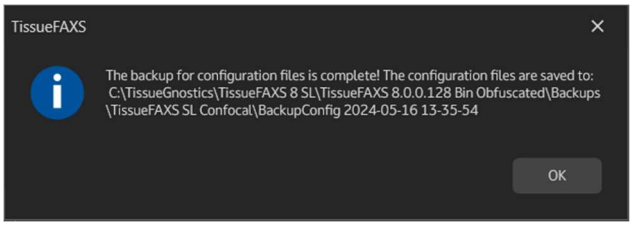


The user can use this feature to load the settings from the previous version of the software.

Press the **Load Settings...** button and a browse window will open for selecting the desired file(s). Once the file(s) are selected, press the **Update configuration files** button to complete the update.



Backup of Configuration Files: pressing this button will perform a backup for all current configuration files and display a message box with the storage details:



Notes:

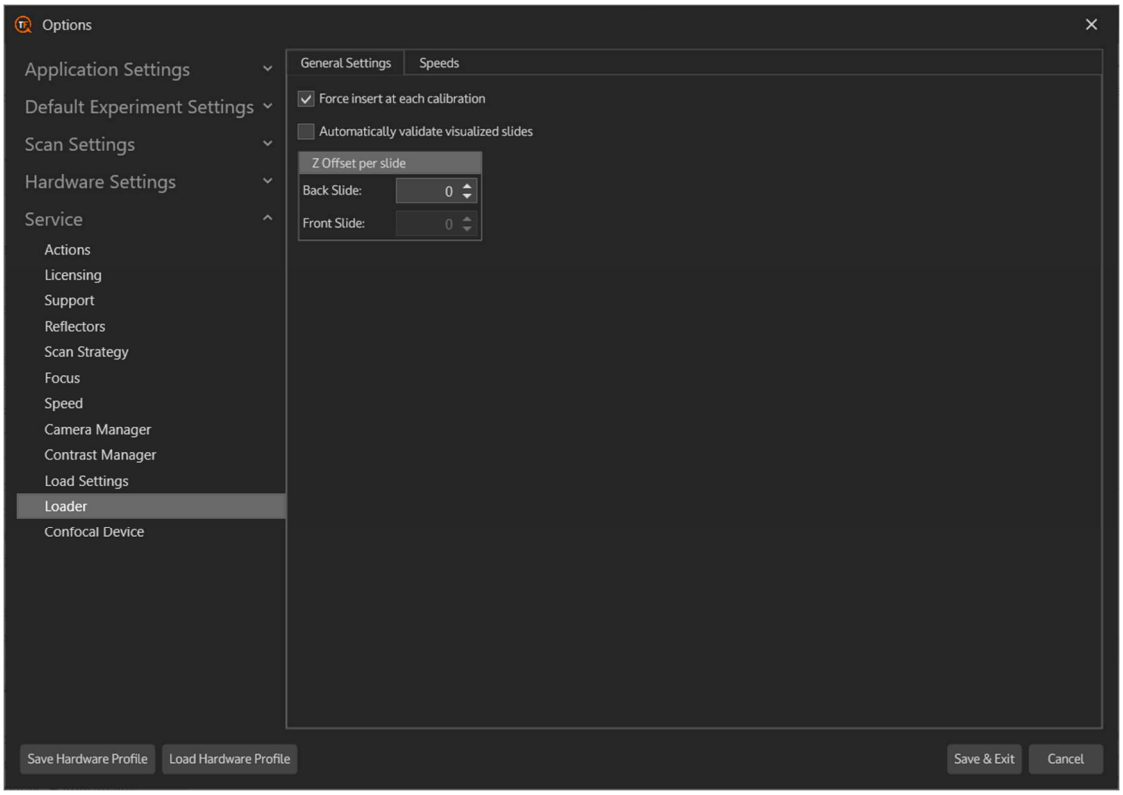
- Use the **Load settings** feature ONLY if instructed by TissueGnostics personnel or authorized distributors.

- Load settings feature is only available if logged as technician or administrator.

- After the update is finished, the application will close automatically.

6.1.5.11. Loader

The **Loader** dialog is meant to adjust the slide loader related settings:



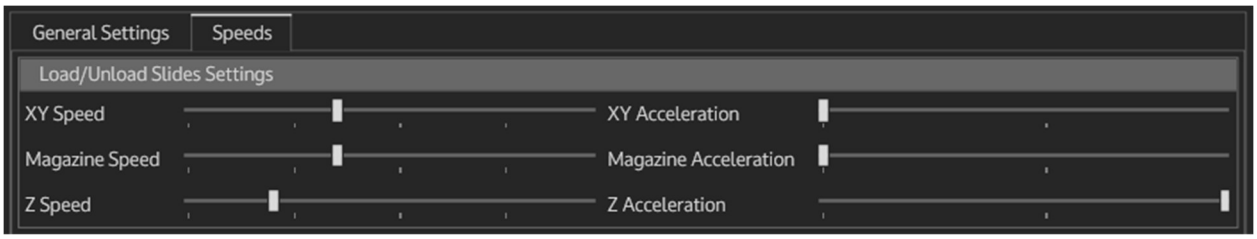
General Settings

- **Force insert at each calibration:** if checked, after each calibration the slide detection will be performed. **TissueFAXS** performs slide loader calibration only if not found on the controller. However, if enabled, the insert (interrogation of the physically available slides of the loader) is done even if found on the controller;
- **Automatically validate visualized slides;**
- **Z offsets per slide**: the slides in the gripper can have a Z offset represented by the difference in focal position between the two slides. Adjust the values for the two slides as needed. This should be set only for the SL 200.

Speeds

In this section you can adjust various speeds and accelerations for stage and magazines.

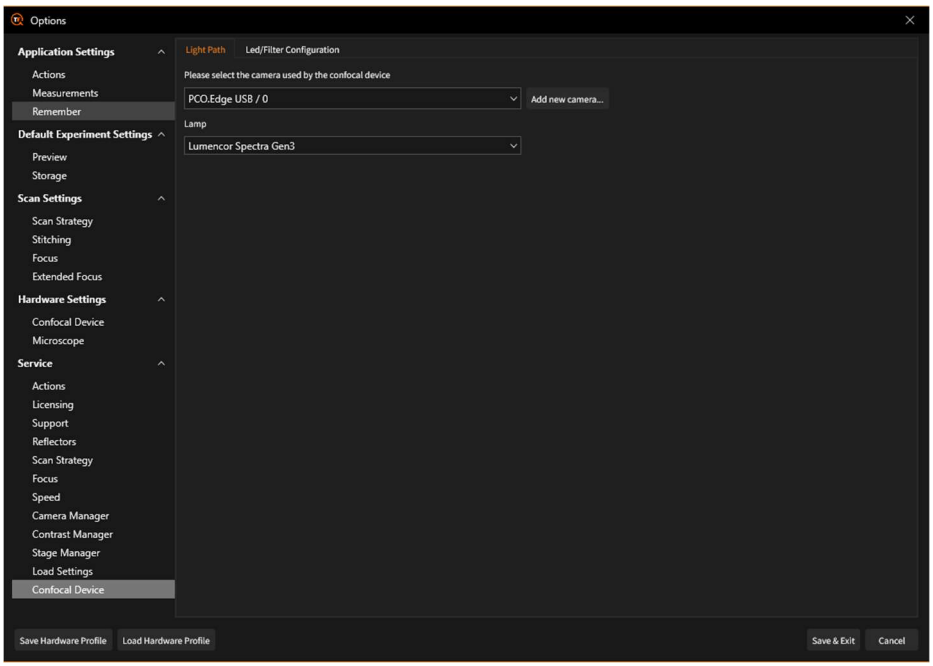
These speeds and accelerations are only applied when loading/unloading slides.



6.1.5.12. Service Confocal Device

Light Path

Select the camera used by the confocal device and the lamp.

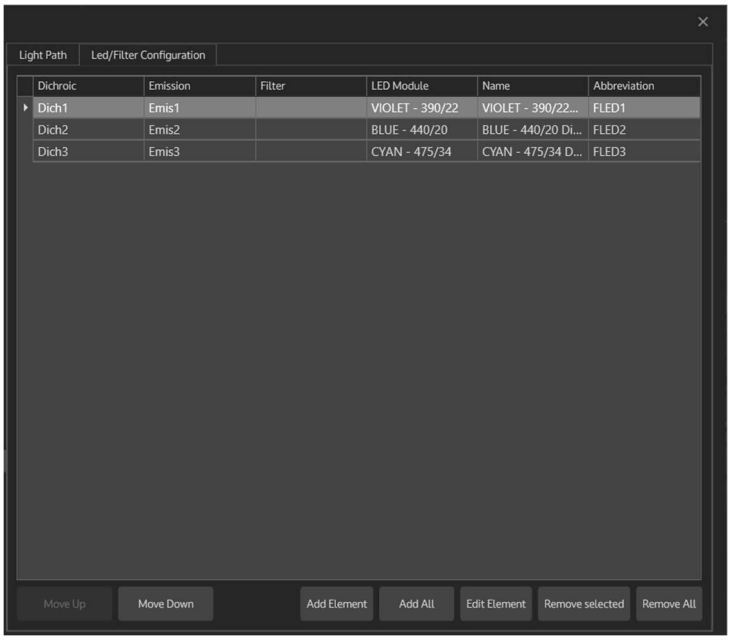


Select the camera used by the confocal device.

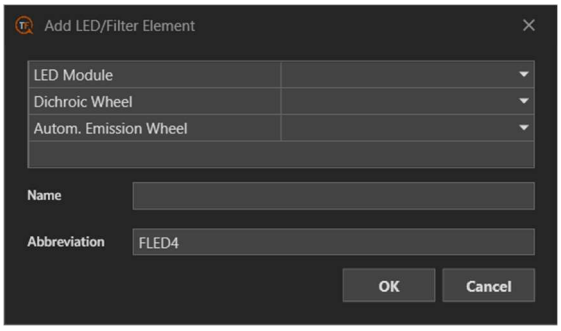
Led/Filter Configuration

Select the led/filter configuration.

Elements can be added one by one (**Add Element**) or all together (**Add All**).



Elements can be added one by one (**Add Element**) or all together (**Add All**).

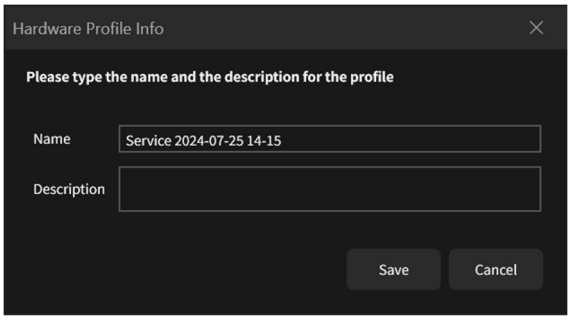


Any element can be modified (**Edit Element**), individually selected elements can be removed (**Remove Selected**), or all elements can be removed (**Remove All**).

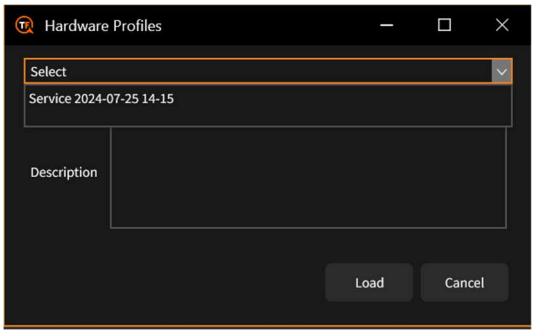
6.1.5.13. Hardware Profile

The **Hardware Profile** section is in the lower part of the **Options** panel. A hardware profile is a profile that will save most of the settings from the **Options** window, depending on a user's requirements (e.g., depending on a sample's type).

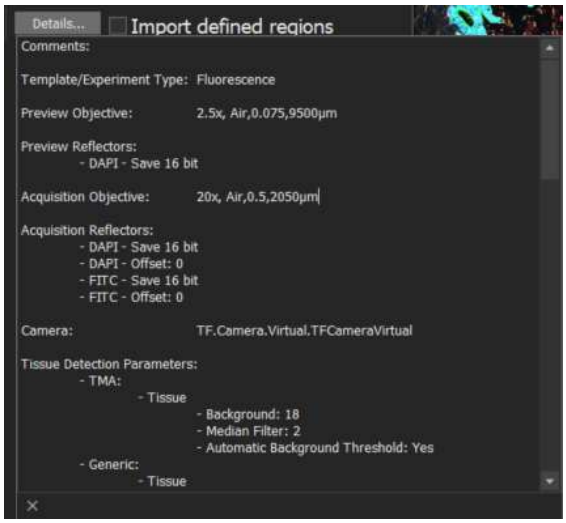
To save a profile, press the **Save Hardware Profile** button. In the dialog that appears, enter a name and (optional) description, then press the **Save** button. The newly created hardware profile will be saved and ready for further use.



To load a previously created hardware profile, press the **Load Hardware Profile** button. In the dialog that appears, select the desired profile from the dropdown list, then press the **Load** button. The hardware profile will be loaded and ready to use.

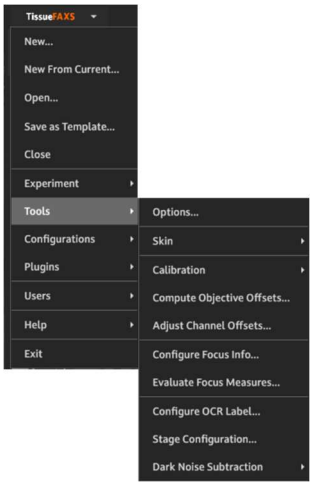


The hardware profile information can also be accessed when creating a new experiment, by pressing **Details** button.



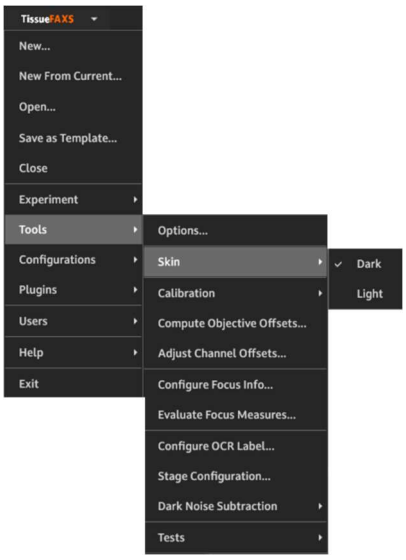
7. TissueFAXS Tools

Access various TissueFAXS tools by going to **TissueFAXS main menu** -> **Tools**.



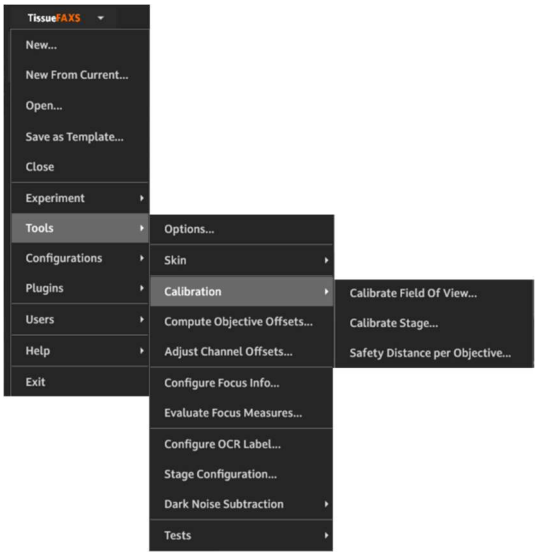
7.1. Skin

TissueFAXS has two visual appearances: dark and light (light will be implemented in the near future). You can switch between the two with a click.



7.2. Calibration

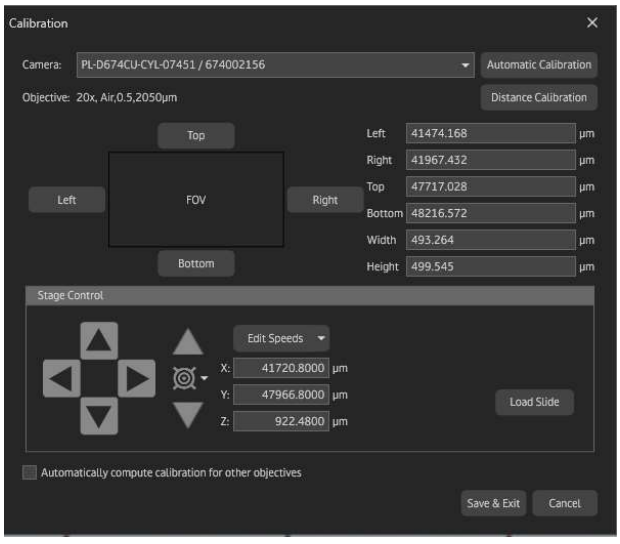
There are more options related to system calibration and hardware safety.



7.2.1. Calibrate Field of View

The *camera calibration* is a basic, but important operation when using **TissueFAXS**, because the quality of the acquired images directly depends on it. **It is therefore essential that calibration be performed with maximum attention and particular care.**

Calibrating the size of the Field of View for each objective from the **Tools → Calibrate Field of View…** button is recommended.



This dialog allows you to compute the size of the Field of View for the current objective.

There are **three ways** accomplish this task:

- manual calibration;

- automatic calibration;

- distance calibration.

Notes:

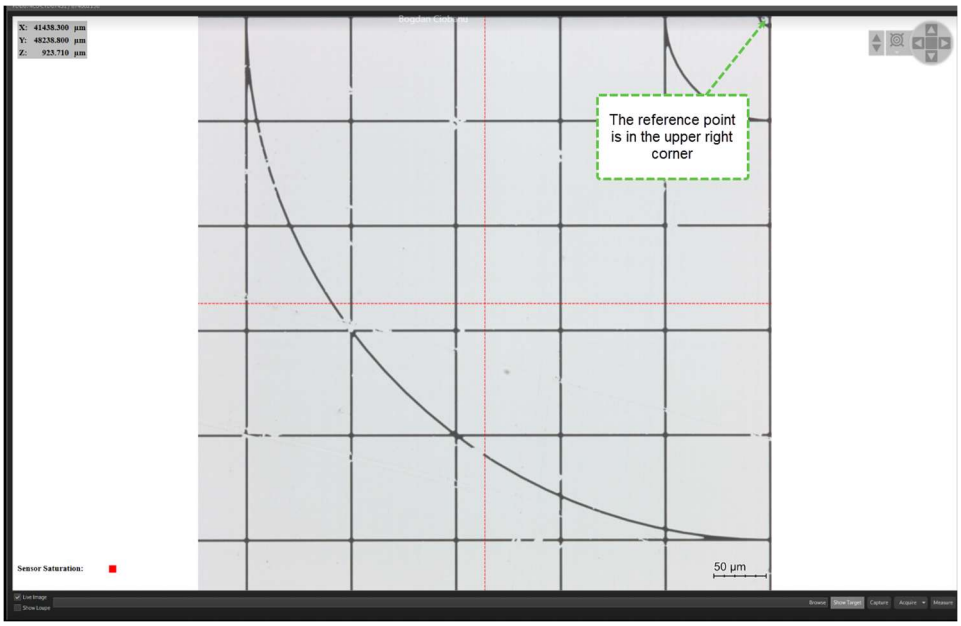
- FOV calibration can only be performed when logged in as Administrator.

- For all FOV calibration methods, it is recommended to use a slide containing a more prominent area, to make tracking easier.

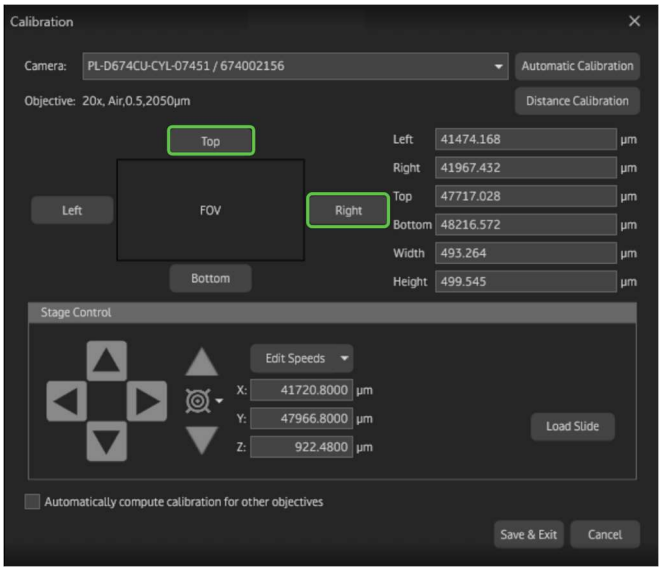
- For optimal results, a calibration slide may be used (the calibration slide is mandatory for the *distance calibration* method).

1. **Manual FOV Calibration**

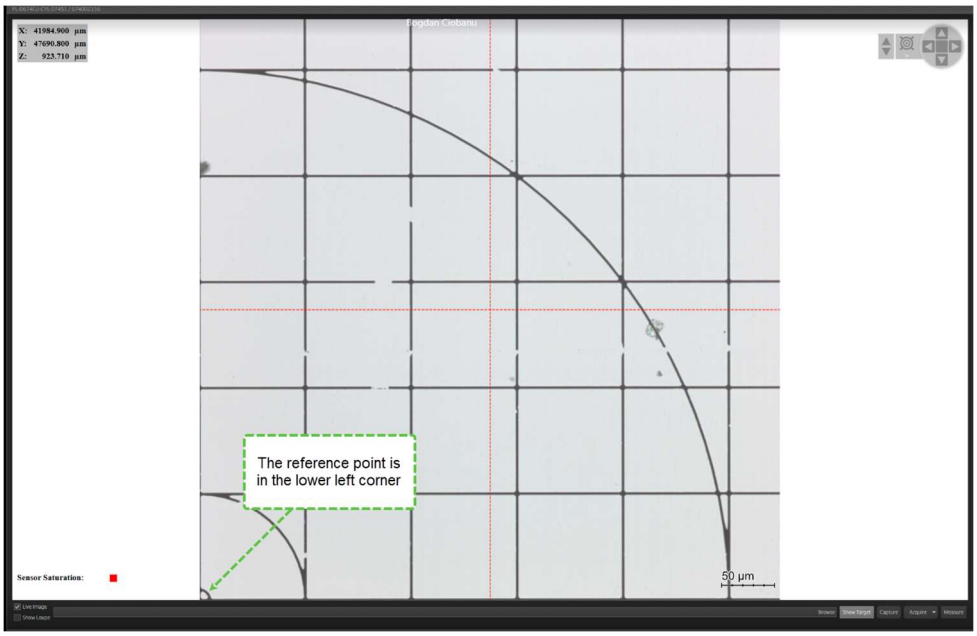
- Choose a *reference point* on the sample. Make sure the whole object is in focus in order to obtain a good evaluation of the reference;
- Move the reference point in the upper-right corner by using the software or hardware joystick;



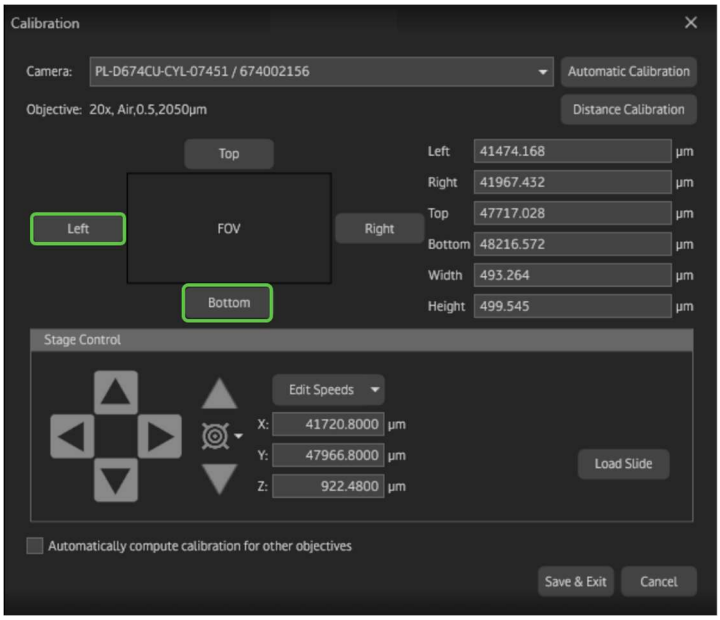
- Press **Top**, then **Right**;



- Using the joystick, move the reference point from the upper-right corner to the lower-left corner;



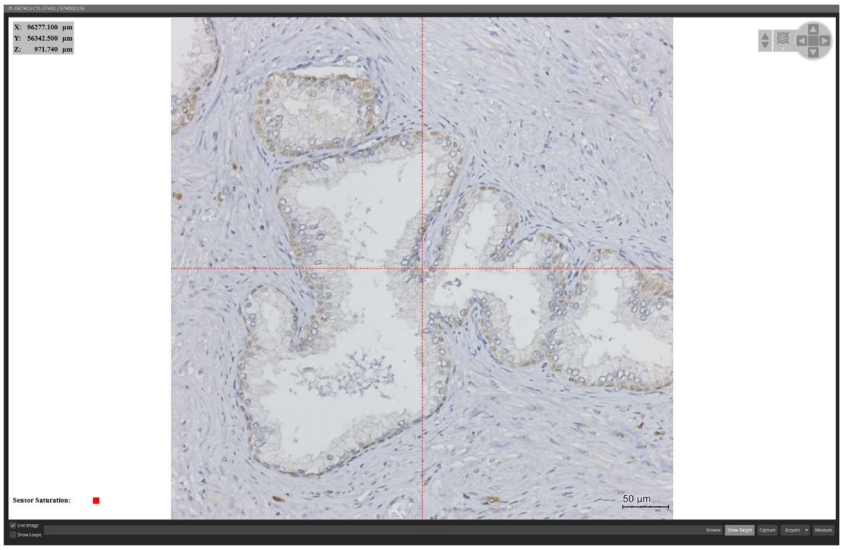
- Press **Bottom**, then **Left**. At this point, the **Width** and the **Height** values will automatically be computed;
- To save the computed values, press **Save and Exit**.



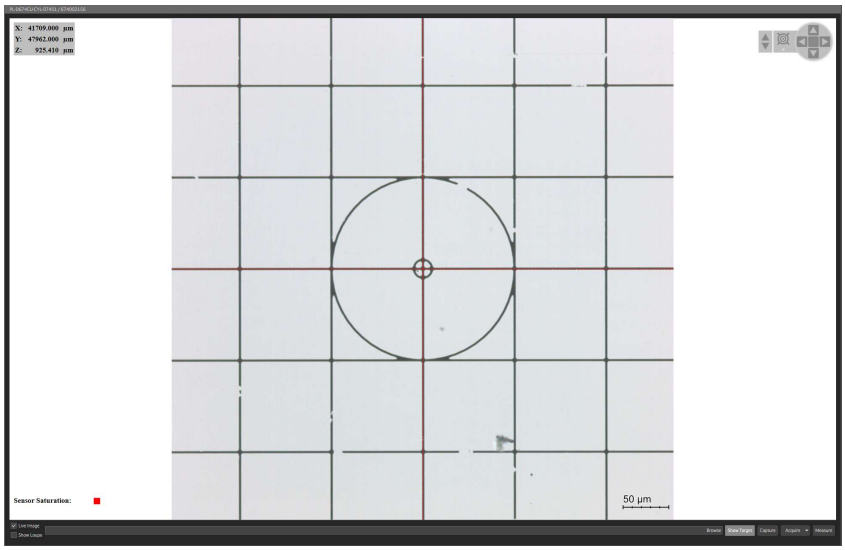
Note: If the FOV size is already known, the Width and Height values can be entered in their corresponding fields.

2. **Automatic FOV Calibration**

- Choose a reference point on the sample. Make sure the whole object is in focus in order to obtain a good evaluation of the reference.



If using a calibration slide, place the center of the calibration slide on the live image as shown in the figure below.



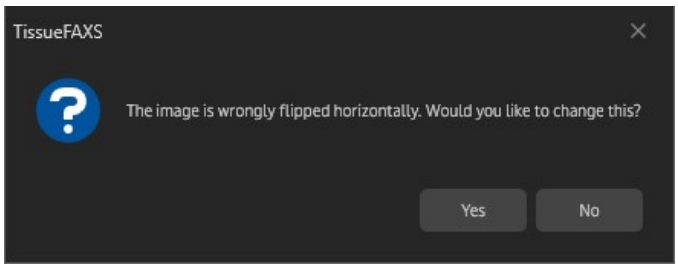
Notes:

- To bring up the red cross (marking the center of the calibration slide) on the live image, use the key combination Alt+C.

- Do not use images with repeating patterns! (For example, slides with fluorescence beads might contain many similar objects and auto calibration will fail or return wrong results.)

- Ensure the speed of the stage is low for better movement precision!

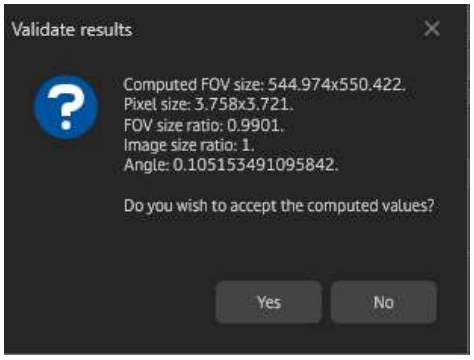
- Press the **Automatic Calibration** button. The stage will be moved and the software will determine the following:
- The correct flip settings. A prompt will appear if the current flip settings are wrong.



- The angle that is formed when the camera is misaligned. Manually rotate the camera and rerun the calibration to recheck this.

Note: The angle value should be around 0.01. Values higher than 0.09 will generate bad quality of stitching.

- The dimensions of the FOV.



Notes:

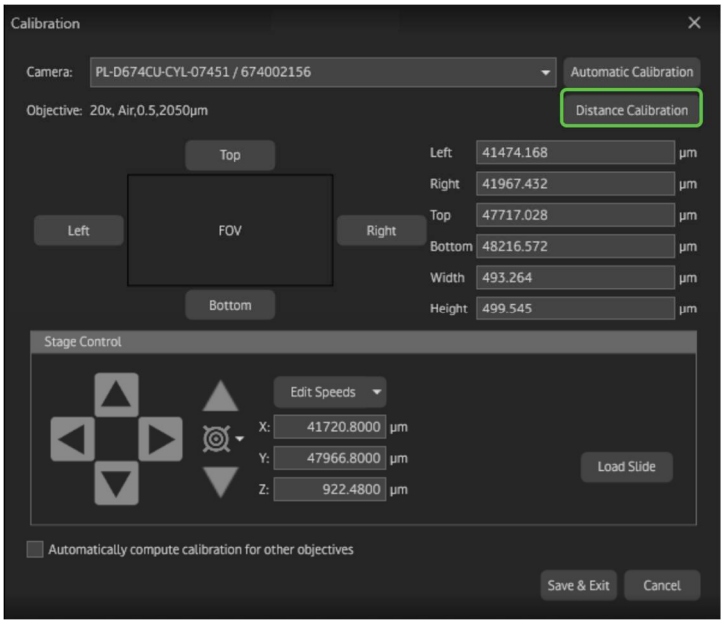
- For best results, set the speed of the stage at a minimum value. When using other slides than calibration slides, you can run this procedure on different areas to ensure the correct value is computed.

- If the image is flipped, the algorithm will detect this and will set the image to the right position.

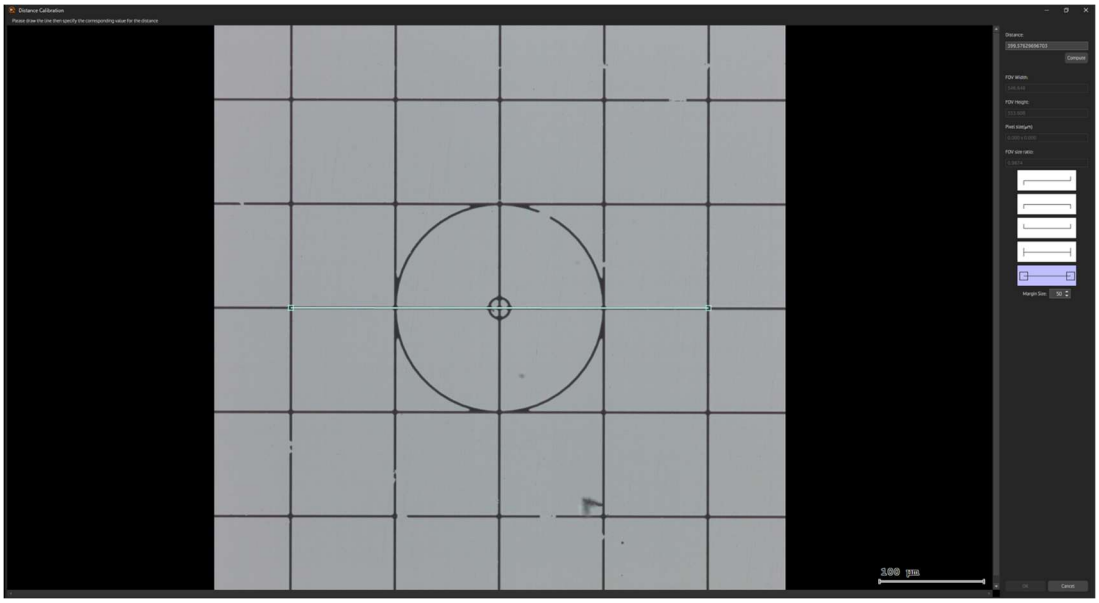
- To save the computed values, press **Save and Exit**.

3. **Distance Calibration**

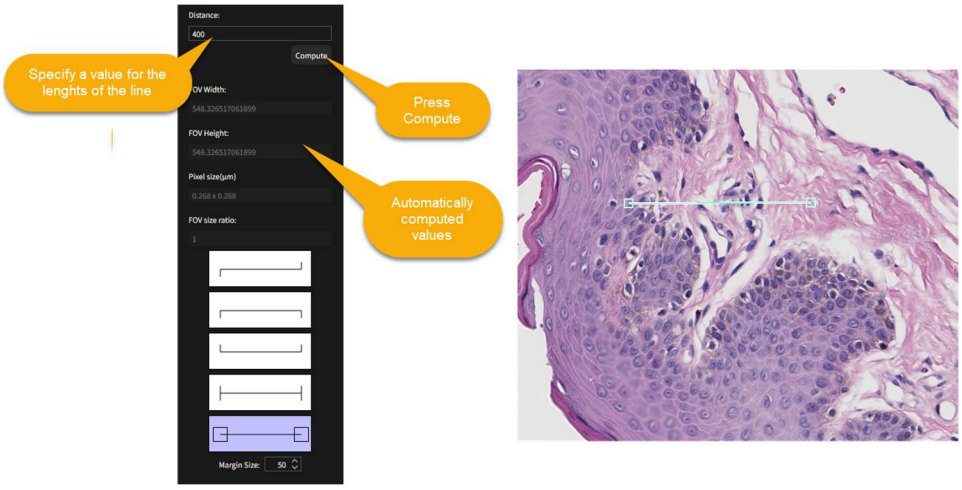
- Insert the calibration slide in an empty insert position;
- Choose the objective for which the calibration will be performed;
- Enable the live image, then focus and center the calibration slide on the live image;
- Access the calibration function: **Tools** -> **Calibrate** -> **Calibrate Field of View…**.
- The **Field of View Calibration** control panel will appear:



- Now press the **Distance Calibration** button and the **Distance Calibration** panel will appear.

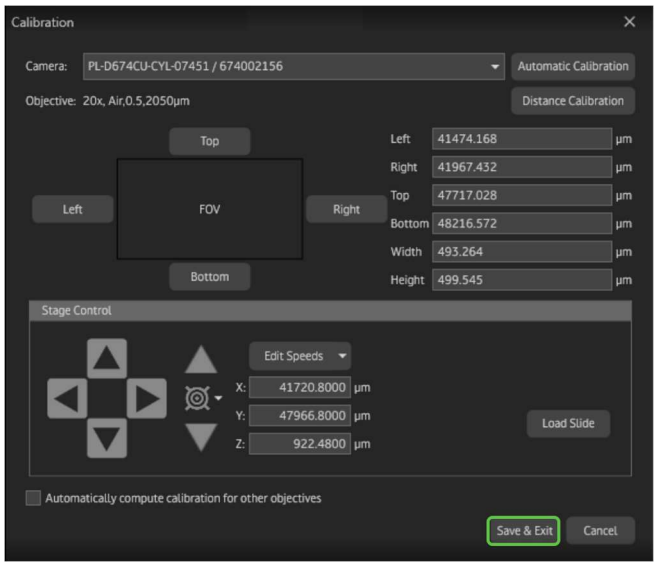


- Draw a line between the two points for which the distance is known. Click in a desired point to start drawing the line, then click again to determine the end of the line. In the example, the squares on the calibration slides are 100m*m*X100m*m*. Use a distance as large as possible to improve results;
- Specify the distance between the two selected points (the value must be in m*m*);
- Press the **Compute** button: FOV Width, FOV Height, Pixel size and FOV size ratio will automatically be computed;
- Press the **OK** button to save the obtained values.



Notes:

- Only the FOV values for the current objective have been calculated / recalculated this far. To compute the FOV sizes for the rest of the objectives, repeat the steps for each objective. For a quick (but less precise) setup, you can select the objective with the medium magnification between your objectives, compute its FOV size and, before pressing **Save and Exit**, select **Automatically Compute calibration for other objectives**.
- Changes can be discarded at any time by pressing the **Cancel** button.



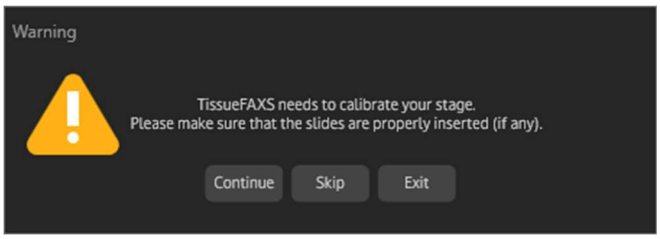
7.2.2. Stage Calibration

The first step of an image acquisition is to calibrate the stage when starting the application. This step may be skipped to simply view previously acquired images in **TissueFAXS.** If acquisition is performed at a later time, there will be prompt to calibrate the stage.

Stage calibration is important for acquiring high-quality stitched images, as well as protecting the hardware components from physical damage. During calibration, the application finds the hardware limits with extreme precision. The objectives and stage will move automatically as the system measures the coordinates of the stage in relation to each objective (in nanometers).

Verify there are no slides in the gripper and the cassettes are in the upper limits.

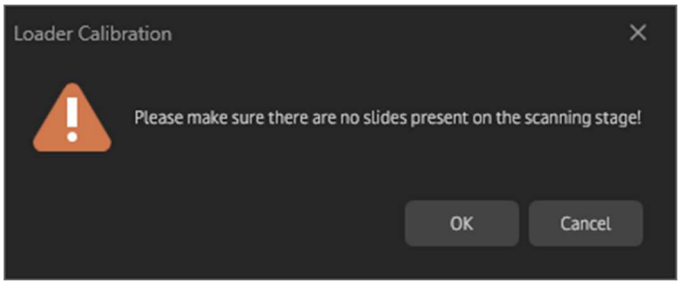
To initiate the calibration procedure, press **Continue**.



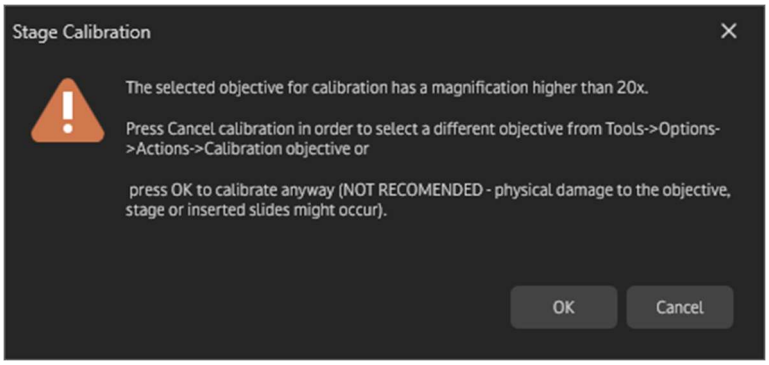
Notes:

- Slides will break if they are in the gripper before beginning the calibration process.

- If any slides are broken during a loader operation, it is important to remove all glass pieces from the loader and the gripper to prevent further damage.



If the magnification of the calibration objective is higher than 20x, a warning message will appear. In this situation there are two options:



- Press **Cancel** calibration, then go to **TissueFAXS Main Menu** → **Tools** → **Options** → **Service** → **Actions** → **Calibration** in order to select another objective from the dropdown list (see Chapter Service Actions). At this point return to calibration process from **Tools** → **Calibration** → **Calibrate stage...**
- Press **OK** to continue calibration.

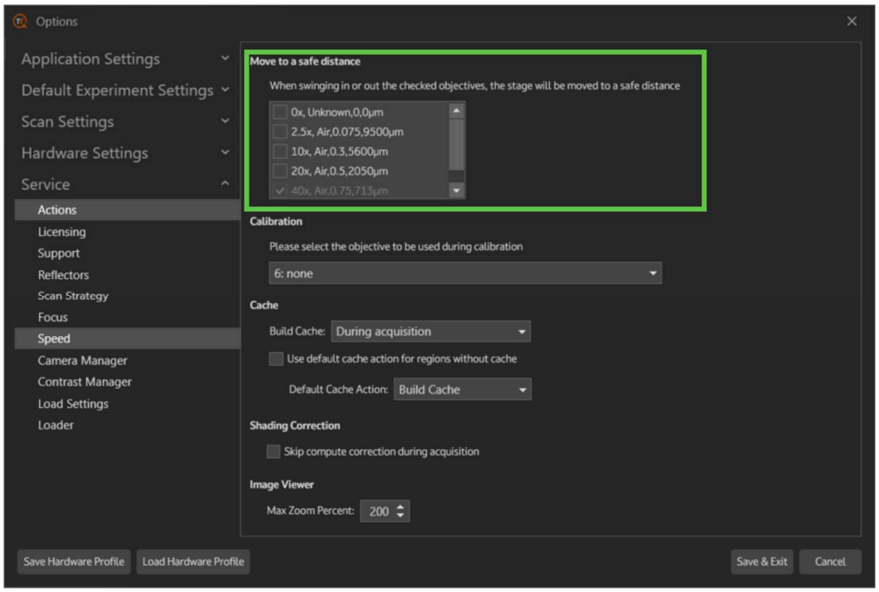
Note: Please consider that if you continue the calibration as described above, you should think about the risks, such as damages to your objective, stage or slides.

After the calibration ends, **TissueFAXS** is ready to be used. You can now create new projects, open existing ones, or simply adjust the microscope components and see live images from the camera, as described in previous chapters.

7.2.3. Safety Distance per Objective

Safety distance

This option can be found in **Options -> Service -> Actions.**



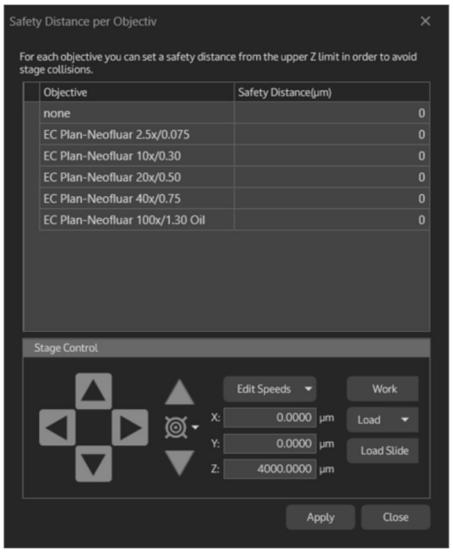
Safety distance means the Z-position to which the stage is lowered in order to ensure that no objective lens collides with the slides or any part of the stage.

When moving between slides or when switching between objectives, the stage will be moved to a safe distance.

Note: By default, for any objective with magnification higher than 20x, **TissueFAXS SL** will lower the stage to the safe distance. For the rest of the objectives, this behavior may be customized in the dialog shown below. Usually, this behavior is enabled for any longer objective or for objectives that come very close to the slide for focus. For inverted microscopes, the best action is to enable it for all objectives.

Safety distance per objective

You can set a safety distance from the upper Z limit for each objective in order to avoid stage collisions. Joystick control is available for an easier interaction.

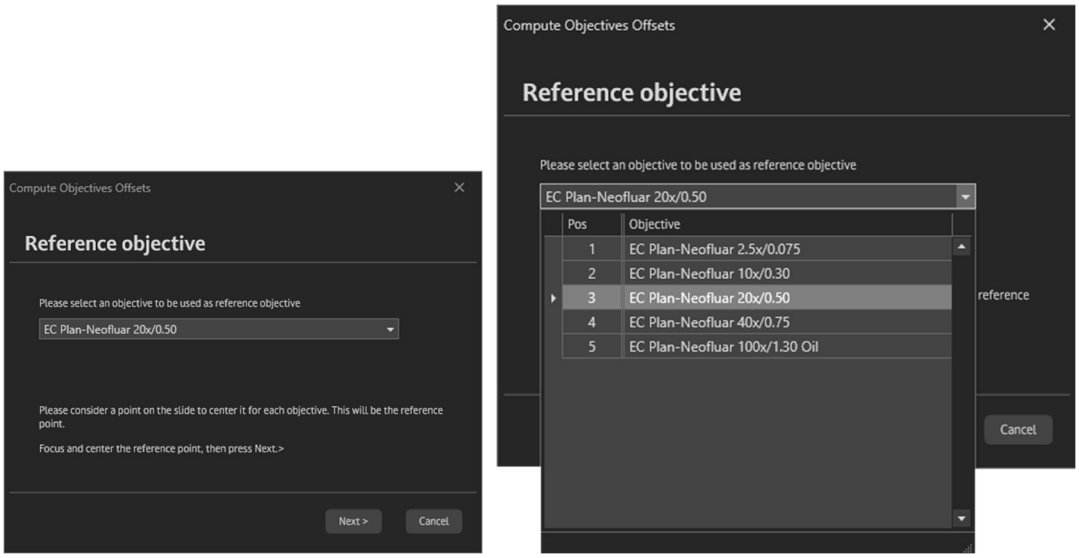


7.3. Compute Objective Offsets

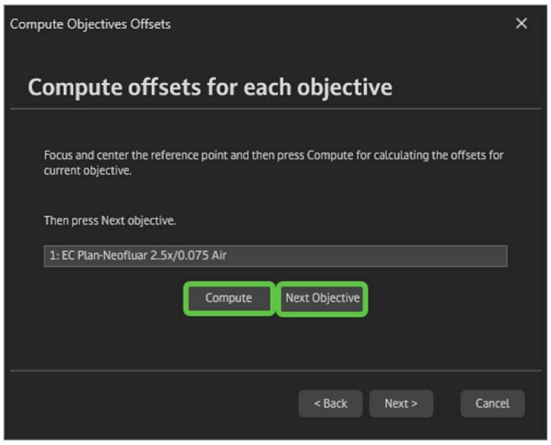
Usually, the objectives present on a microscope are not perfectly centred on the same point. To avoid situations when acquisition images are slightly moved compared with the preview image the objective offsets must be computed.

All **TissueGnostics** systems have this calibration done at delivery time and this function should not be use unless instructed to do so by the support personnel. For best results, please use a calibration slide in the next steps.

The first step is to select the reference objective. The recommended objective is 20x or, if not available, an objective with a medium magnification. Press **Next** after the objective has been selected.



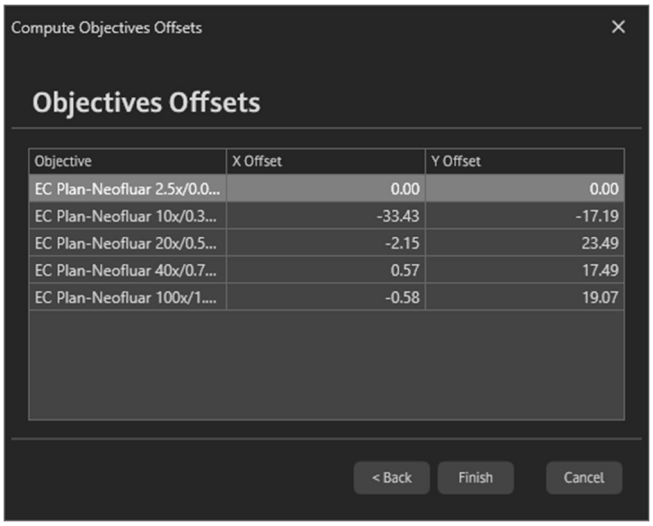
Once this window appears, follow these steps:



1. Focus the image and center the reference object
2. Press Compute
3. Press **Next Objective**

Once you have pressed **Next Objective** the microscope will switch to the next objective and you must do the same steps.

When no more objectives are available, the **Next objective** button will become disabled. Click the **Next** button and the following summary will appear:



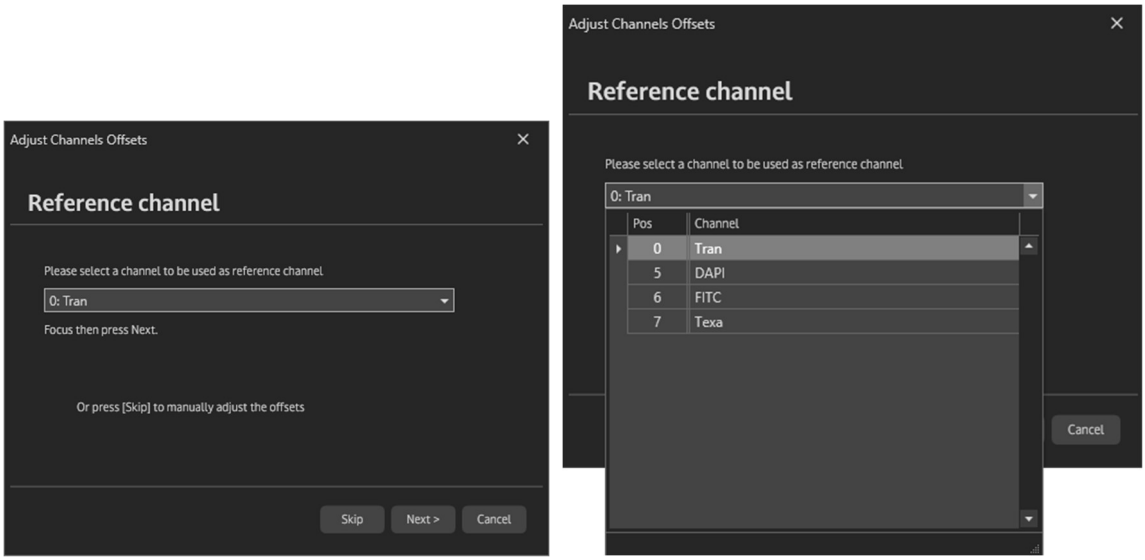
If there are values larger than 200 micrometres something went wrong. Check if the objectives are properly inserted and then rerun this wizard.

Press **Finish** if the values are less than 200 micrometres. Preview a slide to check if the values are correct (do not use existing previews), then define and acquire a small region. If the acquired region matches with the preview, the wizard was successful.

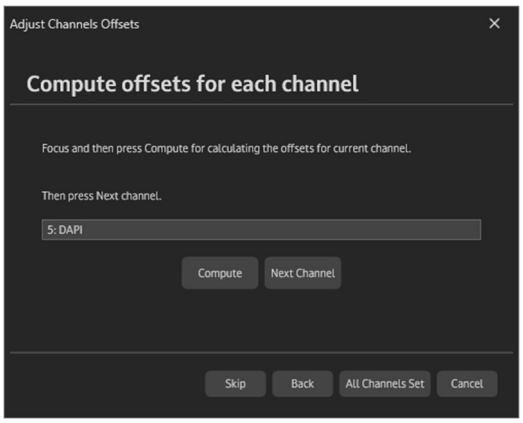
7.4. Adjust Channel Offsets

In fluorescence experiments, there might be instances when the focus plane differs from reflector to reflector. To work around this issue, use the **Adjust Channel Offsets** feature.

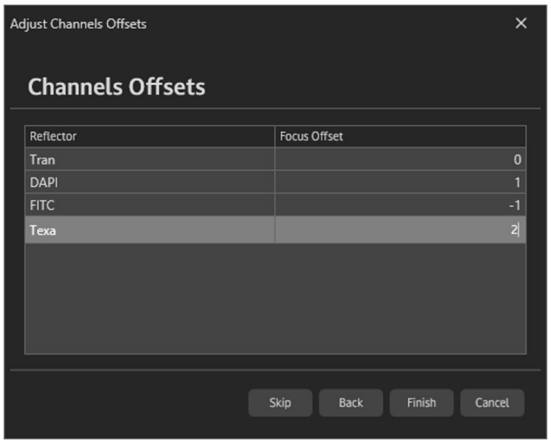
The first step is to select a reference reflector and to focus on it.



After pressing the **Next** button, **TissueFAXS** will switch to the first reflector. Follow the on-screen instructions.



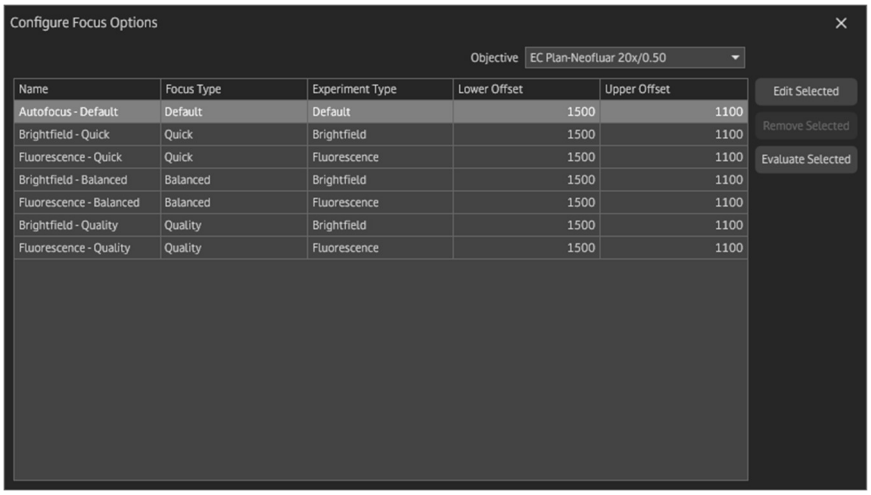
After going through all the reflectors, press **Next** and the following summary will appear.



Review and press **Finish** to complete. To test the results, define a 1x1 region and check the images on each channel to see it the images are in focus.

7.5. Configure Focus Info

Various focus configurations can be edited and stored. For details see Chapter Focus Settings

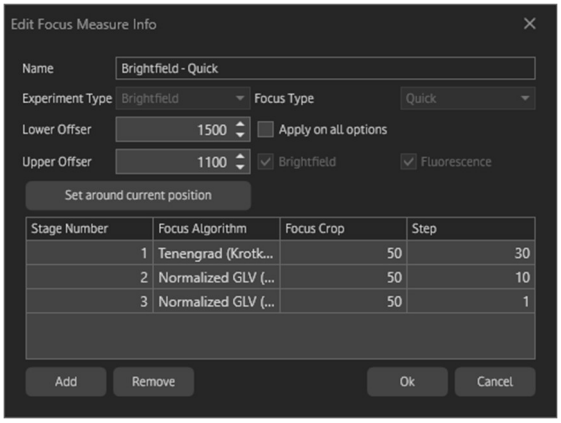


Name	Focus Type	Experiment Type	Lower Offset	Upper Offset
Autofocus - Default	Default	Default	1500	1100
Brightfield - Quick	Quick	Brightfield	1500	1100
Fluorescence - Quick	Quick	Fluorescence	1500	1100
Brightfield - Balanced	Balanced	Brightfield	1500	1100
Fluorescence - Balanced	Balanced	Fluorescence	1500	1100
Brightfield - Quality	Quality	Brightfield	1500	1100
Fluorescence - Quality	Quality	Fluorescence	1500	1100

In addition to the predefined focus options in the list, other focus options can be created using Evaluate Focus Measures Tool (see Chapter Evaluate Focus Measures).

If pressing **Evaluate Selected**, then **Evaluate Focus** window will open, where a new measurement can be done based on the current one.

Press **Edit Selected** to open the **Edit Focus Measure Info** dialog.



Stage Number	Focus Algorithm	Focus Crop	Step
1	Tenengrad (Krotk...	50	30
2	Normalized GLV (...	50	10
3	Normalized GLV (...	50	1

In the **Edit Focus Measure Info**, the following configuration settings can be edited: name, lower/upper offset, focus algorithm, focus crop, step, as well as remove selected focus option.

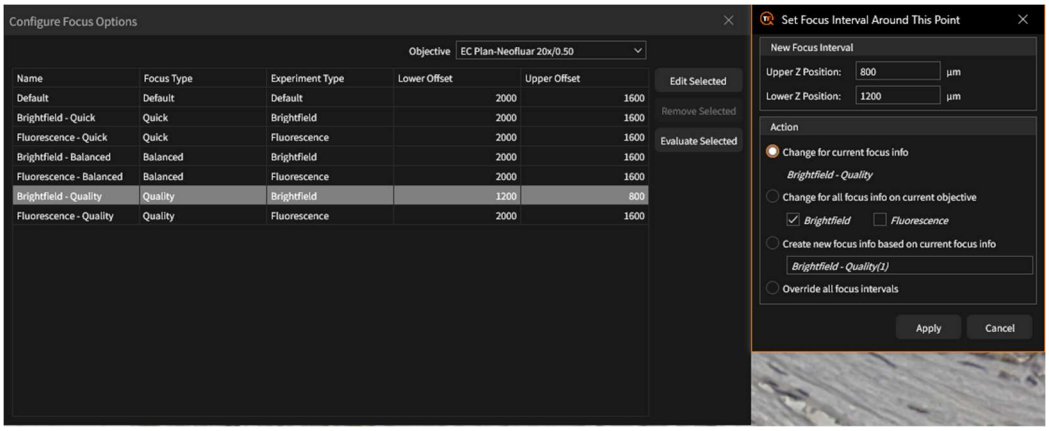
Check **Apply on all options** to apply the selected lower/upper offsets to all the existing focus options.

Customizable focus interval

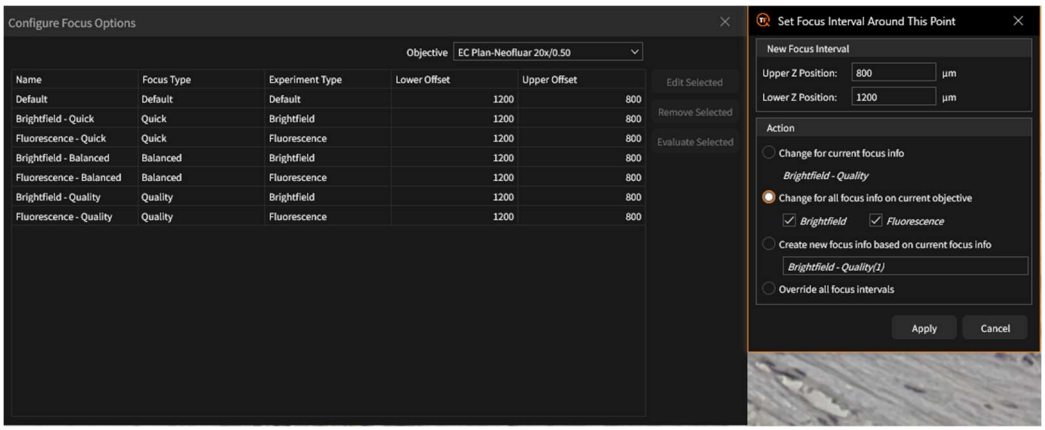
It is now possible to adjust SET FOCUS INTERVAL AROUND THIS POINT from the camera window so that it does not overwrite a predefined focus info.

When setting FOCUS INTERVAL AROUND THIS POINT, the user must select to overwrite predefined focus info or store it under a new name.

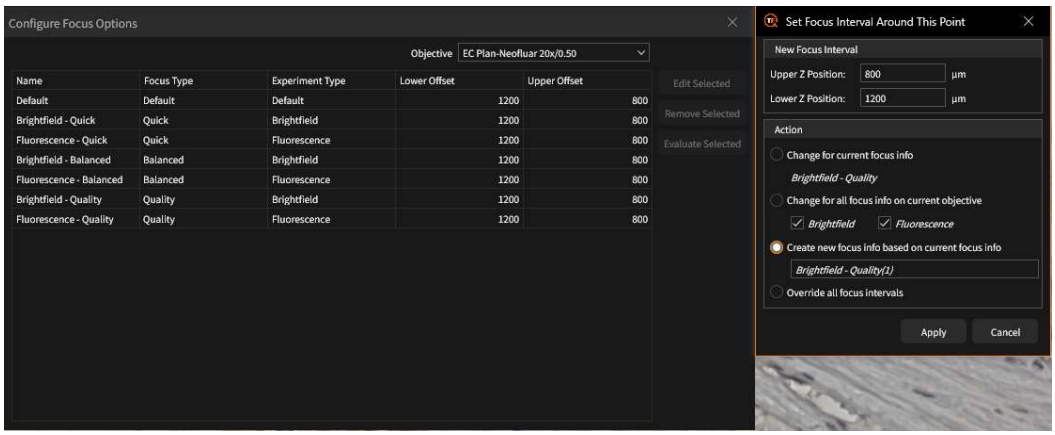
- Change for current focus info: changes the range of the focus interval for the current focus method.



- Change for all focus info on current objective: modifies the focus interval range for all BF, FL focus measures.



- Create new focus info based on current focus info: makes a copy for the current focus measure with new focus ranges. Once created, it is set as the active measure default.



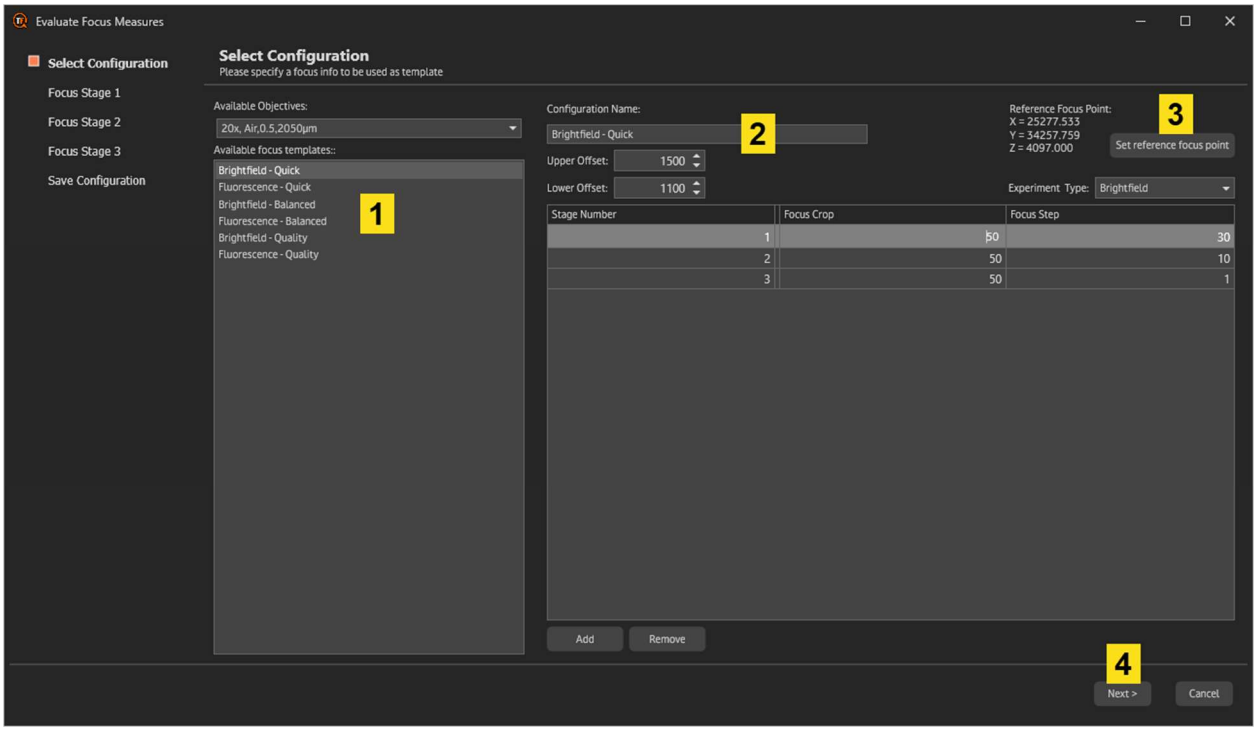
7.6. Evaluate Focus Measures

Evaluate Focus Measures Wizard will open to guide the user through all the focus stages.

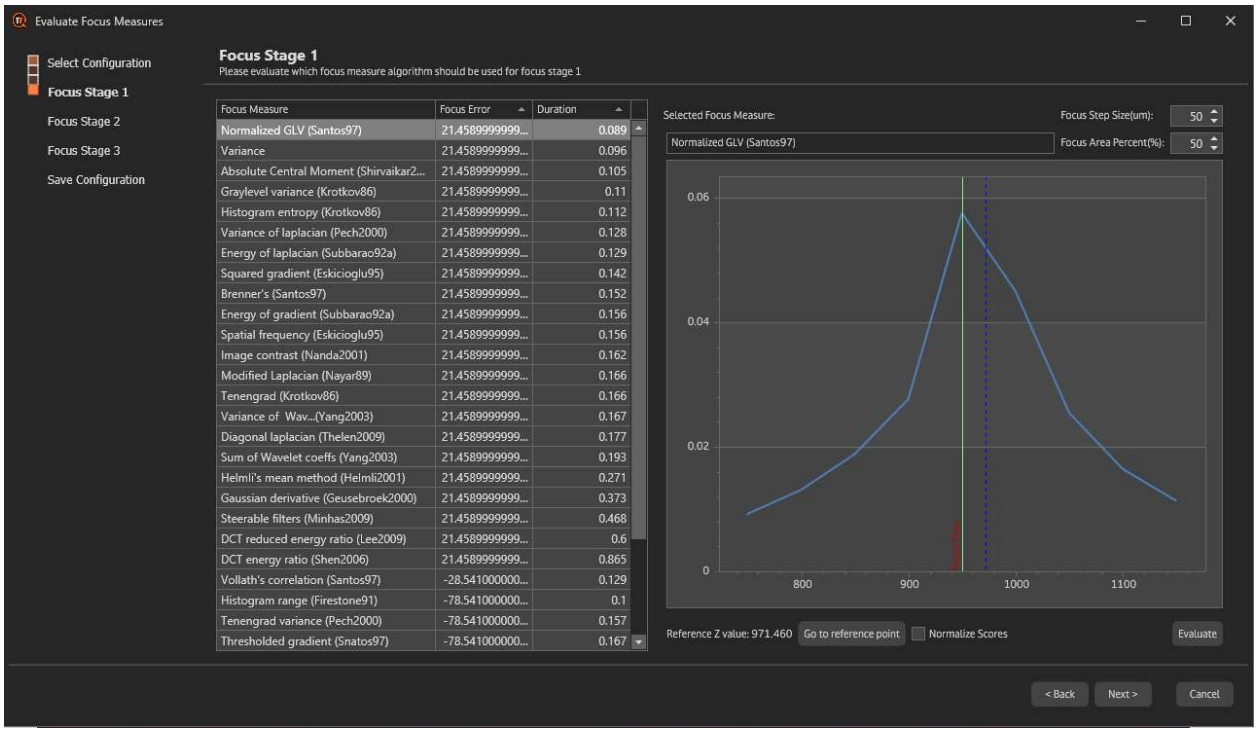
The number of stages is variable. It depends on the objective magnification: a high magnification objective will require more stages because of the small depth of field. Otherwise, a fine tuning of the steps is necessary.

Example

1. **Select configuration**: choose a focus template, give a name to the new configuration, press **Set reference focus point**, then press **Next**.

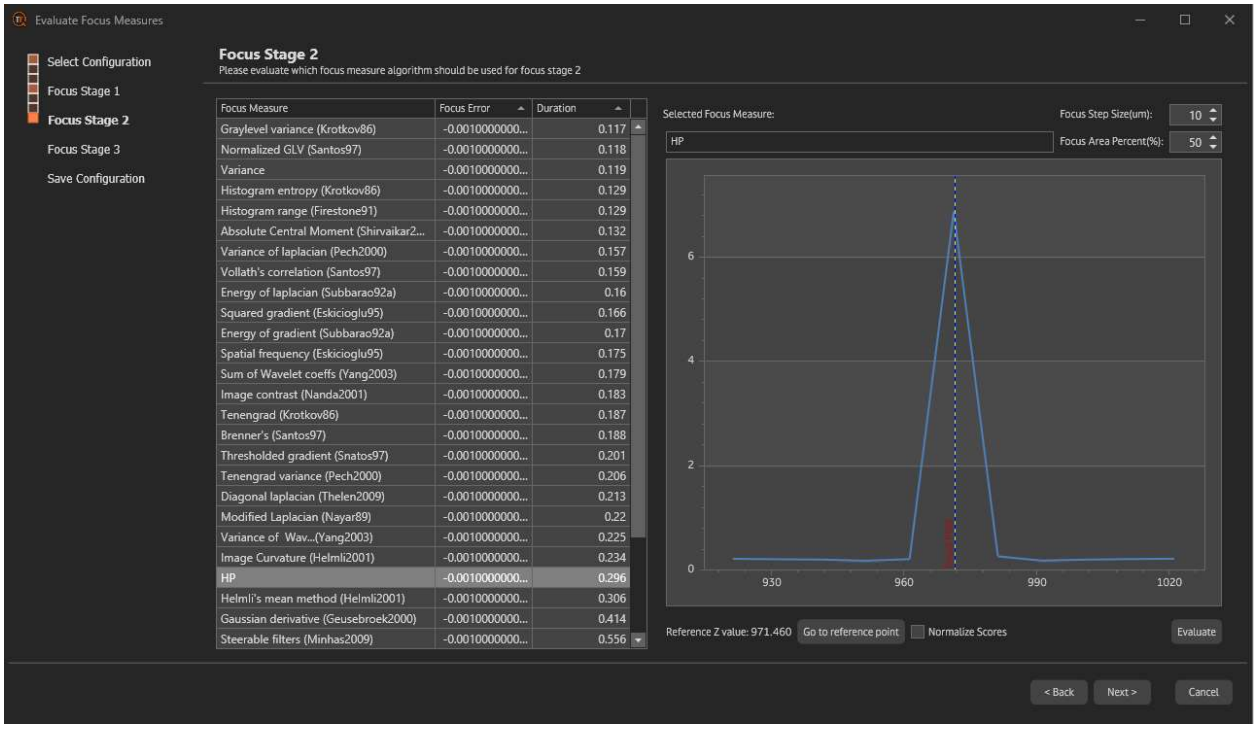


2. **Focus Stage 1**: the application automatically evaluates all the algorithms (focus measures) for the current chosen stack of images in order to select the best focus measure. The first stage will begin to capture images starting from the lower offset to the upper offset with the step of focus step, specified in the current step. The focus step size and the focus area percent can be modified manually, then press **Evaluate**. During the evaluation, the duration for each algorithm will be calculated and possible errors will be detected. The results will return as a list where focus measures will be ranked, beginning with the best. If the automatic ranking is not correct, manually select a focus measure from the list. When done, press **Next**.

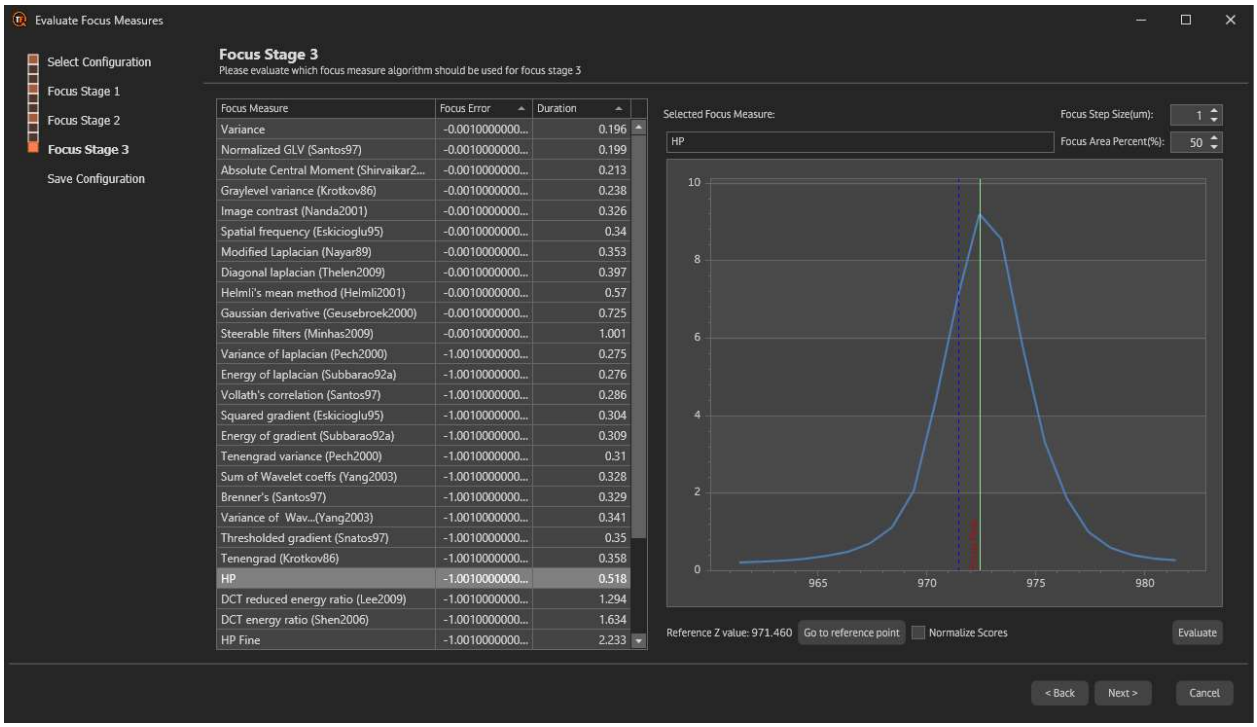


3. **Focus Stage 2**: follow the same procedure for Step 1.

Note: From stages 2 and higher, the lower offset and higher offset is approximated using the previous stage focus step size.

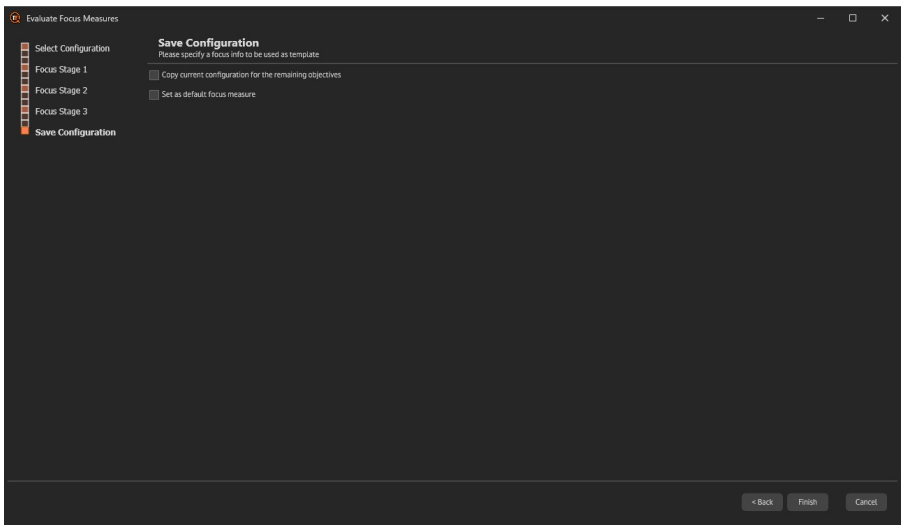


4. **Focus Stage 3**: the same procedure for all remaining stages (the same the first two steps).



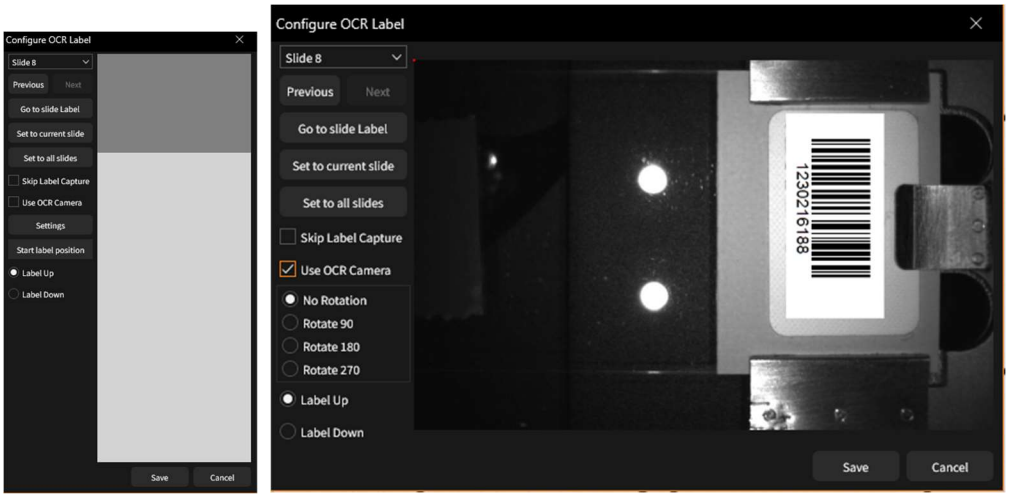
5. **Save Configuration**:

- **Copy current configuration for the remaining objectives**: The newly-created focus method will copy the focus settings (measures) for the other objectives from the initially chosen focus template.
- **Set as default focus measure**: the newly-created focus method becomes the default focus configuration and will be visible in **Tools** -> **Options** -> **Scan Settings** -> **Focus**.
- Press **Finish**.



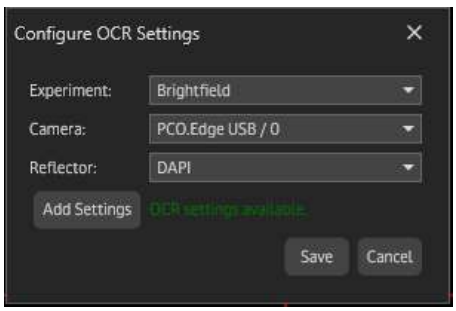
7.7. Slide Labels

Slide labels can be acquired in TissueFAXS. It is possible to adjust the acquisition settings depending on particular situations on your slides labels.

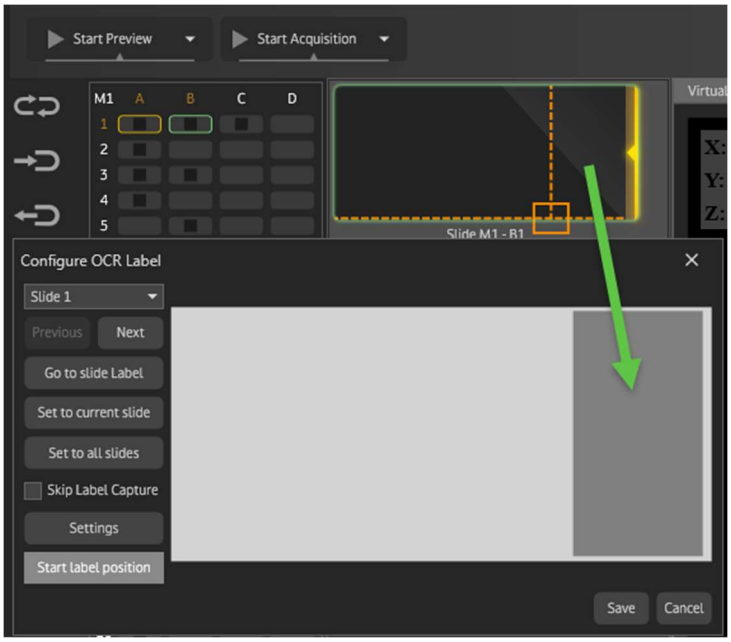


- First of all, select the **slide** where you want to acquire the label. You can navigate between slides by using **Previous/Next** buttons.
- Press **Go to slide label** in order to move the current objective in the label area on the slide.
- **Set to current slide** will set that label position to the current slide.
- **Set to all slides** will set that label position to all existing slides.
- **Skip label capture**: if checked, the label will not be acquired.
- In the **Settings** section, you can select the experiment type, the camera to be used and the reflector for the label acquisition. To save current label acquisition settings, press **Add Settings**.

Note: Settings can be adjusted directly on the live image.



- **Start label position**: current label schema will be erased and you will be able to define a new label area. Move the objective to a corner of the slide to begin defining the new area. Press **Start label position** to begin defining the new label area. Now go in the opposite corner of the label shape to finish drawing the label. To finalize the label definition, press again **Start label position**.

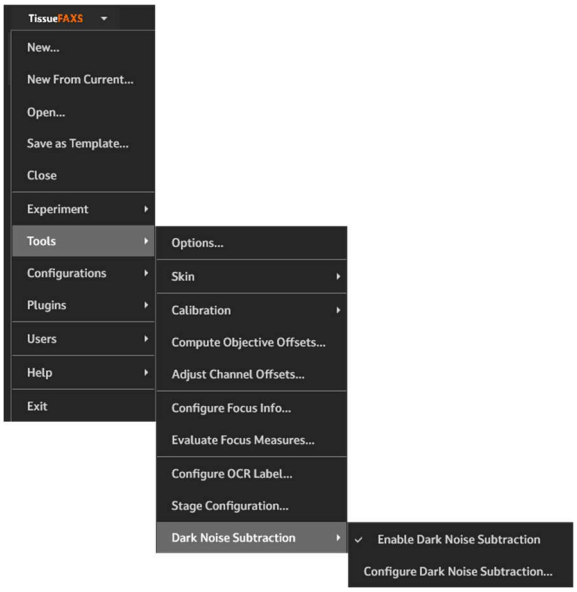


Hover with the mouse over the acquired label to zoom the image for details.

- Draw label using mouse cursor: you can draw a label by pressing SHIFT from your keyboard.

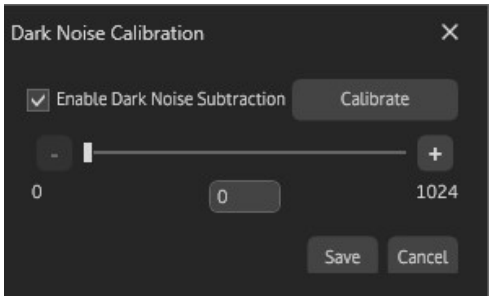
7.8. Dark Noise Subtraction

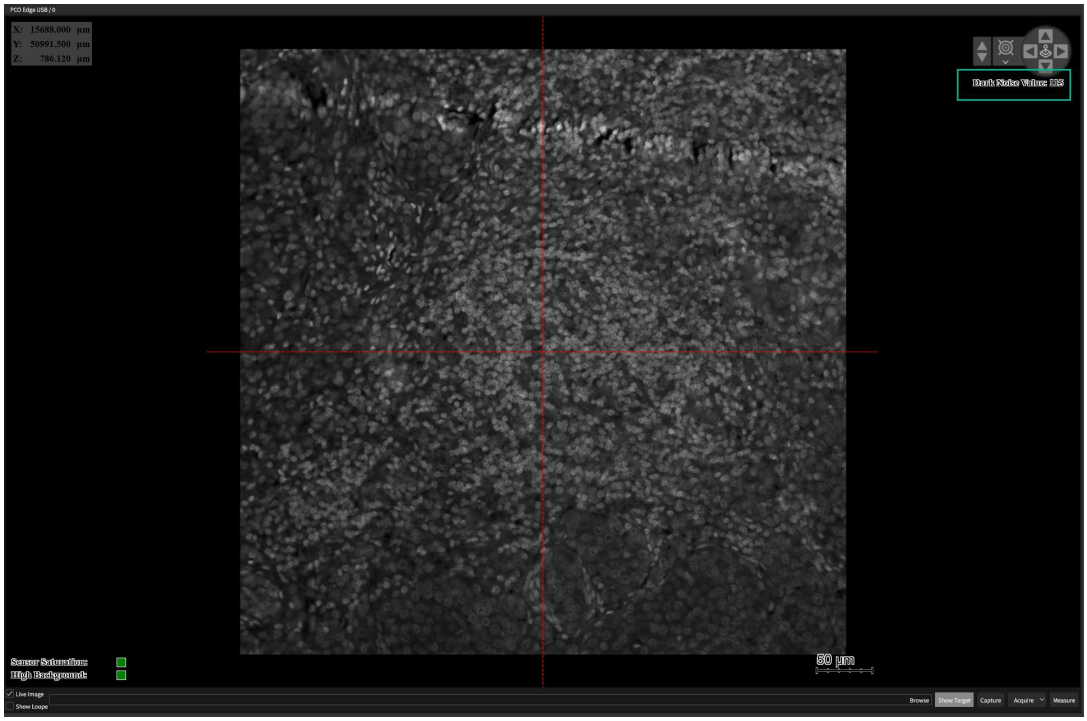
Only available for monochrome cameras, it allows subtracting a black level offset from 16-bit images. This black level offset can be set manually or by computing it based on a set of dark reference images (captured with no light and all shutters closed).



The calibration implies closing all shutters and lights from the microscope. Then, press **Calibrate** to compute the dark noise value.

This way, the further acquisitions will benefit of a dark noise subtraction, generating better quality images.

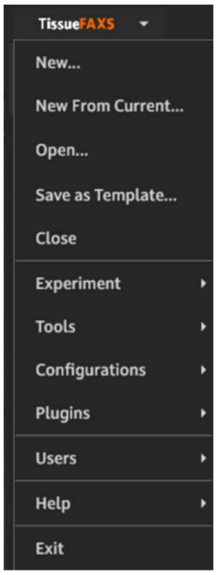




8. TissueFAXS Manager

TissueFAXS Manager is the main operational center of the application. It contains a set of menus and submenus where essential operation can be easily accessed.

The main menu can be accessed by clicking on **TissueFAXS Start** button.



New: create a new experiment;

New from current: create a new experiment from the current one;

Open: opens experiment;

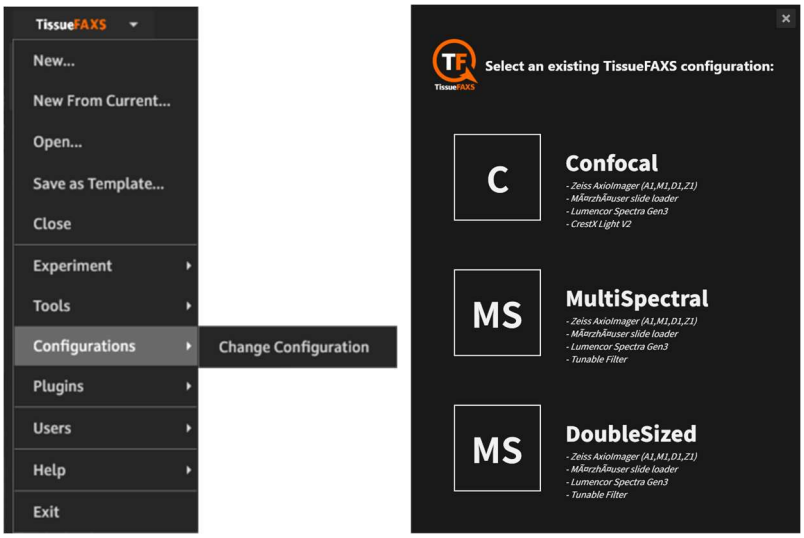
Save as template: opens a browser where you can save current experiment settings as a template, in a dedicated folder.

Close: closes current experiment.

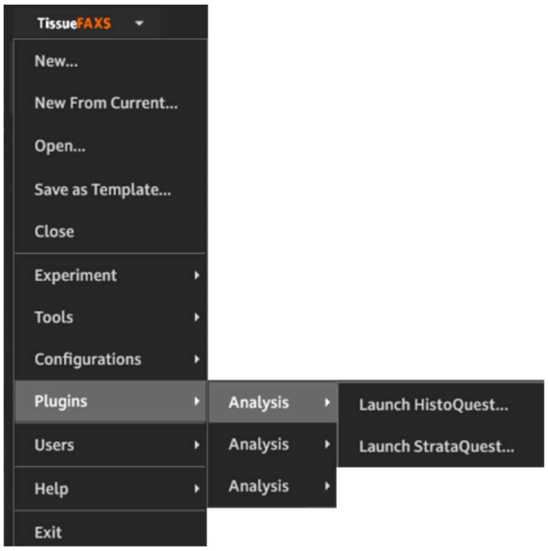
Experiment: opens all experiment related features;

Tools: opens a menu with TissueFAXS essential tools;

Configurations: you can change TissueFAXS configuration using this option, without closing the application. Please remember that the options displayed in this panel depend on your hardware setup.

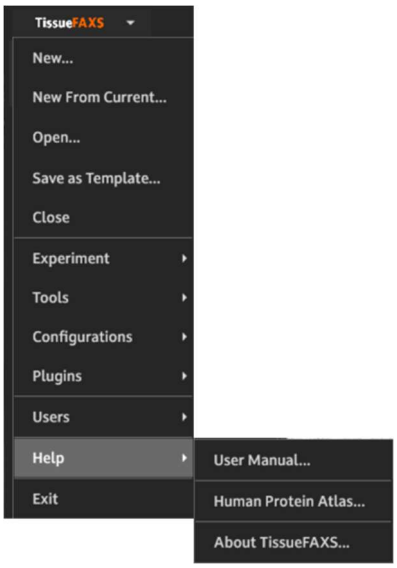


Plugins: from this control you can launch the analysis applications produced by TissueGnostics (StrataQuest, HistoQuest, TissueQuest). The current TissueFAXS project will be opened for analysis in any of the analysis applications.



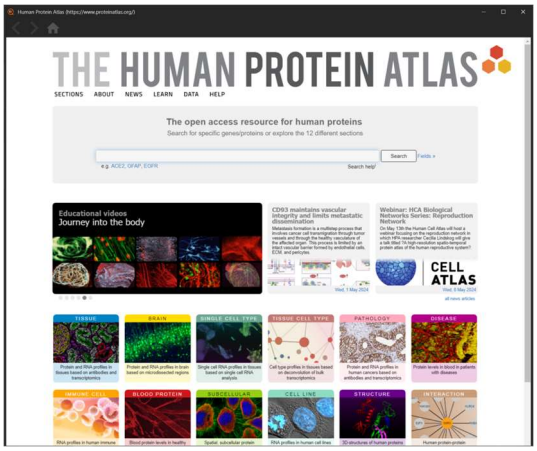
Users: opens users management section;

Help:

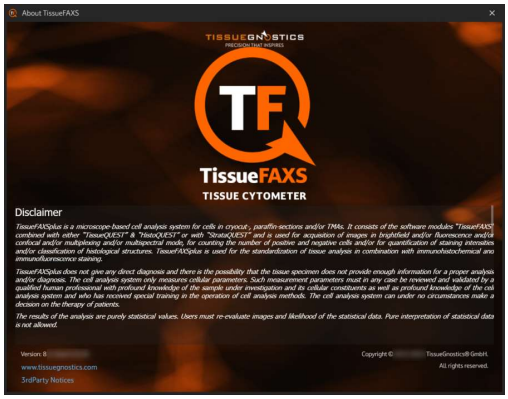


- User Manual: opens TissueFAXS user manual for detailed guidance.

- Human Protein Atlas: opens an online tool called Human Protein Atlas. This Atlas is an open access resource for human proteins.



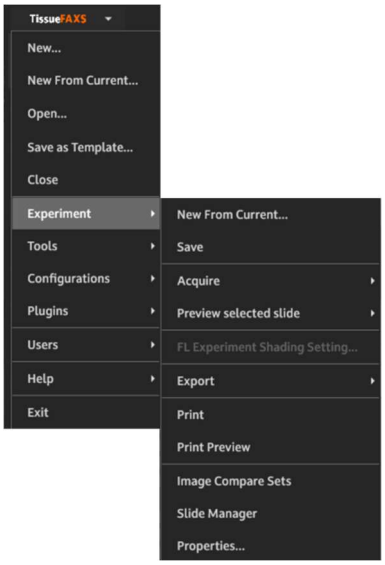
- About TissueFAXS: provides information regarding your current TissueFAXS SL version.



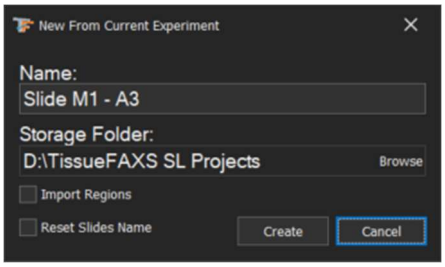
Exit: closes TissueFAXS application

8.1. Experiment Manager

This menu section contains a set of options specific for managing experiments.

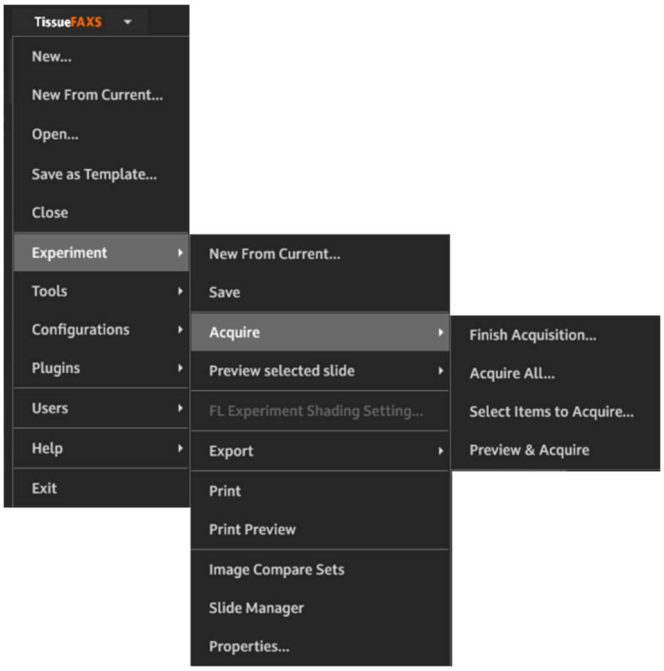


New from current: creates a new experiment that will inherit the settings of the current one. You can browse for a storage folder and decide if you want to import existing regions or reset slide names.

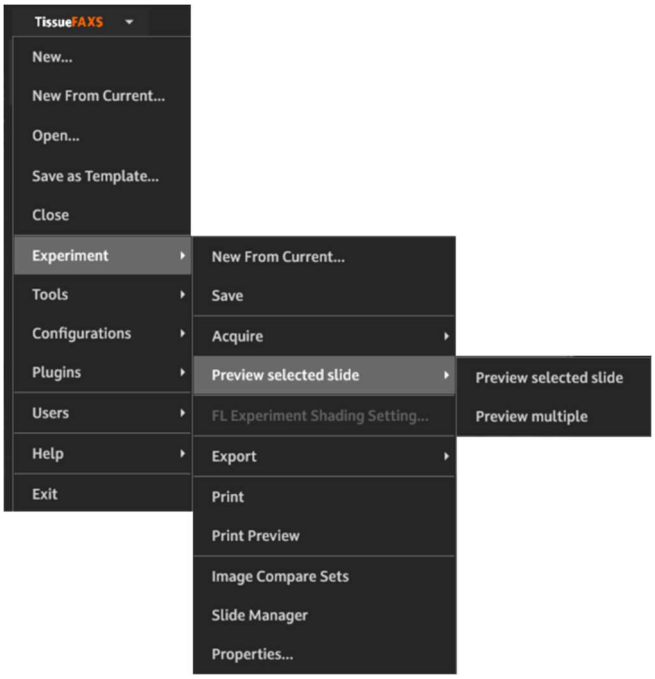


Save: saves current settings within the experiment.

Acquire: main acquisition settings can be found here. For details, see **Chapter** Starting Acquisition

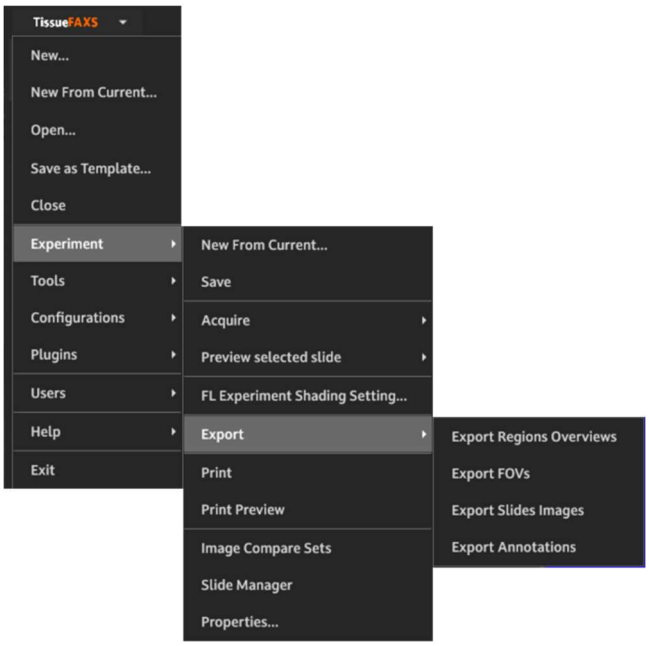


Preview selected slide: you can decide to preview selected slide or choose multiple slides. For details see Chapter Starting a Preview.



FL experiment shading settings: this feature is available only for fluorescence experiments. It allows selecting a correction image that will be used by default in future acquisitions. For details see Chapter Illumination/Shading Correction.

Export: these export options are for *all* regions/FOVs/slide images/annotations from current experiment. For details see Chapter Export Options

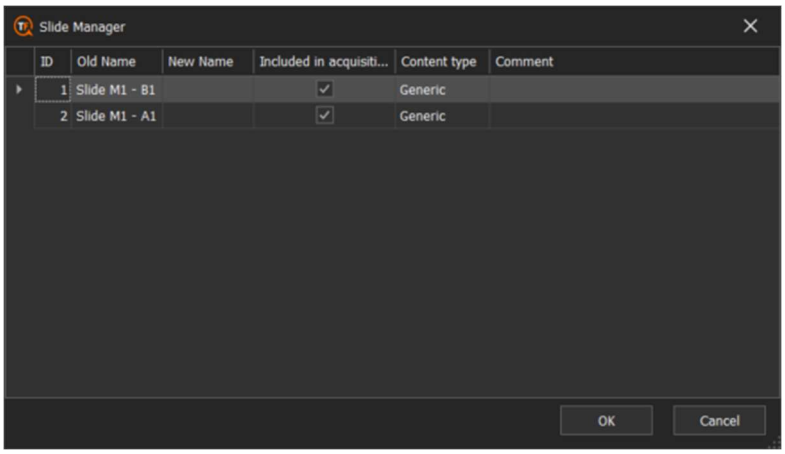


Print: see Chapter Print Experiment.

Print preview: see Chapter Print Experiment.

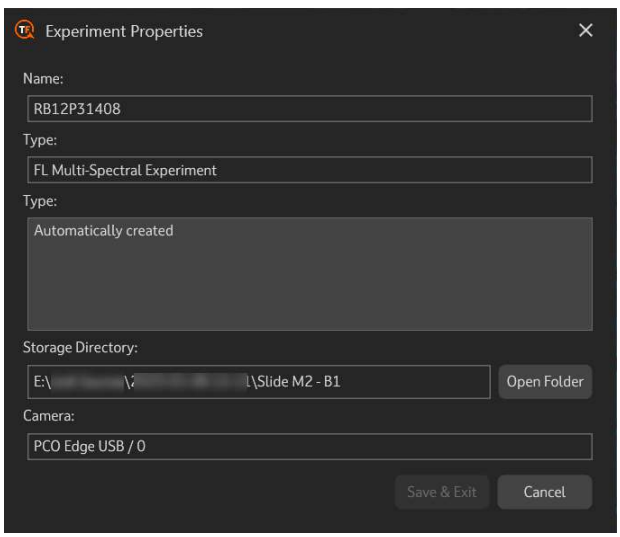
Image compare sets: see Chapter Images Compare Sets

Slide manager: this panel allows editing slide data, as shown below.



- Change the name of the slide: type the new name into the **New Name** field of the desired slide.
- Include the slide in acquisition by checking the corresponding checkbox in the **Included in acquisition** column.
- The **Content type** column identifies each slide's type.
- A comment to each slide can also be added by typing it into the **Comment** field of the desired slide.

Properties: this panel resumes the essential properties of the current experiment.

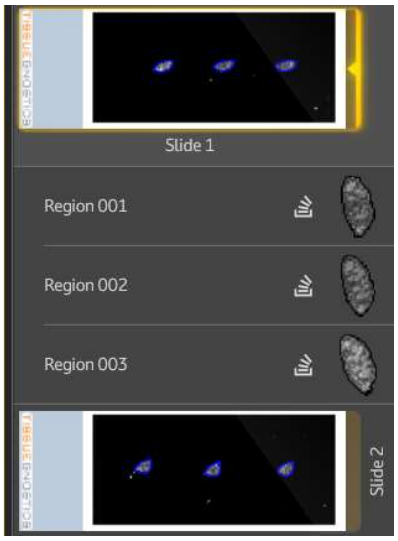


8.2. Slides Management

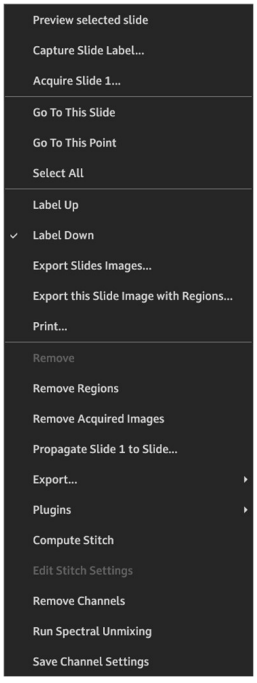
The **Slide Manager** is a feature that allows quick and simple access to the main slide settings. It is located at the left side of the application.

All the slide positions of the stage are listed in the Slide Manager.

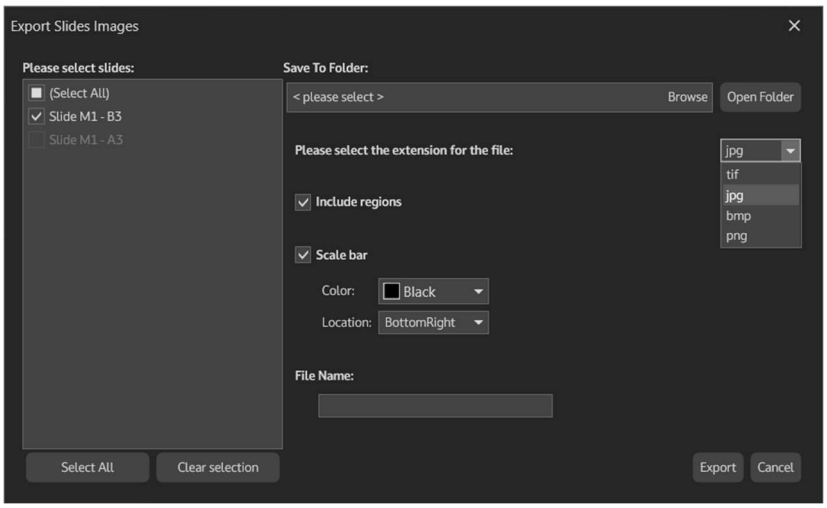
Acquired slides appear as images. Beneath each slide you can find a list of its regions, if any.



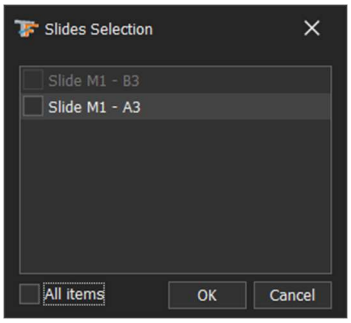
If right clicking on any slide, a contextual menu will be displayed. It contains all the slide related **options and actions**.



- **Preview selected slide**: makes a preview to selected slide;
- **Capture slide label**: select if the digital image will include or not the slide label;
- **Acquire slide**: acquires selected slide;
- **Go to this slide**: the objective goes to selected slide;
- **Go to this point**: the objective goes to selected point on a slide;
- **Select all**: selects all existing slides;
- **Label up/Label down**: select the position of the slide label;
- **Export slides images**: export preview images for selected slides. You can choose the storage folder, output file type, file name, but also decide if you want to include on the images the regions (if any) and the scale bar;



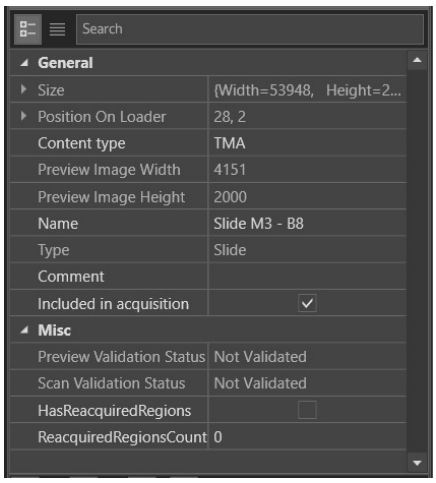
- **Export this slide image with regions**: export selected slide image with its regions;
- **Print**: see Chapter Print Experiment;
- **Remove**;
- **Remove regions**: all regions and groups from the selected slide will be removed;
- **Remove acquired images**: all images from existing regions on the slide will be removed;
- **Propagate slide**: propagates the regions existing on current slide on other slides (that you manually select). The position of the propagated region(s) will be inherited;



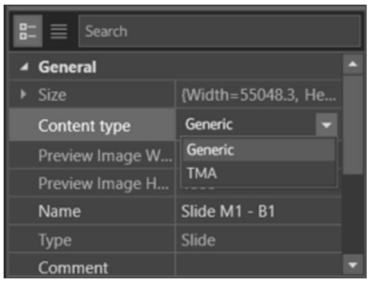
- **Export;**
- **Plugins;**
- **Compute stitch;**
- **Edit stitch settings;**
- **Remove channels;**
- **Run spectral unmixing;**
- **Save channel settings.**

The **properties** section is located below the tree-list. More precisely, it is a property grid that shows all the properties available for a specific project item.

A project item can be a slide, a region, a group, or the experiment itself. Some of the properties displayed in the properties grid are editable, while others are read-only. Here, the user may change/create names for slides, groups, and regions. The user can also choose to include/exclude a region in the acquisition process or determine if the region is already acquired.



Note: The content type of a slide cannot be set if the slide contains any regions or groups.



Some of these properties are just informative, others can be modified.

In the lower part of the slides list, there is a **tool box with display options**:

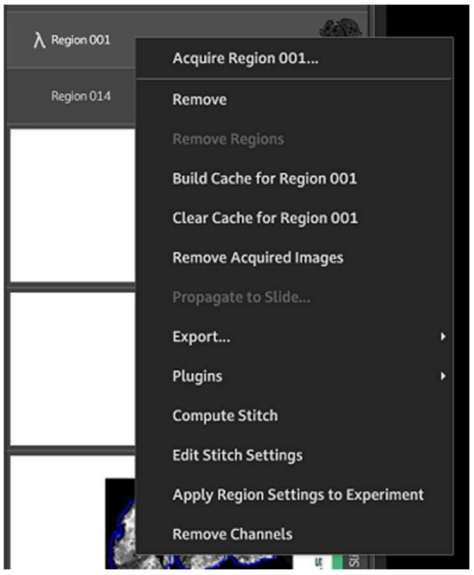
- Display regions on slide
- Display only selected region on slide
- Show stage position target
- Show label on left
- Show properties

Slides Legend

8.3. Regions Management

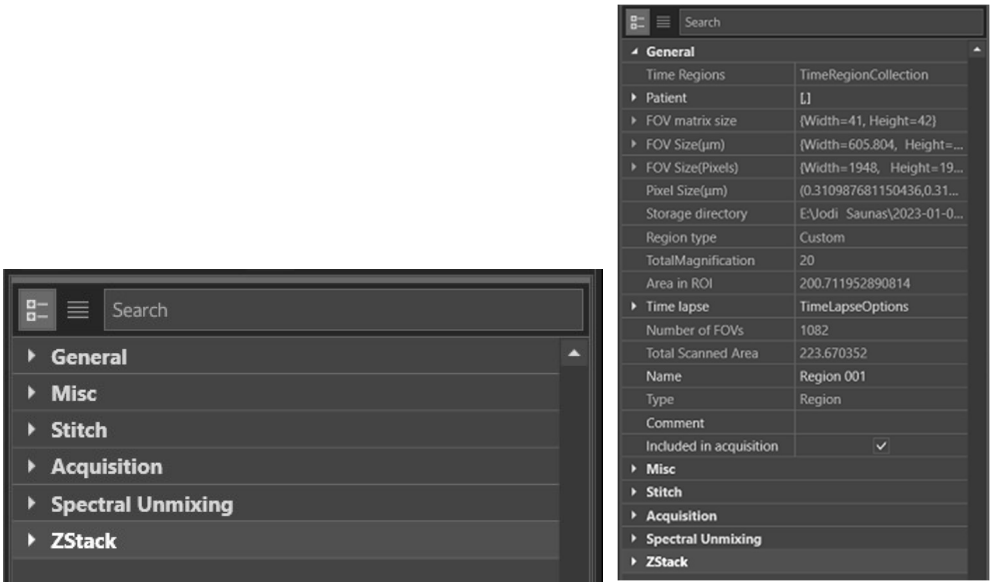
TissueFAXS regions can be managed in more ways.

1. **Using any region's contextual menu**, which provides a consistent set of options:



- **Acquire Region...:** see **Chapter** Starting Acquisition;
- **Remove**: will remove selected region;
- **Build/Clear Cache**: builds/clears cache for selected region;
- **Remove Acquired Images**: deletes all the images from selected region;
- **Propagate to Slide**: propagate selected region to other slide(s);
- **Export:** see Chapter Export Options;
- **Plugins:** launch other TissueGnostics applications;
- **Compute Stitch;**
- **Edit Stitch Settings;**
- **Apply Region Settings to Experiment;**
- **Remove Channels.**

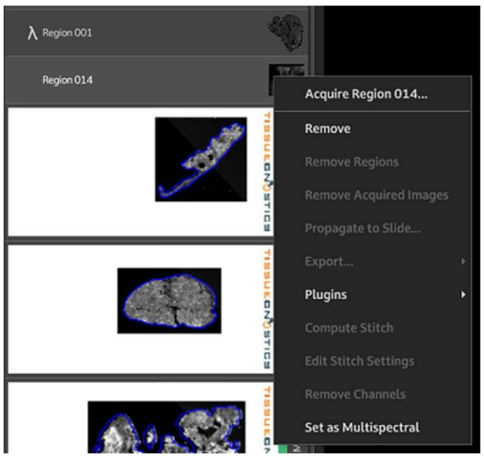
2. Using the **properties** section located below the experiment tree-list. Here, you can see details of selected region and change its name (also see Chapter Generic ROIs).



3. When **scanning**, when you can decide how the region will be scanned.

In scan mode for multi-spectral experiments, regions are scanned as normal FL regions using only the peak wavelengths.

By default, the newly-created regions will be normal FL regions. If they need to be acquired as MS, right click on the region and select **Set as Multispectral region**.



9. TissueFAXS Hardware

9.1. Cameras

TissueFAXS supports the following cameras:

- PixeLINK cameras: select this type of camera for **Brightfield** imaging;

- PCO cameras: select this type of camera for **Fluorescent** imaging;
- Hamamatsu Orca Flash 4: select this type of camera for **Fluorescent** imaging;
- Hamamatsu Fusion: select this type of camera for **Fluorescent** imaging.

9.1.1. Stage Control

The stage is the hardware component of the microscope that allows viewing the slides and their content by moving on X, Y and Z axis.

This section helps accurately control the movement of the stage of the microscope.

Stage control components

- **Joystick control**: The four arrows of the Joystick control can be used to move the stage on the X and Y axis. The adjacent **Up** and **Down** buttons control the Z axis and are used to focus on the current field of view.
- **Autofocus button**: by clicking this button, the software will perform autofocus on the current field of view.

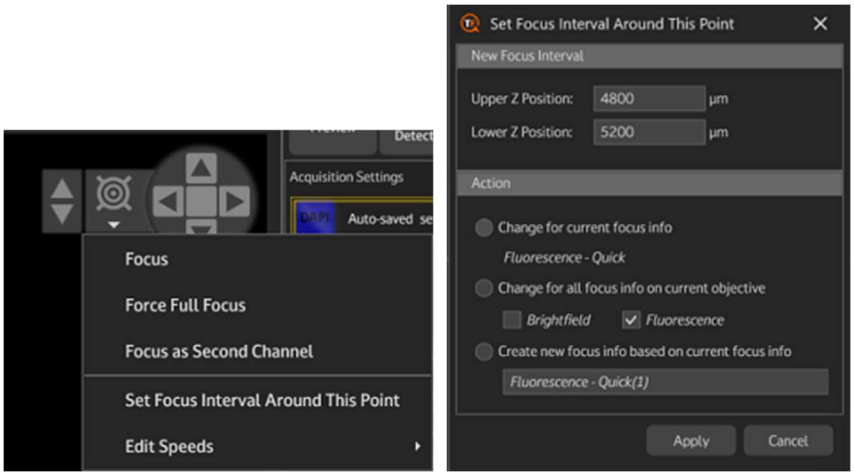
If pressing the arrow, the following options will be available:

- **Focus**: **TissueFAXS** will perform autofocus on the current FOV.

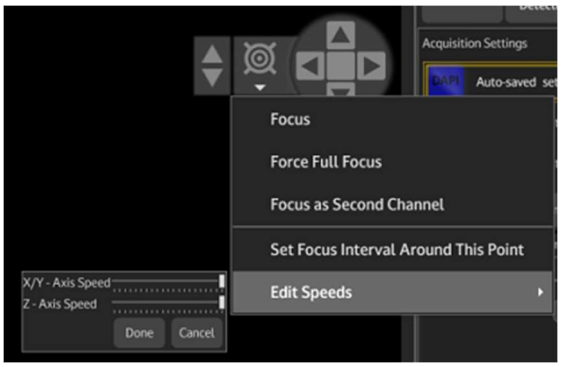
- **Force Full Focus**: The last focus position is ignored and the focus is always searched in the full interval. This method will take more time, but will produce better results for difficult samples and environments (e.g. samples with many tissue folds).

- **Focus as Second Channel**: the focus will be made on the second channel.

- **Set Focus Interval Around this Point**: opens a dialog where Upper and Lower Z Position values can be modified manually. The new values can be applied to the current focus info, to all focus info on current objective (for brightfield or fluorescence), or new focus info can be created based on current focus info.

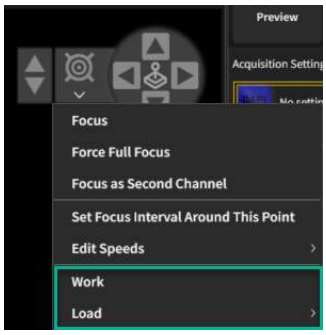


- **Edit Speeds**: this button adjusts the speed at which the stage is moving on X, Y and Z axis when using the hardware joystick, the joystick control or when the following commands are used: **Go to this point**, **Go to this slide** and **Move one FOV**. After the speeds have been adjusted, click the **Done** button to save them. To cancel, press **Esc** or click elsewhere outside the popup. (To adjust the stage speed during acquisition, go to **Tools → Options → Scan Settings → Speed**.)



You can also control the Z of the microscope manually, by using the mouse wheel on the live image:

- Mouse wheel with a step of 50 micrometres
- Ctrl + mouse wheel with a step of 10 micrometres
- Alt + mouse wheel with a step of 1 micrometre
- **Load**: moves the stage down to the load position, to add or remove slides.
- **Work**: moves the stage up to the work position.



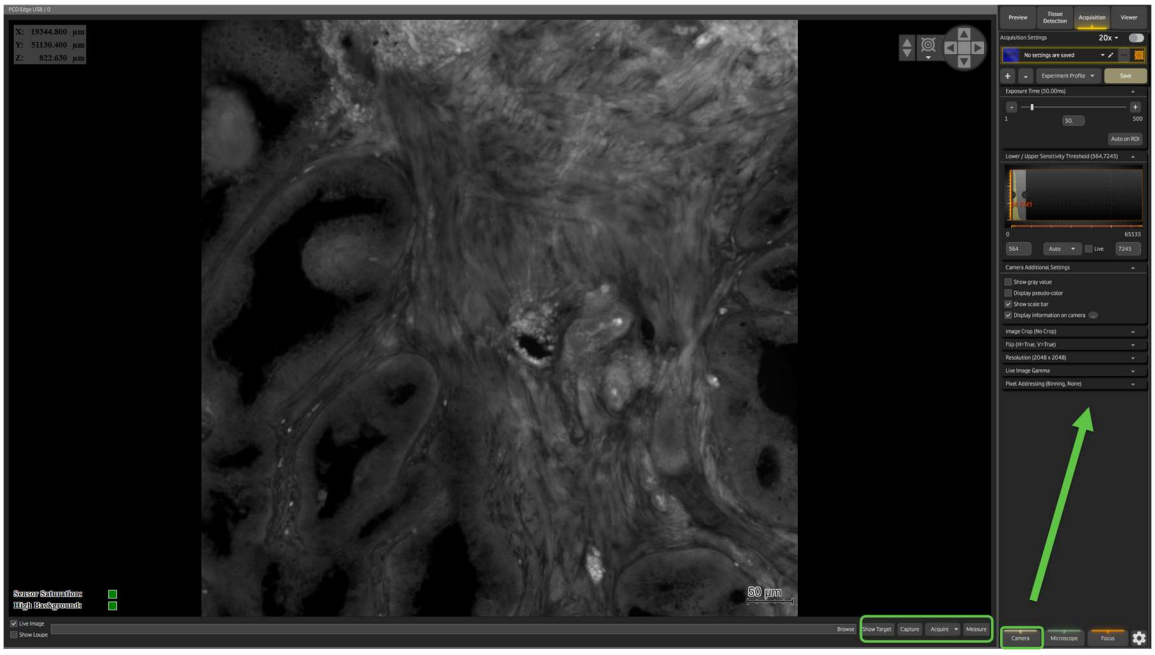
Notes:

- These stage controls only work with a motorized focusing drive.
- When working with a TissueFAXS system, which uses an inverted microscope, the objective revolver will be moved down and up rather than the stage.

9.1.2. Camera Controls

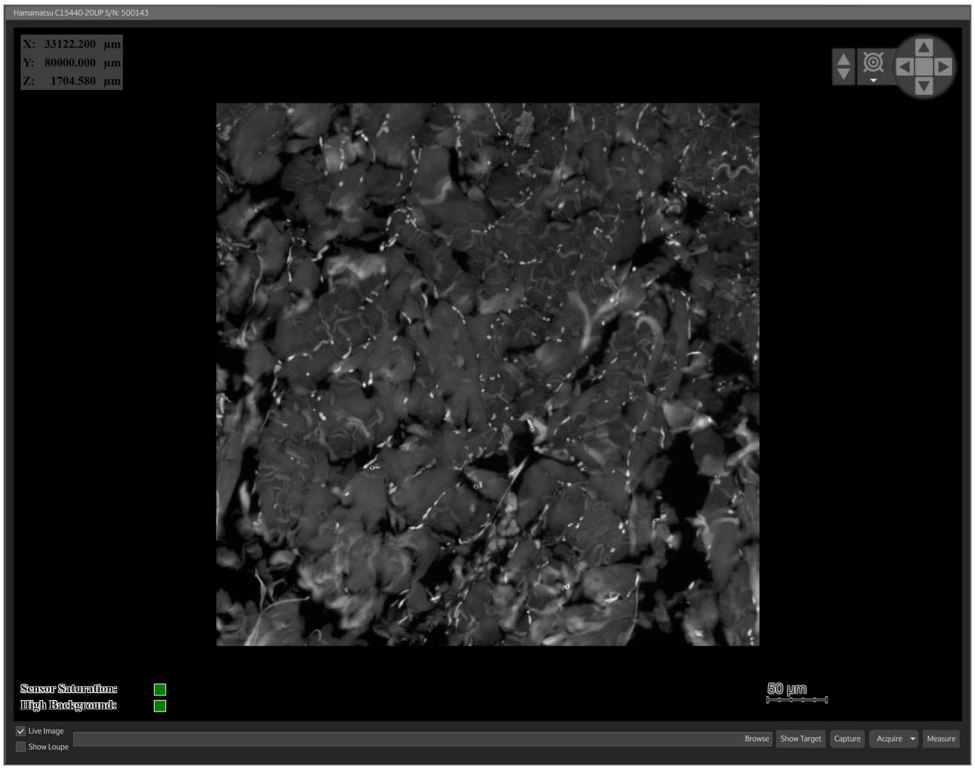
Camera Panel

The **Camera Panel,** one of the essential tools in **TissueFAXS,** contains the stage control, camera controls, and a panel where camera parameters can be added and adjusted.



Camera Control

The camera control can be used to operate the camera to display a live preview, capture images and adjust camera settings.



View live preview

When working with **TissueFAXS**, the **live image** preview can be enabled or disabled by using the checkbox.

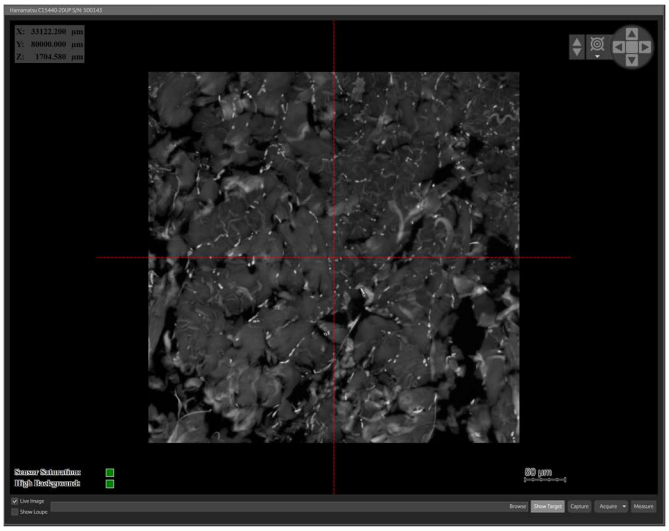
- To enable live preview check **Live Image**;
- To disable live preview uncheck **Live Image**.

Notes

- Any time you press the View button from the channel list, the Live Image will be enabled.
- If the function Live Images is disabled during acquisition, the overall acquisition speed will slightly increase.

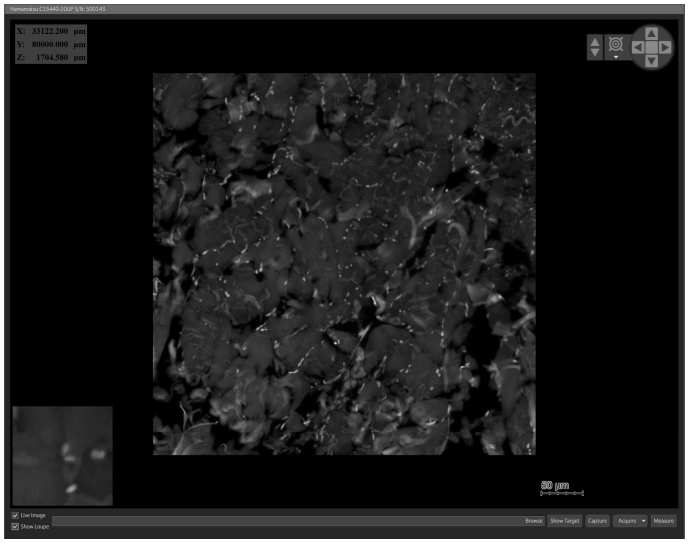
Show Target

Press the **Show Target** button to display the target indicating the center of the image. This feature is useful when computing objective offsets, verifying the accuracy of FOV calibration, etc.



Show Loupe

Show Loupe feature offers a quick display of magnified areas of the live image. Press the **Show Loupe** button, then hover the mouse over the live image to see magnified areas.

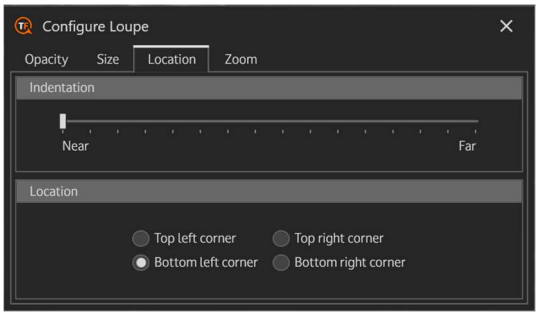


The loupe configuration panel can be accessed by right-clicking on the loupe image. The following **settings** are available:

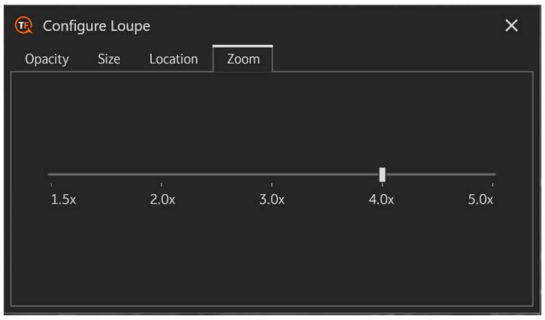
- **Opacity**: sets the opacity of the magnified image.

- **Size**: sets the size of the magnified image.

- **Location**: sets the location of the magnified image on the live image and also the indentation (near/far the viewer's edges).

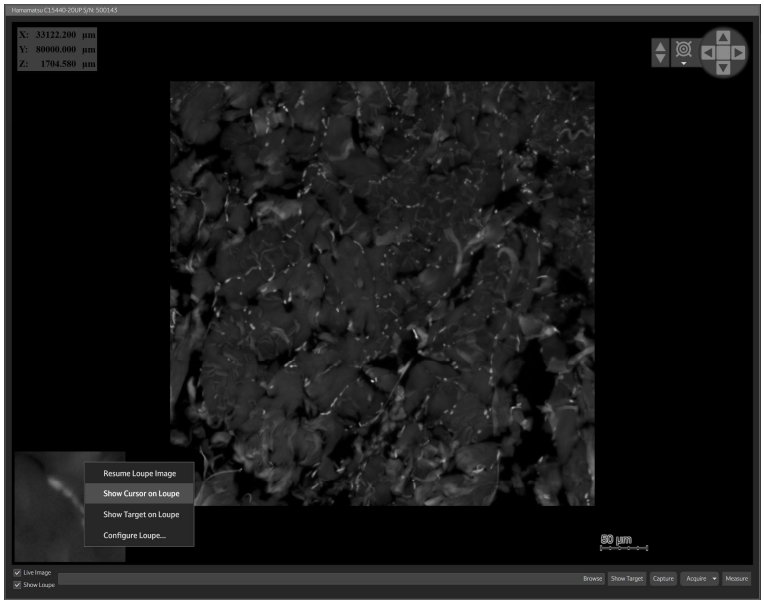


- **Zoom**: sets the zoom level of the magnified image.



- **Show cursor on loupe**

It is possible to show a cursor/target on the loupe image. The option is available in the contextual menu, as shown below.



- **Freeze Position**

Loupe on live camera image has an option to **freeze position**, instead of following the mouse each time it moves.

When hovering with the mouse over the live image, press the **Space** bar on the keyboard in order to freeze the loupe image while continuing to move the mouse over the image.

- **Capture images (manual procedure)**

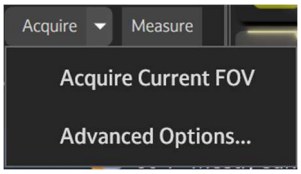
Before manually capturing an image (without using the automatic acquisition feature of the **TissueFAXS** software), choose a location and filename by pressing the Browse button. Next, capture the image by pressing Capture button.

Note: If a desired filename already exists, the TissueFAXS software will not overwrite the existing file. The new filename will be incremented by a numerical extension that will generate a file name that does not already exist. For example, if the filename C:\image.bmp is chosen, the new image will automatically be saved as C:\image.bmp.If that filename already exists, the name will be C:\image 001.bmp.

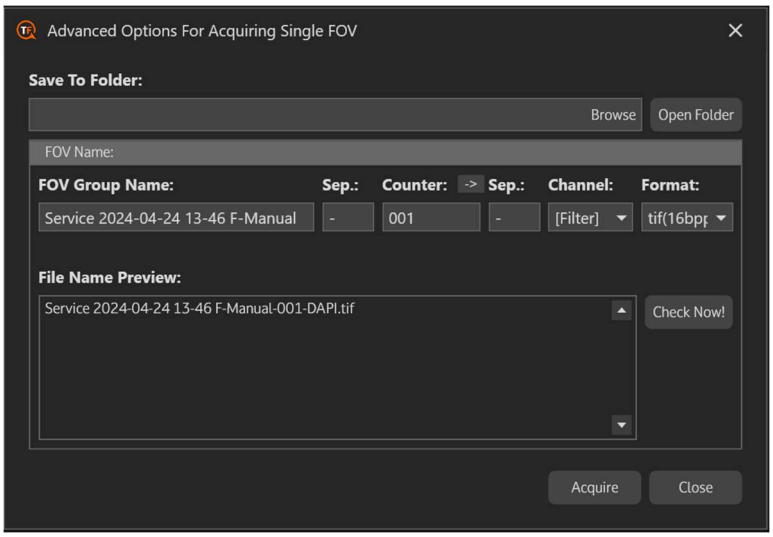
Acquire Single FOV (manual procedure)

Acquiring a FOV means capturing one image (or with multi-color fluorescence - one image per reflector). **Reflectors** can be found in the channels list in the **Acquisition** settings for the current experiment. A current experiment must be created in order to acquire a single FOV. In addition, be sure that the camera settings for all reflectors have been properly adjusted before acquiring an image.

Clicking on the right side of the **Acquire** button will pop-up the following menu:



Pressing the **Acquire** button is the same as choosing **Acquire Current FOV** from the pop-up menu.



The **Advanced Options** can also be adjusted. At the first attempt to acquire, there will be a prompt to go to the window shown above, with a request to enter the following information:

- The **storage folder** for the acquired images;
- **FOV Group Name**, which acts as a naming template for files to be acquired;

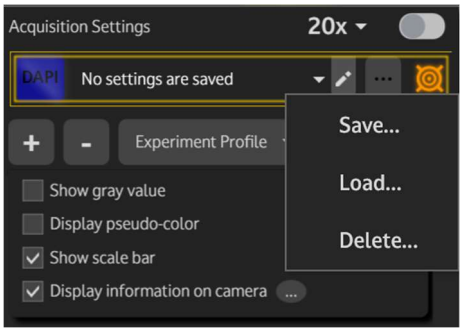
- **Separators** inside the filename (the character used as a separator should not be used as part of the filename provided by the user);
- **Counter** is used to avoid duplicate filenames when pressing the **Acquire** button multiple times;
- **Channel** (usually, the reflector name);
- **Format** of the graphics file.

Note*:* Filename Preview section contains the file or the list of files obtained after the acquisition process. At any time, the Check Now button can be used to verify the validity of a filename in the specified location (e.g., if the name is valid, if an existing folder has been specified, etc.).

Camera Profile

A **Camera Profile** represents a group of settings that can be saved for further use, making different combinations of settings available for the acquisition process.

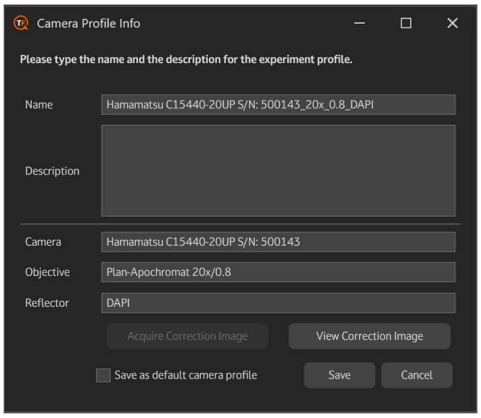
A profile implies a collection of camera-related settings, as well as a collection of microscope settings such as, for example, TL lamp intensity (for Transmission) and UV lamp attenuator settings (for Fluorescence channels).



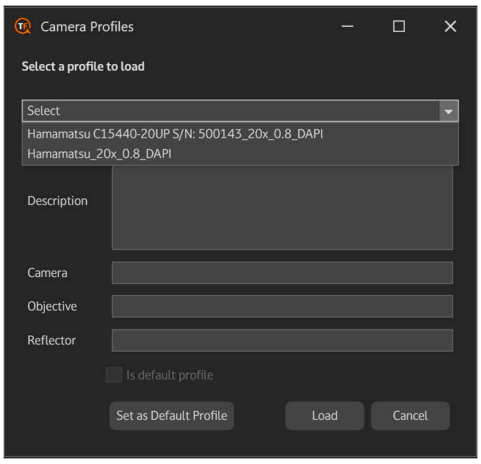
- **Save**: the current camera settings can be saved for further use. A name and a short description must be specified for each profile. A default name is already generated. It contains the camera name and type, the objective magnification, and the reflector selected on the microscope. However, the default name and information can be modified.
- If "**Save as default channel profile**" is checked, the settings from default channel profile will automatically apply to the current filter and objective.

If pressed, the **Acquire Correction Image** button will acquire the correction image for the acquired images. For more details please see Chapter Illumination/Shading Correction.

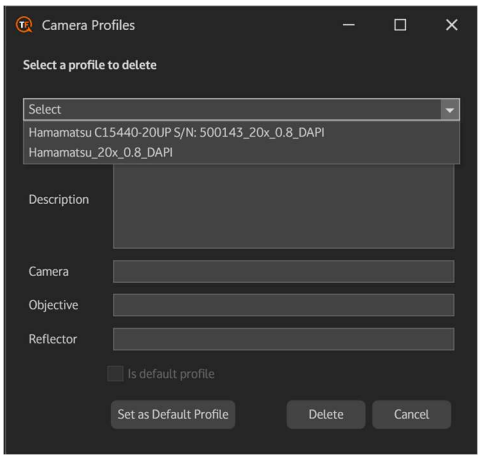
Note*:* Acquire Correction Image button is only available for the Transmission channel.



- **Load**: an existing camera profile can be selected from the list and loaded for a camera.



- **Delete**: if an existing camera profile is no longer needed, it can be deleted by selecting it and then pressing the **Delete** button.



Save

By pressing this button, camera settings will be saved for the channel identified by current objective and current reflector.

Move by One FOV

With these buttons. the live image can be moved exactly one field of view up/down or left/right (only available after stage calibration).

Double click to center

Double-clicking on any point in the live image will center the image at that point.

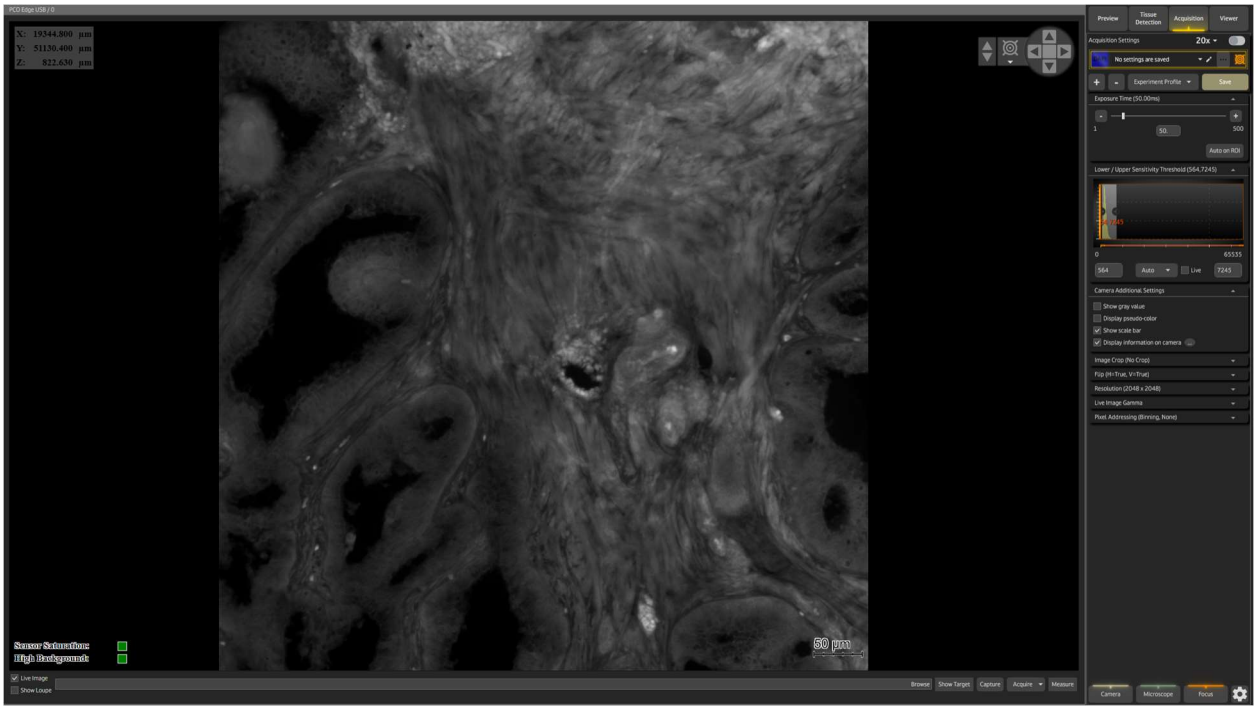
9.1.3. Camera Settings

TissueFAXS compatible cameras each have a set of settings allowing refining the image to obtain optimal results.

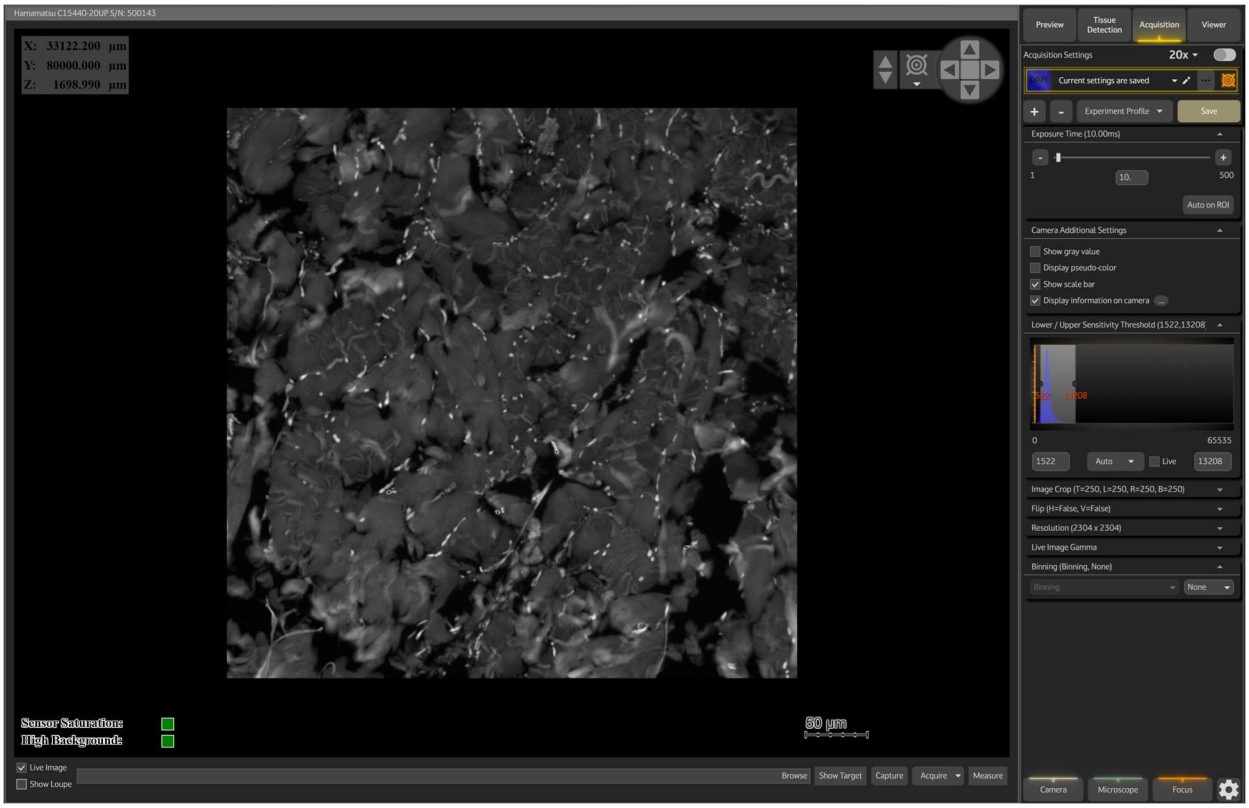
Some of the settings are general for all the cameras, some are specific to certain cameras.

Below, you can find the settings for each camera, with images and explanations for the parameters.

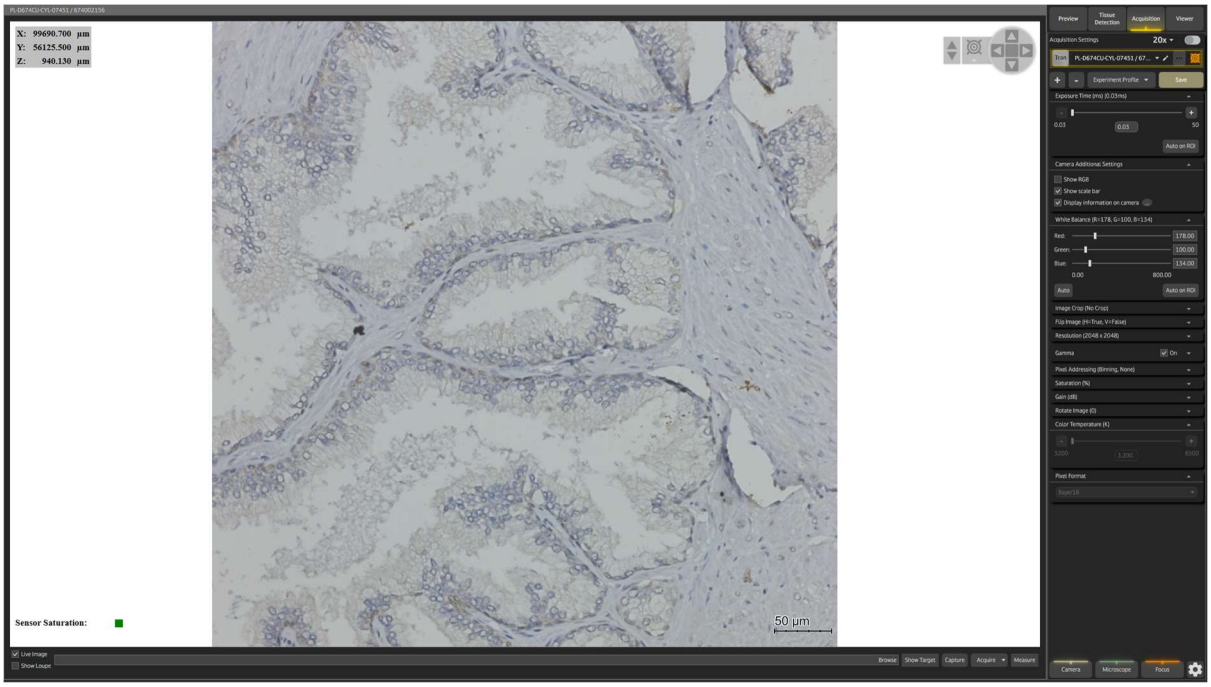
PCO Edge Camera



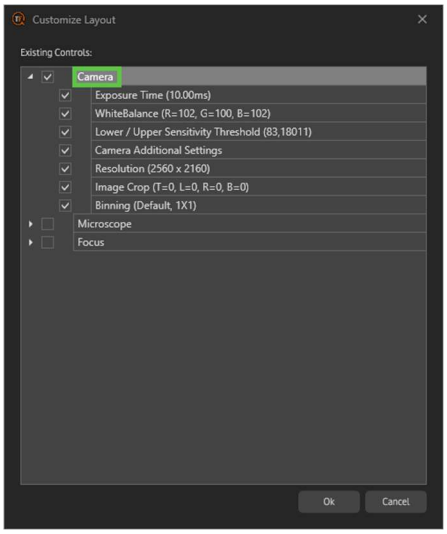
Hamamatsu Camera



PixeLink Camera



You can add or remove camera settings from the **Camera** panel by accessing **Customize Layout** section (press ).

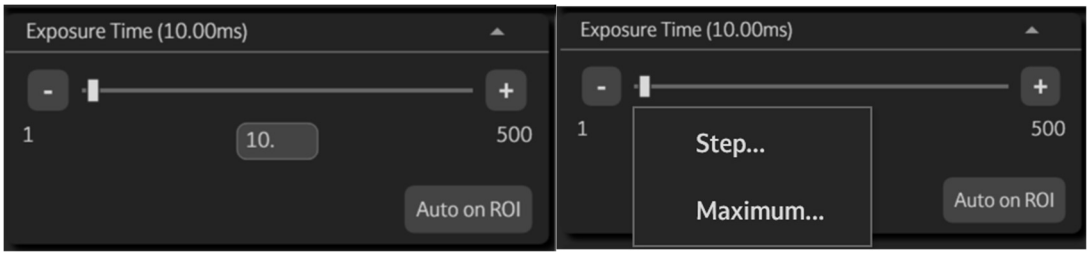


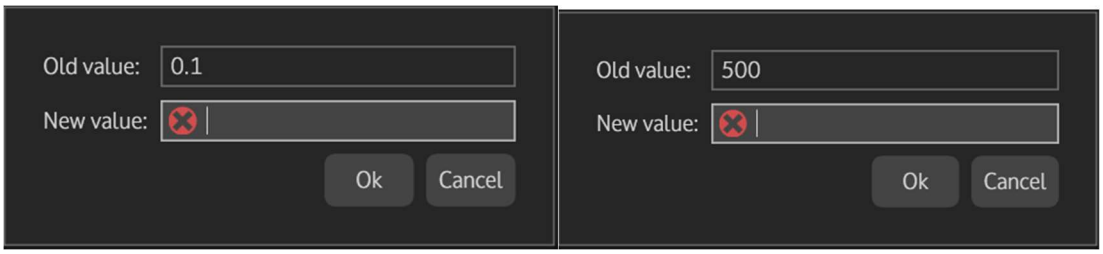
Camera Main Settings

- **Exposure time**

Exposure Time: controls the amount of time available to the sensor for collecting light to form an image.

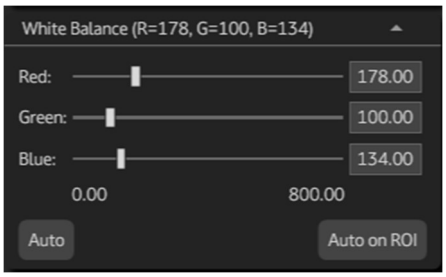
Note*:* Due to the large value (10000) allowed for exposure time for monochrome cameras, this control starts with the maximum value of 500 to increase usability. If a higher exposure time is required, right click on this control and access the Maximum menu.





- **White Balance (Color camera only)**

White balance adjusts the intensity of the Red, Green, and Blue colors in order to render a neutral color correctly (white is usually used as a reference). Press Auto to balance the colors. When the transmitted light is not white, White Balance can be used to correct the hue of the light to obtain the reference white light.



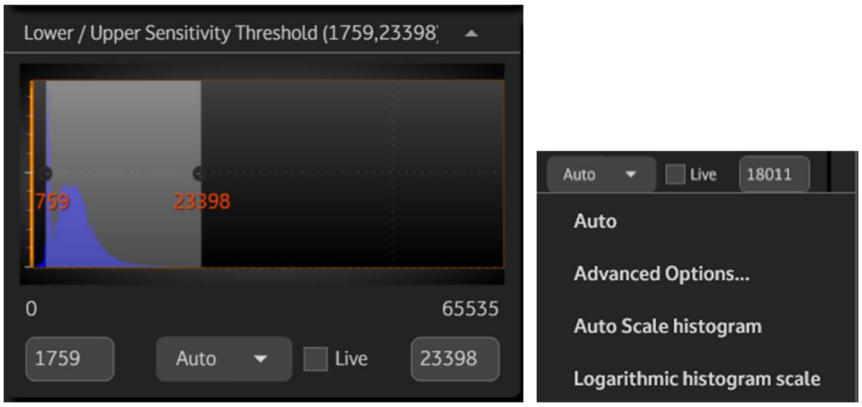
- **Lower/Upper Sensitivity Threshold (Monochrome camera only)**

Specifies the interval that will be scaled to 256 gray levels for display.

- Lower Sensitivity Threshold: by increasing this parameter, the low intensities (like background) in the image will be lost.
- Upper Sensitivity Threshold: by decreasing this value, the shades in the image will become brighter.

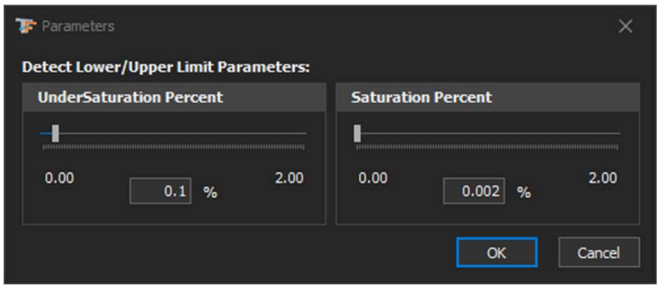
Notes:

- To manually adjust these parameters, they should be set in such a way that the lower parameter limit equals the smallest value for which the **High background** is green and the highest value for the upper parameter for which the **Saturation** indicator is green.



These parameters can be automatically adjusted by pressing the **Auto** button.

By choosing **Advanced Options…** the following dialog will appear:



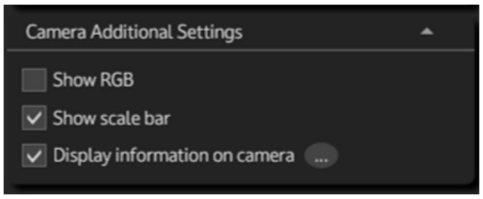
The advanced options control how **TissueFAXS** detects the presence of oversaturation and undersaturation (lack of signal) in the camera image.

UnderSaturation Percent: If the number of pixels with intensity value higher than the (under-) saturation limit is over this percent of the total number of pixels, then the image is considered undersaturated.

Saturation Percent: If the number of pixels with intensity value higher than the (over-) saturation limit is over this percent of the total number of pixel then the image is considered oversaturated.

Camera Additional Settings

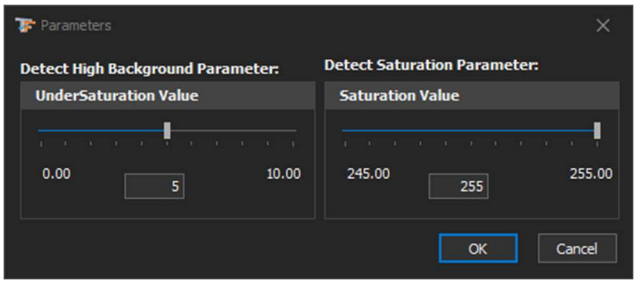
For color cameras



- Show RGB

- Show scalebar

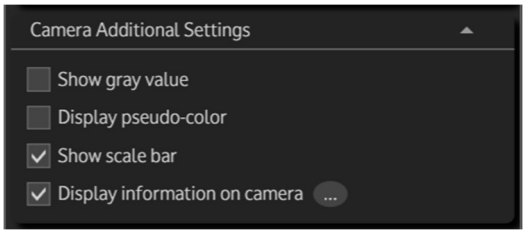
- Display information on camera



UnderSaturation Value: The maximum acceptable intensity value for which a pixel is considered undersaturated.

Saturation Value: The minimum acceptable intensity value for which a pixel is considered oversaturated.

For monochrome cameras



- Show gray value

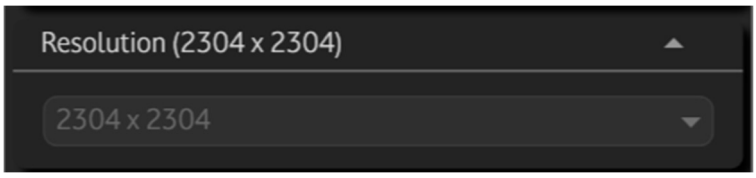
- Display pseudo-color: for the fluorescence gray scale images, this parameter emulates the way they can be seen through the ocular, using the color of the reflector in use.

- Show scale bar

- Display information on camera: displays the histogram, saturation, and high background indicators on the live image.

Warning: The camera settings described below should be modified only if advised by TissueGnostics personnel, in order to avoid failures in the scanning process caused by unappropriated values of the parameters.

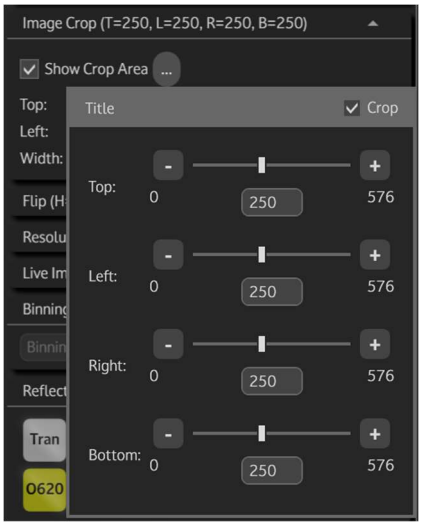
- **Resolution**



- **Image Crop**

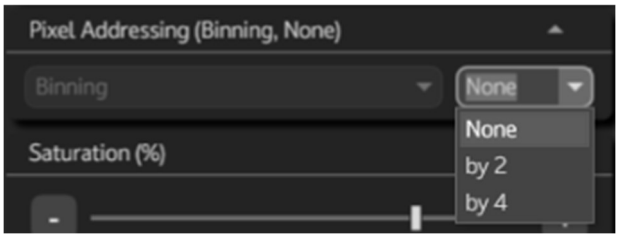
Allows adjusting the display of the image of the viewer by cropping it according to the needs of the operation. The crop area can be displayed by checking/unchecking **Show Crop Area**.

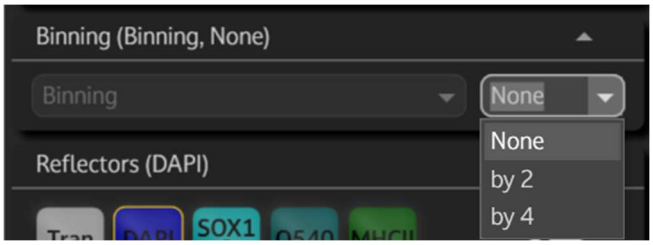
To adjust the crop area (in pixels), press the "..." button and set a value for Top, Left, Right, and Bottom.



- **Binning**

Is used to decrease the size of the captured image and decrease the time needed for the capture operation. This will increase overall acquisition speed, but the image quality will be reduced.

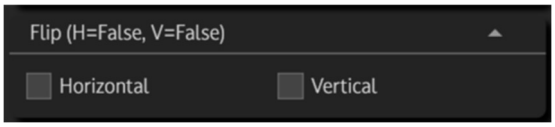




Other Camera Settings

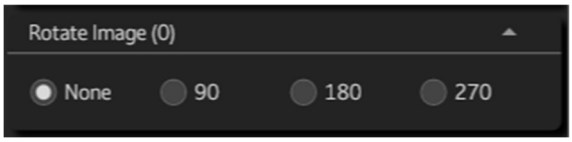
- **Flip**

Used to flip the captured image. This parameter can correct the orientation of the image if the left/right or top/down of the camera are different that those of the stage. This parameter is generally adjusted when the system is installed. The FOV calibration feature can automatically set this parameter to its correct value.



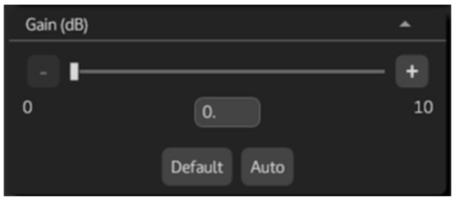
- **Rotate Image**

Used to rotate the captured image. Use this when the camera is placed in a 90/180/270 degree position in relation to the stage.



- **Gain**

Artificially adjusts the intensity of pixels (through software multiplication of pixel values).



- **Pixel Addressing**

Decreases the size of the captured image and the time needed for the capture operation. This will increase overall acquisition speed, but the image quality will be reduced.

- **Frame rate**

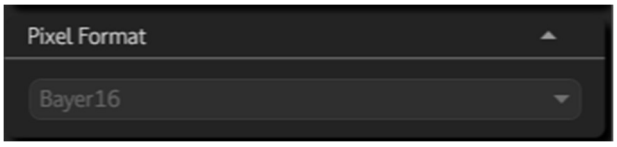
The maximum number of images captured per second.

- **Color Temperature**

Similar to white balance, it makes the picture look colder or warmer.

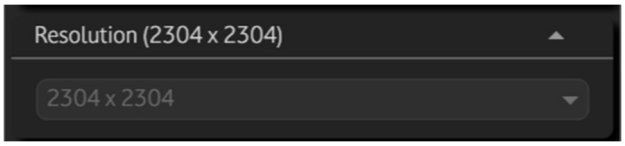
- **Pixel Format**

Used when transferring images from the camera (only experts in imaging should change the settings).



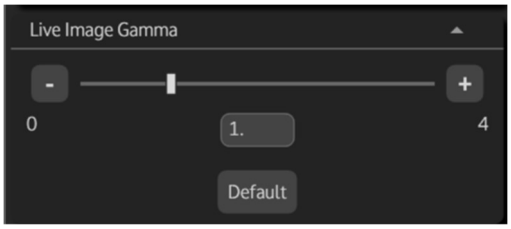
- **Resolution**

Represents the size (in pixels) of the captured images.



- **Gamma**

Adjusts the contrast of the image. This can be used to highlight the differences between the light and dark areas or to minimize these differences.



- **Image Format**

Allows selecting the image format used for the transfer.

Notes:

- Image format is not supported by all cameras.

- This parameter should only be modified at the direction of the support team.

- **Sensor saturation**

This set of parameters has no effect on the final images. The sensor is used to warn of possible oversaturation of images which might have a negative effect on the illumination correction process. The two parameters are:

- Max allowed values: if there is a very bright image that is considered oversaturated (the sensor turns red), this value can be increased so the sensor will accept it.

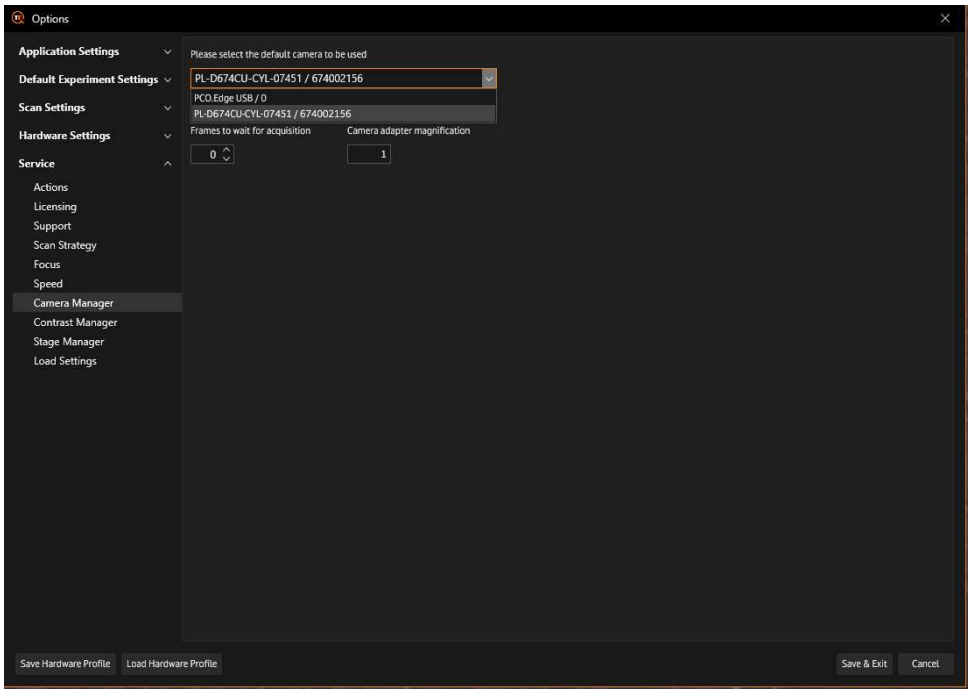
- Sensor saturation area: if the number of bright pixels (with a value above maximum allowed) is lower than Sensor saturation area, the image is considered to be fine; otherwise, it is considered oversaturated. Consider increasing this value when working with samples with many bright areas.

9.1.4. Adding and Removing Camera

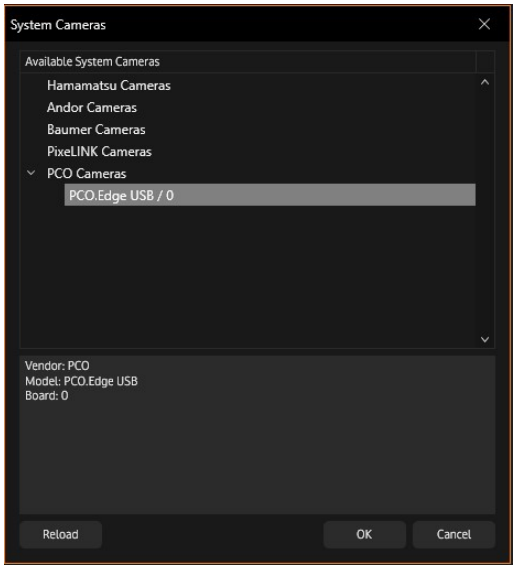
Adding Cameras

Use these steps to add a compatible camera:

- From **TissueFAXS** → **Tools** menu, select **Options…** to open the **Options** dialog box.
- In the **Options** dialog, choose **Hardware Settings** → **Camera Manager**.



- Press the **Add new camera…** button. The **System Cameras** dialog box will appear and it will display the cameras that are supported by **TissueFAXS** and are connected to your computer.



- In the **System Cameras** dialog, select the desired camera and press **OK**. If the camera is not listed there, you can press **Reload** to rescan for connected cameras.

Note*:* A camera already used in TissueFAXS may not be listed, even if you press Reload.

- Now, the selected camera should appear in the combo box.
- **Frames to wait**: This value indicates how many images the camera will skip before the image will be taken and stored. If this value is too low, the images may appear stretched because they were taken before the stage has completely stopped after rapid movement (motion blur). Default values are 1 or 2; higher values may be suitable if working with cell culture dishes.

Note*:* It is recommended to set a certain delay for stage movements and set Frames to wait to 0.

- **Camera adapter magnification**: this is an adapter used to connect the camera to the microscope, having its own magnification factor (written on it); if this information is missing or incorrect in **TissueFAXS**, the total magnification of the experiment will be incorrect.
- Press **Save & Exit** to save the settings and close the **Options** dialog.

Change Default Camera

The camera can be changed at any time by following these steps:

- From **TissueFAXS** → **Tools** menu, select **Options…** to open the **Options** dialog box;
- In the **Options** dialog. choose **Hardware Settings** → **Camera Manager**;
- Select the desired camera from the combo box.
- Press **Save & Exit** to save the settings and close the **Options** dialog.

Operations with Current Camera

The camera that is currently in use allows the following actions:

- Enable or disable the live image for the current camera (depending on what is being worked on, seeing the live image may or may not be desired);

- Capture an image with the current camera (the main purpose when using the camera);
- Adjust the settings for the current camera (to obtain the desired results – in preview or image acquisition – camera settings can be adjusted continually).

Remove Camera

The camera can be changed at any time by following these steps:

- From **TissueFAXS** → **Tools** menu, select **Options…** to open the **Options** dialog box;
- In the Options dialog, choose **Hardware Settings** → **Camera Manager**;
- Select the camera from the combo box and press the **Remove camera** button.

Note: If the camera is not present in the combo box, its removal is not necessary.

9.2. Microscope Controls

9.2.1. Objectives

The **Objectives** panel displays the objectives available in the microscope's Objective Turret.

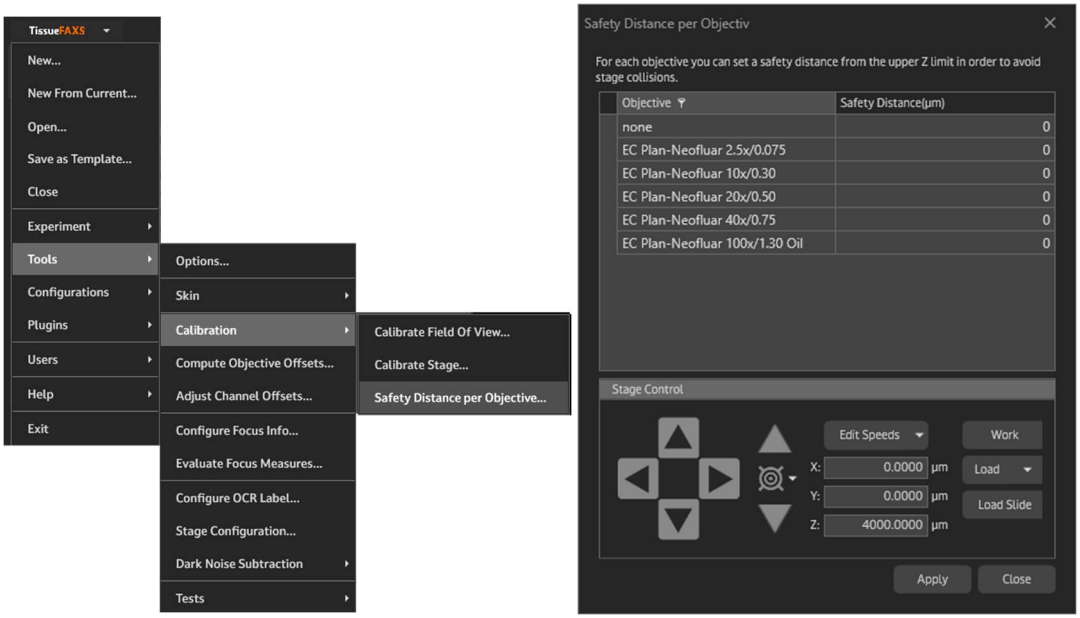
The first listed missing objective is displayed as 0x. The rest of the missing objectives are not displayed.

Select an objective from this panel by clicking on it. This will switch the current objective to the selected one.

The objective in use is always shown in yellow.

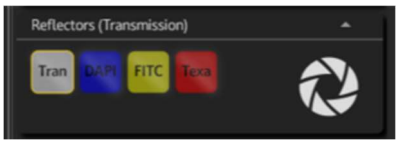
Safety distance per objective

This option can be found in **TissueFAXS main menu** -> **Tools** -> **Safety** distance per objective. The Safety Distance value can be modified for each existing objective in the **Safety Distance per Objective** dialog. A safe distance from the Z upper limit of the microscope can be set for each objective to avoid stage collisions.



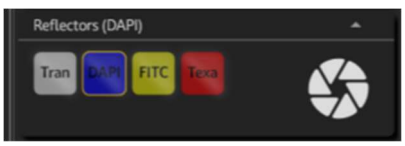
9.2.2. Reflectors

The Reflectors panel displays all the reflectors available in the microscope's Reflector Turret.



The first listed empty position in the reflector turret is displayed as Transmission. The rest of the missing reflectors are not displayed.

Select a reflector from this panel by clicking on it. This will switch the current reflector to the selected one.



The reflector in use is always surrounded by a yellow rectangle.

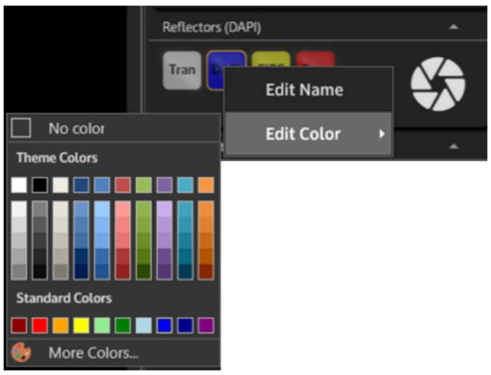
The **RL Shutter** is also present here.

- For the X-cite 120PC Lamp (arc lamp): RL Shutter is used to block or unblock the light coming from RL lamp. This way, the light maintains the same intensity.
- For lamps with multiple light sources (for example LEDs): RL Shutter is used for on/off cycles to increase the lifetime of the lamp.

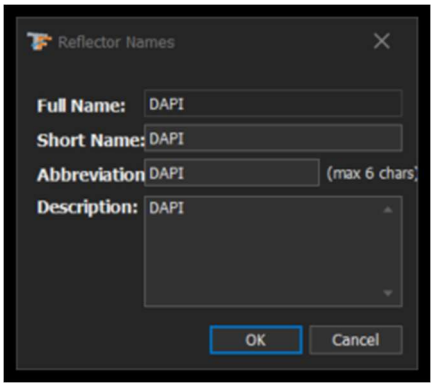
Edit Reflectors

Good management of the reflectors will produce better quality in acquired images and will later assist with image analysis.

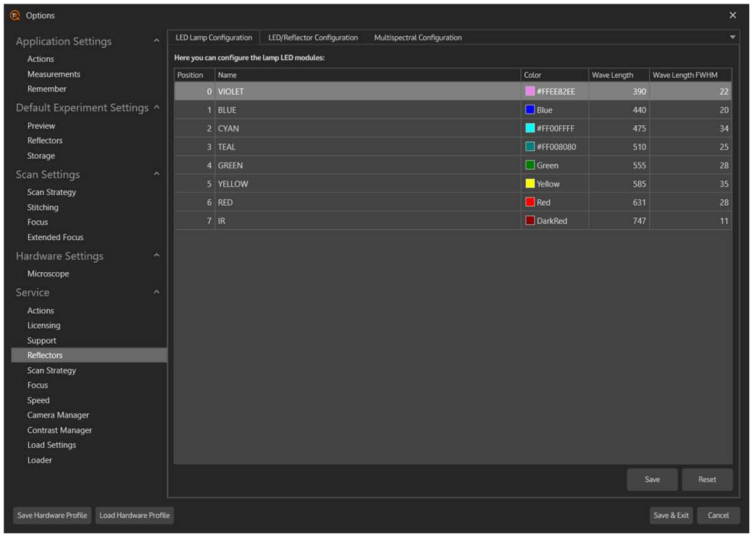
Right clicking on a selected reflector will show a menu with two options:



- Edit Name - change the Full Name, the Short Name, and the Abbreviation (max. 4 characters) for the selected reflector. A short description can be written for the reflector as well.
- Edit color - change the color of the selected reflector.



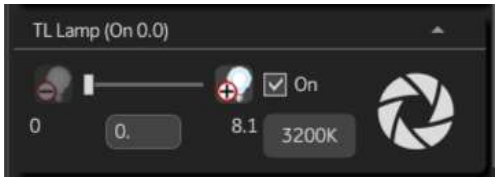
You can choose the displayed names for the reflectors in the application by going to **TissueFAXS main menu →Tools → Options… → Reflectors**.



9.2.3. Lamps

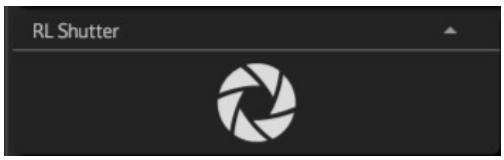
TL Lamp

Use the light switch icon to turn on/off the TL Lamp. The current state of the TL Lamp is displayed (On/Off) to the right of the switch.



- Check the 3200K check box to turn on the color temperature of the TL Lamp directly to 3200K.
- The color temperature can only be set for halogen lamps. This checkbox has no relevance for white-light LED.
- Adjust the light intensity by moving the slider.
- There is a small delay between changing a value on the control and the actual response of the lamp. For instance, when the lamp is turned on, it takes some time before it reaches the luminosity corresponding to the control value.

RL Shutter for reflected light



- For the X-cite 120PC Lamp (arc lamp): RL Shutter is used to block or unblock the light coming from RL lamp. This way, the light maintains the same intensity.
- For lamps with multiple light sources (for example LEDs): RL Shutter is used for on/off cycles to increase the lifetime of the lamp.

Condenser Front Lens

Use the control to swing in or swing out the condenser front lens.

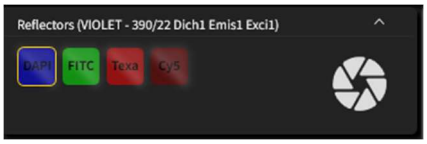
For objective lenses with magnification factors smaller than or equal to 10x and during fluorescence acquisition, the front lens must always be swung out. The front lens is needed for concentrating the light onto the field of view for objective lens magnification factors higher than 10x in transmitted light.

Tube Shutter

Tube Shutter control is used for opening and closing the eyepiece shutter. This setting in available only in the upright motorized microscopes (Axio Imager Z1, Z2).

Confocal Lamp Shutter

Confocal Lamp Shutter is used to block or unblock the light coming from the LED lamp.



Fluo Arc (optional component)

A Fluo arc is a device used to dim the fluorescent lamp. It should be run at 100 % or it will flicker and produce bad results.

White Light Attenuator (optional component)

Adjusts the transmission light intensity using some filters

Fluorescence Attenuator (optional component)

Adjusts the reflected light intensity using some filters.

Confocal Lamp Attenuator

Use the slider to adjust the Confocal Lamp attenuator.

Confocal Disk

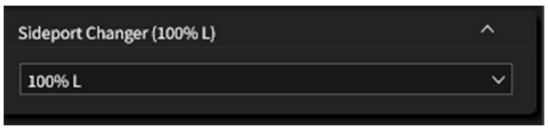
The Confocal Device: pattern configurations are available, depending on the confocal device configuration.

- Double pattern disk 10mm x 10mm FOV each pattern for CCD (40µm and 70 µm).

- Single pattern disk 22mm FOV for large format sCMOS (60 µm).

Sideport Changer

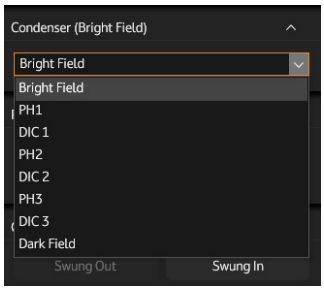
This is used to specify to which port (observation, rear port and front port) the light is directed. Thus, the user can switch between visual observation through the oculars and the digital live image through a camera.



9.2.4. Condenser

Condenser

The **Condenser** combo box allows you to select the desired contrast method.



Notes:

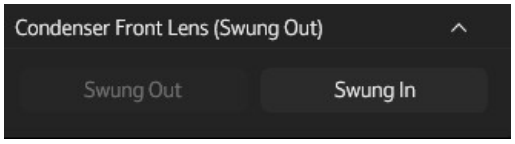
- In fluorescence, it is recommended to move the front lens out of the light path of the condenser.

- Phase Contrast (PH) and Differential Interference Contrast (DIC) are optional components.

Condenser Front Lens

Use the control to swing in or swing out the condenser front lens.

For objective lenses with magnification factors smaller than or equal to 10x and during fluorescence acquisition, the front lens must always be swung out. The front lens is needed for concentrating the light onto the field of view for objective lens magnification factors higher than 10x in transmitted light.



Condenser Aperture

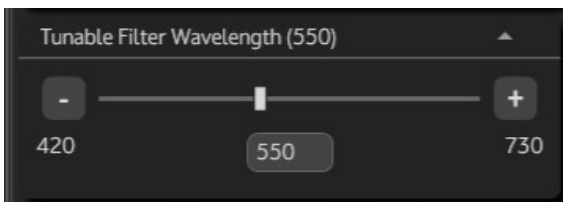
Using the slider, you can open or close the aperture diaphragm.

Note: The condenser aperture determines the contrast in the image. Incorrect contrast settings might impose autofocus functionality and impair the quality of image analysis. As a good starting point, the condenser aperture should be close to the numerical aperture of the objective lens in use. The Light Manager will store individual settings for each objective lens installed.

9.2.5. Filters

Tunable Filter Wavelength

Controls the values of the current wavelength for the liquid crystal tunable filter.



Tunable Filter Bandwidth

Changes the bandwidth on the Tunable Filter device (Kurios).

Liquid Crystal Tunable Filter (LCTF)

Overview

A **liquid crystal tunable filter (LCTF)** consists of a series of liquid crystal cells and polarizers. The LCTF Tunable Filters are directly controlled by the bundled LCTF controller. The drive voltages of the liquid crystal cells are calibrated to provide a specific retardance for each cell. This combination of retardances, along with the used polarizers, result in a bandpass filter in which the passband wavelength can be adjusted by varying the drive voltages. Special signals are applied to optimize the response when switching between wavelengths (voltages for each cell). The filter is internally temperature controlled for consistent performance and fast switching. The LCTF is designed for visible wavelength (VIS) operation and has a tunable wavelength range from 420 nm to 730 nm. A premium shortpass filter, cut-off wavelength at 750 nm, is included.

The full featured controller is custom designed for the LCTF optical heads. There are different modes of operation and bandwidth. The passband wavelength can be controlled in normal manual mode, as well as externally adjusted via an analog control signal or software.

LCTF Front Panel

Controls

WAVELENGTH:

- Adjust the passband wavelength in MANUAL mode.

Status Display

BANDWIDTH:

- WIDE: Default filter operation after power on, work at a wide bandwidth.
- MEDIUM: filter works at medium bandwidth mode.
- NARROW: filter works at narrow bandwidth mode.
- BLACK: Beam blocking mode, minimum transmission.

WAVELENGTH:

- Current passband wavelength. in BLACK mode, it shows the most recent passband wavelength.

MODE:

- MANUAL: Manual mode is the only available mode, as TissueFAXS directly controls this device.

STATUS:

INIT…: Filter is in transition from an idle to operational state. This initialization procedure takes 90 second from power on. The controller screen shows the message:

“INITIALIZING” “Please Wait: --s” where the dashes are replaced by the number of seconds remaining.

WARM UP: Filter is being warmed up to 40 °C. During this time, the filter is operational. However, the passband wavelength might be shifted from the displayed value, and wavelength switching speed is not fully optimized.

READY: Filter has reached 40°C and is now fully functional.

LCTF Optical Head

- All LCTF optical heads have a similar appearance, but different LCTF models have different path thicknesses and clear aperture and threads.

Connector:

- Connects to the optical head connector on the LCTF controller.

Status LED:

- Red: Filter warming up to 40°C.
- Green: Filter is at 40°C and is fully functional.
- T-AXIS: Transmission axis of the filter on each side. The filter may be used in both directions.

However, the input/output polarizations should be aligned to the corresponding T-axis for maximum transmission.

LCTF Controller: Back Panel

OPTICAL HEAD:

Connects to the optical head of the filter. This should be connected before the controller is turned on. If

the cable is not connected while the power is on, then the controller must be restarted. The controller

screen will remind the user to power down and connect the optical head:

- “Power Down To”
- “Connect LC Head”

The maximum current output from the optical head connector is 1 A, and the optical head does not

support hot plugging. Plugging or unplugging the connector while the unit is powered on can cause

damage to the optical head and/or controller.

USB:

- Connects to the computer for software or command-line control.

Technical Terminology

Bandwidth

The LCTF defines the passband bandwidth as the **Full Width at Half Maximum (FWHM)**.

This is the spectral width between the two points where the filter's transmission reaches half of the peak value. LCTF's bandwidths are dependent on wavelength and optical design, and therefore different models and bandwidth modes have different bandwidth values.

Center Wavelength

The center wavelength of LCTF is the center point wavelength between half maximum points.

Note that the center wavelength is not necessarily the same as the peak wavelength (the wavelength with highest transmission), and there may be a slight offset between them.

Transmission

The transmission refers to the peak transmission of the passband profile with a given center wavelength. The transmission value is measured with an incident light beam linearly polarized parallel to the transmission axis of the filter. The LCTF transmission is wavelength-dependent.

Passband

The passband is defined as the range between the first local minimum values on either side of the center wavelength. The passband spectral range is approximately from [Center Wavelength - 1.2 * FWHM] to [Center Wavelength + 1.2 * FWHM].

Out-of-Band Blocking

Out-of-Band Blocking is the maximum transmittance measured from the entire operating wavelength range, but outside of the passband range. LCTF defines blocking range transmittance with optical density OD > 2, which corresponds to transmittance of less than 1%.

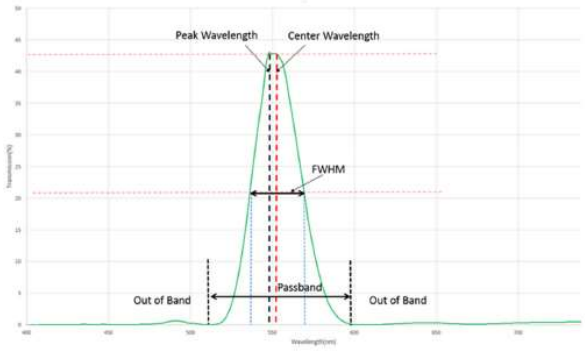


Illustration of Technical Terminology

Switching Speed

LCTFs take a certain amount of time switching between two wavelengths. The time of switching depends on the initial and final wavelength. The switching time data is calibrated for each filter and is saved in its EEPROM memory. This switching time matrix map gives the switching time as a function of the initial wavelength and the final wavelength. Note that when switching from longer wavelengths to shorter wavelengths, the switching time is shorter than switching in the opposite direction. This is due to properties of liquid crystal optics.

Tuning Accuracy

The difference between the wavelength setting and the actual filter output center wavelength is defined as tuning accuracy, and is measured in terms of FWHM at the current set wavelength. LCTF output center wavelength accuracy is corrected to within ±FHWM/10. Tuning Accuracy or wavelength error can be wavelength dependent.

Wavelength Uniformity

Uniformity is defined as the maximum center wavelength shift within the clear aperture in terms of the FWHM bandwidth at the specified wavelength setting. This is measured with a small broadband incident beam through the filter to obtain the filter output center wavelength. The incident beam is then scanned across the entire clear aperture of the filter while the output wavelength at each point is recorded. The wavelength shift at each point is then calculated.

Angle of Incidence (Field of View)

Field-of-view (FOV) is defined as the maximum angle of off-axis rays propagating through the filter while the center wavelength shift is within ±FWHM/2 from the set wavelength. LCTF's visible wavelength filters (-WB1, -VB1, and -WL1), the AOI at 550 nm is measured as reference. For the LCTF near-IR filter (-XE2), the AOI at 850 nm is measured as reference.

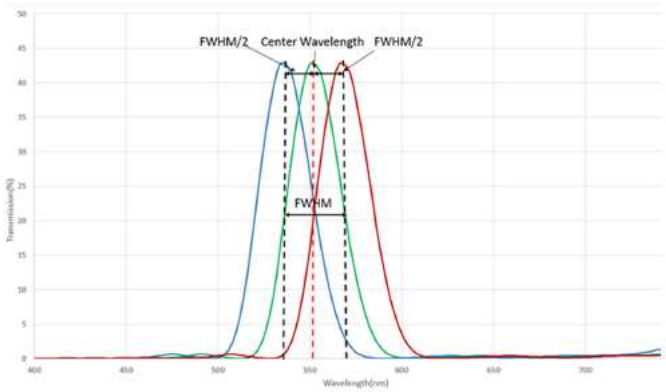


Illustration of Center Wavelength Shift with Off-Axis Rays Propagation through the Filter

Each LCTF comes with a test report including plots of the spectrum, FWHM, transmission, tuning

accuracy, and switching time.

Specifications for Fixed Bandwidth Tunable Filters

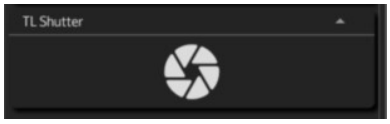
Specifications	Value
Center Wavelength	420 - 730 nm
Bandwidth (FWHM)	35 nm at 550 nm
Polarized Transmission	45% at 550 nm
Out-of-Band Blocking	OD >2

Minimum Incremental Step Size	1 nm
Tuning Accuracy	±FWHM/10
Clear Aperture	20 mm
Field of View	±6
Switching Speed	<40 ms
Uniformity	FWHM/8
Damage Threshold Pulsed (ns)	0.1 J/cm²
Damage Threshold Pulsed (fs)	0.2 J/cm² (532 nm, 76 Hz, 100 fs, Ø162 µm)
Damage Threshold CW	CW: 0.8 W/cm (532 nm, Ø0.471 mm)
Operating Temperature	0 to 40 ºC
Storage Temperature	-15 to 65 ºC

9.2.6. Other Components

TL Shutter for transmitted light

TL Shutter is used to block or unblock the light coming from the halogen or white-light LED lamp.



10. User Management

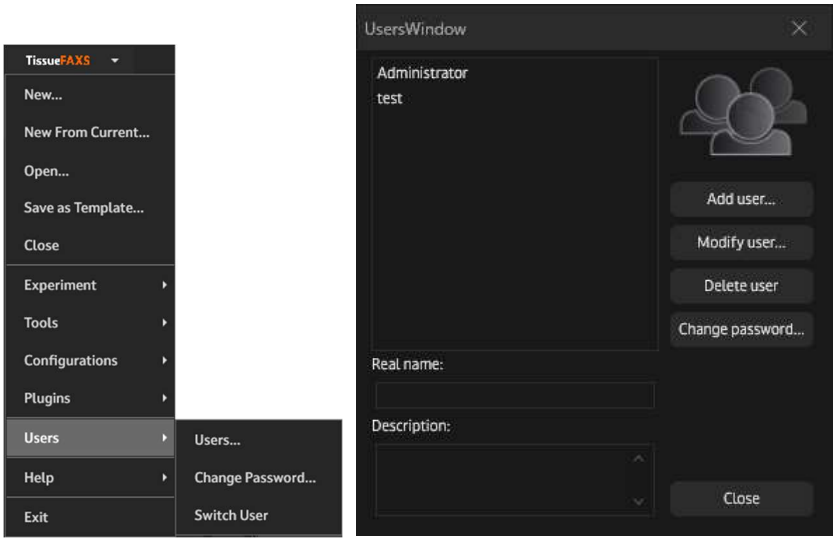
Managing the users in **TissueFAXS** is simple and intuitive.

To use the application, to the user must be authenticated by a username and a password.

1. View Users

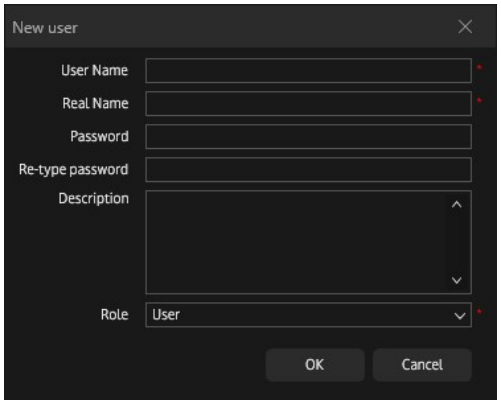
Only the Administrator can manager users. In **TissueFAXS**, there is only one Administrator for the users. The users are managed through the application when the Administrator is logged in.

From TissueFAXS main menu, choose **Users** to access user related operations: Users, Change Password and Switch User



2. Adding Users

Click on the **Add user** button and you will get the following panel:

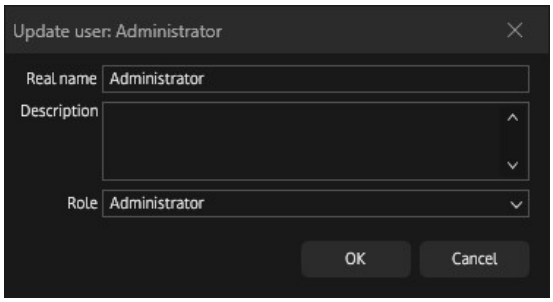


Here, you can enter the necessary information concerning the new user: **User Name**, **Real Name**, **Password**, **Description** and **role**.

Note: All fields, except Description, are mandatory.

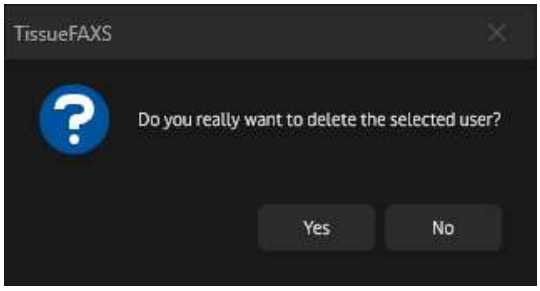
3. Modifying Users

By pressing the **Modify user** button, an Update user panel will appear, where a user's real name, description, or role can be modified.



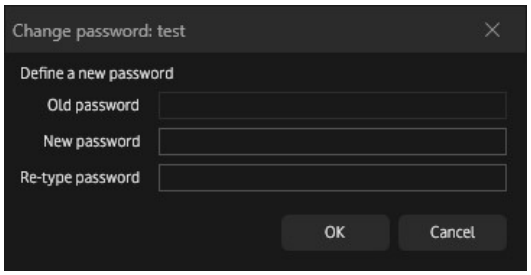
4. Delete Users

To delete a user account, select it in the **User list** dialog, then press **Delete user button**. You will receive a final warning before the effective deletion; it looks like the image below:



5. Change Password (Administrator Mode)

When logged in as Administrator, changing the password for other users is done by using the dialog shown in the image below. To change the password, the application will first request the **old password**, then the **new password** and its confirmation.



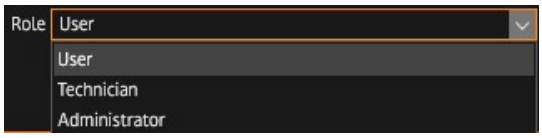
6. Role Based Administration

TissueFAXS has a role-based policy in order to prevent untrained users from accessing advanced controls of the application.

With Role based administration, non-administrators are restricted from accessing advanced controls of the application.

The roles can be assigned in two ways:

- When creating or modifying a new user, choose one of the three available options from the **Role** dropdown box:



User Roles

1. User

- This role cannot modify wide system settings like FOV calibration or scan strategies.

2. Administrator

- This role can modify the list of users and their roles.
- Inherits all the rights of the User role.

3. Technician

- This role can modify wide system settings like FOV calibration.
- Inherits all the rights of the Administrator role.

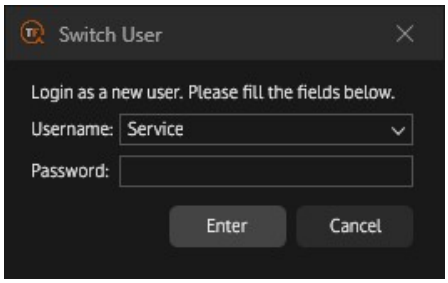
List of settings and the minimum access level:

If using the **Role Based Administration** feature, some settings will be available only for Administrator or Service. Please see table below for more details:

Operation	Minimum Access Level	Behavior for users without access
Run Algorithms	Technician	Hidden
Calibrate Field of View	Technician	Hidden
Test Microscope	Technician	Hidden
Test Stage Precision	Technician	Hidden
Administrate Users	Administrator	Hidden
Options → Default Experiment Settings → Storage → Raw Data	Technician	Disabled
Options → Scan Settings → Scan Strategy	Technician	Disabled
Options → Scan Settings → Speed	Technician	Disabled
Options → Application Options → Actions → Move to a Safe distance	Technician	Disabled
Options → Application Options → Actions → Calibration	Technician	Disabled
Options → Application Options → Support → Connection Details	Technician	Disabled
Options → Application Options → Support → Test Connection	Technician	Disabled
Camera → Advanced	Technician	Hidden
Options → Application Options → Licensing	Technician	Disabled

7. Switch User

Changing from one user to another with a different role can be done without logging off the **TissueFAXS** application. Simply choose the **Switch User** option.



Use the dropdown list to select the new role, then enter the corresponding password to log in with the chosen user.