Software Guide

ZEN 2 (blue edition)

The ZEN slidescan Application

ZEISS

We make it visible.

Content

1 Introduction

Illustration 1: Axio Scan.Z1 system

ZEN slidescan is a software package that offers all tools and functions you need to operate the Axio Scan.Z1. E.g. it includes the possibility of creating z-stack and extended depth of focus images. With the software you can automatically generate high-resolution images (virtual slides) from your specimens. The system is able to perform these scans in brightfield and also multi-channel fluorescence mode The Axio Scan.Z1 is mainly driven by software means. For details regarding the operation of the hardware and other hardware related procedures (like exchange of filter sets, etc.) refer to the latest hardware manual of the Axio Scan.Z1. As the Axio Scan.Z1 includes a built-in magazine with a 12 or 100 slide capacity, the software deals with scanning the specimens, operating the magazine, and handling the slides (including the status of the slides). This is visualized via an intuitive graphical user interface.

This software guide describes the specific operation of the ZEN slidescan software and not the general ZEN functionality; for this, please consult the general ZEN help. The information included in this document is also available via the online help (F1). Here you can conveniently access the information in context within the tabs or the wizards.

2 User Interface

2.1 Scan tab

Here you will find the most important functions needed to operate the **Axio Scan.Z1** system. The tab is closely interlinked with the *Magazine view* [▸ 11], which can be found in the **Center Screen Area**. On top of the tab, you will find the **Default Scan Profile (1)** section where you can select predefined scanning profiles or create new profiles. Additionally, the **Smart Profile Selection (2)** and the **Scan Profile Wizard (3)** will help you with finding a suitable profile or changing and creating a new one. You can start your preview scans or scans with the prominent **Scan buttons (4)**.
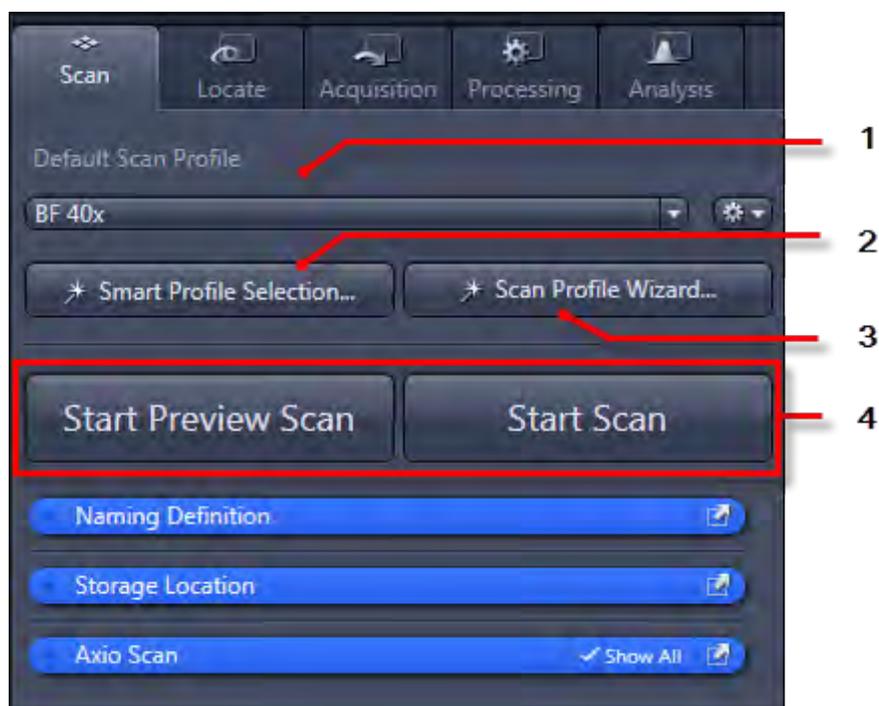
Illustration 2: Scan tab

Default Scan Profile section (1)

From the dropdown list, you can select the default scan profile which will be used if new mounting frames are inserted. The system assigns the selected profile to all newly inserted slides. Click on the **Options** button [⚙▾] to open the *Default Scan Profile options* [▸ 8] context menu.

Smart Profile Selection…(2)

Clicking on this button opens the *Smart Scan Profile Selection wizard* [▸ 19]. This wizard helps you to open predefined profiles. This is the fastest and most intuitive way to assign and generate scanning profiles.

Scan Profile Wizard…(3)

Clicking on this button opens the *Scan Profile wizard* [▶ 20]. This wizard helps you to change/modify existing scanning profiles very easily.

> **ℹ Info**
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> You can change the profile for each mounting frame afterwards in the **Magazine** view. If the default profile is changed, this profile will also be assigned to all unprocessed slides.

Scan buttons (4)

Button	Function
Start Preview Scan	Starts a preview scan of all selected slides.
	To select a slide, go to **Magazine** view and activate the slide's checkbox in the **Process** column. This checkbox will be activated by default if mounting frames are inserted.
	The settings to generate the preview will be used as defined by the assigned profile. For brightfield specimens, the preview consists of a snapshot of the label area (reflected light) and of the specimen area (transmitted light).
	The result will be shown in the **Preview** column of a slide. To enlarge the view, double-click on the image. The software opens the image in a separate tab. To adapt the regions to be scanned, please use the **Advanced Scan Profile Wizard** or the **Tissue Detection Wizard**.
Start Scan	Starts a scan of all selected slides.
	The scan consists of the preview generation (label area and specimen area), tissue detection, focus map, and high resolution scan of the specimen. The settings will be used as defined by the assigned profile. To open an image, double-click on it and it will open in a separate tab in the center of the screen.

2.1.1 Default Scan Profile options

Menu entry	Description
New Scan Profile…	Creates a new profile. If a new profile was created, it will be automatically assigned to all slides in **Magazine** view.
Smart Profile Selection…	Opens a wizard to select a predefined profile. The user will be guided with questions regarding his specimen and the result is a profile which fits best to digitize his specimen. It is recommended to process this profile afterwards with the Scan Profile Wizard
Advanced Scan Profile Wizard…	Opens a wizard to adapt all possible settings of the profile and gives the user the possibility to utilize the full flexibility of the system. This wizard is recommended for experienced users.
Scan Profile Wizard…	Opens a wizard to adapt basic settings of the profile; e.g. Barcode / OCR / Scanning objective / z-stack
Rename	Renames the selected profile.
Save as	Saves the user profile under another file name.
Delete	Deletes the selected profile.

2.1.2 Naming Definition tool
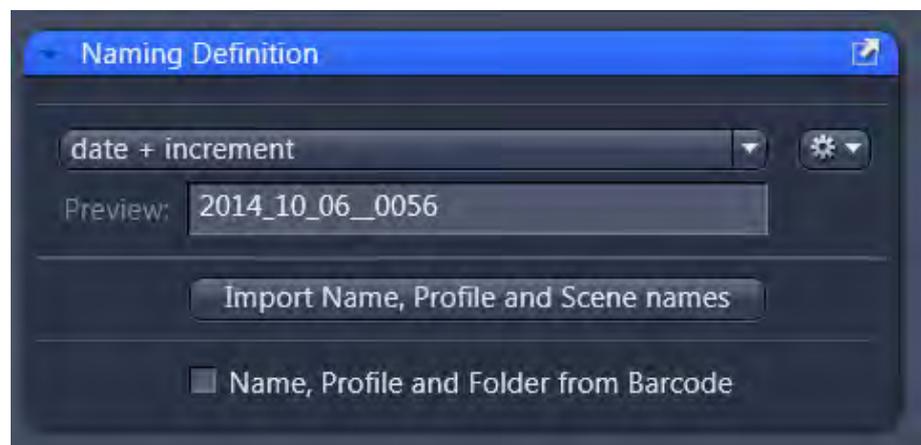
Illustration 3: Naming Definition tool

Here you can define naming definitions for the acquired files/images. You can select several definitions for the file names from the dropdown list. The name automatically contains the detected barcode content if the barcode detection is

active for the active profile. The naming definition contains the command to use the barcode. If you want to use only a sub-set of the barcode as the image / slide name you have to use the Function **Name, Profile and Folder from Barcode**. If you click on the **Options** button ⚙▾ you can define new, edit or delete existing naming definitions.

ℹ **Info**

The **Preview** field shows a preview of the configured file name.

Naming Definition options

List entry	Function
New	Here you can create a new naming definition. The system will ask for the name of the definition and you can then set up the naming definition in the naming dialog.
Edit	Here you can edit the current naming definition.
Rename	Here you can rename the current naming definition.
Save As	Here you can save the current naming definition under another name.
Delete	Here you can delete the currently selected naming definition.

Import Names, Profiles and Scenes names button

Clicking on this button allows you to import the image names and used profiles. You also have the option to apply scene names. When starting an import, it is necessary to insert all your slides beforehand. The software will only apply the imported parameters for slides that have been inserted with the status "**new**". You can read more details on the import under *Importing names, profiles and scenes* [▶ 121].

Name, Profile and Folder from Barcode

Activating this checkbox allows you to use barcode information to name image, assign profiles and sub directories.
You can read more details on the import under *Using a barcode to define name, profile and subfolder* [▶ 126].

2.1.3 Storage Location tool
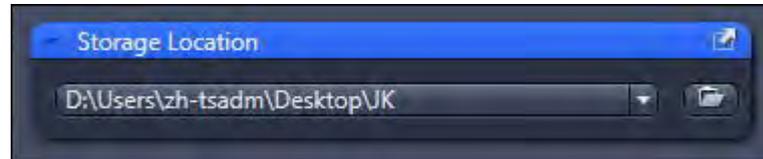
Illustration 4: Storage location tool

Here you can specify the storage location of the created images. A location can be a local path on the computer in use as well as a network share to store the data on a remote server. It is recommended to store the images locally, as depending on the network properties, the connection may not be sufficiently reliable to store the data. This may result in the scan workflow stopping if the network is interrupted, or slowing down if the network connection is performing at a lower rate. If a network share is selected, a transmission rate of at least 100 MB/s is recommended. If they are available, you can select paths that have already been used from the dropdown list. Clicking on the **Create New Folder** button in the **Browse Folder** dialog creates a new storage folder.

2.1.4 Axio Scan tool
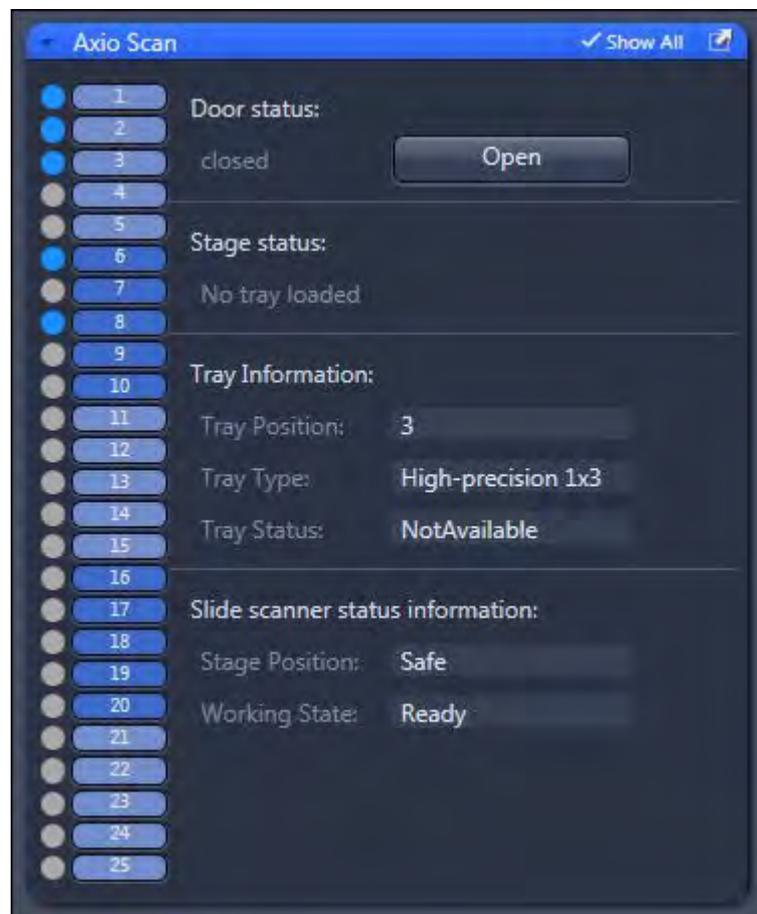
Illustration 5: Axio Scan tool

Door status section

Here you can see whether the door is open or closed. With the **Close/Open** button nearby, you can open or close the door depending on the current status. This function is equivalent to the **Open/ Close** button on the device itself.

Stage status section

Here you see if a tray is loaded or not. You can load a tray by double-clicking on the tray buttons on the left-hand side of the tool. If a tray is loaded, the **Unload tray** button will be shown . Clicking on this button will unload the tray located on the stage into the magazine.

Tray Information section

Here you can see information on the tray and the mounting frame. Hover over the tray buttons on the left to see the information in the display fields. The signal colors in front of the buttons indicate the current status of the trays. This is the same kind of signal that can be found on the device itself. Refer to the operation manual for a detailed description. Double-clicking on the tray button inserts the mounting frame. If another frame is mounted, it will be removed automatically.

- **Tray Position** shows the position of the tray/mounting frame inside the magazine.

- **Tray Type** specifies the inserted mounting frame type (e.g. High-precision 1x3 or High-precision 2x3).

- **Tray Status** shows the status of the frame (e.g. processed, preview done).

Slide Scanner status information section

This sections gives you information on the **Stage Position** (e.g. **safe**) or **Working State** (e.g. **ready**) of the device. It is the same information as shown by the main indicator on the device itself (e.g. ready, processing, warning).

2.2 Magazine view

This view gives you an overview of the **Axio Scan.Z1** magazine. You can assign profiles and naming conventions to slides, show the status, and move trays up and down (using drag and drop) to change the scanning order. The system will start processing slides from top to bottom for all slides that have been activated (via the checkmark in the **Process** column). The view is closely interlinked with the **Scan** tab in **Left Tool Area**.
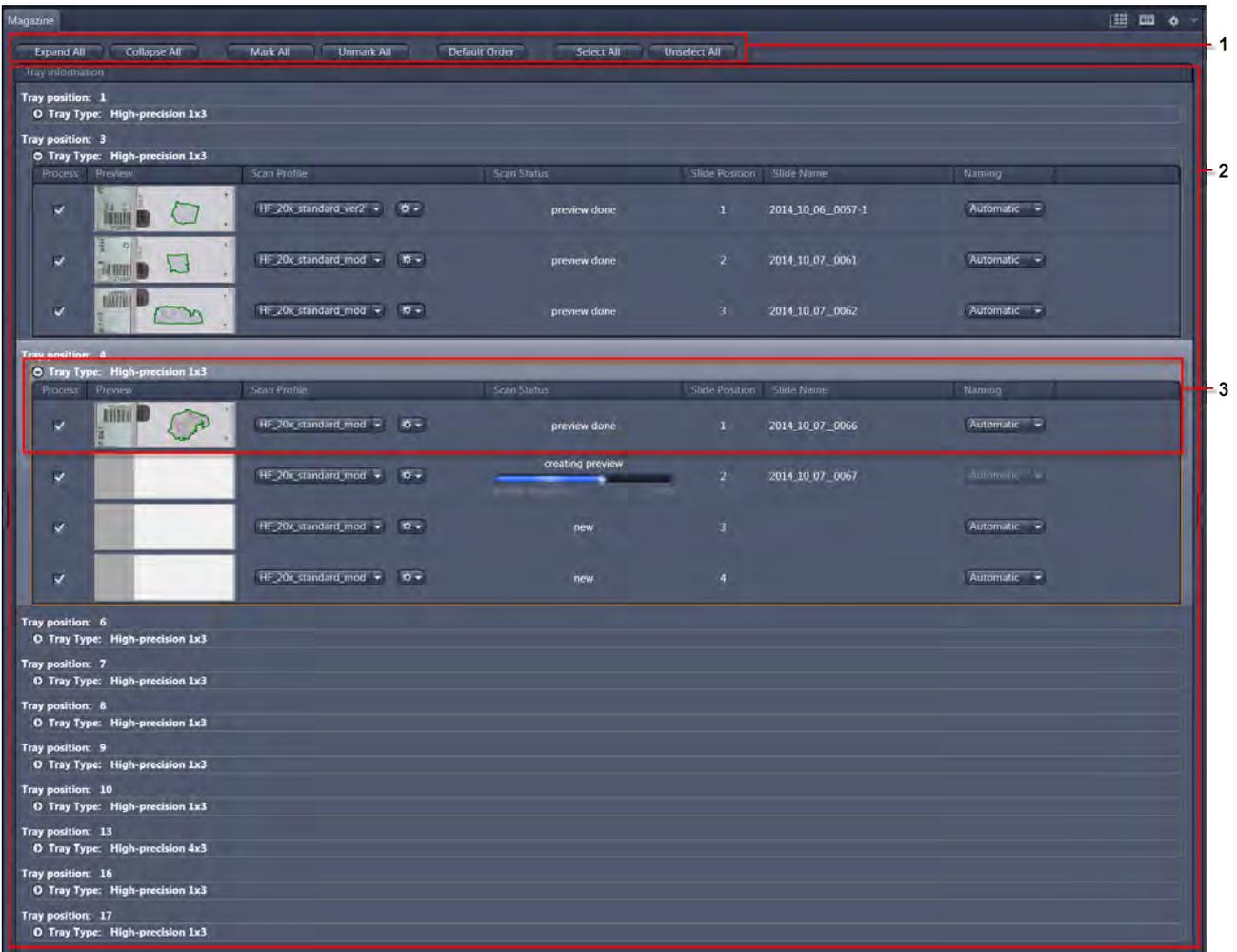
Illustration 6: Magazine view

Notes

- If the door is closed (using hardware or software), the system detects all trays inside the magazine and establishes both the tray type and the positions in the tray that are occupied with a physical slide.

- The system will also check all slides by default (see **Process** column) and assign the **Scan Profile** (defined in the **Scan** tab) to every slide if new slides are inserted. It is still possible to change the profile and other settings afterwards (if the slides are not processed or currently scanned), even if the batch process has already started.

- Only the inserted slides will be shown. Empty places in the tray will not be shown. The loaded tray will be visualized with an orange frame around the mounting frame (in our example this would be Tray 4).

Main buttons (1)

These buttons allow you to adjust the display and settings of the magazine view with ease:

Name	Description
Expand All	Expands all trays so that you can see the slides contained within them.
Collapse All	Collapses all trays so that you can only see the tray positions.
Mark All	Activates all slides for processing.
Unmark All	Deactivates all slides for processing.
Default Order	Resets the default positions of trays and slides. Only if you have changed the order of trays or slides (e.g., by drag and drop).
Select All	Highlights all slides within the magazine
Unselect All	Deactivate the highlighting within the magazine

Tray information section (2)

Here you can see an overview of all trays and the inserted slides. Every tray position has the same structure:

The headline for every tray is the physical **Tray Position** (e.g. **Tray position: 1**). Below the tray position information you will see the **Tray Type** which is inserted (e.g. **High-precision 1x3** or **High-precision 2x3**). Right-clicking on a tray will open the *Magazine View context menu* [▶ 15] with several options for magazine view or the individual trays. If you click on the little expander button in front of the tray type, you can expand or collapse the view to see a tray's **Slides** . All trays are expanded by default.

Slide layout (3)

Each row within a tray represents a slide. Right-clicking on a slide will open the *Slide context menu* [▶ 16] with several options for the selected slide. The slide columns are always the same:

Column	Description
Process	Indicates whether the slide will be scanned (checkbox activated) or not (checkbox deactivated).
Preview	Shows the preview image of the slide if a preview was executed. It shows the label area and the specimen area.

Column	Description
Scan Profile	Assigns a specific scan profile to each slide. If a profile was adapted, the system indicates this with a gearwheel in front of the profile name. If you click on the **Options** button ⚙▼ behind the scan profile, the *Scan Profile Options* [▶ 17] context menu will be opened.
Scan Status	Gives information about the status of the slide scanning process. This can be: ■ **new**: no preview was executed and no scan ■ **preview done**: preview was already executed and will be displayed (in the **Preview** column) ■ **finished**: the scan was successfully executed If an error/warning comes up, this will be shown here. If you click on the error/warning message, it will be displayed.
Slide Position	Represents the physical position of this slide inside the tray.
Slide Name	Represents the name of the image which will be generated. You can insert a name or the system can create a name according to an automated naming rule. The selection of the procedure to be executed depends on the **Naming** column.
Naming	Here you can select whether the system creates the name automatically using a naming rule or specify a name manually. It is also possible to change the name of the image once the preview has been generated by simply clicking on the name and then editing it. Depending on the settings under **Naming Definition** you can also import names via a CSV file or you can use the barcode information to define a name as substring of the barcode.

Tip: To enlarge the label image or the preview image of the specimen area simply click on the image. You will see an enlarged view of this image for a better recognition of the content. If the scanning for a slide is finished you do not see the overview image of the specimen you will see a low magnification representation of the resulting image.

If you want to close the image click on the image again, move the mouse pointer of the image or click another slide to enlarge another preview / scanned image.
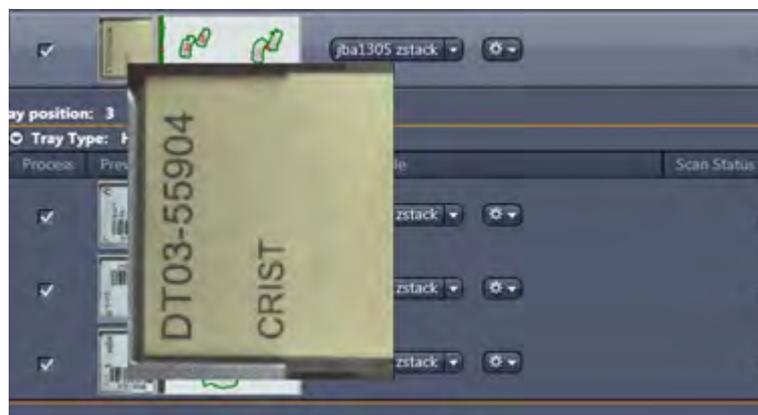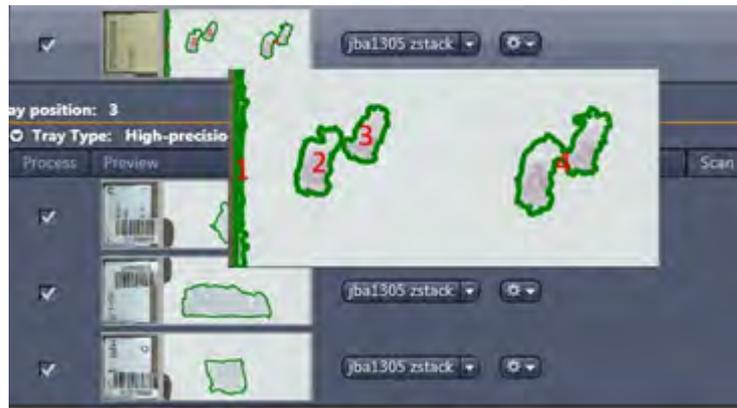
2.2.1 Magazine View context menu

Menu Item	Description
Load tray	The system will move the selected tray from magazine to the stage and an eventually already loaded mounting frame on the stage will be removed before automatically. A loaded tray is marked by an orange rectangle.
Assign scan profile	Opens a dropdown menu where you can assign an existing profile to all highlighted slides.
Mark all slides of highlighted trays for processing	Selects all slides in the highlighted (multi-select possible) trays for processing.
Unmark all slides of highlighted trays for processing	Deselects all slides in the highlighted (multi-select possible) tray for processing.

Menu Item	Description
Expand all highlighted trays	Expands all highlighted trays by on click of he mouse (multi-select possible). To highlight a tray hold Crtl key and click on the trays you want to highlight.
Collapse all highlighted trays	Collapses all highlighted trays by one click of the mouse (multi-select possible).

2.2.2 Slide context menu

Menu Item	Description
Move to Prescan position	Moves the selected slide to the prescan position.
Move to Scan position	Moves the selected slide to the scan position.
Assign scan profile	Here you can select a scan profile and assign it to the selected slide. If you have highlighted several slides the selected profile will be assigned to all highlighted slides.
Open image(s)	Only active if the scan was finished. The software will open the image. If you have selected several slides all images from theses slides will be opened.
Mark all highlighted slides for processing	Marks all selected (highlighted) slides for processing.
Unmark all highlighted slides for processing	Unmarks all selected (highlighted) slides for processing.
Reset Scan status to new	Resets the scan status of the selected (highlighted) slide to "new".
Reset scan status to previewed	Resets the scan status of the selected (highlighted) slide to "previewed".

2.2.3 Scan Profile options

Menu entry	Description
Open advanced wizard	Opens the **Advanced Scan Profile Wizard** (Online Mode). In this mode the system will take the tray containing the slide from the magazine (only if the tray which contains the slide is not already on the stage). You can see a live image from the different cameras (preview camera / scan camera) and you are able to navigate on the slide. This mode is useful if you want to adjust e.g. exposure times and setting windows live and direct. Thus you will always work on a physical glass slide. On the other hand this is can be time consuming because the physical glass slide has to be moved/ loaded.
Open advanced wizard in offline mode	Opens **the Advanced Scan Profile Wizard** (Offline mode). In this mode the system will not move the tray (slide) and you will get no live image from the cameras. This mode is useful if the user wants to change parameters which are not directly associated with a live image; e.g. Z-stack and EDF (extended depth of focus) or focus settings. The adjustment of these settings are independently from any movement of physical glass slides and no slide needs to be loaded from the magazine.
Open tissue detection wizard	Only active if a preview was generated. Opens the **Tissue Detection Wizard**. By the help of this wizard you can change the region which the system will scan afterwards. Within the wizard you can switch directly to the next or previous slides. This makes it fast and convenient to switch between the slides. The adjustment of these settings are independently from any physical glass slide. This wizard can be executed even if the preview generation for other slides is still running.

Menu entry	Description
Save adapted scan profile	Only active if the scan profile was adapted/changed.
	Here you can save an adapted scan profile (an adapted scan profile is visualized via a gear wheel in front of the profile name). After you have activated this function a **Save As…** dialog comes up where you can select an appropriate name for the adapted profile and save it.
Properties	Here you can check the properties of the profile. A XML viewer comes up to present all details of the scan profile. This information is only valuable for very experienced user.

3 Overview Wizards

The software user interface includes the following 6 wizards:

■ *Smart Scan Profile Selection (offline)* [▶ 19]

■ *Scan Profile Wizard (online)* [▶ 20]

■ *Advanced Scan Profile Wizard (online)* [▶ 22]

■ *Advanced Scan Profile Wizard (offline)* [▶ 101]

■ *Tissue detection Wizard (offline)* [▶ 102]

■ *Axio Scan Calibration Wizard (online)* [▶ 104]

Each wizard has a certain functionality and/or provides a certain level of access to the functionality of the Axio Scan.Z1. A wizard always contains several steps. These steps guide you through all necessary settings, e.g., to generate a scan profile or calibrate the scanner. The **Tissue Detection Wizard** contains only one step.

Two principal wizards are implemented – the online type and the offline type. The offline type works without having a physical slide mounted on the stage, meaning that no live image is produced and there is no access to the functionality which is normally associated with a live image. The online type requires a slide to be placed on the stage (or the wizard places the slide on the stage) thereby also generating a live image.

ⓘ Info

For the **Advanced Profile Wizard (online/offline)**, it is not necessary to go through the wizard step by step; it is possible to jump directly to a certain step of the wizard by double-clicking the desired step.

If certain features/settings are missing, this may be because the **Show all** mode is not active. If so, activate the **Show all** checkbox. On the other hand, several settings are only available for very specific applications. If these settings cause confusion, switch off the Show all checkbox.

You can generate new profiles from scratch using the default settings (via the **Scan** tab/**Default profile** section/**New**), but in most cases it is recommended to modify existing profiles. This makes it easier and faster to adapt a profile for a new application without checking each step of the wizard.

If you do not have a adequate profile as a source to modify, it is recommended to use the **Smart Profile Selection** first to select a base profile for a certain application and modify settings afterwards using the **Scan Profile Wizard**.

3.1 Smart Scan Profile Selection (offline)

This wizard will be opened via the **Smart Profile Selection** button on the **Scan** tab.
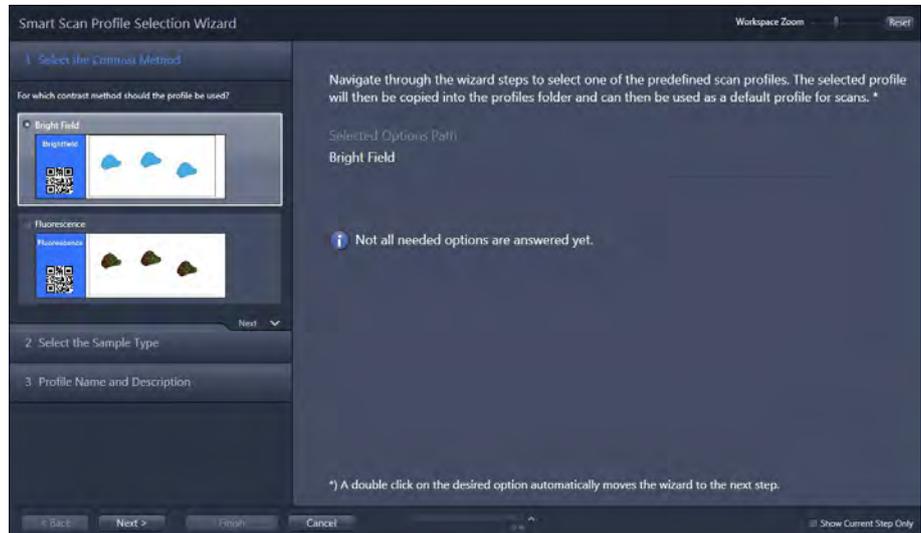
Illustration 7: Smart Scan Profile Selection Wizard

The system has pre-configured profiles (known as pool profiles) for a large number of applications. These profiles are stored in a safe location and cannot be changed by the user.

You will be guided through several steps to find the best profile as a preset for further adaptations. Each choice is followed by a selection of individual options paths. You can choose the desired option by double-clicking on it or by selecting the option and clicking on the **Next** button at the end of the boxes.

When you finish the wizard, the selected profile from the profile pool will be copied to the user accessible folder. From here, you can select it as a scanning profile. In the last step, you can change the name and activate the option that the **Scan Profile Wizard will be started right after the Smart Profile Selection.**

The user can then modify the profile using the **Scan Profile Wizard** or the **Advanced Scan Profile Wizard**.

> **i Info**
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> The default scanning objective for all pool profiles is the 20x objective.

3.2 Scan Profile Wizard (online)

This simplified wizard only has basic functions for the purposes of changing an existing profile.
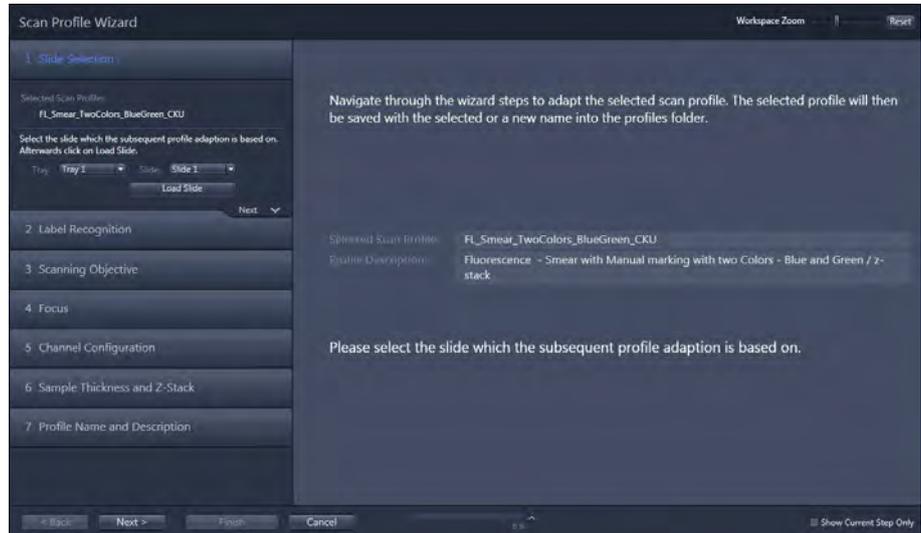
Illustration 8: Scan Profile Wizard

The wizard includes the following settings:

Settings for brightfield profiles

- Grid definition if grid is part of the profile

- Barcode reading

- OCR (optical character recognition) functionality

- Selecting the scanning objective

- Adjusting flash intensity and flash duration

- Z-stack functionality (with the definition of the number of slices and interval automatically via the specimen thickness)

- EDF (extended depth of focus) functionality

Settings for Fluorescence profiles

- Grid definition if grid is part of the profile

- Barcode reading

- OCR (optical character recognition) functionality

- Selecting the scanning objective

- Selecting/deselecting channels

- Defining stitching channel

- Adjusting exposure times

- Adjusting light source intensities

- Z-stack functionality (with the definition of the number of slices and interval automatically via the specimen thickness)

- EDF (extended depth of focus) functionality

3.3 Advanced Scan Profile Wizard (online)

This wizard is an online wizard, thus the system will take the tray containing the slide from the magazine if the tray which contains the slide is not already on the stage. The system will always show the operator a live image from the different cameras (preview camera/scan camera) while also moving the slide according the input from the operator. This mode is important if the user wants to adjust settings such as exposure times and regions of interest including labels and preview areas. This means that the operator will always work on a physical glass slide. On the other hand, this can be time-consuming because the physical glass slide has to be moved. If you want to change settings that are not directly associated with a live image, you can use the Advanced Scan Profile Wizard in offline mode.
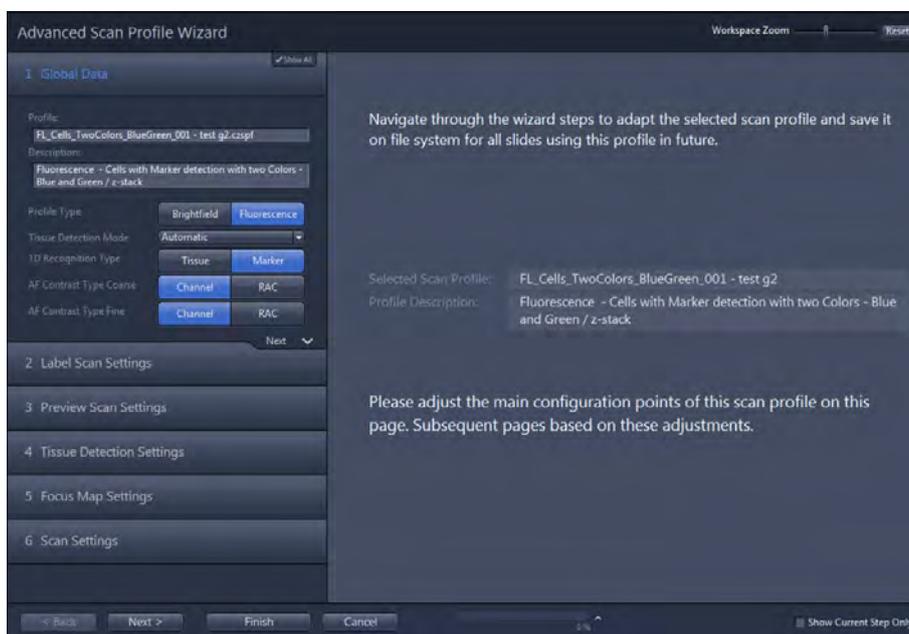

Illustration 9: Advanced Scan Profile Wizard

3.3.1 Fluorescence profiles

3.3.1.1 Global Data

> ℹ️ **Info**
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> To access all of the functions and properties described in this guide, you have to activate "**Show All**" using the checkbox in the upper right-hand corner of the left tool area.

Here you can define global settings for your scanning profile. Depending on these global settings, both the wizard and the workflow will be adapted automatically.

Profile & Description

The name of the selected scan profile is displayed under **Profile**. This name cannot be changed here. A short description of the profile can be found under **Description**. As the information content of the profile name is always limited, you can insert additional information here to highlight the most important profile settings (e.g., magnification/z-stack settings/focus map settings).

Profile Type

Here you can select between **Brightfield** and **Fluorescence profile types.** If you choose brightfield, AF Contrast Type settings will not be shown.

Tissue Detection Mode

This is where you select the kind of tissue detection you want to apply. The following three modes are available:

Mode	Description
Automatic	In this mode, the system detects the tissue automatically.
Interactive	In this mode, the system will pause the scan process if a batch is started and waits for your input to define a region of interest manually. As the system pauses until this interaction is complete, this function has to be used very carefully.
Manually	In this mode, you can define a region of interest to be scanned manually or a grid in a subsequent step of the wizard. This is an option if you only want to select a subset of the tissue on the slide, e.g., if the tissue is very faint, the system cannot detect the specimen reliably, or if the specimen has a regular pattern on the slide. If you use this function, the defined region/regions will always be exactly the same for all slides assigned with this profile, thus it can be only applied if you have a fixed scan region (e.g., PAP smears).

> **i Info**
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> The most common selection here is the **Automatic** mode with **Marker** as it works independently of the kind of specimen and specimen preparation.

TD Recognition Type

Not available if you set **Tissue Detection Mode** to manual.

This is where you select whether the tissue detection is carried out via thresholds (**Tissue**) or if you want to use a **Marker** to encircle the specimen manually on the physical slide. In this case, the threshold will be used to detect the marker. Everything inside this marker will be scanned.

If **Marker** is selected, the system will always generate a preview using the separate Preview camera.

If **Tissue** is selected, the user is able to define whether the input image is created by the preview camera (**Preview cam**) – i.e., low resolution with no special contrast method – or with the scan camera (**Scan cam**) and a selected objective with the Ring aperture contrast (RAC). This is defined under "**based on image from**". These settings are only available if **Tissue** is selected.

The **Preview cam** option can be used if the specimen is visible on the slide (e.g., if the specimen is very thick). If the specimen is faint (i.e., hard to see with the naked eye) **Scan cam** has to be selected.

AF Contrast Type Coarse & Fine

Here you can define what contrast type is used to perform the Coarse and Fine focus. Only available for **Fluorescence** Profile Types.

Channel: The system will use a fluorescence signal to perform the focus. This is the best choice if the specimen provides a fluorescent counterstain (e.g., DAPI); you must ensure that the fluorescent stain is evenly distributed over the sample so that the system detects enough signals at the focus point to preform a reliable autofocus.

RAC: The system will use the Ring aperture contrast (RAC) to perform the focus. This principle is recommended if no fluorescent counterstain is available and/or if the fluorophores are very sensitive to bleaching. It is also important for the kind of specimen to be visualized using this brightfield contrast method. This means that cells spreads cannot be visualized with this contrast method.

3.3.1.2 Label Scan Settings
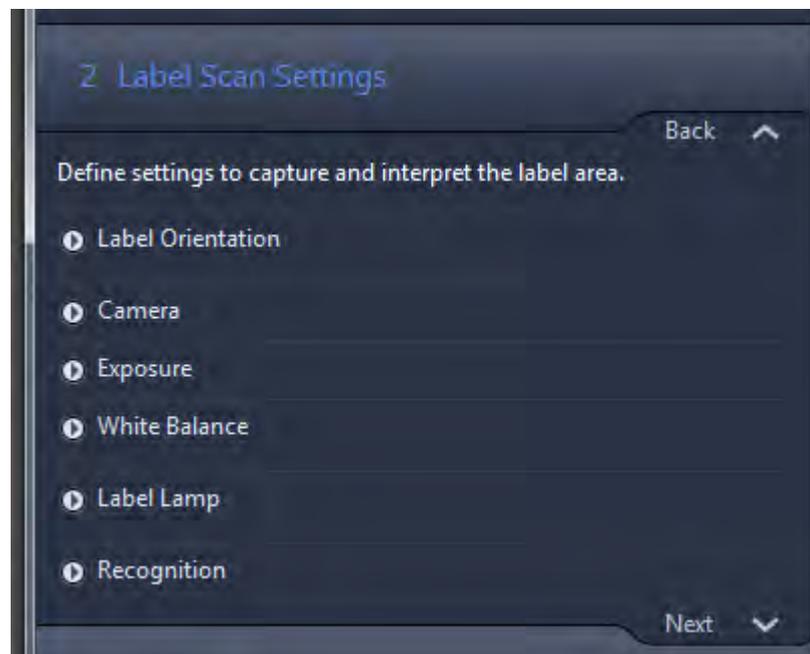
Here you can select the capture parameters of the label area.

The label area ROI (Region Of Interest) is defined by the red rectangle shown in the live image. It is possible to adjust the frame in size and position freely to fit with the label on your slide. The label area is captured with reflected light and a separate camera (called **AxioCam IC**).
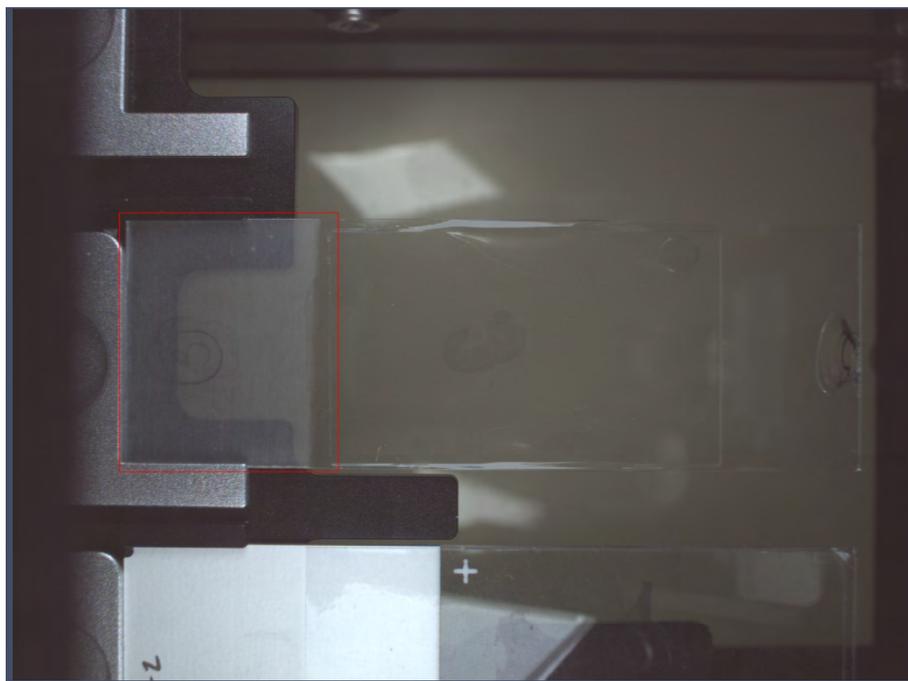
Illustration 10: Label Area

> **i Info**
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> It is not usually necessary to adapt the camera settings and/or light source as these settings are factory defaults and should represent the optimal settings. For this reason, these settings (**Camera**, **Exposure**, **White Balance** and **Label Lamp**) are not described in detail within this section.

Label Orientation

This is where you select how the label is aligned on a slide.

You can rotate the label image in 90° steps clockwise (CW) or keep the original orientation (**Original**). If you select **Use Barcode Orientation**, a barcode sticker has to be attached on the slide. The software will then determine the orientation of the barcode and apply this to the orientation of the label area of the current slide. To use this function, a barcode label has to be attached and you also have to activate **Barcode Recognition active** in the **Recognition** expander.

You can also change the label orientation later on in the ZEN software if you browse through the slides.If all of the slides have a rotated label, however, it is recommended to change the label orientation here.

Recognition

■ **Barcode**

If Barcode Recognition is activated, the system interprets the barcode and saves the barcode information as metadata within the image. If you want to use the barcode information as part of the image name, you have to apply automated naming and use the keyword "**RecognizedCode**" or short cut " **%N**" to make the barcode part of the image name.

By default, the software will check for all implemented barcode types and all orientations, thus it is not necessary to define a specific barcode. If the label contains two or more barcodes with a different barcode type, we recommend that you define the specific barcode type you are looking for. To activate a specific barcode, open the expander for **Barcode types** and activate only the barcode you want to be read.

■ **OCR (Optical Character Recognition)**

■ The OCR works with **English** text only. Be aware that OCR works best with clearly printed numbers and/or letters. We recommend using OCR_A or OCR_B as the font for the OCR. We do not recommend using underscores (_) or special characters within the text. You can select different options to optimize the recognition quality, but the standard parameter should work. The printed text should be very precise in terms of its orientation. There should be minimal text tilt after the image orientation.

To check if the barcode or characters can be recognized, click on the **Test** button. The system will now show the region of interest with the orientation given and apply the barcode or OCR recognition. The result will be presented in the **Recognized** field.

> **i Info**
>
> You have the possibility to adjust the Display curve (see Display tab on the bottom of the window). The display settings will stored within in the profile! This has also an impact on the display of the label in the Magazine view. This has also an impact on how the label is displayed in the Magazine view!

Please read also the instruction under *Using Label Scan Settings for 4''x3'' slides* [▶ 123].

3.3.1.3 Preview Scan Settings

Depending on the settings under **Global Data** the appearance and functions on the user interface can be different. In the following 2 sections we describe the two most common settings for a preview scan with an objective (prescan) or the preview camera (preview).

3.3.1.3.1 Preview Scan with Objective (Prescan)
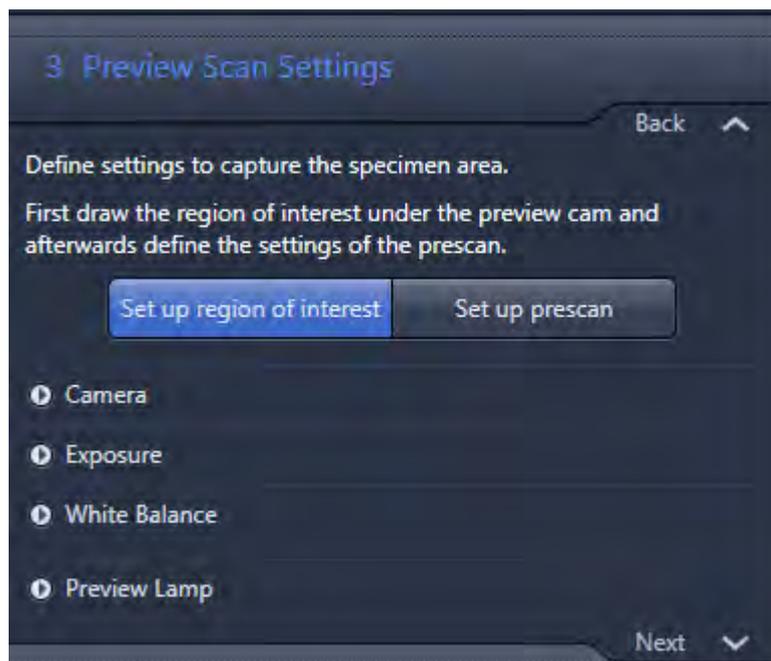
Prerequisites

■ In Step 1 **Global Data** under **Tissue Detection Mode**, you must have activated either the **Automatic** or **Interactive** setting.

■ Under **Tissue Recognition Type**, you must have activated the **Tissue** setting and the **Scan cam** setting for **based on image from**.

With these settings, the system will perform a preview scan with the scanning objective (known as Prescan) and will also determine the **Coarse Focus**. The following buttons are available:

Set up region of interest **button**

If you have selected this button, you can use the **Center Screen Area** to set up the area for the prescan by changing the position and the size of the red frame which defines the prescan region. As a prescan is much more time-consuming than a preview, it is recommended to select a rather small area.

Set up prescan button

If you have selected this button, you can set up how the system will scan the region of interest using a prescan.

You have the choice of RAC or fluorescence (channel). The settings depend on the setting for **Coarse Focus** under **Global Data**. If the coarse focus is set to **RAC**, the prescan will be carried out with RAC; if it is set to **Channel**, the prescan will be carried out with fluorescence. This principle is based on the connection between prescan and coarse focus. For an in-focus prescan, the system needs an autofocus. As this autofocus is already carried out using the prescan, it is not necessary to carry out any subsequent steps for the coarse focus. For this reason, the coarse focus will be disabled under **Focus Map Settings** if a prescan is activated. This is where you set up the acquisition parameters for the prescan and the settings for the z-stack (for the autofocus). As the process for creating the acquisition parameters is similar to that for setting up the *Focus settings* [▶ 37], please refer to this section for more details.

Camera settings (Camera, Exposure, White Balance)

This is where you can adjust the settings for the camera and the illumination; however, we recommend that you do not change these settings for the sake of producing reproducible results. The camera settings are described in the appropriate chapters in the ZEN Online Help. It is not possible to change the camera for the preview as this is fixed.

Preview Lamp

Here you can adjust the intensity of the preview lamp for the specimen area (transmitted light). Once again, we recommend that you do not change the value.

3.3.1.3.2 Preview Scan with Preview Camera (Preview)
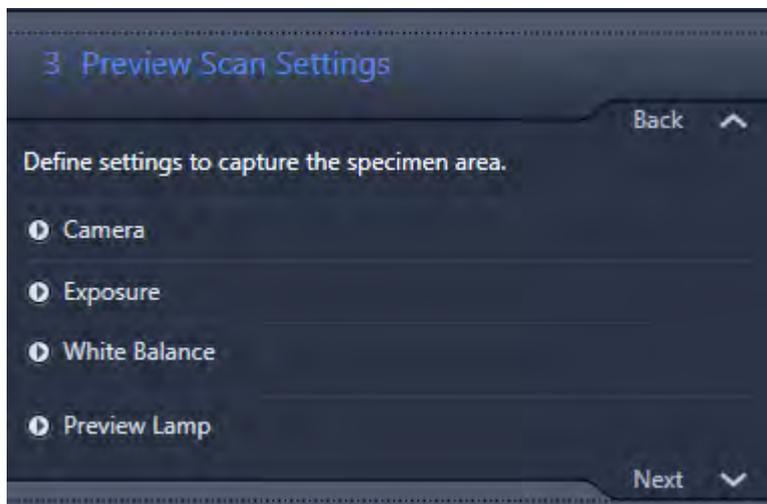
Prerequisites

■ In Step 1 **Global Data** under **Tissue Detection Mode** you must have selected the **Automatic** or **Interactive** option.

■ Under **Tissue Recognition Type**, you must have activated the **Tissue** button, as well as the **Preview cam** button under **based on image from**.

Or:

■ In Step 1 **Global Data** under **Tissue Detection Mode**, you have selected the **Manually** option.

■ Under **Tissue Recognition Type**, you must have activated the **Marker** button.

In all cases, the system will perform a preview taken with the separate preview camera, thus the system generates the preview image (called preview) with one snapshot.

Camera Settings (Camera, Exposure, White Balance)

This is where you can adjust the settings for the camera and the illumination; however, we recommend that you do not change these settings for the sake of producing reproducible results. As the controls are similar to the standard ZEN controls for cameras, please refer to the appropriate chapters in the Online Help. It is not possible to change the camera for the preview (under **Camera**), as this is fixed.

Preview lamp

Here you can adjust the intensity of the preview lamp for the specimen area (transmitted light). Once again, we recommend that you do not change the value.

3.3.1.4 Tissue Detection Settings

The appearance of the TD (Tissue Detection) settings depends on the settings which you have made in the first step of the wizard under **Global Data**.

> **i Info**
>
> You have the possibility to implement his own tissue detection algorithm and make this algorithm part of the workflow (for more details see below under Further information)

Case A Prerequisites

If you have selected the **Automatic** mode in Step 1 **Global Data** under **Tissue Detection Mode**, as well as the **Tissue** button under **TD Recognition Type**, the TD settings will appear as follows:

Region of Interest for Tissue Detection

The red rectangle in the live image which you can see in the **Center Screen Area** represents the ROI for the Tissue Detection. Only the regions that are inside the ROI will be presented as regions which can be scanned. Other regions outside the ROI and/or touching them will be ignored. You can change the size of the ROI by adapting the size of the red rectangle.

Using the **Test** button, the software will execute the inserted parameters and show the results in the middle part of the window. The detected objects (every object is a single scene) will be presented with a green border:

All objects are listed in the **Graphics** tab. The user can delete objects and even add new ones; however, the result will be only valid for this slide.

To achieve a continuous update, you can activate **Live update** via the checkbox. Please keep in mind that a refresh can take some seconds after changing a parameter.

Mode & Method display fields

This is where you can see the mode and method for tissue detection which you selected under **Global Data**.

Region dilation size

Here you can enter a value for the spacing of the border around the detected tissue.

Specimen

Here you define the upper and lower borders of the specimen via the thresholds. By clicking on one of these borders and pressing the right mouse button you can move the borders up and down. If you have activated the **Live update**, you will see the result toward the left of center with a certain delay. The upper border (right side) is the border for the lighter stains and the lower border (left side) is the border to influence the darker stains. Thus moving the right side further to the right marks the lighter stains. Moving the left side further to the left marks the darker stains.

Min Region size

Here you can enter the minimum region size to be scanned. The system will not detect regions if they are smaller than this value.

Air border dilate

The air border is the border around the physical glass slide, i.e., the area outside the glass slide and inside the red frame. This value determines the dilation of this air border. Everything inside this dilated air border will not be recognized as tissue/a marker. This is a measure for ensuring that slide edges and coverslip edges are not detected as needing to be scanned.

Over the peak factor

This parameter is only visible if the **Automatic** checkbox is activated. It defines the distances between the glass peak of the slide and the upper threshold value for the specimen detection. Thus if **Automatic** is activated, the upper value will be determined automatically.

Max elongation

This setting is important to remove coverslip edges. Coverslip edges are normally structures with a large number for the ratio of the longer side and the shorter side. This means that every structure that exceeds this parameter for the ratio between the longest side and the shortest side will be excluded.

If you click on the **Reset** button, the values will revert to their default settings.

Once you have determined the appropriate settings, you can save these settings by selecting **New** via a gearwheel in the **Settings** line. These settings can then be used in another profile, for example, by selecting the Settings name from the dropdown menu within the **Settings** line. It is not essential to save these settings as they will be saved within the profile. It is only necessary if the settings are to be used in another profile.

---

i Info

If you draw your own regions, make sure that these contain tissue. Empty space could lead to an incorrect focus map as the software will use the entire drawn region to create the focus point distribution.

> **ℹ Info**
>
> If two or more objects are very close to each other and thus the two or more objects are seen as one objects it is perhaps advisable to split these objects with the **Split** function which is availabe in the context menu seen after a right-mouse click.

Case B   Prerequisites

If you have selected the **Automatic** mode in Step 1 **Global Data** under **Tissue Detection Mode**, as well as the **Marker** button under **TD Recognition Type**, the TD settings will appear as follows:

The settings are limited to the definition of the marker threshold (comparable to the tissue threshold described above). The only further adjustable value is the **Min region size**, whose functionality is the same as described above.

Prerequisites
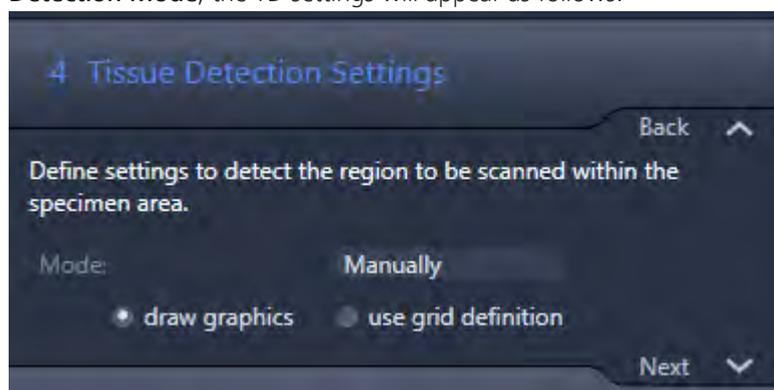
> **i Info**
>
> If you use the marker on the physical glass slide, make sure that the regions marked contain specimens. Empty space could lead to an incorrect focus map as the software will use the entire marked region to create the focus point distribution.

Case C Prerequisites

If you have selected the **Manually** mode in Step 1 **Global Data** under **Tissue Detection Mode**, the TD settings will appear as follows:

Drawing in TD ROI manually or using Grid Definition

Here it is possible to draw regions manually within the region of interest if you activate draw graphics. To draw regions, you can use the tools on the **Graphics** tab in the general view options of the **Center Screen Area**. You can use it in the same way you would generally annotate images within ZEN. The drawn regions will be saved and the same areas will be used for the entire time the profile is applied, hence this option can only be used if the regions are always defined precisely on the slide (e.g., PAP smears).

For the grid definition, see chapter *Grid definiton* [▶ 36].

> **i Info**
>
> If you want to mark a specimen that is very faint, you can use the **Display** curve to change the display settings (particularly by adjusting the gamma curve) to make it possible to see even unstained specimens of a reasonable thickness.

> **i Info**
>
> You have the possibility to adjust the Display curve (see Display tab on the bottom of the window). The display settings will stored within in the profile! This has also an impact on the display of the label in the Magazine view. This has also an impact on how the specimen area is displayed in the Magazine view!

3.3.1.4.1 Grid definiton

If the **Tissue Detection Mode** under **Global Data** is set to **manually**, it is possible to mark the tissue by drawing in graphics or by using a grid definition.

You can define a grid with rectangles or circles as **Element Types** in a regular pattern. The reference point for this grid is the upper right-hand corner of the specimen area (red rectangle). Based on this, you can define on offset from the upper left-hand corner in x (A) and y (B) in mm and also the pitch of the grid in x (C) and y (D).

Based on the selected geometrical shape (circle or rectangle), you can define a radius or the dimensions in x (F) and y (E). The number of elements in x (in this example, 6) and y (in this example, 3) defines the number of elements.

When you click on the **Create** button, the grid will be generated. You will see the grid as an overlay in the image. It is also possible to make manual adjustments such as those used for standard graphics in ZEN.

3.3.1.5 Focus Map Settings
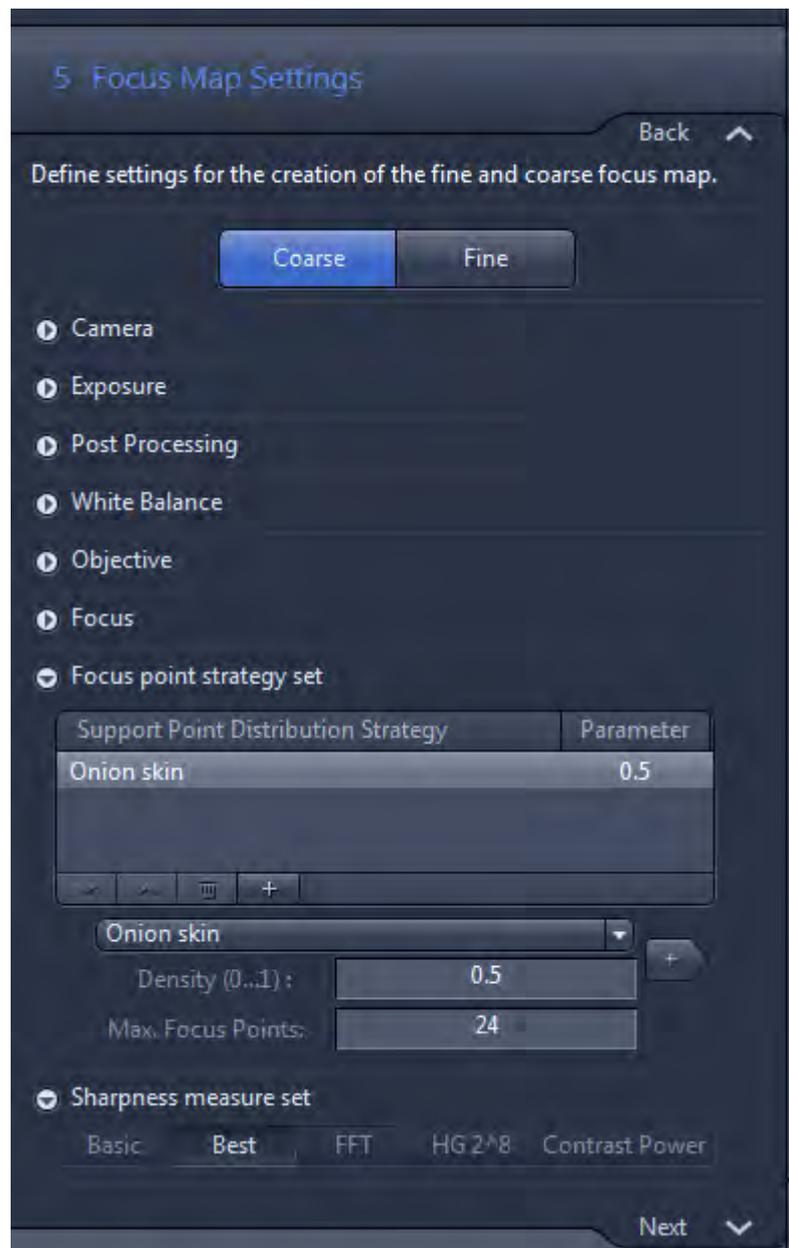
Appropriate focus map settings are important for ensuring good focus quality throughout the specimens. The focus map generation is done in two steps:

■ With a low magnification objective to generate the **Coarse** focus map. For this coarse focus map, only some focus points are necessary. We recommend the use of the 5x Fluar for the Coarse focus as the offset between the 5x and the scanning objective (mostly 20x) is minimal.

■ With a high magnification objective (normally the scanning objective) to generate the **Fine** focus map. In this case, more focus points are needed and this is normally defined via a density. The Fine focus map is based on the coarse focus map.

The focus is not a traditional autofocus that needs to hit the sharpest z-value precisely. The system will acquire a z-stack (defined in the next step) and calculate a curve through the focus value (e.g., contrast value). This curve is used to determine the peak value. This makes it possible to cover a large range while still maintaining fast focusing.

If the profile is configured in a way that involves a prescan (scan with an objective) being carried out, the **Coarse** focus is disabled as this is already carried out while prescanning the region of interest.

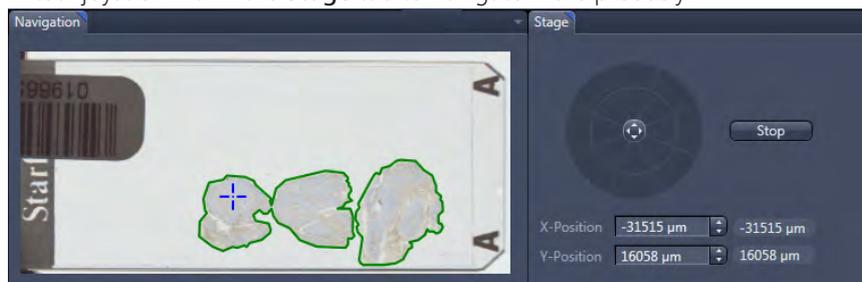#### 3.3.1.5.1 Coarse focus (Channel mode)

The layout of the coarse focus settings depends on the selected **Autofocus Contrast type** selected under **Global Data**. If you selected **Channel** for the **Autofocus Contrast type** under **Global Data**, the following layout will come up:

Procedure **1** Start **Smart Setup** and define the channel you want to use for focusing. For more information on **Smart setup**, see *Smart Setup* [▶ 137]. A good channel for coarse focus is defined by a short exposure time (to minimize bleaching and reduce the focus time) and it is very important that the fluorophore is evenly distributed over the specimen so that the software will always find a signal to focus on. After the configuration with **Smart Setup**, the software will automatically calculate the **Channel configuration**.

2 By default, the system will insert the objective in the first position of the objective revolver as a coarse focus objective inside the Light Path Settings. You can change the objective by opening the expander for **Light Path Settings** and activating the **Before Experiment: Before Exp.** You can now see the light path configuration. If you want to use another recommended objective (5x or 10x), select this and click on **Go!** so that the system will put the selected objective in the light path.

3 As the image quality is not important for the focus, it is recommended to change the binning from 1x1 to 2x2 or higher as this will decrease the exposure time and thereby minimize the bleaching and the time for focus afterwards. This settings can be changed under the expander **Acquisition Mode**.

4 Now navigate to the specimen with the tools you have in the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the virtual joystick within the **Stage** tab to navigate more precisely.

5 Under **Channels** in the list, you can see all defined channels. If you highlight a certain channel, the hardware will change the light path.

6 To select a channel as Focus Reference highlight the channel and click on the button **Focus Ref.** and make sure that this channel is selected via the checkbox and the others are de-selected. You can still select the light source if you have two (e.g., Colibri.2 and HXP 120 V), as well as the intensity of the light source. Lower light source intensities normally result in less bleaching even if the exposure time is longer.

7 The next step is to **Focus** on the specimens and adjust both the exposure time (**Time**) and light source intensities. To see a live image from the camera, you have to activate **Live**, which will display a live image, or else click on **Snap** to take a single snapshot of all selected channels. It is important to take extra care in Live mode as this can result in bleaching of the sample. It is therefore recommended to use this feature with caution and, if possible, to use a representative region outside the scanning region.

8 You can use the **Find Focus** (**AF)** button to perform the autofocus with the selected (highlighted) channel. If the focus is off and/or the exposure time is not appropriate, you should use the manual focus control (expander **Focus**).

You may also need to adjust the exposure time. (**Time**) and/or the intensity of the light source(s).

9 It is not necessary to increase the exposure, thus the complete dynamic range of the camera is covered. You can adjust the Display curve in the histogram to also show the signal on the screen, even if only 5% of the range is covered, for example.

10 Once the focus has been established, you can then adjust the exposure time (**Time**). This can be done manually by moving the **Time** slider or automatically by clicking the **Set Exposure** button. The exposure time will be determined so that the range of the camera will be covered by the value stated under "Shift".

11 The next step is to set up the autofocus parameters, which can be done in the **Software Autofocus** expander. The setting under *Quality* [▶ 61] defines the type of autofocus algorithm in use. The **Range Coverage** defines whether the complete z-stack is performed (**Full**) or if it is only captured until a local maximum is detected first (**Smart**). **Full** is the more robust principle but the **Smart** is the faster one. It is an option if the system detects two maxima with **Full**. The **Sampling** will define the step size of the z-stack. The currently selected value can be seen under **Step size** (the available range and steps depend on the objective and other optical parameters). A step size of approx. 20 µm is recommended for 2.5x and 5x, and approx. 15 µm for 10x. The **Sharpness Measure** defines whether the systems are using the average **Contrast** for determining the autofocus or **Intensity**, or if the software decides which of the two are used (**Auto**).

12 Look up the current z-position (under Position, in our case 4550 µm). This z-position should be in the range specified under **Set First** and **Set Last**. In our case, it fits very nicely in this range. If it does not fit within the range, you have to adjust the range. You can increase the number of slices (under **Slices**). This

will increase the range, thus the focus will take longer. Otherwise, you can shift the range by changing **Set First** and **Set Last**.

13 The next step is to adjust the *Focus point strategy set* [▶ 58]. The default setting (**Number of points** = **6**) is the best value for a wide variety of specimen (in the table view you can see only one parameter if you want to see also the other parameters (if available; e.g. for Onion skin) double-click on the entry). An exemption could be Tissue Micro Arrays, for which **Center of gravity** is recommended.
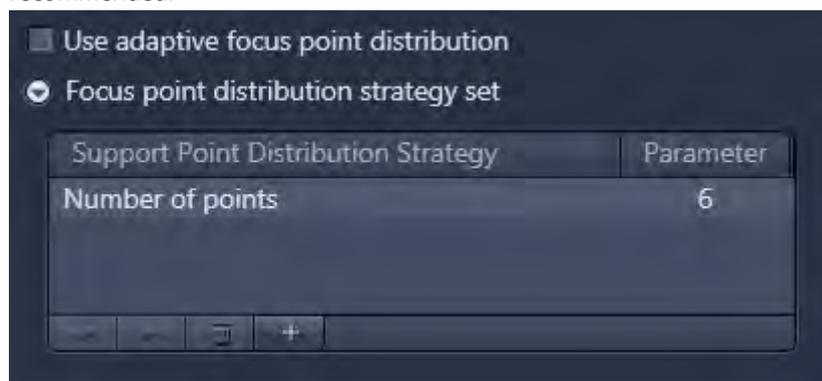
14 To change the **Focus point distribution strategy set**, double-click on the entry. A window comes up, in this window you can select another **Focus point distribution strategy set** via the dropdown menu and you can adjust the parameters for this setting. With a click on **Modify** the changes will be applied.

---
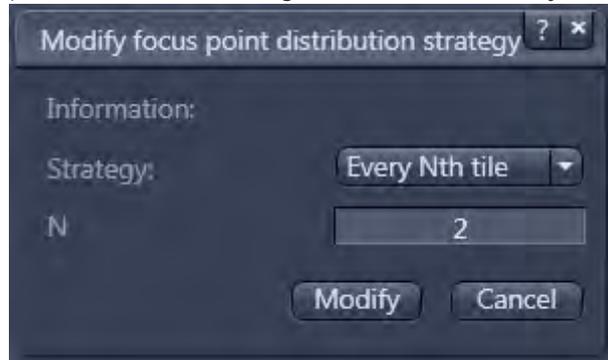
i Info

The described procedure is applicable for specimens where the objects have an equal size. As the Focus point distribution strategy is depending on the object size the system offers the possibility of the so-called Adaptive focus point distribution, please refer to the chapter *Adaptive focus point distribution* [▶ 85] for more detailed information on this topic.

Procedure **1** To double-check, click on **Find focus**. If all settings are correct, the system should focus on the specimen without providing any warnings/errors. If you still experience problems, please double-check the above settings.

2 By default, the next step would be to adjust the **Fine Focus**.

3.3.1.5.2 Fine focus (Channel mode)

The layout of the fine focus settings depends on the selected **Autofocus Contrast type** selected under **Global Data**. If you have selected **Channel** for the **Autofocus Contrast type** under **Global Data**, the following layout will come up:
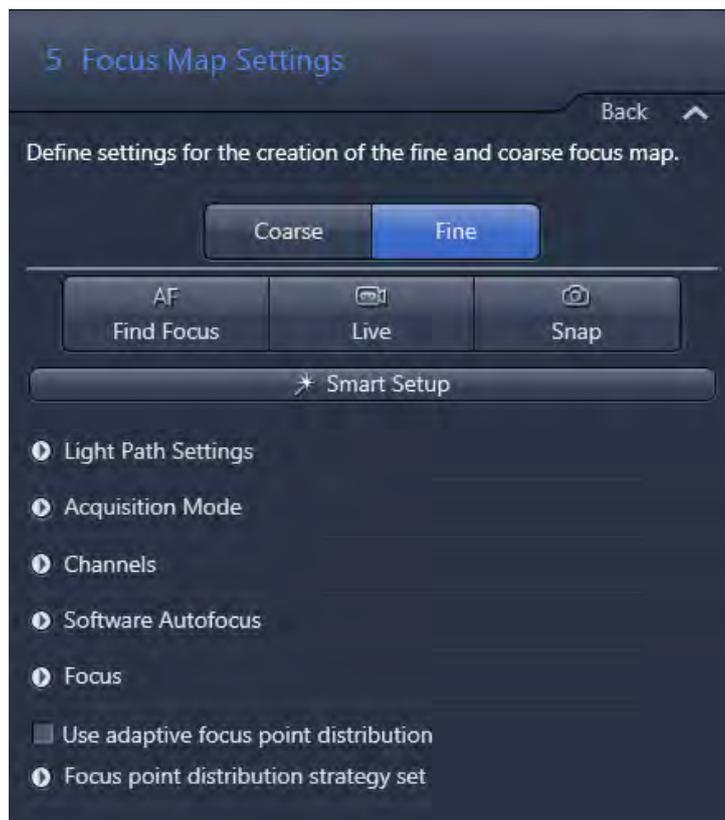
Prerequisites ■ You should adjust the **Coarse Focus** settings in advance.

Procedure 1 Start **Smart Setup** and define the channel you want to use for focusing. For more information on **Smart setup**, see *Smart Setup* [▶ 137]. For **Fine Focus**, use the same channel as you used for the **Coarse Focus**. After the configuration with **Smart Setup**, the software will automatically calculate the **Channel configuration**.

2 By default, the system will insert the 20x as a fine focus objective inside the **Light Path Settings**. You can change the objective by opening the expander for **Light Path Settings** and activating the **Before Experiment: Before Exp.** You can now see the light path configuration. If you want to use another objective, select this and click on **Go!**. The system will now put the selected objective in the light path. The objective for the **Fine Focus** should be the same as the one you want to use for scanning.

3 As the image quality is not important for the focus, it is recommended to change the binning from 1x1 to 2x2 or higher as this will decrease the exposure time and thereby minimize the bleaching and the time for focus

afterwards. This settings can be changed under the expander **Acquisition Mode**.

4   Now navigate to the specimen with the tools you have in the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the virtual joystick within the **Stage** tab to navigate more precisely.

5   Under **Channels** in the list, you can see all defined channels. If you highlight a certain channel, the hardware will change the light path.

6   To select a channel as Focus Reference highlight the channel and click on the button **Focus Ref.** and make sure that this channel is selected via the checkbox and the others are de-selected. You can still select the light source if you have two (e.g., Colibri.2 and HXP 120 V), as well as the intensity of the light source.

Lower light source intensities normally result in less bleaching even if the exposure time is longer

7 The next step is to **Focus** on the specimens and adjust both the exposure time (**Time**) and light source intensities. To see a live image from the camera, you have to activate **Live**, which will display a live image, or else click on **Snap** to take a single snapshot. It is important to take extra care in Live mode as this can result in bleaching of the sample. It is therefore recommended to use this feature with caution and, if possible, to use a representative region outside the scanning region.

8 You can use the **Find Focus** (**AF)** button to perform the autofocus with the selected (highlighted) channel. If the focus is off and/or the exposure time is not appropriate, you should use the manual focus control (**Focus** section). You

may also need to adjust the exposure time. (**Time**) and/or the intensity of the light source(s).

9   It is not necessary to increase the exposure, thus the complete dynamic range of the camera is covered. You can adjust the Display curve in the histogram to also show the signal on the screen, even if only 5% of the range is covered, for example.

10   Once the focus has been established, you can then adjust the exposure time (**Time**). This can be done manually by moving the **Time** slider or automatically by clicking the **Set Exposure** button. The exposure time will be determined so that the range of the camera will be covered by the value stated under "Shift".

11   The next step is to set up the autofocus parameters, which can be done in the **Software Autofocus** expander.

The setting under *Quality* [▶ 61] defines the type of autofocus algorithm in

use. The **Range Coverage** defines whether the complete z-stack is performed (**Full**) or if it is only captured until a local maximum is detected first (**Smart**). **Full** is the more robust principle but the **Smart** is the faster one. The **Sampling** will define the step size of the z-stack. The currently selected value can be seen under **Step size** (the available range and steps depend on the objective and other optical parameters). A step size of approx. 2 μm is recommended for 20x. The **Sharpness Measure** defines whether the systems are using the average **Contrast** for determining the autofocus or **Intensity**, or if the software decides which of the two are used (**Auto**). The **Range** defines the search range of the autofocus based on the coarse focus found in the first step. If you use a 2.5x for the coarse focus, a search range of up to 250 μm for the fine focus is needed. If you use a 5x or 10x, the search range can go down to around 80 μm. This also depends on the focus settings and the evenness of the sample.


NOTICE

Risk of damaging the objective!

Based on the working distance of the objectives the maximum focus range should be for a Plan-Apo 40x 100 μm and for every other objective a focus range of below 600 μm is acceptable. This considers a typical cover slip thickness (170 μm) and a thickness of a specimen of around 10 μm. If you use only a fine focus range of 60 μm, the maximum thickness of the specimen can be 50 μm. A larger working range could lead a damage of the objective and in extreme cases to a damage of the slide.

12 The next step is to adjust the *Focus point strategy set* [▶ 58]. The default setting (**Onion skin** with a density of 0.1 and a maximum number of 24 focus points; in the table view you can see only the parameter for the Density if you want to see also the other parameters (if available) double-click on the entry) is the best value for a wide variety of slides with large specimens. An exemption could be Tissue Micro Arrays, for which **Center of gravity** is recommended.
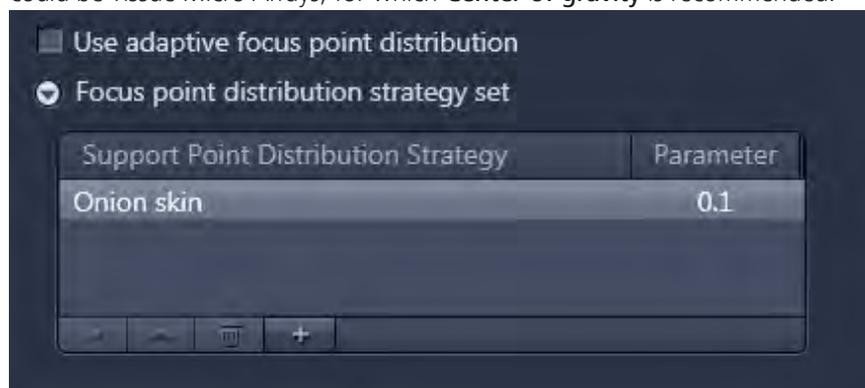
13 To change the **Focus point distribution strategy set**, double-click on the entry. A window comes up, in this window you can select another **Focus point distribution strategy set** via the dropdown menu and you can adjust the parameters for this setting. With a click on **Modify** the changes will be applied.
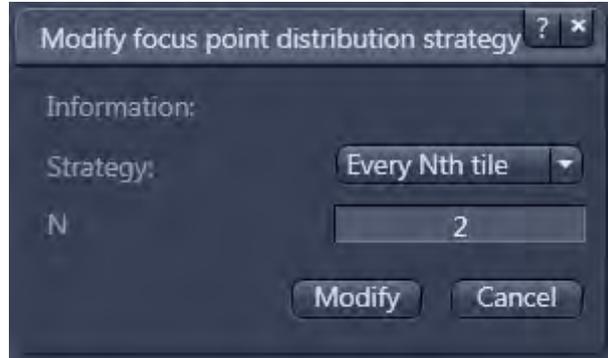

14 To double-check, click on **Find focus**. If all settings are correct, the system should focus on the specimen without providing any warnings/errors. If you still experience problems, please double-check the above settings.

---

i Info

The described procedure is applicable for specimens where the objects have an equal size. As the Focus point distribution strategy is depending on the object size the system offers the possibility of the so-called adaptive Focus point distribution, please refer to the chapter *Adaptive focus point distribution* [▶ 85] for more detailed information on this topic.


3.3.1.5.3 Coarse focus (RAC mode)

The layout of the coarse focus settings depends on the selected **Autofocus Contrast type** selected under **Global Data**. If you selected **RAC** for the **Autofocus Contrast type** under **Global Data**, the following layout will come up:
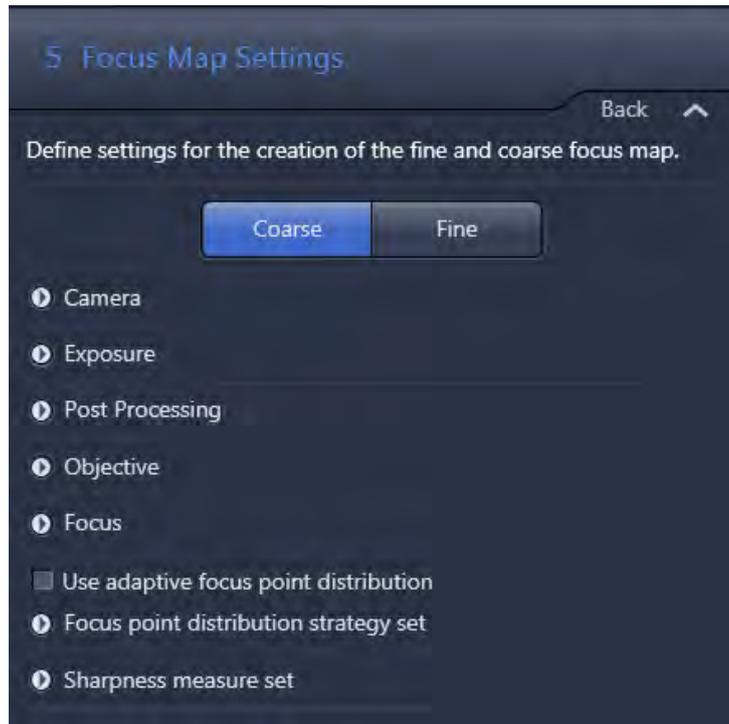
As the settings for the **Exposure** and **Post Processing** are the same as used for the standard ZEN controls, please refer to these chapters for more information.

Procedure

1 In the **Camera** section, it is possible to select the active camera; however, only select a scan camera (Orca flash or AxioCam MR) and not the AxioCam IC as the selection will not result in a working profile!
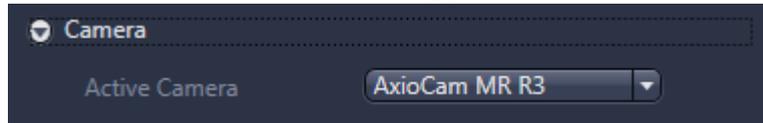
2 Select the appropriate objective in the **Objective** section. In this case, a 5x objective is recommended (if a 5x is not available, use the following objectives in the recommended order: 10x, 2.5x).
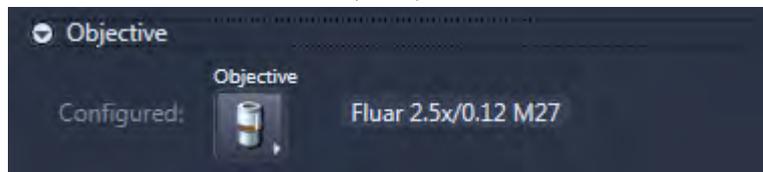
3 Now navigate to the specimen with the tools you have in the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the virtual joystick within the **Stage** tab to navigate more precisely.
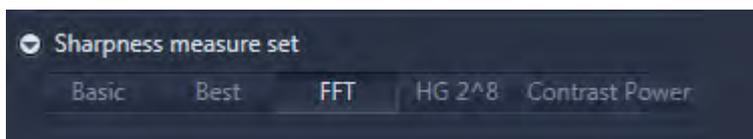

4 The next step is to adjust the *Sharpness measure set* [▶ 61]. For the 2.5x objective **FFT** is the best choice for the coarse focus in RAC for the other objectives (5x and 10x) use Basic or Best only in case if this does not work use **FFT**.

5 Focus on the specimen. You can use the **Run Auto Focus** button in the **Focus** section to do so. If the system does not find a focus, you may have to focus manually, e.g., via the slider next to the virtual representation of the z-stack. You can stop the focus run if you click on **Stop Auto Focus**.

6 Once the specimen is in focus, double-check that the image is not too bright or too dark. If you need to adjust one of these parameters, please change the settings under **Exposure**. By clicking on the **Measure** button, the system will automatically determine the best **Flash intensity** and **Flash duration**. For best

results, move the stage to a position that keeps specimens out of the light
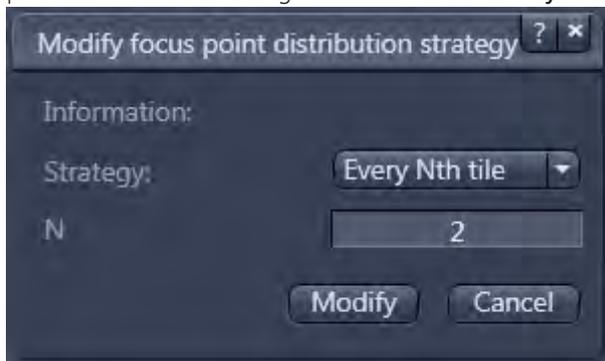path.

> **i** **Info**
>
> For the 5x objective, you will see a dark-field-like image, which is based on the
> ring size in use and is an expected result. For the other objective, you will see
> the typical phase-contrast-like image.

7 Look up the current z-position (under Position, in our case approx. 3500 µm).
This z-position should be in the range specified under **Set First** and **Set Last**. In
our case it fits very nicely in this range. If it does not fit within the range, you
have to adjust the range. You can increase the number of slices (under **Slices**).
This will increase the range, thus the focus will take longer. Otherwise, you can
shift the range by changing **Set First** and **Set Last**.

8 The next step is to adjust the *Focus point strategy set* [▶ 58]. The default
setting (**Number of points** = **6**) is the best value for a wide variety of specimen
(in the table view you can see only one parameter if you want to see also the
other parameters (if available; e.g. for Onion skin) double-click on the entry).
An exemption could be Tissue Micro Arrays, for which **Center of gravity** is
recommended.
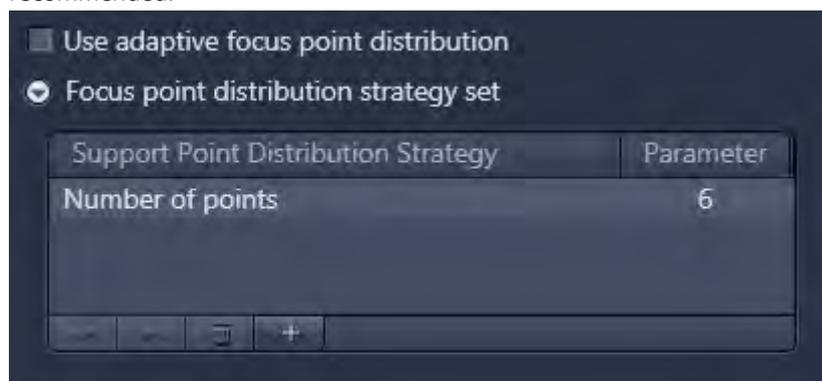
9 To change the **Focus point distribution strategy set**, double-click on the entry. A window comes up, in this window you can select another **Focus point distribution strategy set** via the dropdown menu and you can adjust the parameters for this setting. With a click on **Modify** the changes will be applied.


10 To double-check, click on **Run Auto Focus**. If all settings are correct, the system should focus on the specimen without providing any warnings/errors. If you still experience problems, please double-check the above settings. Please try at different positions to be sure that for every spot on the specimen the focus range is sufficient.

11 By default, the next step would be to adjust the **Fine Focus**.

> **i** **Info**
>
> The described procedure is applicable for specimens where the objects have an equal size. As the Focus point distribution strategy is depending on the object size the system offers the possibility of the so-called adaptive Focus point distribution, please refer to the chapter *Adaptive focus point distribution* [▶ 85] for more detailed information on this topic.

3.3.1.5.4 Fine focus (RAC mode)

The layout of the fine focus settings depends on the selected **Autofocus Contrast type** selected under **Global Data**. If you selected **RAC** for the **Autofocus Contrast type** under **Global Data**, the following layout will come up:
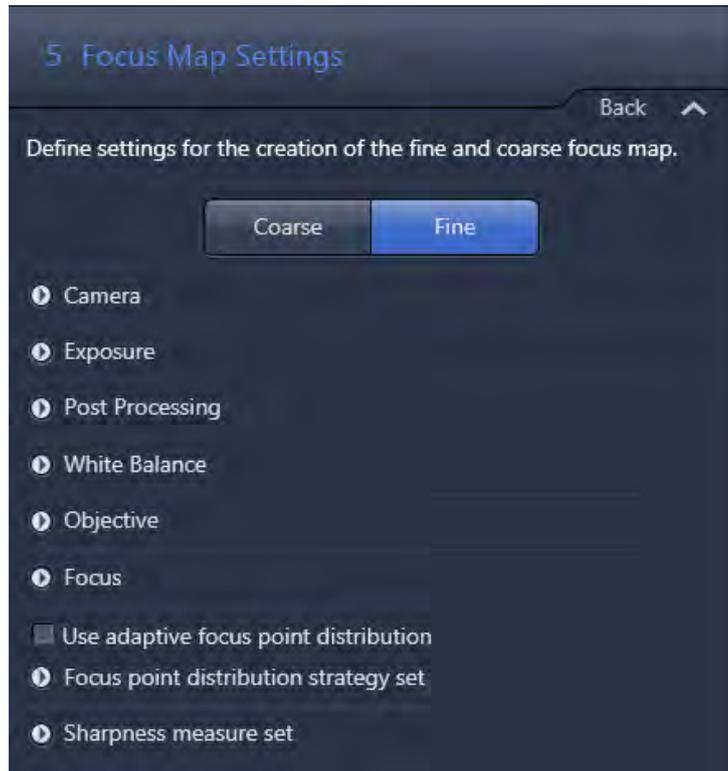
As the settings for the **Exposure** and **Post Processing** are the same as used for the standard ZEN controls, please refer to these chapters for more information.

Prerequisites ■ You should adjust the **Coarse Focus** settings in advance.

Procedure **1** In the **Camera** section, it is possible to select the active camera; however, only select the monochrome camera (Orca flash or AxioCam MR) and not a color camera or the AxioCam IC as the selection will not result in a working profile!
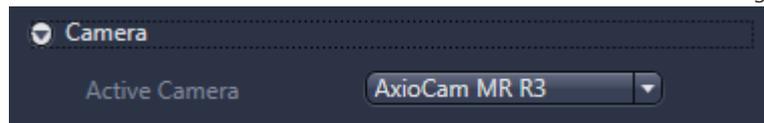
2 Select the appropriate objective in the **Objective** section. In our case, the desired scanning objective should be the objective for the fine focus (the software will select the 20x objective by default).
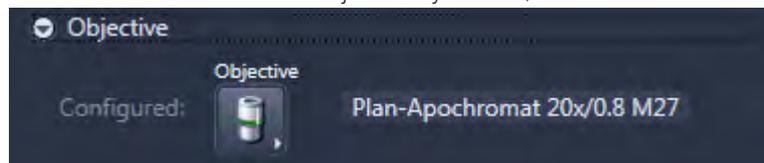
3 Now navigate to the specimen with the tools you have in the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the virtual joystick within the **Stage** tab to navigate more precisely.

4 The next step is to adjust the *Sharpness measure set* [▶ 61]. In our case, **Best** or **Basic** are the best choices for the fine focus in RAC.

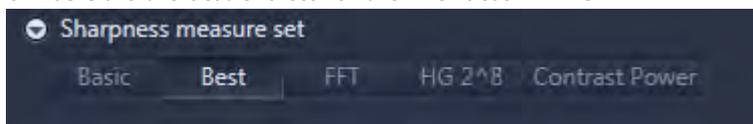**5** Focus on the specimen. For this reason, use the **Run Auto Focus** button in the **Focus** section. During a focus run you can interrupt the run by clicking on the button **Stop Auto Focus**. If the system does not find a focus, you may have to focus manually, e.g., via the slider next to the virtual representation of the z-stack.
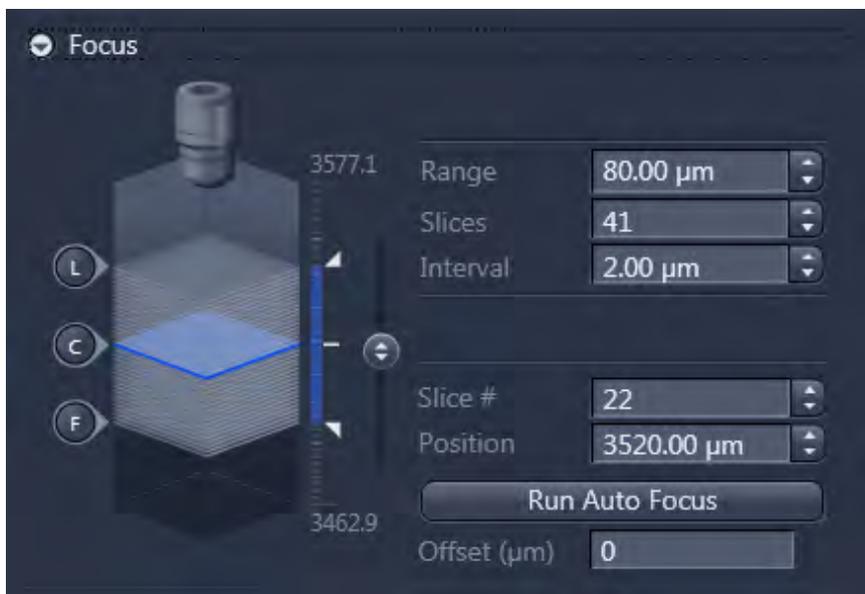
6 Once the specimen is in focus, double-check that the image is not too bright or too dark. If you need to adjust one of these parameters, please change the settings under **Exposure**. By clicking on the **Measure** button, the system will automatically determine the best **Flash intensity** and **Flash duration**. For best

results, move the stage to a position that keeps specimens out of the light
path.

7 You now have to adjust the **Range** in the expander **Focus**. You could change
the **Interval**, but it is not normally recommended to make any significant
changes: The following settings are recommended for the interval: 2.5x: 20
µm; 5x: 8 µm; 10x: 4 µm; 20x: 2 µm; 40x: 1.5 µm), thus it is advised to change
the number of **Slices**. The final optimal range depends on several parameters,
such as the number of focus points, specimen evenness, and the objective used
for the Coarse Focus. If 5x or 10x is used as the objective for Coarse focus,
then the search range for the Fine focus can be around 80 µm. If you use 2.5x
as the Coarse focus objective, the range should be at least around 150 µm.
With the **Offset** in µm, you can apply a fixed offset to the focus map. This
offset value will be added to all z-values of the focus map.

NOTICE

Risk of damaging the objective

Based on the working distance of the objectives the maximum focus range
should be for a Plan-Apo 40x 100 µm and for every other objective a focus
range of below 600 µm is acceptable. This considers a typical cover slip
thickness (170 µm) and a thickness of a specimen of around 10 µm. If you use
only a fine focus range of 60 µm, the maximum thickness of the specimen can
be 50 µm. A larger working range could lead a damage of the objective and in
extreme cases to a damage of the slide.

8 The next step is to adjust the *Focus point strategy set* [▶ 58]. The default
setting (**Onion skin** with a density of 0.1 and a maximum number of 24 focus
points; in the table view you can see only the parameter for the Density if you
want to see also the other parameters (if available) double-click on the entry) is

the best value for a wide variety of slides with large specimens. An exemption could be Tissue Micro Arrays, for which **Center of gravity** is recommended.

9   To change the **Focus point distribution strategy set**, double-click on the entry. A window comes up, in this window you can select another **Focus point distribution strategy set** via the dropdown menu and you can adjust the parameters for this setting. With a click on **Modify** the changes will be applied.

The described procedure is applicable for specimens where the objects have an equal size. As the Focus point distribution strategy is depending on the object size the system offers the possibility of the so-called adaptive Focus point distribution, please refer to the chapter *Adaptive focus point distribution* [▶ 85] for more information.

10   To double-check, click on **Run Auto Focus**. If all settings are correct, the system should focus on the specimen without providing any warnings/errors. If you still experience problems, please double-check the above settings.

3.3.1.5.5 Focus point strategy set

> **i Info**
>
> The focus point strategy set will be applied for each scene, i.e., as a separate detected object in each case. If you select six fixed points for the Coarse focus, the software will apply 6 focus points for each scene.

Item	Description
Onion skin	This is a density focus schema. The resulting focus points are displayed as layers of onion skin, providing an even distribution and also ensuring that the border in particular has enough focus points. This is the standard setting for the fine focus map. The parameter is a density with a standard setting of 0.1. This setting means that 10% of the total number of viewing fields will be used as focus points to calculate the focus surface. For large specimens this number can get very large, but a larger number of focus points does not always automatically mean a better quality calculation of the focus surface. For this reason, the user can also define a maximum number of focus points. This number is normally in the range of 24 to 36 points. This focus point strategy set is recommended for mid-sized to large objects.

Example (Density: 0.1; max number 24):

Item	Description
Every Nth tile	The system will put a grid over the specimen with a distance between two focus points of the specified value (N) for the number of camera viewing fields in the x and y direction. This is a clearly defined way to create the focus point, even it creates more focus points and will take longer to create the focus map. The focus point strategy set is recommended for mid-sized and smaller objects.
	Example (every 5th tile):
Every tile	The system will focus on every tile. This is particularly time-consuming for scans with a large magnification and/or large samples; however, it yields the best results for very uneven specimens.
Number of focus points	Here the user defines a fixed number of focus points per object (scene). The default setting for the Coarse focus is six focus points per scene.
	Example (n = 24):

Item	Description
Density	The density is similar to that of an onion skin; the difference is that the pattern is not distributed like the layers of an onion. The distribution is more evenly spread over the specimen/scene. This setting can also be used to define a maximum number of focus points. The creation of this focus map is more random – as shown in the example – and thus the onion skin is recommended over the density. Example (density: 0.1; max number 24):
Grid cell size	This setting defines a fixed grid over the specimen to establish the focus points for the focus surface. The value is in µm. This setting is independent from the current frame/sensor size of the camera used. If you want to use a setting that is based on the chip size of the camera, please use "Every Nth tile". As seen on the sample, this schema is not as strict as Every Nth tile, thus Every Nth tile is recommended over the Grid cell size. Example (1000 µm 1000 µm):

| Item	Description
Center of Gravity	The system will place one focus point within each scene/object. The position of this focus point is the center of gravity, thus the whole object has only one focus point. This is a useful setting for small objects (e.g., Tissue micro arrays). Be aware that for certain structures (such as a half moon), the center of gravity may lie outside the tissue, hence the system has nothing to focus on. In such cases, select "Number of focus points" with 1 as the parameter. The center of gravity is a valid option, e.g., for Tissue Micro Arrays to place only one focus point in each core.

3.3.1.5.6 Sharpness measure set

| Item	Description
Basic	The autofocus is contrast-based, hence the algorithm will focus on the highest contrast along the z-stack. It is a simple but fast autofocus.
Best	The autofocus is also contrast-based in this case, thus the algorithm will focus on the highest contrast along the z-stack. Compared to Basic, the focus is slightly slower but more accurate for certain samples. Please be aware that Best does not mean that this algorithm is always superior to Basic, as this depends on the kind of tissue/specimen.
FFT	This autofocus setting applies to the Fourier transform algorithm to determine the best focus value. This algorithm is slower than the other algorithms and should be used only in the case of the Ring aperture contrast. It is not ideal for standard brightfield contrast or fluorescence as this algorithm looks for two contrast peaks and the local minimum between them.
HG 2^8	This is an advanced algorithm compared to Basic and Best. This algorithm focuses less on the larger stained structures and more on the smaller local structures, thus this algorithm will focus more on nuclei, for example, than on unstained structures (e.g., immunohistochemistry stains). Based on the current status of evaluation, this means that HG2^8 is a good autofocus method if a user wants to focus on nuclei. **Drawback: The weight of dirt also increases, hence it should only be used for the fine focus, not the coarse focus.**

Item	Description
Contrast Power	This algorithm is comparable to the HG 2^8.

3.3.1.6 Scan Settings
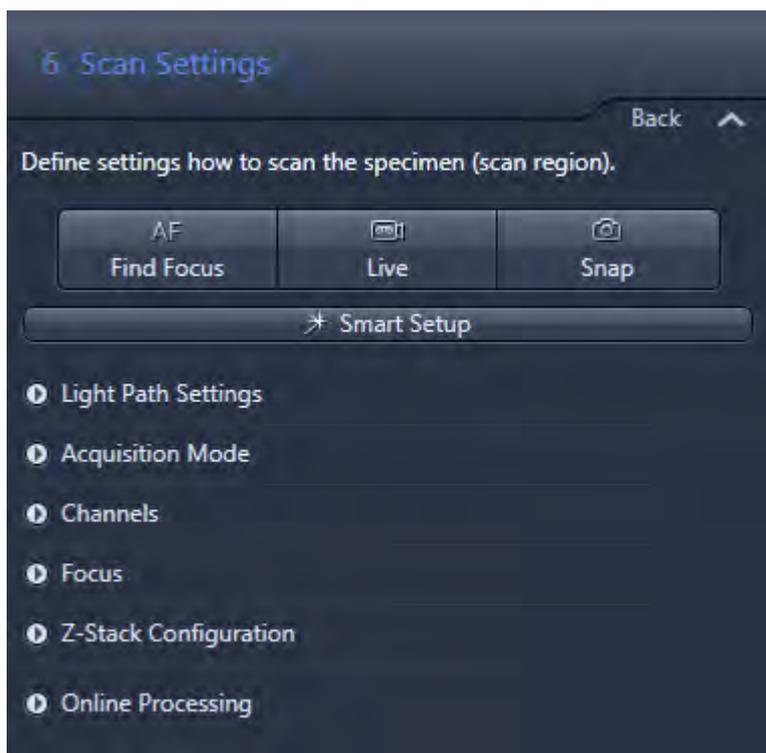
Procedure

1 The first step is to create the **Light Path Settings**. This can be done manually or with the help of the **Smart Setup**. For more information on Smart Setup, see *Smart Setup* [▶ 137].

It is recommended to use the **Smart Setup** as it not only includes double-checks, but is also very easy to use. Once you open the **Smart Setup**, you add your dyes by double-clicking the dyes from within the extensive list available. The system then suggests one or more potential configurations. The number of suggestions provided by the system depends on your hardware configuration. If you select a setup (e.g., Best Signal) the system will populate the **Light Path Settings**. **Smart Setup** allows you to define up to four channels. If you want to define more than 4 channels, you have to access the **Light Path Settings** and add additional channels manually.
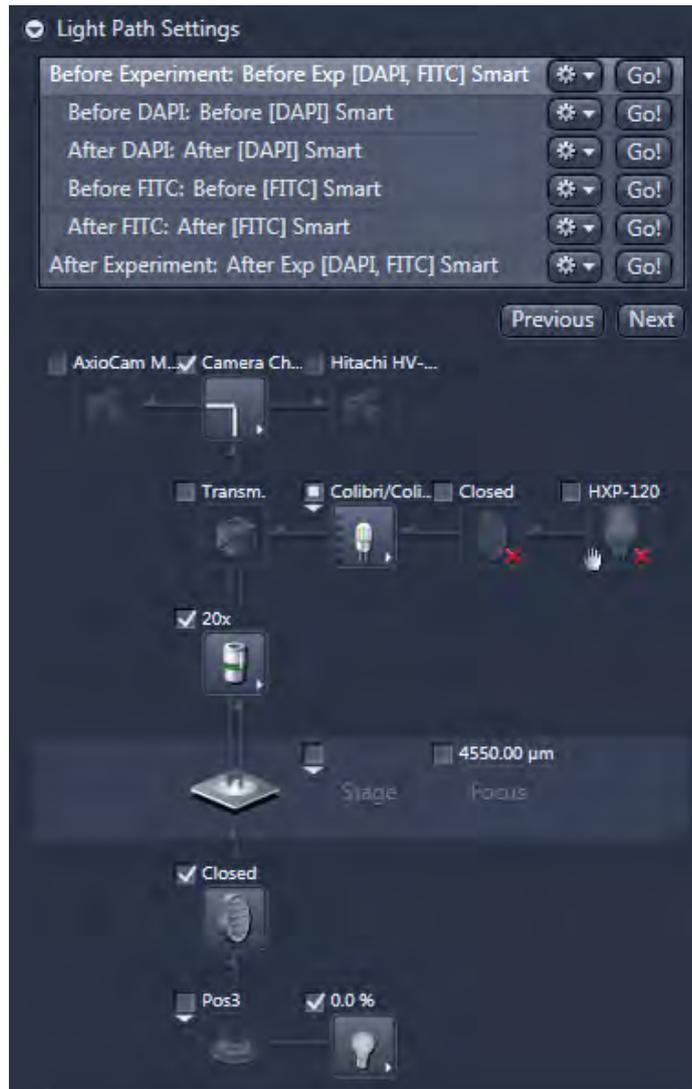
By default, the system will insert the 20x as a scanning objective inside the Light Path Settings. You can change the objective by opening the expander for Light Path Settings and activating the Before Experiment: Before Exp. You can now see the light path configuration. The objective should be checked and the 20x should be selected as this is the default setup. If you want to use another objective, select this and click on the **Go!** button. The system will then put the selected objective in the light path.

NOTICE

Risk of Damaging the device

Use only the **Colibri** or the **HXP 120** in one scan, thus in the Scan Settings setup. The switch within the Colibri (switching between the LEDs and the HXP) is not laid out for a large number of cycles as we have to expect if the switch is used for every tile.

> **i Info**
>
> You can select more channels than you actually need for the experiment to make the profile more universal, and you can select and deselect single channels at a later stage.

> **i Info**
>
> If you change the objective, ensure that you also use the same objective for your **Fine focus**.

2   The settings in the **Acquisition Mode** section offer advanced settings, but these are normally set to the optimal levels and do not require any manual intervention. Here you have the possibility to decrease settings such as the exposure time to minimize bleaching or even decrease the scan time. This can achieved by increasing the **Gain** of the camera (if available for the specific camera) or increasing the **Binning**. However, it must be noted that this will decrease the resolution of the image.


3   Now navigate to the specimen with the tools you have in the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the virtual joystick within the **Stage** tab to navigate more precisely.

4   Under **Channels** in the list, you can see all defined channels. You can activate (or deactivate) each channel using the checkbox in front of each one. If you highlight a certain channel, the hardware will be changed the optical light path. This is where you have to define the channel to be used for Stitching. The

Channel is marked with "Ref.". To change the stitching channel, highlight a channel and click on "Stitching Ref.". Make sure that the selected stitching channel is activated. An appropriate stitching channel is one in which the dye covers the specimen evenly (thus the system has enough structure for stitching). Large structures with fine details (such as cytoskeletons) are preferable if they are evenly distributed across the specimen. Under channels, you can still select the light source if you have two (e.g., Colibri.2 and HXP 120 V), as well as the intensity of the light source.
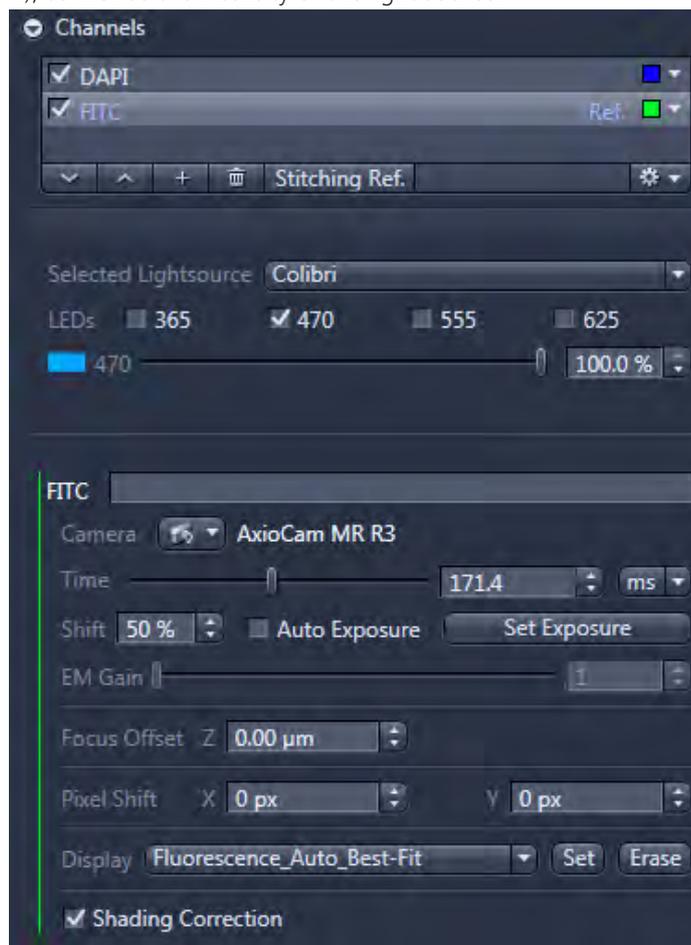
5   The next step is to **Focus** on the specimens and adjust both the exposure time (**Time**) and light source intensities. To see a live image from the camera, you have to activate **Live**, which will display a live image, or else click on **Snap** to take a single snapshot of all selected channels. It is important to take extra care in Live mode as this can result in bleaching of the sample. It is therefore recommended to use this feature with caution and, if possible, to use a representative region outside the scanning region.

6   You can use the **Find Focus** (**AF)** button to perform the autofocus with the selected (highlighted) channel. If the focus is off and/or the exposure time is not appropriate, you should use the manual focus control (expander **Focus**). You may also need to adjust the exposure time. (**Time**) and/or the intensity of the light source(s).

It is not necessary to increase the exposure, thus the complete dynamic range of the camera is covered. You can adjust the Display curve in the histogram to also show the signal on the screen, even if only 5% of the range is covered, for example.
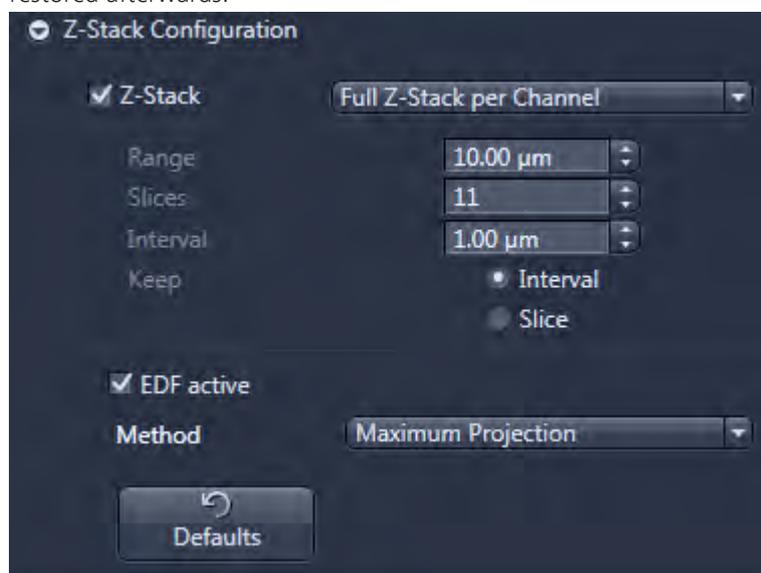
7   Once the focus has been established, you can then adjust the exposure time (**Time**). This can be done manually by moving the **Time** slider or automatically by clicking the **Set Exposure** button. The exposure time will be determined so that the range of the camera will be covered by the value stated under "Shift".

8   Make sure to check several regions of the specimen (using Navigator or Stage control) to establish whether certain regions have a much higher fluorophore concentration. Repeat this step to determine the exposure time for all other checked channels.

> **i Info**
>
> If the illumination of the light source is very even you can deselect Shading Correction as a shading correction could make the resulting image even worse. If Shading Correction is activated you have to make sure that an appropriate shading reference is available. You can perform a manual shading correction via the Locate tab with the standard ZEN measures. You can also generate shading reference images directly from an existing image (which was created without a shading correction active). To do so you have to use the processing function **Shading Reference From Tile Image**, for instructions see *Creating a shading reference from tile image* [▶ 131].

9   The **Z-Stack Configuration** is important if you want to automatically acquire a z-stack with or without extended depth of focus (EDF). This can be considered for uneven specimens or very thick samples. Please note that – depending on the objective, condenser, and camera – a 20x Plan-Apo has a depth of focus of around 1 µm and the 40x Plan-Apo has a depth of focus of around 0.5 µm. The unevenness can be the result of a sub-optimal preparation or if the user aims to scan thick or cytological specimens.
The best way to evaluate the optimal settings is to go to the representative region of the specimen, autofocus from a blurry image to an image where the

first structures come into focus, and note the z-value. Go through the specimens until the last regions of the specimens in the field of view are switching from being focused to blurry, then note the z-value once again. The difference between the z-values is the height of the z-stack. It is recommended to repeat this procedure for different regions of the specimen. The largest z-range will be used. Empirical research has shown that it is recommended to add 20% to the z-range as the autofocus is not always exactly in the middle of the specimen. It is now necessary to determine the step size based on the z-range. Theoretically, the step size is between the depth of focus and half of it, thus 0.5 to 1 μm for a 20x Plan-Apo objective. However, this would result in a larger number of z-steps and a low scanning speed. If you do not want to preserve the z-stack and work with smaller image sizes, you can activate the extended depth of focus (**EDF active**). If active, the software will use the captured images in different focus planes for each image field and combine the regions of strongest contrast within given z-stack images into an image that contains the maximum contrast portions of all images recorded for this image field. This method guarantees maximum depth of sharpness. It is strongly recommended to use the standard parameters as these have been selected for their optimal fit. For a detailed description of the single parameters, please refer to the help for standard ZEN functionality of EDF. A scan with EDF is slightly faster than the same scan with a Z-stack. It is important to note that an extended depth of focus can also be applied at a later stage; however, if the extended depth of focus is applied in the profile then the z-stack cannot be restored afterwards.

> **NOTICE**
>
> Risk of damaging the objective
>
> Based on the working distance of the objectives the maximum Range should be for a Plan-Apo 40x 50 µm and for every other objective a Range of below 300 µm is acceptable. This considers a typical cover slip thickness (170 µm). A larger working Range could lead a damage of the objective and in extreme cases to a damage of the slide.

10 The final step to check is the **Online Processing** expander. Here you can define whether the stitching is carried out **Online**, i.e., during the acquisition, **Offline**, or if no stitching (**None**) will be applied at all. The standard setting is online stitching as this provides the best performance in terms of the processing time of the slide. The **Offline** mode will initially perform the scan, with the stitching applied at a later stage. This procedure is slower than the Online stitching. The difference is relative to the size of the specimen, i.e., if a specimen is very large, the offline mode is very slow compared to the online mode. For smaller specimens, there is barely any difference in processing time. It is only possible to use no stitching (**None**) if the stitching is to be applied afterwards using ZEN measures or a separate program after an export. As stitching for fluorescence is critical, offline mode should at least be considered for small samples. If the offline mode is selected, the user has the possibility to activate **Fuse**. With **Fuse**, the system creates new tiling and thus the shading improves.
Pyramid active should always be activated, as this will speed up the viewing of the image afterwards. However, a pyramid can also be created afterwards. If a non-pyramid image of a certain size is opened, the software will always ask if the pyramid should be created.
These final settings allow you to adjust the compression which will be applied as part of the online processing. If **JpegXR active** is deactivated, the image will be saved uncompressed. If the **JpegXR active** is activated via the checkbox, you can save them lossless (**Lossless compression**), thus the image will only be compressed to the extent that no information is lost. If **Lossy compression** is activated, the compression always involves a loss of information. If this checkbox is activated, you can determine the degree of **Quality**. You will find more information on compression vs. file size here *Comparison compression regime vs. file size* [▶ 101]
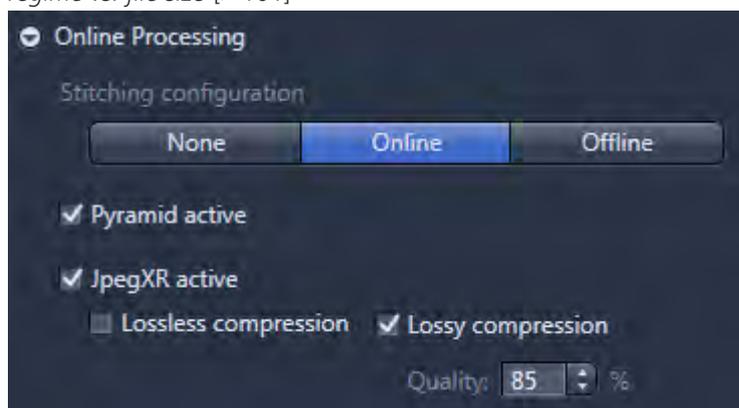
3.3.2 Brigthfield profiles

3.3.2.1 Global Data

---

i Info

To access all functions and properties described in this help, you have to activate the **Show All** mode.

From here, you can define global settings. Depending on these global settings, both the wizard and the workflow will be adapted automatically.

Profile & Description

The name of the selected scan profile is displayed under **Profile**. This name cannot be changed here. A short description of the profile can be found under **Description**. As the information content of the profile name is always limited, you can insert additional information here to highlight the most important profile settings (e.g., magnification/z-stack settings/focus map settings).

Profile Type

Here you can select between **Brightfield** and **Fluorescence** profile types. If you choose brightfield, AF Contrast Type settings will disappear.

Tissue Detection Mode

This is where you select the kind of tissue detection you want to apply. The following three modes are available:

Mode	Description
Automatic	In this mode, the system detects the tissue automatically.
Interactive	In this mode, the system will pause the scan process if a batch is started and waits for your input to define a region of interest manually. As the system pauses until this interaction is complete, this function has to be used very carefully.
Manually	In this mode, you can define a region of interest to be scanned manually or a grid in a subsequent step of the wizard. This is an option if you only want to select a subset of the tissue on the slide, e.g., if the tissue is very faint, the system cannot detect the specimen reliably, or if the specimen has a regular pattern on the slide.

i Info

The most common selection here is the **Automatic** mode with **Tissue**.

TD Recognition Type

Not available if you set **Tissue Detection Mode** to manual.

This is where you select whether the tissue detection is carried out via thresholds (**Tissue**) or if you want to use a **Marker** to encircle the specimen manually on the front of the physical slide. In this case, the threshold will be used to detect the marker. Everything inside this marker will be scanned. If **Marker** or **Tissue** is selected, the system will always generate a preview with the separate preview camera.

3.3.2.2 Label Scan Settings
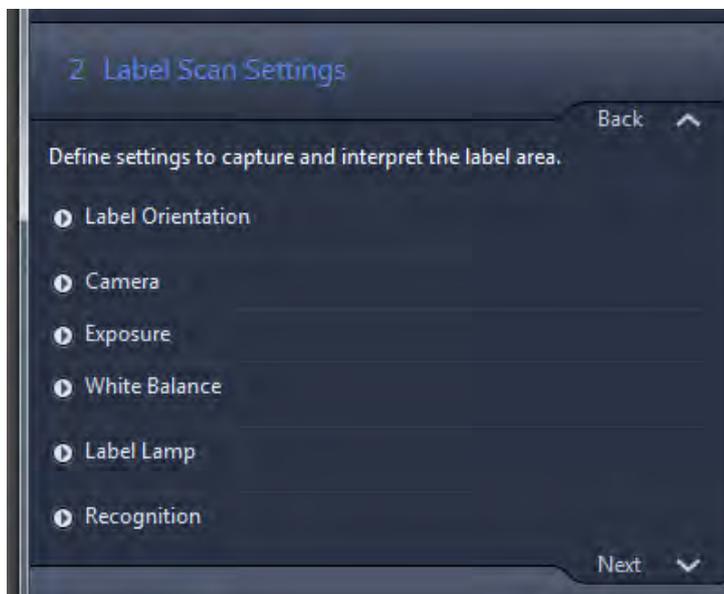
Here you can select the capture parameters of the label area.

The label area ROI (Region Of Interest) is defined by the red rectangle shown in the live image. It is possible to adjust the frame in size and position freely to fit with the label on your slide. The label area is captured with reflected light and a separate camera (called **AxioCam IC**).
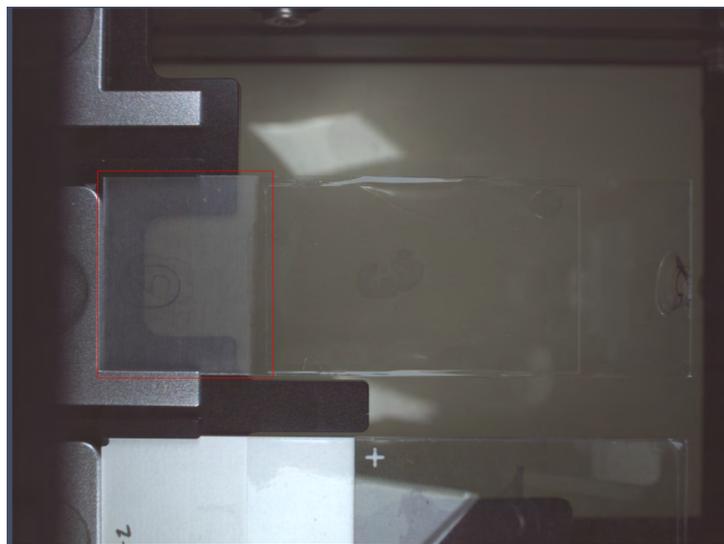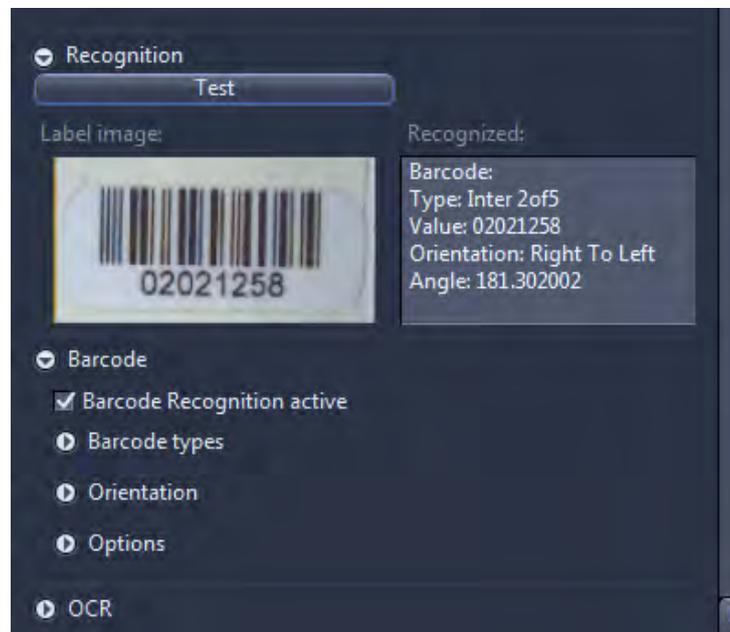

Illustration 11: Label Area

> **i Info**
>
> It is not usually necessary to adapt the camera settings and/or light source as these settings are factory defaults and should represent the optimal settings. For this reason, these settings (**Camera**, **Exposure**, **White Balance** and **Label Lamp**) are not described in detail within this section.

Label Orientation

This is where you select how the label is aligned on a slide.

You can rotate the label image in 90° steps clockwise (CW) or keep the original orientation (**Original**). If you select **Use Barcode Orientation**, a barcode sticker has to be attached on the slide. The software will then determine the orientation of the barcode and apply this to the orientation of the label area of the current slide. To use this function, a barcode label has to be attached and you also have to activate **Barcode Recognition active** in the **Recognition** expander.

You can also change the label orientation later on in the ZEN software if you browse through the slides.If all of the slides have a rotated label, however, it is recommended to change the label orientation here.

Recognition

■ **Barcode**

   If Barcode Recognition is activated, the system interprets the barcode and saves the barcode information as metadata within the image. If you want to use the barcode information as part of the image name, you have to apply automated naming and use the keyword "**RecognizedCode**" or short cut "**%N**" to make the barcode part of the image name.

   By default, the software will check for all implemented barcode types and all orientations, thus it is not necessary to define a specific barcode. If the label contains two or more barcodes with a different barcode type, we recommend that you define the specific barcode type you are looking for. To activate a specific barcode, open the expander for **Barcode types** and activate only the barcode you want to be read.

■ **OCR (Optical Character Recognition)**

■ The OCR works with **English** text only. Be aware that OCR works best with clearly printed numbers and/or letters. We recommend using OCR_A or OCR_B as the font for the OCR. We do not recommend using underscores (_) or special characters within the text. You can select different options to optimize the recognition quality, but the standard parameter should work. The printed text should be very precise in terms of its orientation. There should be minimal text tilt after the image orientation.

To check if the barcode or characters can be recognized, click on the **Test** button. The system will now show the region of interest with the orientation given and apply the barcode or OCR recognition. The result will be presented in the **Recognized** field.

---

i Info

You have the possibility to adjust the Display curve (see Display tab on the bottom of the window). The display settings will stored within in the profile! This has also an impact on the display of the label in the Magazine view. This has also an impact on how the label is displayed in the Magazine view!

Please read also the instruction under *Using Label Scan Settings for 4''x3'' slides* [▶ 123].

3.3.2.3 Preview Scan Settings
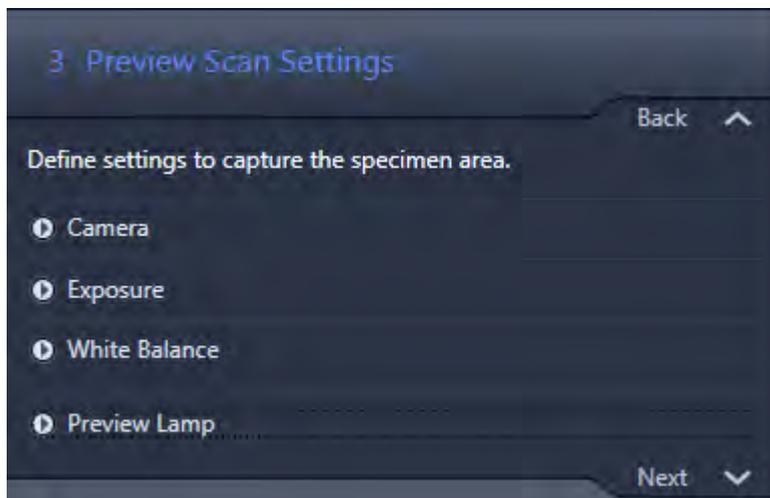
The preview for brightfield scans will always be taken with the separate preview camera, thus the system generates the preview image with one snapshot.

If you are using 4"x3" slides the graphical user interface has additional entries, see *Using Preview Scan Settings for 4"x3" slides* [▶ 127]

Camera settings (Camera, Exposure, White Balance)

This is where you can adjust the settings for the camera and the illumination; however, we recommend that you do not change these settings for the sake of producing reproducible results. As the controls are similar to the standard ZEN controls for cameras, please refer to the appropriate chapters, see ZEN 2 Online Help / Camera tool. It is not possible to change the camera for the preview (under **Camera**), as this is fixed.

> **i Info**
>
> Please avoid any kind of over exposure of the image. An overexposed image can result in a sub-optimal shading correction.

Preview lamp

Here you can adjust the intensity of the preview lamp for the specimen area (transmitted light).

For detailed instructions please read also the chapter *Setting up the ROI for the preview image* [▶ 129].

3.3.2.4 Tissue Detection Settings

The appearance of the TD (Tissue Detection) settings depends on the settings which you have made in the first step of the wizard under **Global Data**.

> **i** **Info**
>
> You have the possibility to implement his own tissue detection algorithm and make this algorithm part of the workflow (for more details see below under Further information)
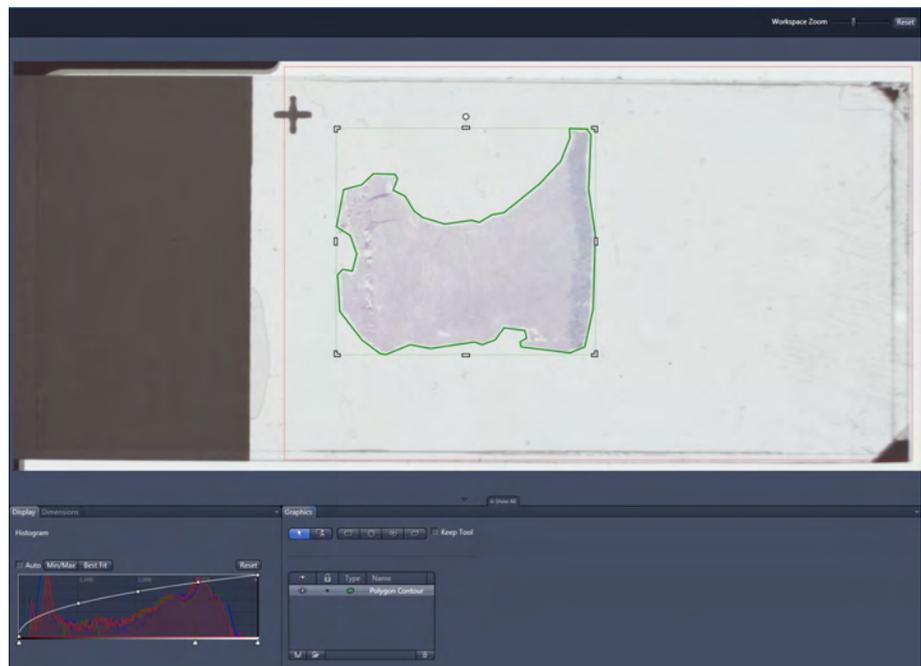
Case A Prerequisites

If you have selected the **Automatic** mode in Step 1 **Global Data** under **Tissue Detection Mode**, as well as the **Tissue** button under **TD Recognition Type**, the TD settings will appear as follows:

Region of Interest for Tissue Detection

The red rectangle in the live image which you can see in the **Center Screen Area** represents the ROI for the Tissue Detection. Only the regions that are inside the ROI will be presented as regions which can be scanned. Other regions outside the ROI and/or touching them will be ignored. You can change the size of the ROI by adapting the size of the red rectangle.

Using the **Test** button, the software will execute the inserted parameters and show the results in the middle part of the window. The detected objects (every object is a single scene) will be presented with a green border:

All objects are listed in the **Graphics** tab. The user can delete objects and even add new ones; however, the result will be only valid for this slide.

To achieve a continuous update, you can activate **Live update** via the checkbox. Please keep in mind that a refresh can take some seconds after changing a parameter.

Mode & Method display fields

This is where you can see the mode and method for tissue detection which you selected under **Global Data**.

Region dilation size

Here you can enter a value for the spacing of the border around the detected tissue.

Specimen

Here you define the upper and lower borders of the specimen via the thresholds. By clicking on one of these borders and pressing the right mouse button you can move the borders up and down. If you have activated the **Live update**, you will see the result toward the left of center with a certain delay. The upper border (right side) is the border for the lighter stains and the lower border (left side) is the border to influence the darker stains. Thus moving the right side further to the right marks the lighter stains. Moving the left side further to the left marks the darker stains.

Min Region size

Here you can enter the minimum region size to be scanned. The system will not detect regions if they are smaller than this value.

Air border dilate

The air border is the border around the physical glass slide, i.e., the area outside the glass slide and inside the red frame. This value determines the dilation of this air border. Everything inside this dilated air border will not be recognized as tissue/a marker. This is a measure for ensuring that slide edges and coverslip edges are not detected as needing to be scanned.

Over the peak factor

This parameter is only visible if the **Automatic** checkbox is activated. It defines the distances between the glass peak of the slide and the upper threshold value for the specimen detection. Thus if **Automatic** is activated, the upper value will be determined automatically.

Max elongation

This setting is important to remove coverslip edges. Coverslip edges are normally structures with a large number for the ratio of the longer side and the shorter side. This means that every structure that exceeds this parameter for the ratio between the longest side and the shortest side will be excluded.

If you click on the **Reset** button, the values will revert to their default settings.

Once you have determined the appropriate settings, you can save these settings by selecting **New** via a gearwheel in the **Settings** line. These settings can then be used in another profile, for example, by selecting the Settings name from the dropdown menu within the **Settings** line. It is not essential to save these settings as they will be saved within the profile. It is only necessary if the settings are to be used in another profile.
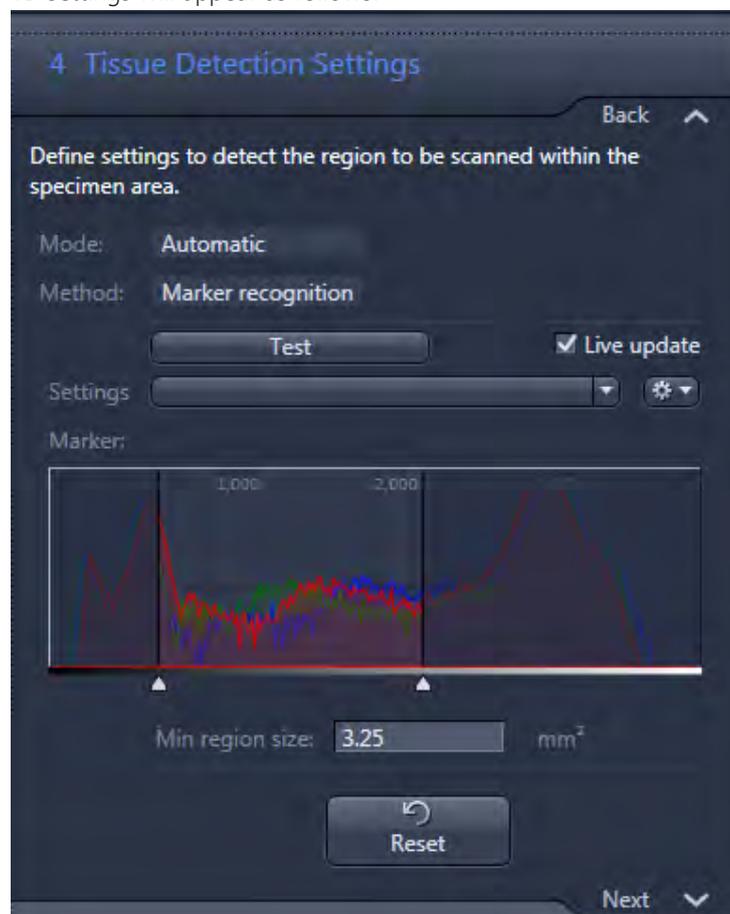
---

i Info

If you draw your own regions, make sure that these contain tissue. Empty space could lead to an incorrect focus map as the software will use the entire drawn region to create the focus point distribution.

> **i Info**
>
> If two or more objects are very close to each other and thus the two or more objects are seen as one objects it is perhaps advisable to split these objects with the **Split** function which is availabe in the context menu seen after a right-mouse click.

Case B   Prerequisites

If you have selected the **Automatic** mode in Step 1 **Global Data** under **Tissue Detection Mode**, as well as the **Marker** button under **TD Recognition Type**, the TD settings will appear as follows:

The settings are limited to the definition of the marker threshold (comparable to the tissue threshold described above). The only further adjustable value is the **Min region size**, whose functionality is the same as described above.
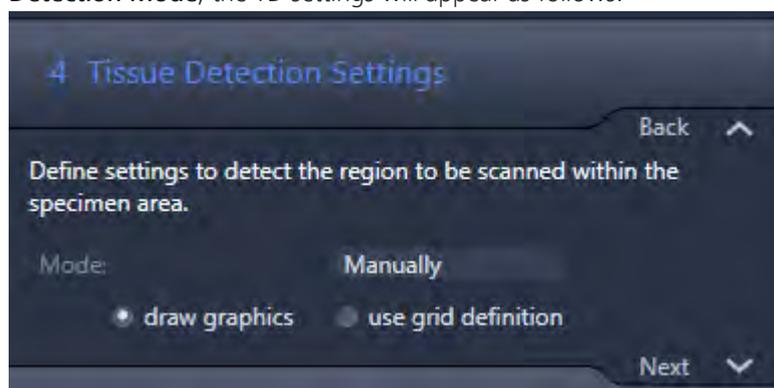
Prerequisites

> **i Info**
>
> If you use the marker on the physical glass slide, make sure that the regions marked contain specimens. Empty space could lead to an incorrect focus map as the software will use the entire marked region to create the focus point distribution.

Case C   Prerequisites

If you have selected the **Manually** mode in Step 1 **Global Data** under **Tissue Detection Mode**, the TD settings will appear as follows:

Drawing in TD ROI manually or using Grid Definition

Here it is possible to draw regions manually within the region of interest if you activate draw graphics. To draw regions, you can use the tools on the **Graphics** tab in the general view options of the **Center Screen Area**. You can use it in the same way you would generally annotate images within ZEN. The drawn regions will be saved and the same areas will be used for the entire time the profile is applied, hence this option can only be used if the regions are always defined precisely on the slide (e.g., PAP smears).

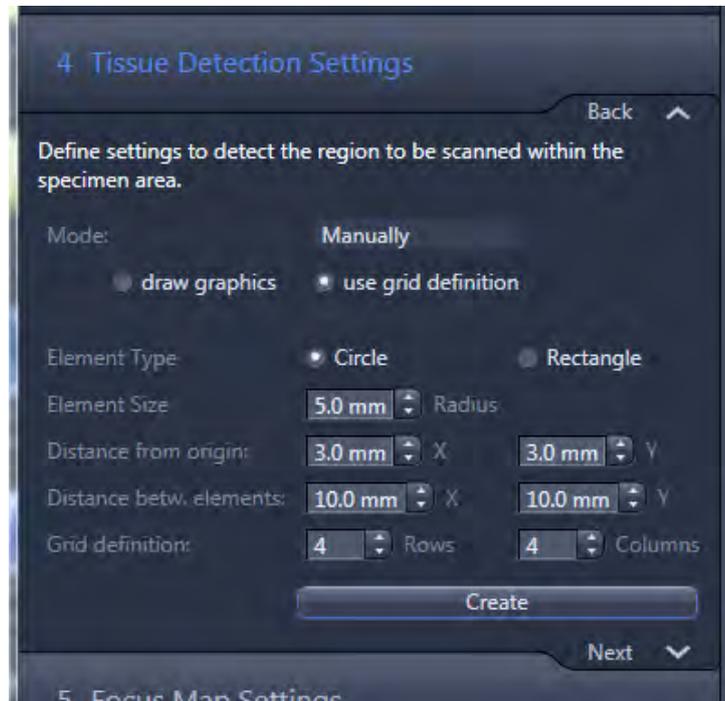For the grid definition, see chapter *Grid definiton* [▶ 80].

> **i Info**
>
> If you want to mark a specimen that is very faint, you can use the **Display** curve to change the display settings (particularly by adjusting the gamma curve) to make it possible to see even unstained specimens of a reasonable thickness.

> **i Info**
>
> You have the possibility to adjust the Display curve (see Display tab on the bottom of the window). The display settings will stored within in the profile! This has also an impact on the display of the label in the Magazine view. This has also an impact on how the specimen area is displayed in the Magazine view!
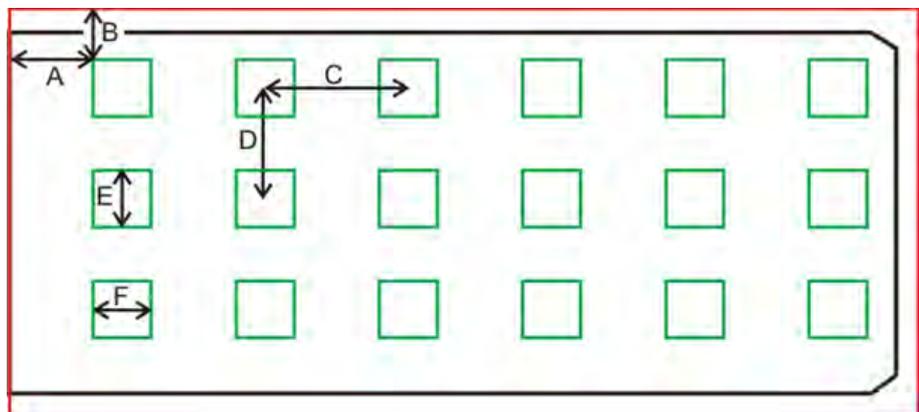
3.3.2.4.1 Grid definiton

If the **Tissue Detection Mode** under **Global Data** is set to **manually**, it is possible to mark the tissue by drawing in graphics or by using a grid definition.


You can define a grid with rectangles or circles as **Element Types** in a regular pattern. The reference point for this grid is the upper right-hand corner of the specimen area (red rectangle). Based on this, you can define on offset from the upper left-hand corner in x (A) and y (B) in mm and also the pitch of the grid in x (C) and y (D).

Based on the selected geometrical shape (circle or rectangle), you can define a radius or the dimensions in x (F) and y (E). The number of elements in x (in this example, 6) and y (in this example, 3) defines the number of elements.

When you click on the **Create** button, the grid will be generated. You will see the grid as an overlay in the image. It is also possible to make manual adjustments such as those used for standard graphics in ZEN.

3.3.2.5 Focus Map Settings
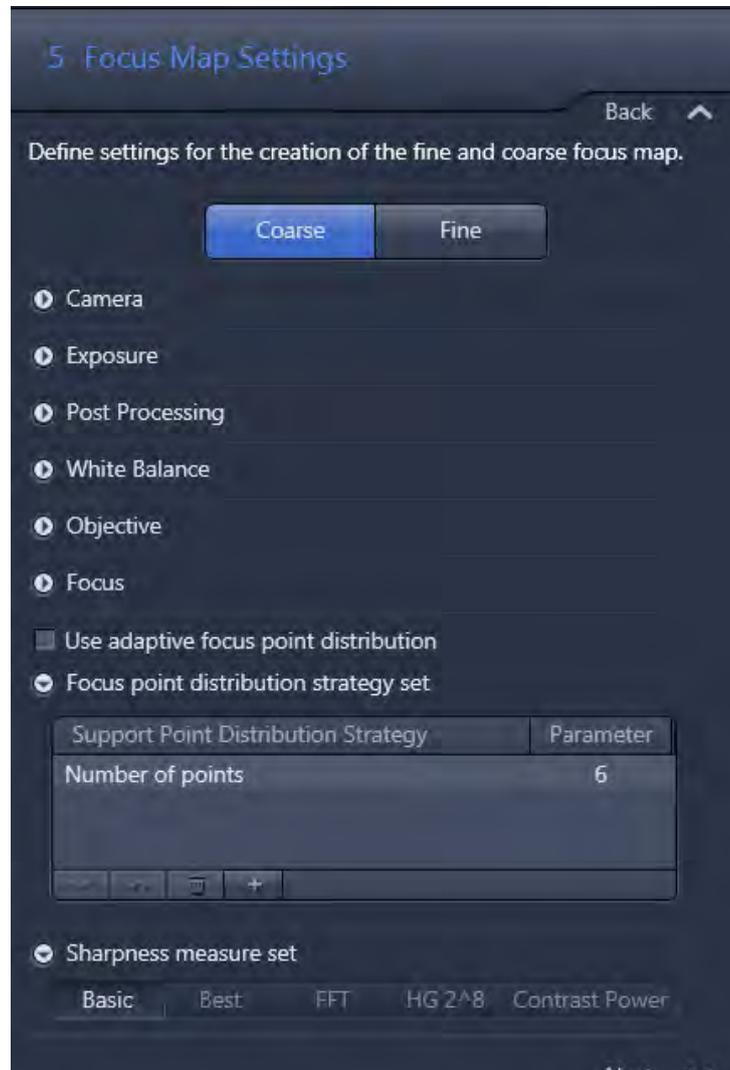
Appropriate focus map settings are important for ensuring good focus quality throughout the specimens. The focus map generation is done in two steps:

- With a low magnification objective to generate the **Coarse** focus map. For this coarse focus map, only some focus points are necessary. We recommend the use of the 5x Fluor for the **Coarse** focus as the offset between the 5x and the scanning objective (mostly 20x) is marginal.

- With a high magnification objective (normally the scanning objective) to generate the **Fine** focus map. In this case, more focus points are needed and this is normally defined via a density. The Fine focus map is based on the coarse focus map.

The focus is not a traditional autofocus that needs to hit the z-value with the sharpest image precisely. The system will acquire a z-stack (defined in the next step) and calculate a curve through the focus value (e.g., contrast value). This curve is used to determine the peak value. This makes it possible to cover a large range while still maintaining fast focusing.

As we recommend leaving most of the settings untouched, here we will focus on the two most important settings, which are the **Focus Point Strategy** and **Sharpness Measure sets**. You will find a detailed description of these two settings in the following chapter.

3.3.2.5.1 Coarse focus
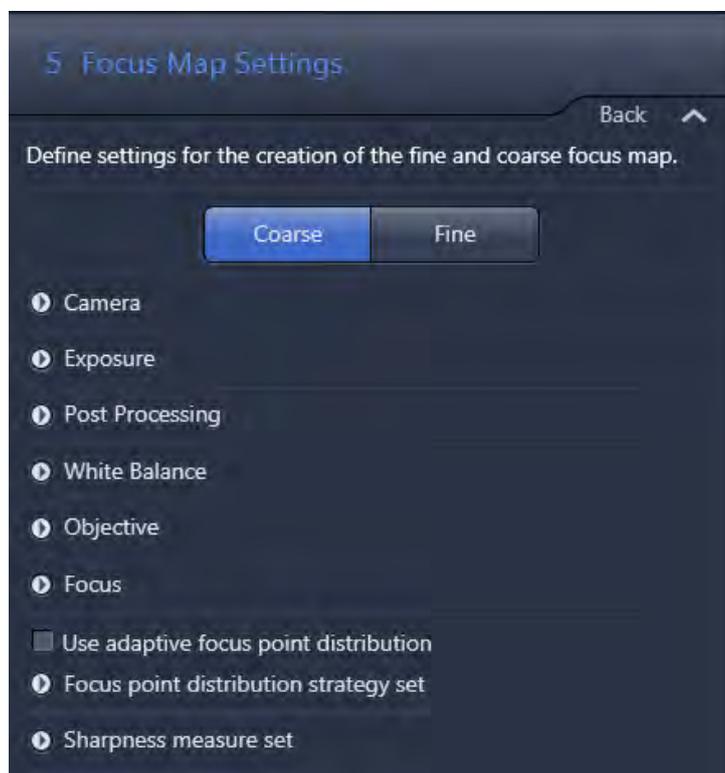
The settings for the **Exposure**, **Post Processing**, and **White Balance** are the same as used for the **Scan settings**.

Procedure
1. Under the **Camera** expander, it is possible to select the active camera; however, only select the color camera (Hitachi) and not a monochrome camera or the AxioCam IC as the selection will not result in a working profile!

2. Select the appropriate objective under the **Objective** expander. In this case, a 5x objective is recommended. If a 5x is not available, use the 10x; If no 10x is installed, use the 2.5x objective.

3. Navigate to the specimen using the tools you have on the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the

virtual joystick within the **Stage** tab to navigate more precisely. The main part of the window contains a LIVE image from the camera.


4   Autofocus on the specimen. To do so, you can use the **Autofocus** button under the **Focus** expander. If the system does not find a focus, you may have to focus manually, e.g., via the slider next to the virtual representation of the z-stack.

5   Once the specimen is in focus, double-check that the image is not too bright or too dark and that the white balance is correct.

6   Look up the current z-position (under Position, in our case 4200 µm). This z-position should be in the range specified under **Set First** and **Set Last**. In our case it fits very nicely in this range. If it does not fit within the range, you have to adjust the range. You can increase the number of slices (under **Slices**). This will increase the range, thus the focus will take longer. Otherwise, you can shift the range by changing **Set First** and **Set Last**. The following settings are recommended for the **Interval**: 2.5x: 20 µm; 5x: 20 µm; 10x: 15 µm.
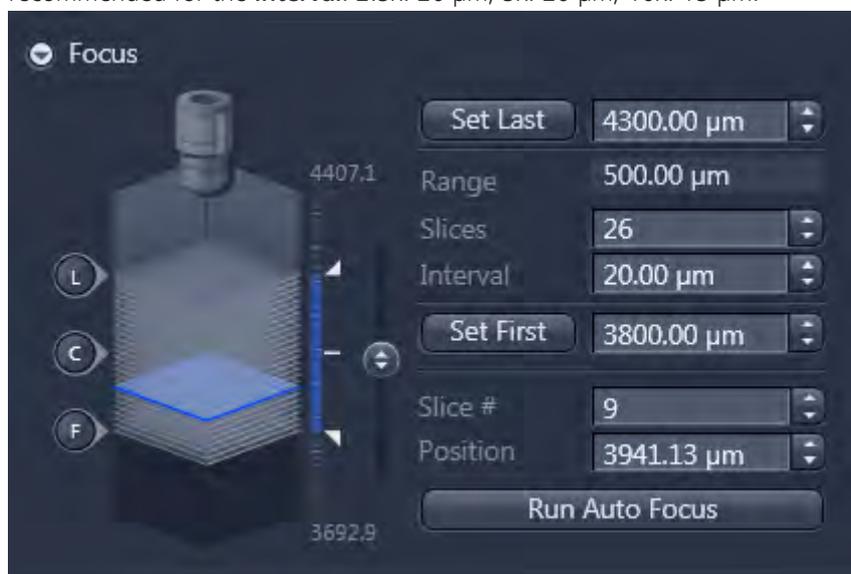

7   The next step is to adjust the *Focus point strategy set* [▶ 91]. The default setting (**Number of points** = **6**) is the best value for a wide variety of specimen (in the table view you can see only one parameter if you want to see also the other parameters (if available; e.g. for Onion skin) double-click on the entry).

An exemption could be Tissue Micro Arrays, for which **Center of gravity** is recommended.
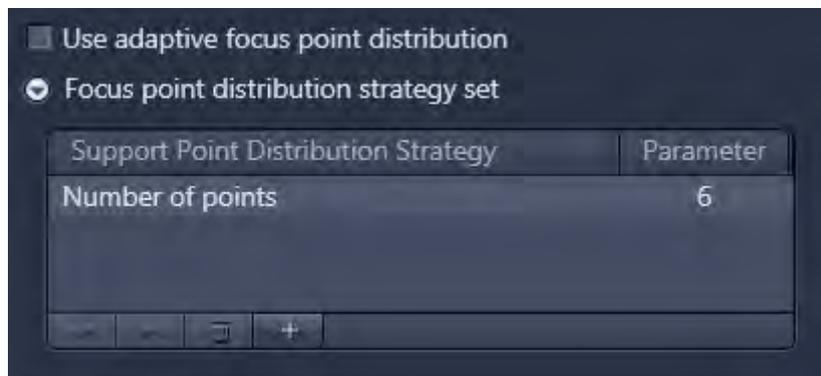
8 To change the Focus point distribution strategy set (see *Adaptive focus point distribution* [▶ 85]), double-click on the entry. A window comes up, in this window you can select another **Focus point distribution strategy set** via the dropdown menu and you can adjust the parameters for this setting. With a click on **Modify** the changes will be applied.
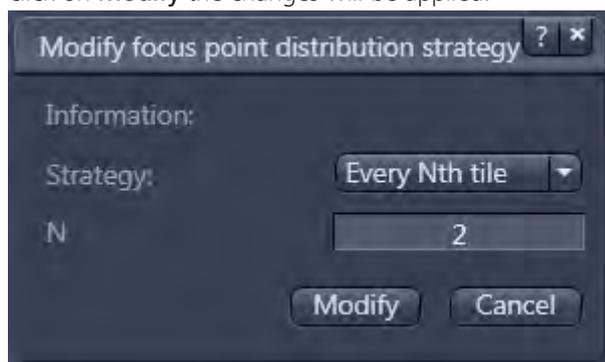
9 The last step is to adjust the Sharpness measure set. In our case, **Best** or **Basic** are the best choices for the coarse focus in brightfield. For more information, see *Sharpness measure set* [▶ 94]

10 To double-check, click on **Run Auto Focus**. If all settings are correct, the system should focus on the specimen without providing any warnings/errors. If you still experience problems, please double-check the above settings. You can interrupt the focus run by clicking on **Stop Auto Focus**.

**11** By default, the next step would be to adjust the **Fine Focus**.

---

i **Info**

The described procedure is applicable for specimens where the objects have an equal size. As the Focus point distribution strategy is depending on the object size the system offers the possibility of the so-called adaptive Focus point distribution, please refer to the chapter *Adaptive focus point distribution* [▶ 85] for more detailed information on this topic.

3.3.2.5.1.1 Adaptive focus point distribution

If on a slide are objects with different sizes it is not possible to use only Focus point distribution set as for smaller samples like Tissue Micro Arrays the set Center of Gravity would be the best choice and for mid-sized specimens Every Nth tile (with n = 3) and for larger objects the Onion skin is the best choice.

To overcome this issue ZEN offers a so-called Adaptive Focus point distribution. With this feature you can define for certain object sizes different Focus point distributions. To activate the function you have to activate the checkbox Use adaptive focus point distribution. This checkbox is located over the Focus Point Distribution expander.
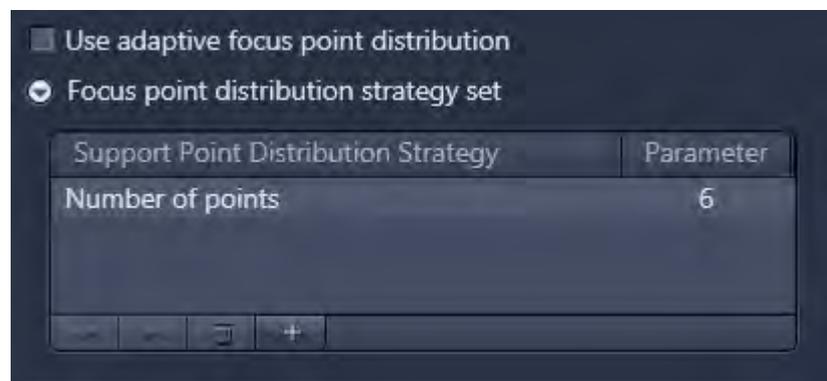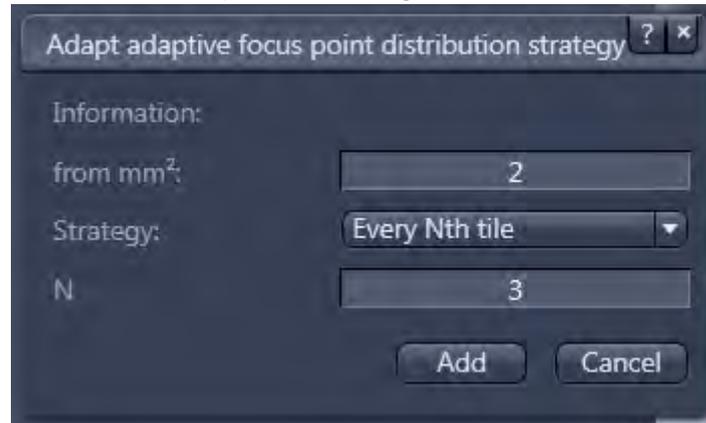

Procedure 1 If you activate the function the Focus Point Distribution list is normally empty or populated with the default entry.

2 To add a new focus point strategy click on the **+** button at the bottom of the list.

A window comes up with the setting for the new Focus Point distribution.

**3** Insert the start size of the object (the first should always start with 0).From the **Strategy** dropdown list you can select the desired strategy. Based on the selected strategy the window will present the possible settings for this Strategy (e.g. for Every Nth tile the value for N).

4 Click on the **Add** button.

The system will now add the selected strategy to the list. You can continue to add more strategies, if necessary.

5 Once the last strategy is added click on the **Cancel** button.

Now the list is populated with all the added strategies.
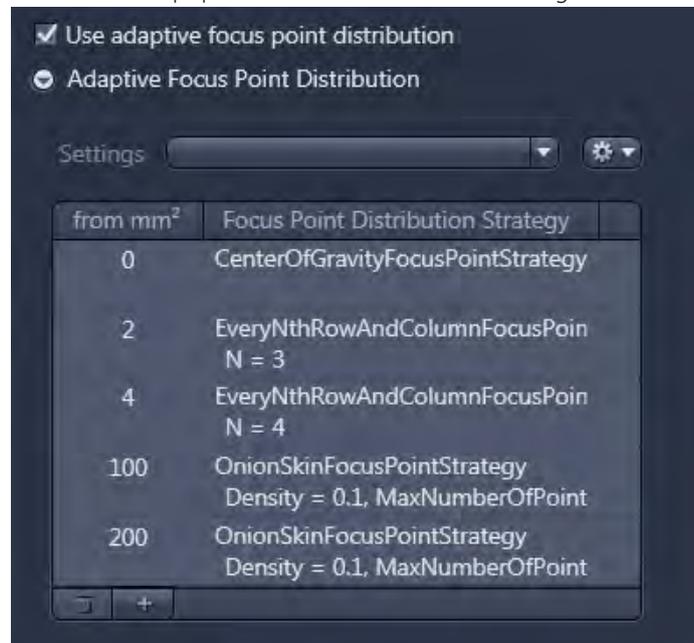
In the example above the system will apply following settings:

■ From 0 to <2 mm the **Center of Gravity** strategy

■ From 2 to <4 mm the **Every Nth tile** strategy with (N = 3)

■ From 4 to <100 mm the **Every Nth tile** strategy with (N = 3)

■ From 100 to <200 mm the **Onion skin** strategy with a density of 0.1 and a maximum of focus points of 24

■ From 200 to endless the **Onion skin** strategy with a density of 0.1 and a maximum of focus points of 36

Notice, that you can still modify the settings. If you double-click on an entry a window comes up, in which you can change the settings.

You can add an other entry by clicking on the **+** button.

As such kind of set-ups (settings) can reach a certain complexity, it is important that you save the settings from time to time.

If you want to save a set-up it is important to create at first a name for the set-up and afterwards add the entries, as if you create a new name the system will start with the default entry.

To create a new settings file click on the **Options** button to see the dropdown list entries and select the**New** entry. Now the software will ask you for a name for this set-up. Type in the name and click on the little **Save** button to create the settings file.

Now you can add the new strategy set. The modification is shown with an asterisk behind the name. To save the modification open the dropdown list again and click on **Save** entry.

3.3.2.5.2 Fine focus
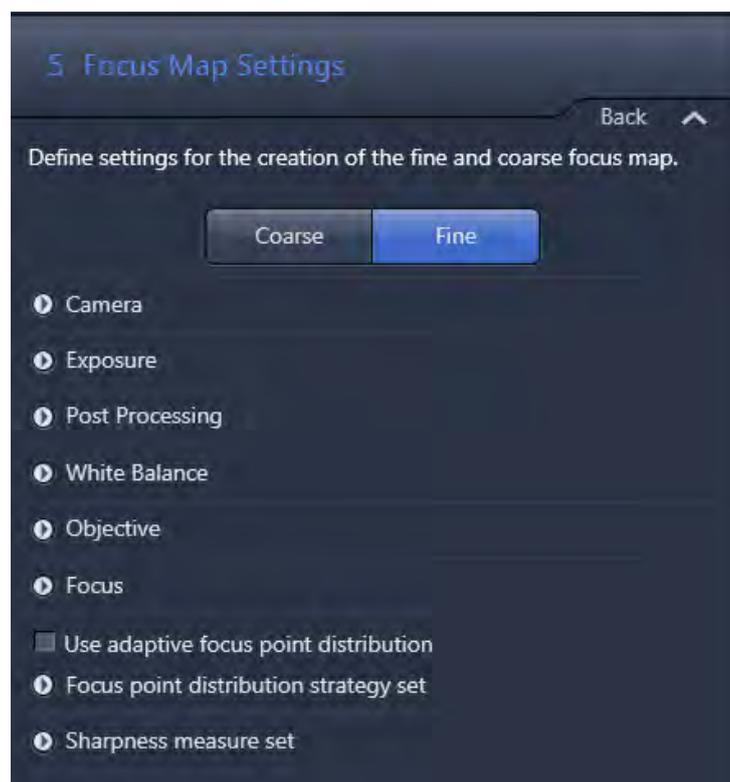
As the settings for the **Exposure**, **Post Processing**, and **White Balance** are the same as used for the Scan settings, please refer to the *Scan Settings* [▶ 95] chapter.

Prerequisites ■ You should adjust the **Coarse Focus** settings in advance.

Procedure 1 Under the **Camera** expander, it is possible to select the active camera; however, only select the color scan (Hitachi) and not a monochrome camera or the AxioCam IC as the selection will not result in a working profile!

2 Select the appropriate objective in the **Objective** section. This is the same objective that you would use for scanning the specimen.

3 Navigate to the specimen using the tools you have on the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the virtual joystick within the **Stage** tab to navigate more precisely. The main part of the window contains a LIVE image from the camera.

4 Autofocus on the specimen. To do so, you can use the **Autofocus** button. If the system does not find a focus, you may have to focus manually, e.g., via the slider next to the virtual representation of the z-stack. If the Coarse focus step was executed previously and the software does not find a focus, this suggests that the focus parameters for the fine focus are not correct.

5 Once the specimen is in focus, double-check that the image is not too bright or too dark and that the white balance is correct. If you need to adjust one of these parameters, refer to **Scan settings** section.

6 You now have to adjust the **Range**. You could change the **Interval**, but it is not normally necessary to make any significant changes. The following settings are recommended for the **Interval**: 2.5x: 20 µm; 5x: 8 µm; 10x: 4 µm; 20x: 2 µm; 40x: 1.5 µm), thus it is advised to change the number of **Slices**. The final optimal range depends on several parameters, such as the number of focus points, specimen evenness, and the objective used for the **Coarse Focus**. If 5x or 10x is used as the objective for Coarse focus, then the search range for the Fine focus can be around 70 µm. If you use 2.5x as the Coarse focus objective, the range should be at least around 150 µm.
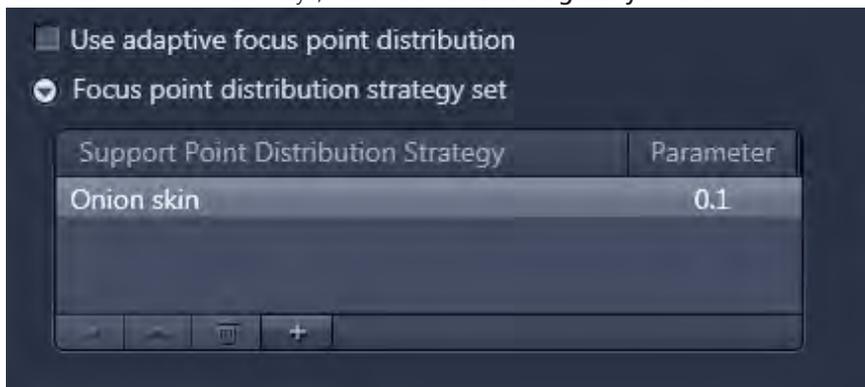
NOTICE

Risk of damaging the objective

Based on the working distance of the objectives the maximum focus range should be for a Plan-Apo 40x 100 µm and for every other objective a focus range of below 500 µm is acceptable. This considers a typical cover slip thickness (170 µm) and a thickness of a specimen of around 10 µm. If you use only a fine focus range of 60 µm, the maximum thickness of the specimen can be 50 µm. A larger working range could lead a damage of the objective and in extreme cases to a damaged of the slide.
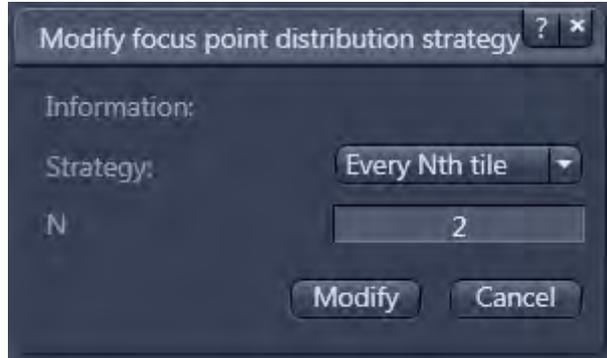
7   With the **Offset** in µm, you can apply a fixed offset to the focus map. This offset value will be added to all z-values of the focus map.

8   The next step is to adjust the *Focus point strategy set* [▶ 91]. The default setting (**Onion skin** with a density of 0.1 and a maximum number of 24 focus points; in the table view you can see only the parameter for the Density if you want to see also the other parameters (if available) double-click on the entry) is the best value for a wide variety of slides with large specimens. An exemption could be Tissue Micro Arrays, for which **Center of gravity** is recommended.

9 To change the **Focus point distribution strategy set**, double-click on the entry. A window comes up, in this window you can select another **Focus point distribution strategy set** via the dropdown menu and you can adjust the parameters for this setting. With a click on **Modify** the changes will be applied.

10 The last step is to adjust the *Sharpness measure set* [▶ 94]. In our case, **Best**, **Basic**, or **HG 2^8** are the best choices for the fine focus in brightfield. The latter focuses more on the nuclei.

11 To double-check, click on **Run Auto Focus**. If all settings are correct, the system should focus on the specimen without providing any warnings/errors. If you still experience problems, please double-check the above settings. You can interrupt the focus run by clicking on **Stop Auto Focus**.

---

i Info

The described procedure is applicable for specimens where the objects have an equal size. As the Focus point distribution strategy is depending on the object size the system offers the possibility of the so-called adaptive Focus point distribution, please refer to the chapter *Adaptive focus point distribution* [▶ 85] for more detailed information on this topic.

3.3.2.5.3 Focus point strategy set

> **i Info**
>
> The focus point strategy set will be applied for each scene, i.e., as a separate detected object in each case. If you select six fixed points for the Coarse focus, the software will apply 6 focus points for each scene.

Item	Description
Onion skin	This is a density focus schema. The resulting focus points are displayed as layers of onion skin, providing an even distribution and also ensuring that the border in particular has enough focus points. This is the standard setting for the fine focus map. The parameter is a density with a standard setting of 0.1. This setting means that 10% of the total number of viewing fields will be used as focus points to calculate the focus surface. For large specimens this number can get very large, but a larger number of focus points does not always automatically mean a better quality calculation of the focus surface. For this reason, the user can also define a maximum number of focus points. This number is normally in the range of 24 to 36 points. This focus point strategy set is recommended for mid-sized to large objects.

Example (Density: 0.1; max number 24):

Item	Description
Every Nth tile	The system will put a grid over the specimen with a distance between two focus points of the specified value (N) for the number of camera viewing fields in the x and y direction. This is a clearly defined way to create the focus point, even it creates more focus points and will take longer to create the focus map. The focus point strategy set is recommended for mid-sized and smaller objects. Example (every 5th tile):
Every tile	The system will focus on every tile. This is particularly time-consuming for scans with a large magnification and/or large samples; however, it yields the best results for very uneven specimens.
Number of focus points	Here the user defines a fixed number of focus points per object (scene). The default setting for the Coarse focus is six focus points per scene. Example (n = 24):

Item	Description
Density	The density is similar to that of an onion skin; the difference is that the pattern is not distributed like the layers of an onion. The distribution is more evenly spread over the specimen/scene. This setting can also be used to define a maximum number of focus points. The creation of this focus map is more random – as shown in the example – and thus the onion skin is recommended over the density. Example (density: 0.1; max number 24):
Grid cell size	This setting defines a fixed grid over the specimen to establish the focus points for the focus surface. The value is in µm. This setting is independent from the current frame/sensor size of the camera used. If you want to use a setting that is based on the chip size of the camera, please use "Every Nth tile". As seen on the sample, this schema is not as strict as Every Nth tile, thus Every Nth tile is recommended over the Grid cell size. Example (1000 µm 1000 µm):

| Item	Description
Center of Gravity	The system will place one focus point within each scene/object. The position of this focus point is the center of gravity, thus the whole object has only one focus point. This is a useful setting for small objects (e.g., Tissue micro arrays). Be aware that for certain structures (such as a half moon), the center of gravity may lie outside the tissue, hence the system has nothing to focus on. In such cases, select "Number of focus points" with 1 as the parameter. The center of gravity is a valid option, e.g., for Tissue Micro Arrays to place only one focus point in each core.

3.3.2.5.4 Sharpness measure set

Item	Description
Basic	The autofocus is contrast-based, hence the algorithm will focus on the highest contrast along the z-stack. It is a simple but fast autofocus.
Best	The autofocus is also contrast-based in this case, thus the algorithm will focus on the highest contrast along the z-stack. Compared to Basic, the focus is slightly slower but more accurate for certain samples. Please be aware that Best does not mean that this algorithm is always superior to Basic, as this depends on the kind of tissue/specimen.
FFT	This autofocus setting applies to the Fourier transform algorithm to determine the best focus value. This algorithm is slower than the other algorithms and should be used only in the case of the Ring aperture contrast. It is not ideal for standard brightfield contrast or fluorescence as this algorithm looks for two contrast peaks and the local minimum between them.
HG 2^8	This is an advanced algorithm compared to Basic and Best. This algorithm focuses less on the larger stained structures and more on the smaller local structures, thus this algorithm will focus more on nuclei, for example, than on unstained structures (e.g., immunohistochemistry stains). Based on the current status of evaluation, this means that HG2^8 is a good autofocus method if a user wants to focus on nuclei. **Drawback: The weight of dirt also increases, hence it should only be used for the fine focus, not the coarse focus.**

| Item	Description
Contrast Power	This algorithm is comparable to the HG 2^8.

3.3.2.6 Scan Settings
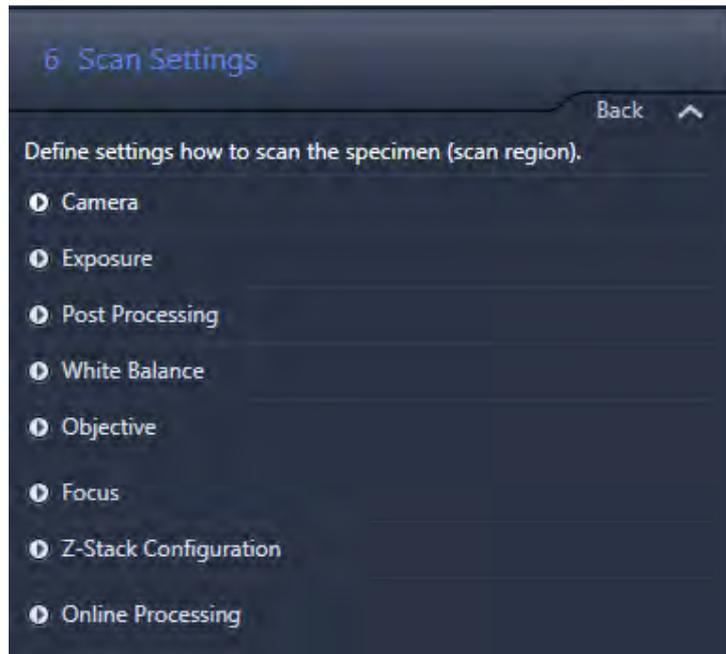
Here you can set up the scan settings. These settings are important as they determine the image quality that is subsequently experienced by the user in terms of exposure time, white balance, etc. The settings under **Post Processing** and **White Balance** are similar to the standard ZEN tools.


ℹ **Info**

It is possible to change the camera in the **Camera** section; however, the Hitachi HV-F202SCL is the best choice for brightfield scanning. Other cameras can result in unexpected behavior.

In the case of the **Hitachi-HV-F202** camera, you can select whether you want to capture the image in 8-bit, 10-bit or 12-bit. Please note that a bit depth above 8 bits will result in images with a much larger image size.
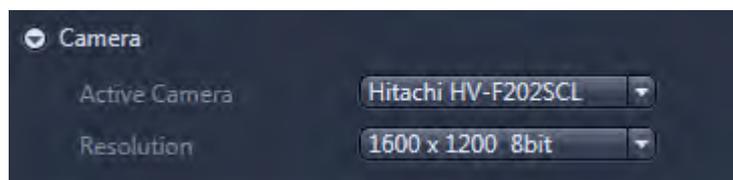
Procedure **1** To begin with, check whether the correct objective is activated under **Objective**. This should be the same as the objective used for the fine focus or at least an objective with a lower magnification.
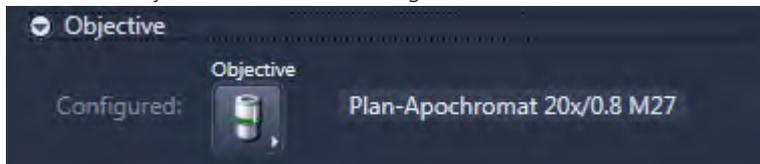
2 Navigate to the specimen using the tools you have on the lower part of the window. You can click inside the slide shown in the **Navigation** tab and the software will move the specimen to the selected position. You can also use the virtual joystick within the **Stage** tab to navigate more precisely. The main part of the window contains a LIVE image from the camera.


3 To be able to judge the quality, you first have to focus (using the **Focus** expander). This section is only for focusing and has no influence on the focusing in the subsequent scan as the settings have already been made in the previous step. Use the **Run Auto Focus** button to focus automatically. If the autofocus is running you can interrupt the focus run by clicking on **Stop Auto Focus**.


4 To adjust the **White Balance** and the **Exposure** time, navigate to a region outside the specimen. Use the **Measure and White Balance** button in the **Exposure** expander to adjust the exposure and white balance automatically (the software will only determine the exposure automatically using **Measure**).

If the intensity is inadequate, you can adjust the exposure under the **Exposure** expander. It is not possible to adjust the exposure time for the camera as the exposure time is fixed. You can only change the lamp settings. As the system is equipped with a flashlight LED, you can adjust the **Flash duration** and the **Flash intensity**. The flash duration is the duration for each flash, and the flash intensity is the intensity of the flash. The flash intensity can be increased up to 300%. With the flash intensity, you have a measure to make very fine adjustments to the resulting intensity. Changes to the flash duration constitute a coarse adjustment. In the case of manual adjustment, it is therefore advisable to start with a flash intensity of around 250% and adjust the flash duration to achieve a decent signal before increasing the flash intensity as appropriate.
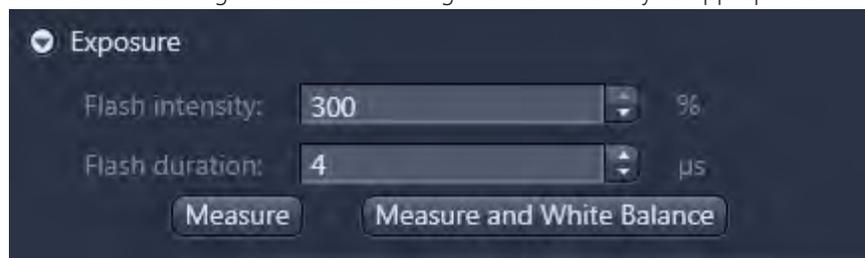
5 For the description of the settings for **White Balance** and **Post Processing**, please refer to the general ZEN description of these features as these are the same. In the **Post Processing** section, you can enable **Unsharp Mask**. The unsharp mask will increase the contrast of the resulting images, but has to be applied carefully to prevent the images from looking artificial. This can be a particularly useful tool for a 40x magnification, as the 40x magnification provides oversampling and an unsharp mask could enhance the structures. If you apply sharpening, the scan speed will decrease slightly. Shading should always be activated.
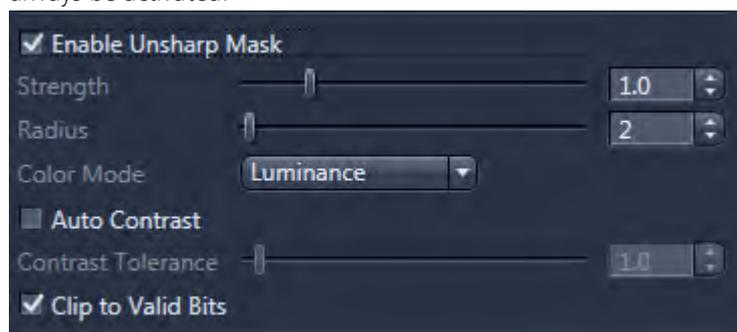
6 In the **Post Processing** section, it is also possible to activate color correction if you activate **Apply Color Profile**. When you activate the **On** checkbox, you can select a color profile. The **srgb** color profile will be applied by default. If the software does not find a profile, it is very likely that the color profile was deleted or no color calibration was executed. In this case, you have to repeat the color calibration with the help of the **Axio Scan Calibration Wizard**.
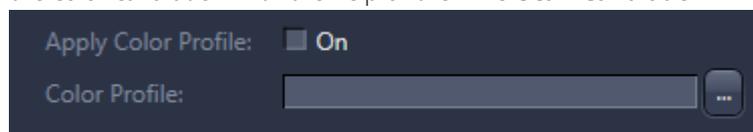
7 The Z-Stack Configuration is important if you want to automatically acquire a z-stack with or without extended depth of focus (EDF). This can be considered for uneven or thick specimens. Please note that – depending on the objective, condenser, and camera – a 20x Plan-Apo has a depth of focus of around 1 µm and the 40x Plan-Apo has a depth of focus of around 0.5 µm. The unevenness can be the result of a sub-optimal preparation or if the user aims to scan thick or cytological specimens. The best way to evaluate the optimal settings is to go to the representative region of the specimen, focus from a blurry image to an image where the first structures come into focus, and note the z-value. Go through the specimens until the last regions of the specimens in the field of view are switching from being focused to blurry, then note the z-value once again. The difference between the z-values is the height of the z-stack. It is recommended to repeat this procedure for different regions of the specimen. The largest z-range will be used.

Empirical research has shown that it is recommended to add 20% to the z-range as the autofocus is not always exactly in the middle of the specimen. It is now necessary to determine the step size based on the z-range. Theoretically, the step size is between the depth of focus and half of it, thus 0.5 to 1 µm for a 20x Plan-Apo objective. However, this would result in a larger number of z-steps and a low scanning speed. If you do not want to preserve the z-stack and work with smaller image sizes, you can activate the extended depth of focus (**EDF active**). If active, the software will use the captured images in different focus planes for each image field and combine the regions of strongest contrast within given z-stack images into an image that contains the maximum contrast portions of all images recorded for this image field. This method guarantees maximum depth of sharpness. It is strongly recommended to use the standard parameters as these have been selected for their optimal fit. For a detailed description of the single parameters, please refer to the help for standard ZEN functionality of EDF. A scan with EDF is slightly faster than the same scan with a Z-stack. It is important to note that an extended depth of

focus can also be applied at a later stage; however, if the extended depth of focus is applied in the profile then the z-stack cannot be restored afterwards.



NOTICE

Risk of damaging the objective

Based on the working distance of the objectives the maximum Range should be for a Plan-Apo 40x 50 μm and for every other objective a Range of below 300 μm is acceptable. This considers a typical cover slip thickness (170 μm). A larger working Range could lead a damage of the objective and in extreme cases to a damage of the slide.

---

8   The final step to check is the **Online Processing** section. Here you can define whether the stitching is carried out **Online**, i.e., during the acquisition, **Offline**, or if no stitching (**None**) will be applied at all. The standard setting is online stitching as this provides the best performance in terms of the processing time of the slide. The Offline mode will initially perform the scan, with the stitching applied at a later stage. This procedure is slower than the Online stitching. The difference is relative to the size of the specimen, i.e., if a specimen is very large, the offline mode is very slow compared to the online mode. For smaller specimens, there is barely any difference in processing time.
No stitching (**None**) should only be applied if the stitching is to be applied afterwards using ZEN measures or by a separate program after an export.
Pyramid active should always be activated, as this will speed up the viewing of the image afterwards. However, a pyramid can also be created afterwards. If a non-pyramid image is opened by ZEN, the software will always ask if the pyramid should be created.

These final settings allow you to adjust the compression which will be applied as part of the online processing. If **JpegXR active** is deactivated, the image will be saved uncompressed. If the **JpegXR active** is activated via the checkbox, you can save them lossless (**Lossless compression**), thus the image will only be compressed to the extent that no information is lost. If **Lossy compression** is activated, the compression always involves a loss of information. If this checkbox is activated, you can determine the degree of **Quality**.
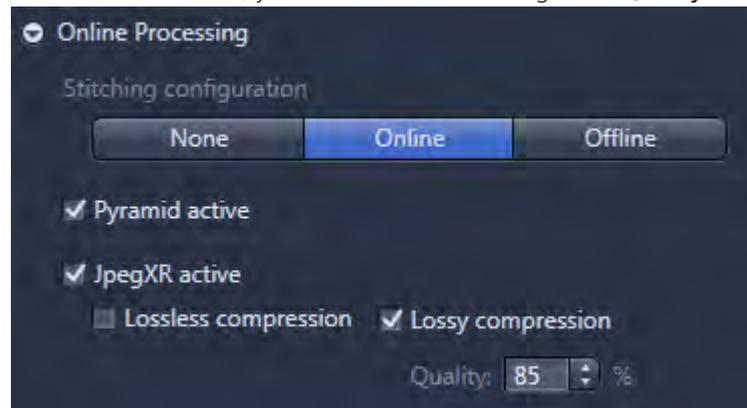
9 In the lower part of the window below the area of the live image, you have the tab **Display**. In this tab you can adjust the display curve. The adjusted display will be saved along with the generated image, thus if you open the image this display curve will be applied. This has no influence on the image data itself, but it is e.g. a possibility to suppress the grayish background.
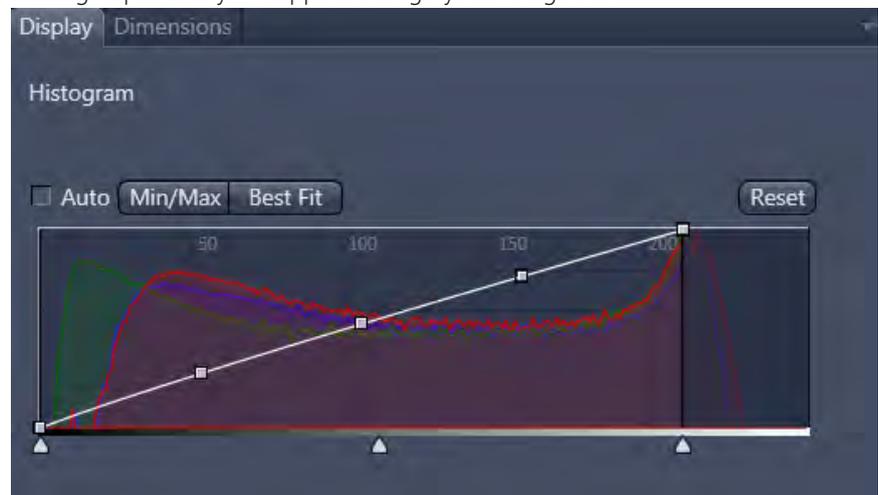
> **i Info**
>
> The checkbox for the shading correction in the **Post Processing** section should always be activated. The applied shading correction workflow depends on the magnification of the objective, e.g., if the magnification <20x, the software will capture the shading correction image outside the slide, i.e., through the air. The advantage of this is that the shading correction image is not influenced by dirt or other objects.
>
> If the magnification is >=20x, the system will capture the shading correction image through the glass. To do so, the software will determine an empty region with approx. 100 fields of view. The software will scan this region and determine an average image as the shading correction image. If the system cannot find an empty region (for example, if the slide is completely covered with the specimen; e.g., blood smears), the system will use the most recently captured shading correction image and will display a warning.
>
> In the **Post-Processing** section you can create a shading correction image manually, but this will be overwritten by the automated shading correction procedure.

3.3.2.6.1 Comparison compression regime vs. file size

Compression regime	File size
No compression	9124 MB / cm^2
Lossless (JPEG XR)	4997 MB / cm^2
90 % JPEG XR quality	916 MB / cm^2
80 % JPEG XR quality	392 MB / cm^2
60 % JPEG XR quality	263 MB / cm^2
40 % JPEG XR quality	198 MB / cm^2
30 % JPEG XR quality	157 MB / cm^2

3.4 Advanced Scan Profile Wizard (offline)

This wizard is an offline wizard, which means that the system will not move the tray (slide) and the user will not be shown any live images from the cameras. The user is still able to change the parameters. This is a mode that is appropriate if the user wants to change parameters that are not directly associated with a live image; e.g., z-stack and EDF (extended depth of focus) settings. It is not necessary to move the physical glass slides to adjust these settings. As a result, the functionality is limited compared to the Advanced Profile Wizard (online) but does not contain more settings than this Advanced Wizard. Please refer to the Advanced Wizard for help with certain functions.

3.5 Tissue detection Wizard (offline)

i Info

This wizard is only active if a preview scan was generated previously. Once the system has started the preview generation, you can start the Tissue detection wizard immediately and work in parallel.

The wizard is accessed via the *Scan Profiles options* [▶ 17] menu.

Here you can change the region that the system will scan afterwards in a single step. It is not possible to change any settings other than tissue detection; however, the option is available to navigate directly to the next slide (**Next slide**) or previous slide (**Previous slide**). This makes the switch between the slides fast and convenient.

This option does not exist for the other wizards. It is not necessary to move the physical glass slides to adjust these settings.

Generally speaking, you will not change the settings in the wizard on the left-hand side. Instead, you will normally work directly in the image of the specimen area to mark the region(s) of interest or delete regions of interest that have been wrongly detected. To delete a region of interest, simply select the region and hit "**Delete**" on the keyboard. You can also use the multi-select function.
The hotkey CTRL+Z allows you to undo the most recent actions.
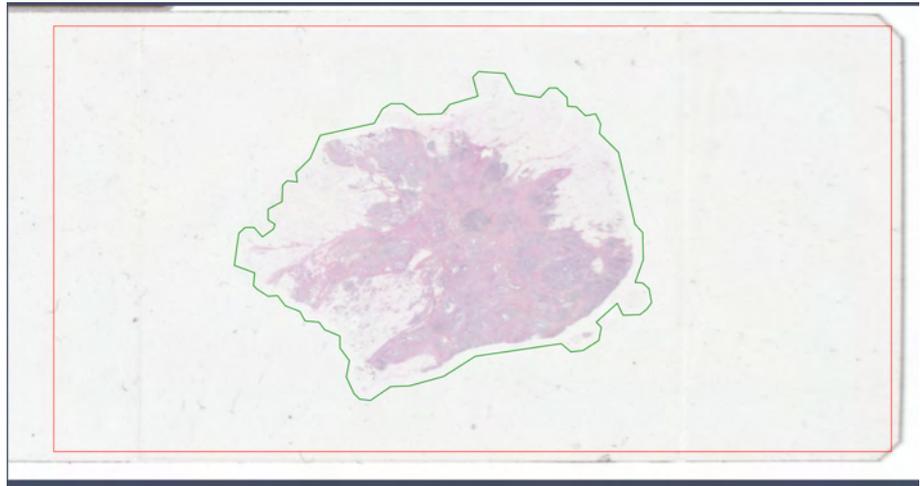
The red rectangle is not a graphic; it is the frame to define the region where the automated tissue detection is looking for specimens. It is possible to draw graphics manually outside this frame. In the **Graphics** tab in the lower part of the window, you can view and manage the graphics (regions of interest) that you have drawn.
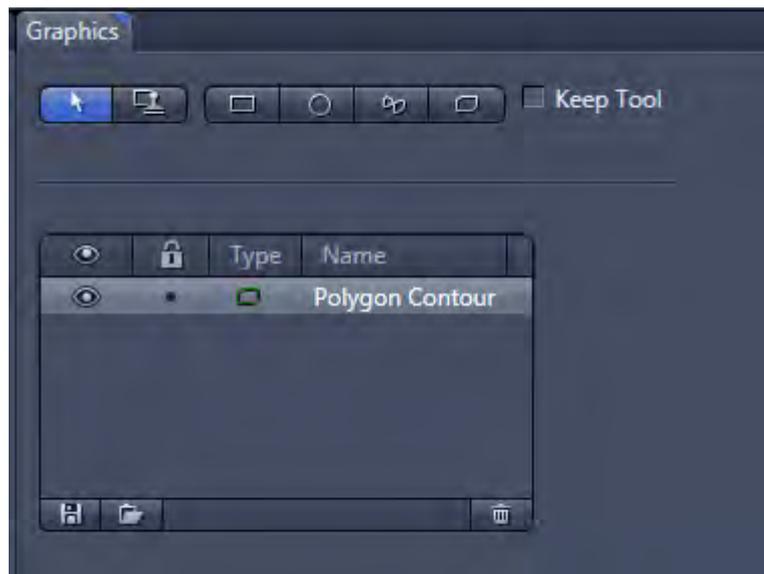
As the regions of interest are standard graphics within ZEN, you have access to the advanced editing options depending on the graphics type, such as Split, Merge, Edit points, etc.

The tissue detection settings are basically the same as described in the **Advanced Scan Profile** wizard in the Tissue Detection Settings chapter.

3.6 Axio Scan Calibration Wizard (online)

> **i Info**
>
> To perform the calibration, you must at least use the geometric calibration slide (474029-9030-000).

The following settings can be calibrated or checked:

- Parfocality of the objectives

- Parcentricity of the objectives

- Adjustment between preview camera and scan camera

- Scaling of the objectives

- Determination of the camera rotation (this is a double-check); if the camera rotation is not in the expected range (<= 0.5°), please contact ZEISS service

- Color calibration (you need the Color calibration slide (474029-9041-000) for this calibration) – this step can be skipped

The wizard guides you through the process step-by-step. The steps do not normally require user interaction (only the color calibration step needs an user interaction, loading the calibration, thus this calibration should be available). It is only necessary to start the action for each step. Once the action is complete, this will be indicated by a green light. You can then continue with the next step.

The last step is to save the data to the system.

The tray with the calibration slides must be placed in the first position within the magazine.

The first position of the tray must hold the **Geometric calibration slide (474029-9030-000)**, the second position the **Color calibration slide (474029-9041-000)**.

4 Working with ZEN slidescan

4.1 Setting up a brightfield profile

The following steps will show you how to set up a brightfield profile starting with the **Smart Profile Selection** followed by the **Scan Profile Wizard**.

4.1.1 Step 1: Smart Profile Selection

Procedure **1** Click on the **Smart Profile Selection…** button on the **Scan** tab (the button is located in the top half of the tab).

You will now be guided through a decision tree. The steps are illustrated with pictograms to simplify the visualization process. Once this is complete, the software will present you with the most appropriate profile for your application/specimen. You can change the name and description of the profile if necessary.

> **i Info**
>
> The profiles used in the **Smart Profile Selection** are fixed and part of a profile pool which cannot be changed by the user.

2 Click on the **Finish** button.

The profile will be copied to the profile folder. You can see the profile as an additional item in your profile list.

The profile can now be used as it is. It is recommended to execute the **Scan Profile Wizard** to adapt this profile to your specific needs. This can be done directly after the Smart Profile Selection by activating the corresponding checkbox.

4.1.2 Step 2: Scan Profile Wizard

Prerequisites ■ Open the door of your **Axio Scan.Z1** e.g., via the **Open/Close** button. Put a tray with a typical glass slide (the specimen should represent a larger group of similar specimens) inside the magazine and close the door. Wait until the main status indicator on the device is continuously green.
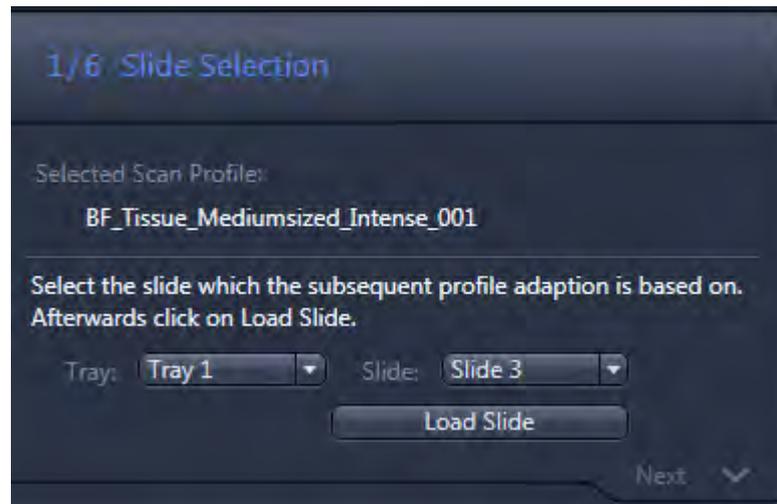
Procedure **1** On the **Scan** tab in the **Default Scan Profile** section, select the profile you want to modify. We recommend using a profile from the **Smart Profile Selection**.

2 Click on the **Scan Profile Wizard…** button.

The Scan Profile Wizard opens. You will see the description of the profile (e.g., what kind of preview detection is used). This is important for preparing the slide correctly (e.g., if the marker is needed, the slide has to have a marker on it).

3 The first step is the **Slide Selection**. Select the **Tray** and **Slide** position and click on the **Load Slide** button.


The system will now move the tray to the operating position. If the tray is not already on the stage, the software will load the tray from the magazine.

> **i Info**
>
> If you see a view to set up a grid in the next step and the profile contains the information that a grid should be used, please refer to the Grid definition.

4 The next step is the **Label Recognition**. Here you can select if the system uses **Barcode recognition** or **OCR** (Optical Character Recognition). If barcode recognition is selected, the system will check for all possible barcode types by

default. For detailed settings (e.g., if only a specific barcode should be read) use the **Advanced Scan Profile Wizard**.
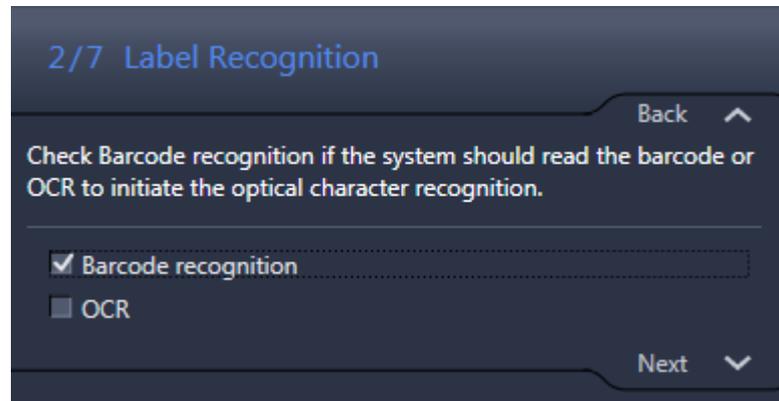
5   In the next step, you have to select the **Scanning Objective**.
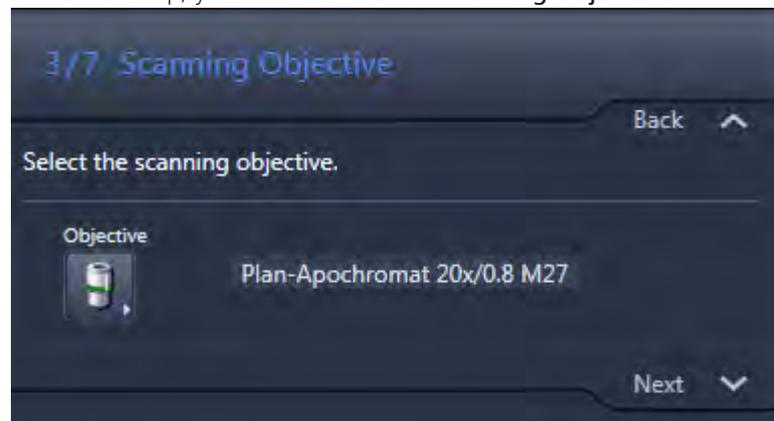
6 The next step is to adapt the image brightness in the **Exposure settings**. This can be influenced by setting the **Flash intensity** and the **Flash duration**.

To adapt these settings (this is particularly necessary if the scanning objective has been changed) you should move the stage containing the specimen into

the light path (e.g., by clicking inside the preview image in the **Navigation** tab or using the virtual joystick in the **Stage** tab).

7   You now need to focus within the specimen, e.g., by clicking on the **Smart Focus** button. You can also use the manual focus adjustment options.

> ℹ **Info**
>
> In most cases, the **Smart Focus** (which is the combination of the Coarse Focus and the Fine Focus) should work. If the **Smart Focus** does not work, check the detailed focus settings in the **Advanced Profile Wizard**.

8   Once the specimen is focused, you should visit a region that does not include part of the specimen inside the field of view. You can now adapt the settings until you achieve a suitable appearance, meaning you have no overexposure and no significant underexposure. Please refer to the Display curve to check the intensity distribution.
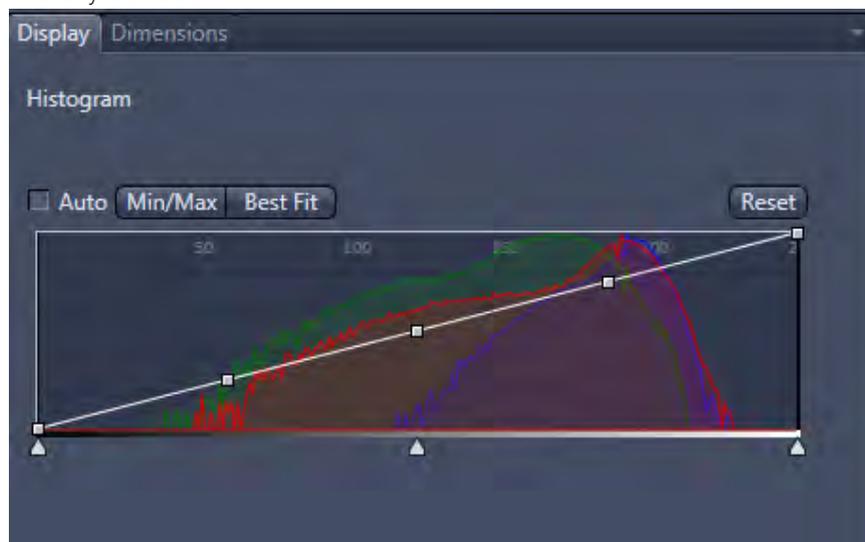
> ℹ **Info**
>
> Please keep in mind that adjustments to the flash intensity are fine, while those to the flash duration are coarse. The flash duration should not exceed 10 μs.

9   The next step is to select the **Sample Thickness** and **Z-Stack**. To use a z-stack, activate the **Z-Stack** checkbox. The thickness is used to calculate not only the z-stack parameter, but also the fine focus range. This means that even if no z-stack is activated, this value has to be double-checked and adjusted.

10 If the user was already working on a profile from this pool in the smart profile selector, the z-stack can be activated if this is necessary based on the application. Nevertheless, the parameters also have to be double-checked. In this case, the parameter is the thickness of the specimen.


Based on this thickness, the software will calculate the number of focus levels and the distance of these levels based on the objective used.
Finally, you can decide if the z-stack should be conserved or if an **EDF**

(Extended Depth of Focus) should be applied. If so, the **EDF** checkbox must be checked.
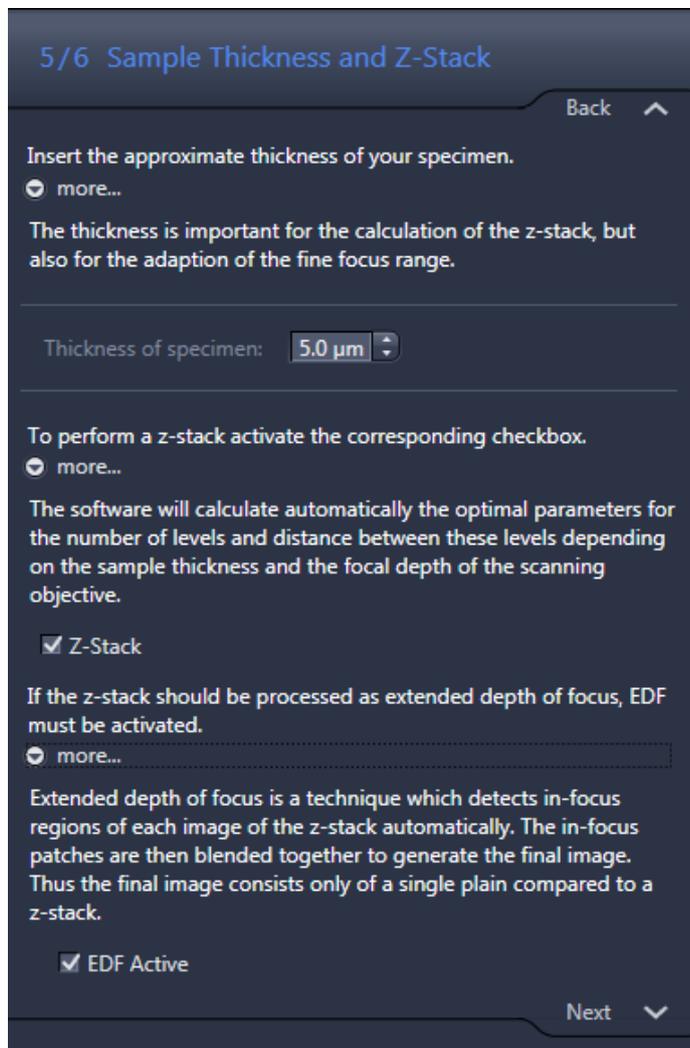
11 The final step is **Profile Name and Description**. Here you can save the scan profile under a new name if the **Save as New Profile** function is activated. You can also change the description or provide a new one. It is recommended to

change at least the description of the profile so that it is possible to track the basic setup of the profile with ease.
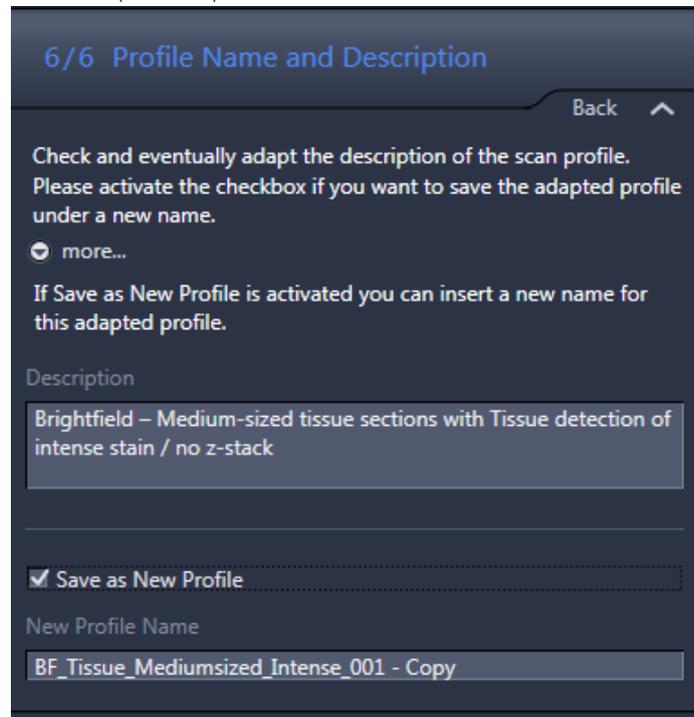
12 Click on the **Finish** button to save the changes and close the wizard.

You have successfully set up a brightfield scan profile.

4.2 Setting up a fluorescence profile

4.2.1 Step 1: Smart Profile Selection

Procedure **1** Click on the **Smart Profile Selection…** button on the **Scan** tab (the button is located in the top half of the tab).

You will now be guided through a decision tree. The steps are illustrated with pictograms to simplify the visualization process. Once this is complete, the software will present you with the most appropriate profile for your application/specimen. You can change the name and description of the profile if necessary.

> **i Info**
>
> The profiles used in the **Smart Profile Selection** are fixed and part of a profile pool which cannot be changed by the user.

2 Click on the **Finish** button.

The profile will be copied to the profile folder. You can see the profile as an additional item in your profile list.

The profile can now be used as it is. It is recommended to execute the **Scan Profile Wizard** to adapt this profile to your specific needs. This can be done directly after the Smart Profile Selection by activating the corresponding checkbox.
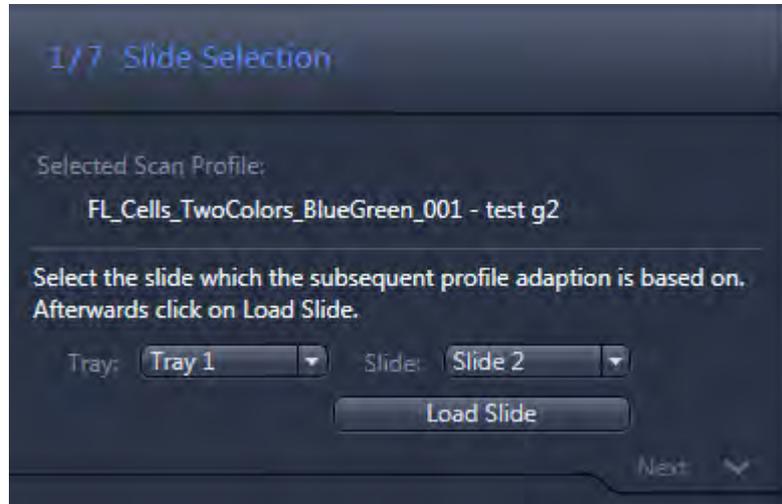
4.2.2 Step 2: Scan Profile Wizard

Prerequisites ■ Open the door of your **Axio Scan.Z1** e.g., via the **Open/Close** button. Put a tray with a typical glass slide (the specimen should represent a larger group of similar specimens) inside the magazine and close the door. Wait until the main status indicator on the device is continuously green.

Procedure **1** On the **Scan** tab in the **Default Scan Profile** section, select the profile you want to modify. We recommend using a profile from the **Smart Profile Selection**.

2 Click on the **Scan Profile Wizard…** button.

> ＊ Scan Profile Wizard…

The Scan Profile Wizard opens. You will see the description of the profile (e.g., what kind of preview detection is used). This is important for preparing the slide correctly (e.g., if the marker is needed, the slide has to have a marker on it).

3   The first step is the **Slide Selection**. Select the **Tray** and **Slide** position and click on the **Load Slide** button.


The system will now move the tray to the operating position. If the tray is not already on the stage, the software will load the tray from the magazine.

> **i Info**
>
> If you see a view to set up a grid in the next step and the profile contains the information that a grid should be used, please refer to the Grid definition.

4   The next step is the **Label Recognition**. Here you can select if the system uses **Barcode recognition** or **OCR** (Optical Character Recognition). If barcode recognition is selected, the system will check print for all possible barcode types by default. For detailed settings (e.g., if only a specific bar code should be read) use the **Advanced Scan Profile Wizard**.
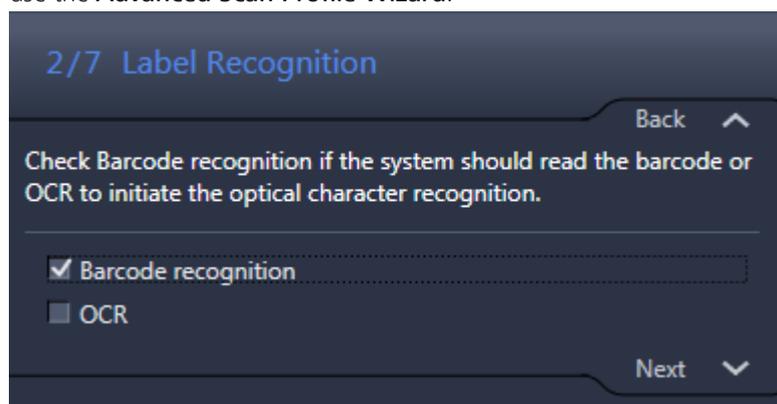
5   In the next step, you have to select the **Scanning Objective**.

> ℹ **Info**
>
> By default, the coarse focus objective is the objective in the first position of the objective revolver. To change this setting, use the **Advanced Scan Profile** wizard.

6   In the next step, you have to focus within the specimens. To do so, you should move first the stage containing the specimen into the light path (e.g., by clicking on "Navigation" inside the preview image or using the virtual joystick under "Stage").

7   Then click on the **Smart Focus** button. If the predefined settings are not suitable, (e.g., exposure times) the focus may fail. If you experience issues, please continue to the next step where you can change the exposure times and perform a manual focus.

8   The next step involves adapting the exposure times for the channels. You will see list of all defined channels. You should first select all channels you want to use. It is also necessary to double-check that the selected stitching channel is appropriate. To select the stitching channel, highlight a selected channel and

click on **"Stitching Ref"**. It is very important to ensure that the stitching channel is selected as needing to be scanned!
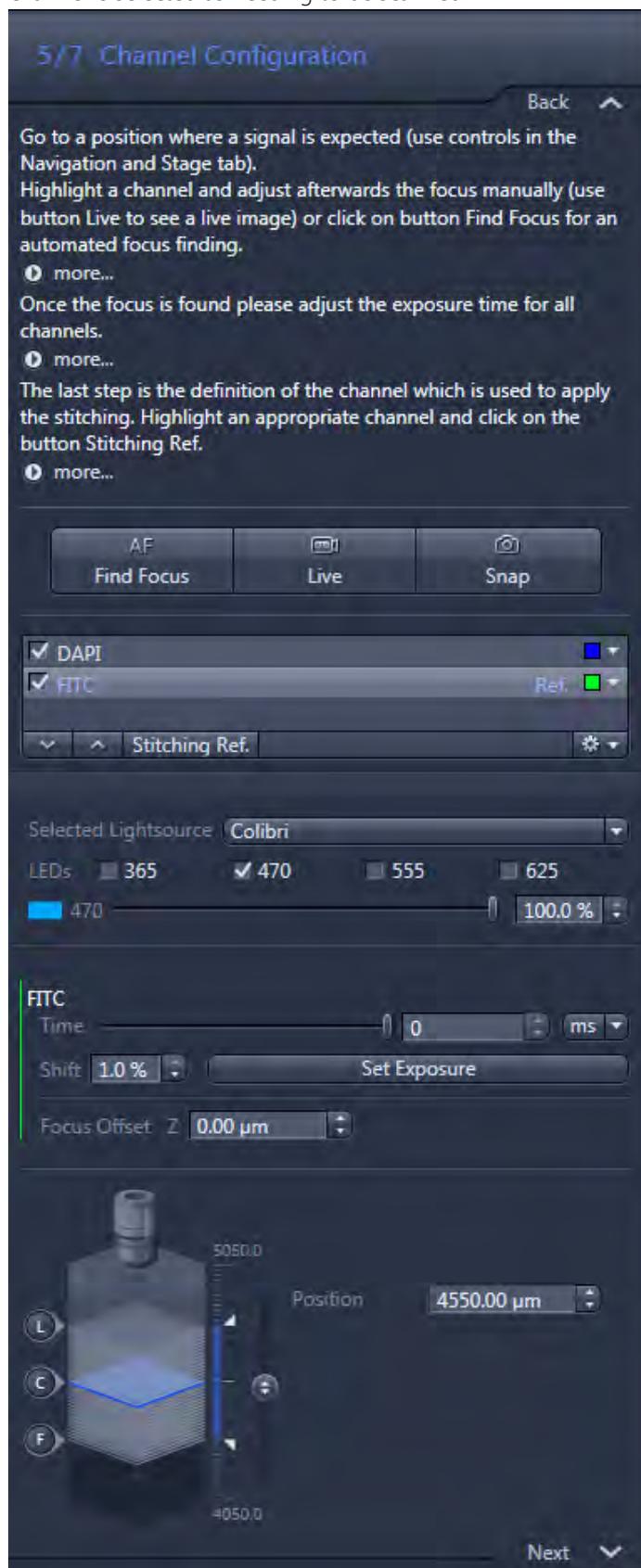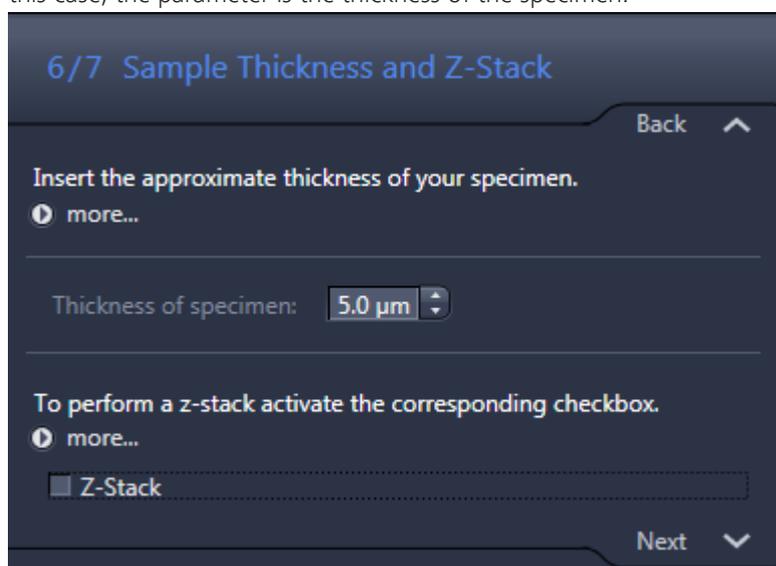

> **i Info**
>
> An appropriate stitching channel is one which visualizes a structure that is bright, has decent contrast, and is evenly distributed over the sample. The latter prerequisite is the most important.

9 To adapt the exposure times, you have to move the specimen inside the light path if this has not already been done in one of the previous steps. You then have to double-check the focus to get a sharp image. You can use the slider next to the virtual Z-Stack control to perform a manual focus. The highlighted channel is always the one that is activated and has the hardware set up to display the correct visualizations. You can adapt the exposure time directly (**Time**) or you can change the intensity of the light source. If you want to set up or change the optical configuration for a channel or want to add more channels, you must use the **Advanced Scan Profile Wizard**. The software will derive the exposure time for this objective from the type of scanning objective and the exposure time set for this objective. If this estimation leads to issues, the exposure time can be changed with the **Advanced Scan Profile wizard**.

10 The next step is to select the **Sample Thickness** and **Z-Stack**. To use a z-stack, activate the **Z-Stack** checkbox.

> **i Info**
>
> The thickness is used to calculate not only the z-stack parameter, but also the fine focus range. This means that even if no z-stack is activated, this value has to be double-checked and adjusted.

11 If the user was already working on a profile from this pool in the smart profile selector, the z-stack can be activated if this is necessary based on the application. Nevertheless, the parameters also have to be double-checked. In this case, the parameter is the thickness of the specimen.

> **i Info**
>
> The z-stack parameter settings will be applied to all selected (scanned) channels.

12 Based on this thickness, the software will calculate the number of z-stack levels and the distance of these levels based on the objective used.
Finally, you can decide if the z-stack should be conserved or if an **EDF** (Extended Depth of Focus) should be applied. If so, the **EDF** checkbox must be checked.
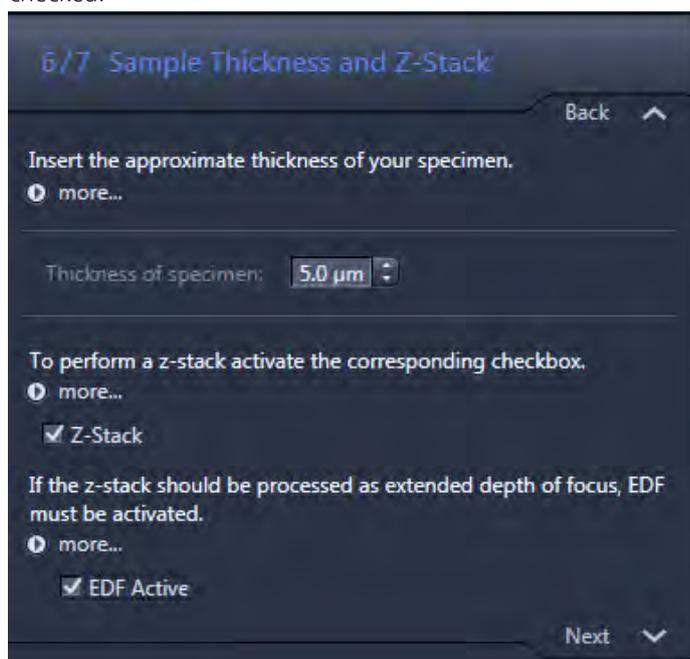
13 The final step is **Profile Name and Description**. Here you can save the scan profile under a new name if the **Save as New Profile** function is activated. You can also change the description or provide a new one. It is recommended to

change at least the description of the profile so that it is possible to track the basic setup of the profile with ease.

14 Click on the **Finish** button to save the changes and close the wizard.

You have successfully set up a fluorescence scan profile.

4.3 Importing names, profiles and scenes

> **i Info**
>
> Before you start the import you have to insert the trays with the slides first in the magazine as only with this information the system can make logical double-checks.

The import is done via a comma-separated values file (csv). For each slide one row has to be used. Each line for each defined slide should have following format:

- Tray number (absolute),

- slide number (absolute),

- image name,

- profile name,

- scene1, scene2, scene3, etc.

Tray and slide number must be inserted the other parameters are optional. It is possible to skip certain entries and these entries will not be changed by the import. The naming of the scenes starts at the upper left object and continues to the right side. After all objects are assigned with a specified scene name the software will start at left at the second line of objects and continues to the right side, etc.

Example for the scene name order assignment:

1	2	3	4
5	6	7	8
9	10	11	12

Example A

1,1,Mouse1,Profile001,brain1,brain2,brain3

For the first slide of the first tray the system will define Mouse1 as image name and will apply the Profile001. If the preview detects several scenes the system will name the first scene as brain1, the second scene as brain2, etc.

Example B

1,1,Mouse1

1,2,Mouse2

1,3,Mouse3

Here the system will only apply the image names to the specified tray and slide position.

Example C

1,1,,Profile001

1,2,,Profile002

1,3,,Profile003

Here the system will only apply the profile names to the specified tray and slide position.

Notes

■ If in the import file a profile is specified which is not in the list of the available profiles, the software will generate a warning and the profile will not be applied.

■ If in the import file a position is defined which is not occupied by a slide the software will generate a warning and will skip the import for this slide position.

■ If you defined more scenes than the system detects during the preview the system will not generate a warning, the system will stop the naming assignment after the last detected scene.

■ If you defined less scenes than the system detects during the preview the system will not generate a warning, the system will name all the scene where names are available and will stop the naming but all scenes will be scanned.

■ By default the comma (,) is used as separator. It is possible to select another separator (tabulator, semicolon, space) via Tools -> Options -> Acquisition -> Scan

4.4 Using Label Scan Settings for 4''x3'' slides

If you inserted 4"x3" slides the Label Scan Settings section will have some additional functions. This is based on the fact that the preview image for the label will be created by two images from the preview camera.

---

i Info

In this mode for the 4"x3" slides the **Recognition** and **Label Orientation** expander is only available in the **Snap** mode, thus the two stitched images are seen.

If you open the step the system will activate automatically the **Live** mode for the upper image.

With buttons **Upper Side** and **Lower Side** you can toggle between a live view from the two views.

Here you can judge the exposure time, white balance, light intensity and adapt these settings which are listed in the expanders below the graphical user elements. This handling is the same as for the other type of slides (1"x3" and 2"x3").

Once the settings are done (normally the standard settings should work and an adjustment is not necessary) you can switch now to the Snap mode by clicking on the **Snap** button.

Now the system will create two images and stitch these two images together. This image will be presented and the user can adapt the captured area via the red frame. The content of the red frame will be captured during the preview / scan of the slide and will be stored together with the image.

> **i Info**
>
> The other settings like **Recognition** are the same as for the other slide types.

4.5 Implementing your own tissue detection algorithm

You have the possibility to integrate your own tissue detection algorithm with in the standard workflow. This realized via an extension written in C# the extensions has to be provided as a dynamic linked library (DLL). The wrapper has to be in C#, the code underneath for the tissue detection can be of a programming language of your choice.

The basic workflow will be that the system created a CZI and provide this image to your extension. Based on this image your algorithm should perform the tissue detection and as a feedback for the standard workflow you will provide the image with annotations. These annotations will mark the regions to be scanned.
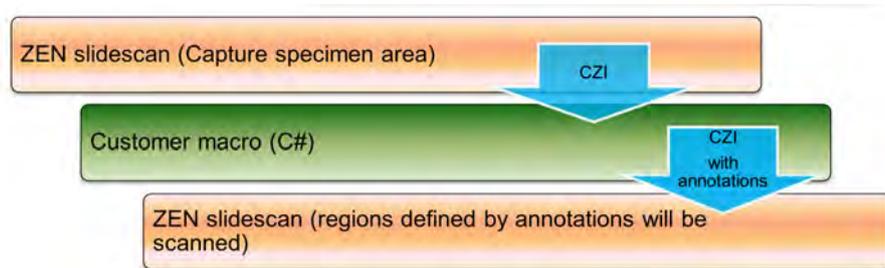

Illustration 12:

> **i Info**
>
> Steps 1 to 3 has to be executed only the first time.

Procedure **1** Close ZEN

2 If necessary create a directory with the name extensions under C:\Program Files \Carl Zeiss\ZEN 2\ZEN 2 (blue edition)

3 Copy your DLLs in this directory

4 Open ZEN

5 Now you will see a new entry under **Tissue Detection Processor** with in the tissue detection section of the wizard as pull-down menu (this pull-down menu is only seen if the software finds DLLs in the directory).
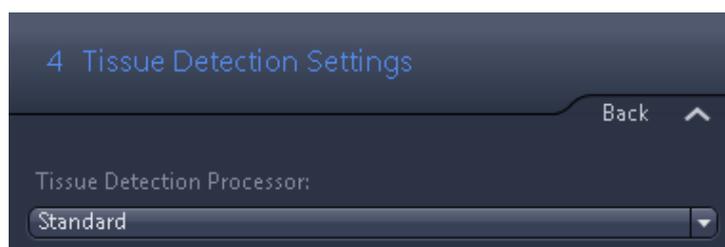
6 Standard is the default tissue detection built-in with the ZEN slidescan, thus you will see still the tools from the specific tissue detection set-up

7   Now you have to select your tissue detection algorithm via the pull-down menu.
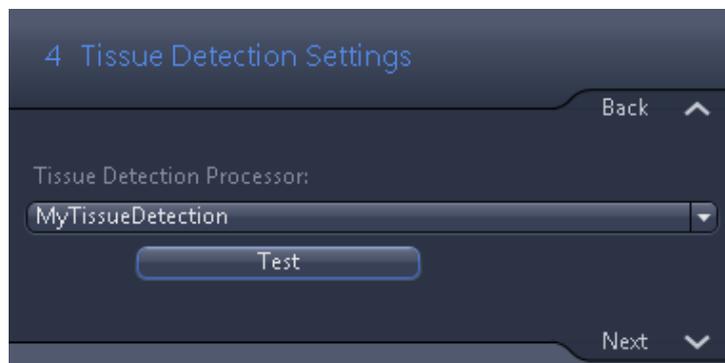
8   If you select your extension all the other possibilities to adjust the tissue detection settings will disappear.

9   With the button **Test** you can execute a test with your algorithm to check the performance of your algorithm and if the implementation works.

---

i Info

For further information regarding our file format please visit **www.zeiss.com/czi** and register to receive the necessary information.

4.6   Using a barcode to define name, profile and subfolder

If the you want to use the information from a barcode to define the name of the image, the profile used or the sub-path, you have to activate the function **Name and Profile from Barcode**. It is located in the **Naming Definition** tool in the Left Tool Area.

A barcode can be used to define the output subdirectory, the name and the profile to be used. To do this, the positions of the characters in the string (=barcode) are defined in the mask fields. It is possible to define areas using the character –. Furthermore, characters located at different places of the string may be combined by means of a comma.

Example A

Character string: AXIO_SCAN_123

Mask: 1-4 AXIO
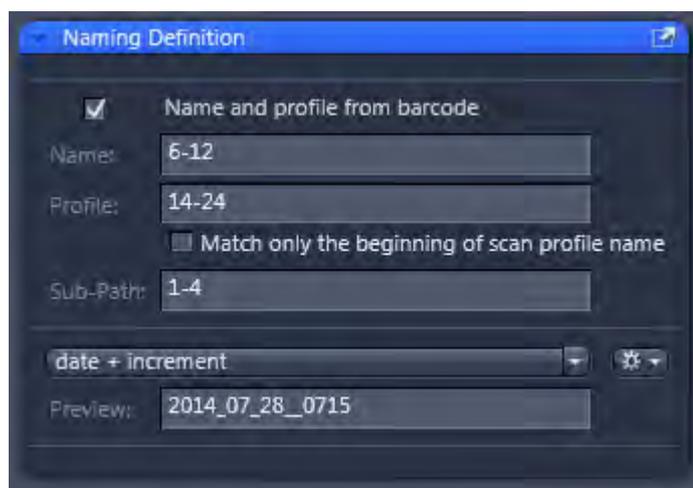
Mask: 6-9 SCAN

Mask: 1-4,11-13 AXIO123

Example B

Barcode: Path_DoeJohn_003_FAST_HE

Path is the path for saving

DoeJohn is the name of the image

003_FAST_HE is the profile name

The barcode control for the example above would have to look like this:

It is not necessary to utilize all three possibilities, you can also use only one or two of these possibilities. If you do not want to use a certain setting please leave this setting simply empty.

If under **Name** a range is defined the software will insert under **Naming** (in the **Magazine** view) the text **Name from barcode**, as the system does not know until now the barcode once the barcode of this slide is read the system insert the correct name. The same applies for the profile, in this case the text is **Profile from barcode**.

If the profile is derived from the barcode the system will take the label capture information (this includes the kind of barcode, etc.) from the profile selected under **Default Scan Profile** on the Scan tab.

In administrating and creating profiles, you will prefer descriptive names to recognize them easier (e.g.: FF04_CF08_HE_23). However, coding of such profiles in the barcode is very memory-intensive and also prone to errors. For this case, the option **Match only the beginning of scan profile name** may be enabled. Then, the system will use only the first characters to identify a profile. The name may begin, for example, with an unequivocal number used as identification. The profile could be named, for example, 005_FF04_CF08_HE_23. You would then enter 1-3 into the profile mask and enable the option *Match only the beginning of the profile name*. Now, only the number 005 would be used to identify the profile. Make sure that only one profile name beginning with the characters 005 exists.

4.7 Using Preview Scan Settings for 4"x3" slides

If you insert 4"x3" slides the section will have some additional functions. This is based on the fact that the preview image for the specimen area will be created by two images from the preview camera.

If you open the step the system will activate automatically the LIVE mode for the upper image.

With buttons **Upper Side** and **Lower Side** you can toggle between a live view from the two views.

Here you can judge the exposure time, white balance, light intensity and adapt these settings which are listed in the expanders below the graphical user elements. This handling is the same as for the other type of slides (1"x3" and 2"x3").

Once the settings are done (normally the standard settings should work and an adjustment is not necessary) you can switch no to the Snap mode by clicking on the button **Snap**.

Now the system will create two images and stitch these two images together. This image will be presented and the user can adapt the captured area via the red frame. The content of the red frame will be captured during the scan of the slide and will be stored together with the image.

<table>
<tr><td>i Info</td></tr>
</table>

The other settings like Recognition are the same as for the other slide types.

4.8 Setting up the ROI for the preview image

An important adjustment to be made is the definition of the ROI (Region Of Interest) for the preview image of the specimen area. The system will take this region of interest and capture it. The tissue detection (if selected) will also be applied to this region of interest. This includes the saved image along with the scanned image and the image of the label area. The region of interest can be adjusted in the **Live** image via the red rectangle:
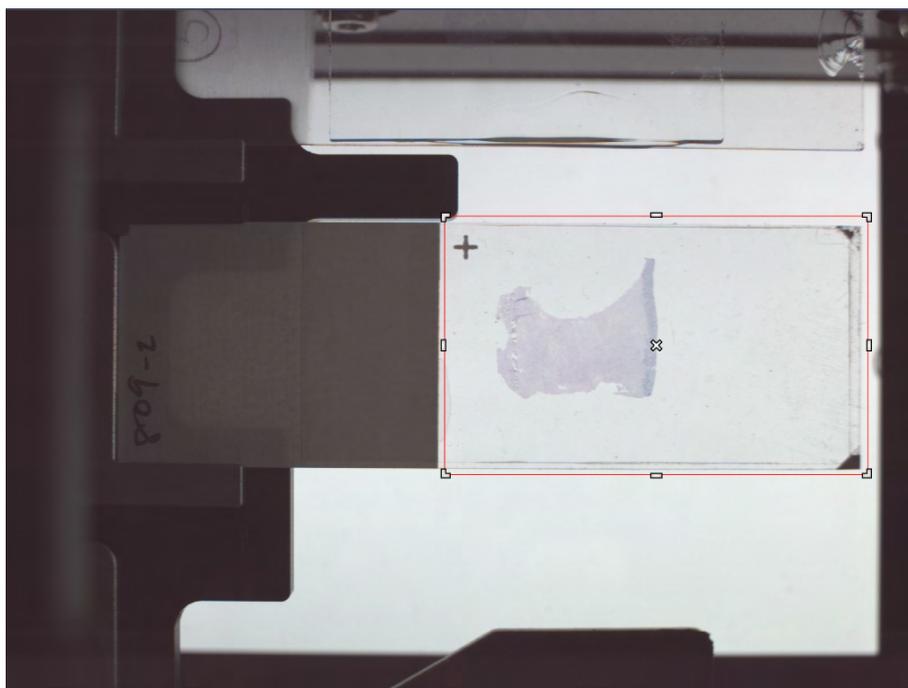
The red rectangle can be moved by dragging it. The size can be changed by moving the cursor to the edge or the borders, clicking and holding the left mouse button, and moving the edges or borders of the rectangle until the appropriate size is achieved.

> **ℹ Info**
>
> For best results the red frame should cover the complete slide and some air around. Especially for the detection of an empty area on the slide, for the automated shading correction on glass, it is important that the red frame is not set too small (e.g. around the tissue only). Otherwise the sample could be considered as empty region. This would result in a sup-optimal shading correction. If you want to limit the range for the tissue detection use the red frame in the **Tissue Detection Settings** section in the sub-sequent step. Avoid that the red frame covers the label area of the slide.

> ℹ **Info**
>
> You have the possibility to adjust the Display curve (see Display tab on the bottom of the window). The display settings will stored within in the profile! This has also an impact on the display of the label in the Magazine view. This has also an impact on how the specimen area is displayed in the Magazine view!

4.9 Creating a shading reference from tile image

The function **Shading Reference From Tile Image** is used to generate shading reference images. The function will create for every channel of the processed image a separate shading correction image. This image can be stored directly to the Calibration manager.

> ℹ **Info**
>
> This function can only be used for cameras which are not part of the Axiocam portfolio (like Orca flash 4.0).

> ℹ **Info**
>
> As we need around 300+ tiles this function is limited mainly to a calibration with an objective with a magnification of 20 and higher. Other objectives (especially 2.5x and 5x) need still the manual calibration via the **Locate** tab.

Prerequisites
- You have created an image with all the channels for which you want to create a shading reference image for. For a good reference image it is necessary to have a number of over 300 tiles per image / scene.

- Avoid any over- or underexposure in the resulting image. If the signal is only very dim it is possible to increase the background to get a good signal. Depending on the camera type you need a certain degree of coverage of the dynamic range. **For the Orcaflash it should be at least 25% of the total dynamic range. Otherwise the resulting reference image will not be accepted as a valid reference image. For color cameras used for brightfield the minimum value would be 40%.**

- Be aware that the acquisition must be done **without** activated Shading correction. As the function can process only one scene, it is important to select a specimen with a large scene and not with many small ones.

- The use of fluorescence slides for the creation of reference images for fluorescence has sometimes disadvantages (e.g. sparse distribution of the signal, bleaching). Thus it has to be considered to use at least for standard fluorescence channels (like DAPI, GFP, Cy3) a standard H&E sample and utilize the autofluorescence of this sample.

Procedure **1** Open the image with ZEN software.

2 In the **Processing** tab under **Method** open the function **Shading Reference From Tile Image**.

3 In the **Input** tool open the created image.
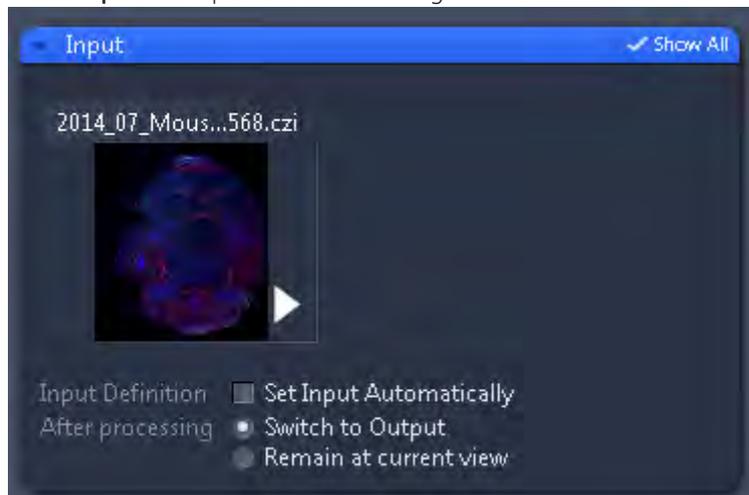
4 Set the desired parameters. For a detailed description of the parameters , see *Shading Reference From Tile image* [▶ 133].

5 Independent from the imaging modalities you can see a slider / spin box if the image contains more than one scene. The processing function can only process one scene per reference image.

6 In the **Output** tool define the name of the created files (this has no influence on the naming if the files are stored directly to the calibration manager). The functionality is the same as for the other image processing functions offering the naming possibility under **Naming…**.

7 Click on **Apply** button to start the processing function.

---

i Info

If you have a multi channel image and you define for at least one channel the attribute Skip Channel you will still see under **Output** all channels from the selected image.

4.10 Shading Reference From Tile image

With this method you can create shading reference images for multi-channel tile images.

Parameters

For a **Brightfield** image you can select following parameters:

Parameters	Description
Save directly as Shading Reference	If activated , the software will create the shading reference image and will store this directly to the Calibration Manager (Shading Reference).
	The software provides no possibility to check the image before they will be saved, thus it is recommended to deactivate the checkbox and execute this function. The system will create the reference images and present it to you. If the images have a good quality, you can activate the checkbox and run the function again.

Parameters	Description
Channel-specific	If activated, the software will perform channel-specific shading correction. In this case the fluorescence filter block used is saved with the shading file. The following components will be considered:
	Contrasting method and condenser, fluorescence filter, magnification: Objective and Optovar; camera bit depth and RGB/BW mode, camera type and port position.
	If the checkbox is not activated the system will create an **All Channel** calibration and perform an objective specific shading correction.
	The following components will be considered: magnification (Objective and Optovar); Camera bit depth and RGB/BW mode and camera type and port position

i Info

If you activate **Save directly as Shading Reference** it is important that you check afterwards the system messages (the "i" in the lower part of the screen). If e.g. the resulting shading reference image is too dim the system will not use it as valid shading reference image, this will be shown under the system messages. Also if the import of the reference database were successful this will be shown!

For a **multi channel-image** (e.g. fluorescence image) the system will show additional settings:

Parameters	Description
Adjust per Channel	If activated, you can apply the settings **Save directly as Shading Reference** and **Channel-specific** separately for each channel. If you want to use the same settings for all channels deactivate the checkbox.
Channel specific dropdown menus	If you have activated **Adjust per Channel** you can adjust for every channel the settings separately. Additionally you can select in the dropdown menus fo each channel, if you want to process the channel (select **Process Channel**) or skip the channel (select **Skip Channel**).

5 Smart Setup

Smart Setup offers you support when configuring multichannel acquisition experiments. Select the fluorescent dyes and contrast techniques that you want to include in your experiment from a large dye database. Smart Setup takes the configuration of your microscope hardware and the properties of the selected dyes into account. Based on this information it makes one or more suggestions for acquisition. You can adopt these into your experiment as required and make further changes to them there.Smart Setup also configures the motorized components of your system for the acquisition of multichannel images. It does not, however, influence any camera parameters (e.g. exposure time or resolution) and also does not change any parameters of other acquisition dimensions (e.g. Z-stack, time series or multiposition acquisitions).

To start the **Smart Setup** dialog simply click on the **Smart Setup button**
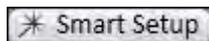
$\boxed{\text{✳ Smart Setup}}$

on the **Acquisition** tab.

5.1 Smart Setup dialog
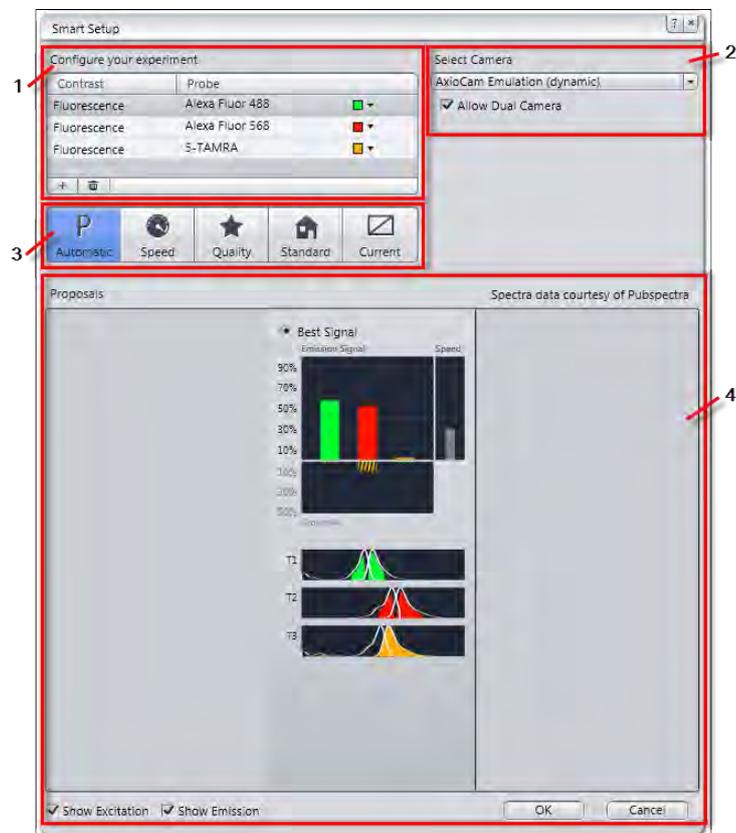
Illustration 13: Smart Setup dialog

1 Configure your experiment	**3** Motif buttons
2 Select Camera	**4** Proposals

Configure your experiment (1)

Add up to four reflected light fluorescence channels and one transmitted light contrast technique to your experiment. The added dyes or the contrast technique are shown in the **Contrast /Probe** list below.

Add button   `+`

Opens the Add Dye or Contrast Technique dialog.

Select camera (2)

Only visible, if two or more cameras are configured for the system.

First select the camera which Smart Setups gives a proposal for from dropdown list.

Motif buttons (3)

Here you can optimize image acquisition regarding particular requirements like speed or quality. All parameters e.g. camera resolution or dynamic range in **Acquisition Mode** or **Channels** tool were set automatically. They will influence basically camera, detector and lightning settings.

Button	**Function**
Automatic	■ Sets the optimal resolution for the camera in **Acquisition Mode** tool. Resolution will be calculated from camera parameters, numeric aperture, Microscanning will not be applied even if the camera would support this mode.
	■ Sets the dynamic range for all fluorescence channels to 50% or for all transmitted light channels to 80 %.

Button	Function
Speed	■ If binning is supported, one binning category under the optimal resolution will be set for the camera in **Acquisition Mode** tool.
	■ Sets dynamic range of all fluorescence channels to 20% or 50 for transmitted light channels.
	■ Sets power of all Colibri-LEDs to 100%.
	■ Sets EMGain of the camera (if available) to half of Gain max. Sets read mode of camera to fastest.
	■ Creates an acquisition configuration which removes all reducers or neutral filters.
	■ Changes acquisition sequence for dimensions to fastest. Only effective with 2 or more dimensions.
Quality	■ Sets the optimal resolution for the camera in **Acquisition Mode** tool. Microscanning will be applied if the camera supports this mode.
	■ Sets the dynamic range for all fluorescence channels to 90% or for all transmitted light channels to 100 %.
	■ Sets power of all Colibri-LEDs to 75%.
	■ Sets EMGain of the camera (if available) to 10% of Gain max. Sets read mode of camera to slowest.
Standard	Sets all parameters in **Channels** and **Acquisition Mode** tool to the default values. All changes will be overwritten and reset.
Current	No changes are made. Only the necessary hardware settings for acquisition are applied by **Smart Setup**.

> **i Info**
>
> If you changed hardware settings in **Acquisition mode** tool manually and don't want to loose them, make sure you select **Current** button.

Proposals (4)

> **i Info**
>
> If **Smart Setup** is unable to make a proposal, acquisition using the selected dyes or contrast technique and the current microscope hardware is not possible. Select other dyes or another contrast technique or configure your acquisition experiment using the **Acquisition Mode** tool and the **Channels** tool.

Here you see the proposals made by **Smart Setup** displayed graphically. The number of proposals (max. 3) depends on the microscope hardware being used, the selected dyes and the contrast technique:

Proposal	Description
Best Signal	This proposal results in the best signal strength.
Fastest	This proposal results in the fastest acquisition.
Best Compromise	This proposal results in the best compromise of signal strength and fastest acquisition.

Emission Signal, Speed and Crosstalk
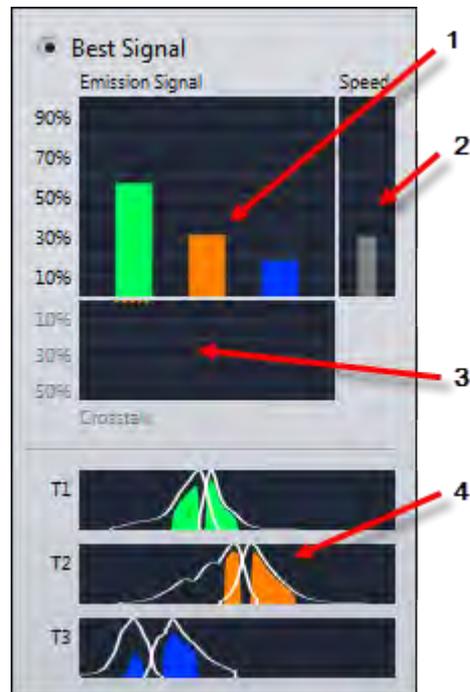
Illustration 14: Emission Signal, Speed, Crosstalk and Tracks

Number	Name	Description
1	Emission Signal	A filled, colored bar in the **Emission Signal** display field shows the relative emission signal to be expected for the corresponding channel. The channel color corresponds to the color of the selected dye in the **Configure Experiment** section.
2	Speed	A gray bar in the **Speed** display field represents the approximate acquisition speed that can be expected. This is the time required for the movement of microscope hardware during multichannel acquisition. Camera exposure times or parameters of other acquisition dimensions are not taken into account here.
3	Crosstalk	A hatched bar in the **Crosstalk** display field shows the relative crosstalk originating from one or more dyes of other channels that can be expected.
4	Tracks	Only visible if the **Show Excitation** and **Show Emission** checkboxes are activated. The various tracks are labeled with **T1, T2,** etc. and the various channels with **C1, C2,** etc. The white lines show the excitation and emission spectra of the dyes schematically. The spectra are filled in color in the places that will be acquired by the acquisition configuration suggested by **Smart Setup**. Transmitted light channels are displayed as a filled white field.

---

i Info

The bars in the graphs only show relative values. The actual strength of the emission signal and the crosstalk in the image can deviate substantially from this estimate, as Smart Setup has no knowledge of the strength with which the sample has been dyed with the individual dye components.

Show Excitation checkbox

Shows the excitation spectrum of the selected dyes in the **Tracks** display field.

Show Emission checkbox

Shows the emission spectrum of the selected dyes in the **Tracks** display field.

OK button

Adopts the suggestion displayed as the current acquisition experiment. The suggestion overwrites existing experiments on the **Acquisition** tab.

Cancel button

Ends **Smart Setup**. The suggestions are not adopted into the experiment.

Z