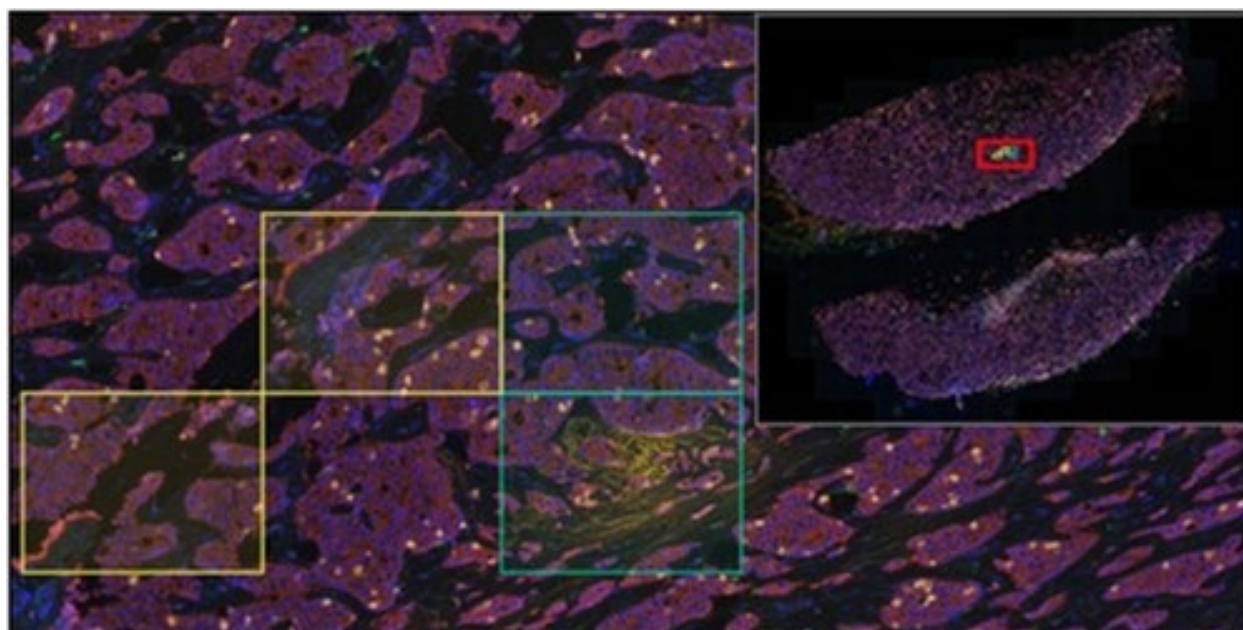


PhenoChart User Guide

WHOLE SLIDE CONTEXTUAL VIEWER FOR ANNOTATION & REVIEW

2.0.1



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Chapter 1 Overview

Phenochart™ is a slide viewer for scans created by the Phenolmager HT™ and Phenolmager™ Fusion Quantitative Pathology Imaging Systems that enables users to:

- View and navigate around whole slide images.
- Select areas of interest for further review, multispectral field imaging, or analysis using inForm® Tissue Finder™.
- Identify cores for imaging or analysis.

Phenochart is freely available and can be installed on multiple machines at your site.

Phenochart is for Research Use Only. Phenochart is not for use in diagnostic procedures.

Chapter 2 Loading Slides

The Slide Browser allows you to select and load slides. It shows a list of available studies and the slides within them.

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2.1 SELECT A SLIDE

1. Click the Load Slide button in the upper left to bring up the Slide Browser. (See Figure 2.1.)

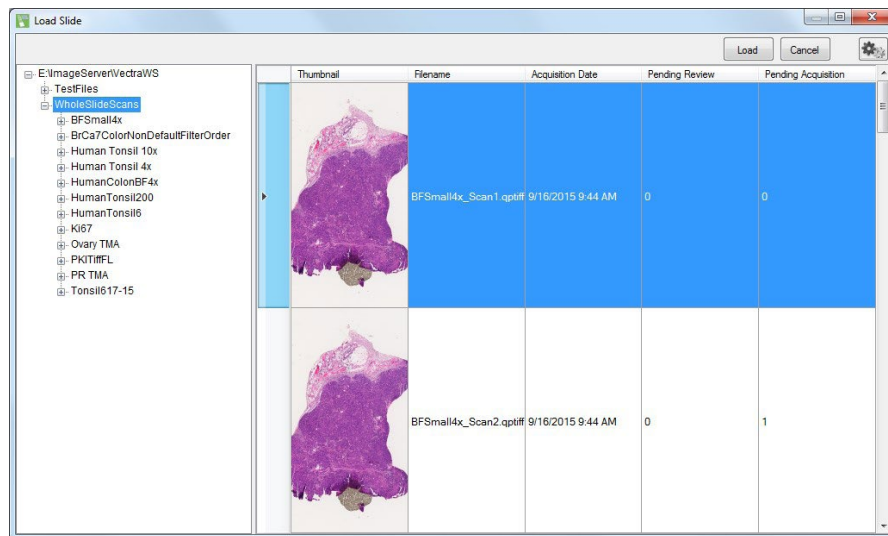


Figure 2.1. Slide Browser.

- a. The list of studies is on the left.
 - b. You can browse deeper to see slides within a study.
2. Select a study on the left; the right side shows the scans in that study. If you select a slide within a study, the right side will show scans of that slide only.

Click a column header to sort by it. This may help locate a slide with a specific name, a slide acquired on a certain date, etc.

Double-click any scan on the right-hand side to load that scan. You can also highlight the scan on the right-hand side and click Load. Click Cancel to close the Slide Browser without loading anything.

By default, the browser will show the slide thumbnail, a summary column, and columns showing the number of annotations pending acquisition or review. Note that the thumbnail

can be used for qualitative sample identification but should not be used to assess relative brightness of different images. You can add other columns. Select Set Column Visibility from the browser's Gear menu to select which columns are shown in the table. (See Figure 2.2.)

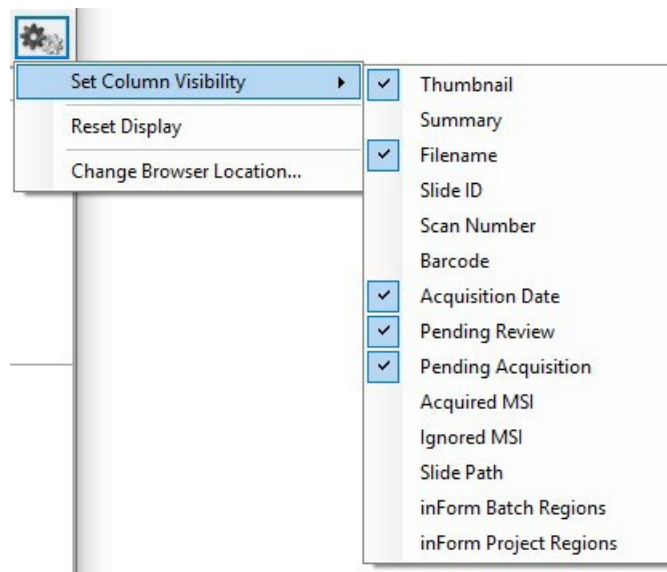


Figure 2.2. Gear menu.

You can also resize the columns by dragging the column dividers (e.g., if you want to make the thumbnail bigger). Select Reset Display from the browser's Gear menu at any time to restore the original column sizes.

2.2 INITIALIZE THE BROWSER

The very first time you launch the Slide Browser, you may need to locate your PhenoImager HT or Fusion data. You locate the data by selecting an example scan within it. PhenoChart obtains the example by asking you to open a QTIFF file (.qptiff). (See Figure 2.3.)



Figure 2.3. Select QTIFFSPTIFF file window.

The next time you click Load Slide, you will be able to use the Slide Browser.

NOTE: You will only see this prompt the very first time you load the Slide Browser or if you reset the browser in the menu.

If you need to change which data the browser looks at, use the browser's Gear Menu to change the location. (See Figure 2.4.)

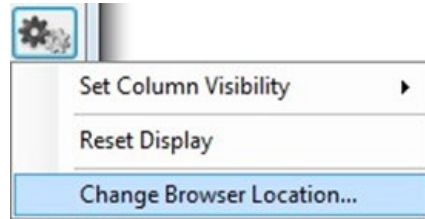


Figure 2.4. Gear menu > Change Browser Location...

Phenochart will again ask for an example QPTIFF file (.qptiff). This allows you to access data that may have been archived or data in a different location.

2.3 PHENOIMAGER HT AND FUSION DATA

These systems store scan data by study. (See Figure 2.5.)

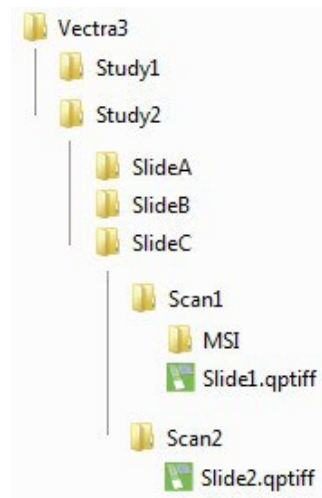


Figure 2.5. PhenoImager HT and Fusion studies.

A study is a group of slides that belong together; this could be an actual study (like Ki67 markers in breast cancer), all slides from one customer, etc.

Each study will contain one or more slides. Each slide can be scanned more than once if needed. Any acquired Multispectral fields from a slide scan will be stored within the scan folder.

Chapter 3 Viewing Slides

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3.1 NAVIGATION

The Navigation functions allow you to move around the slide and to view the image in more detail. This may be useful to review the image for focus quality or exposure or to confirm the correct area of the slide was captured by the scan.

The Navigation Tool is the arrow button located in the toolbar at the top of the screen. (See Figure 3.1.)



Figure 3.1. Navigator tool.

When first launching PhenoChart, this button is selected as the default setting for your mouse. It allows you to click-and-drag to move the image on your display or to use the mouse wheel to zoom in and out on the image.

The Navigator Window is the small image of your slide located at the top-right of the screen. (See Figure 3.2. and Figure 3.3.)

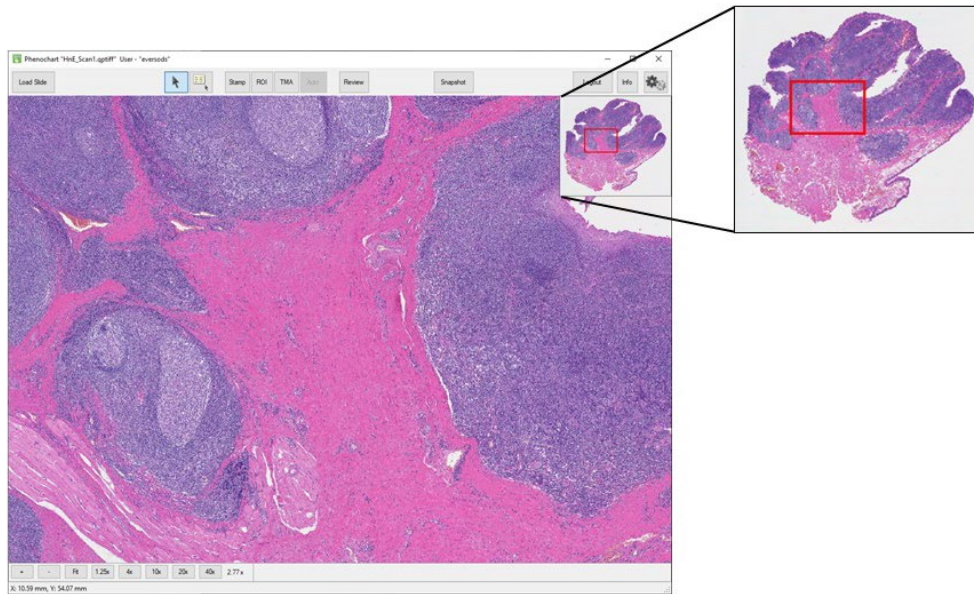


Figure 3.2. Navigator window.

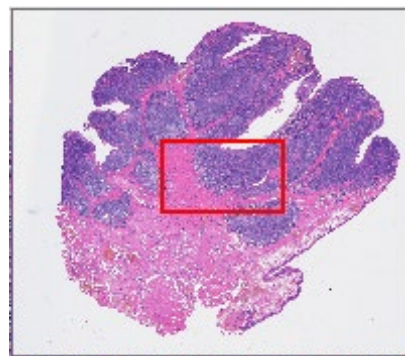


Figure 3.3. Navigator window inset.

The Navigator Window contains a small image of the entire slide and a red box within that image. The red box shows you what part of the slide is being displayed on the main image on your screen. You can click-and-drag the red box in the Navigator Window to change which area of the slide to display. (Note: The main image will not update until you release the mouse once you have moved the box to your desired location). Or, click on any area of the image in the Navigator Window and the red box—and the main image on the screen—will move to that section of the slide.

The Navigator Window is displayed on your screen by default. However, you can hide this window at any time by deselecting Show Navigator in the Gear Menu at the top right of your screen. (See Figure 3.4.)

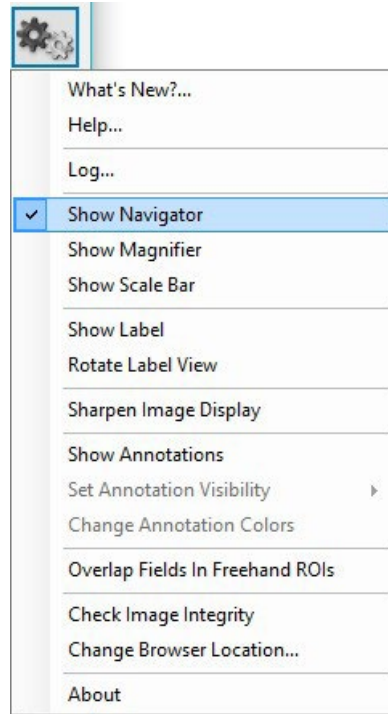


Figure 3.4. Gear menu > Show Navigator.

To redisplay the Navigator Window, select Show Navigator again in the Gear menu. This Navigator preference (show vs. hide) is saved across sessions.

You can zoom in and out on the slide. As you zoom in and out, the red box in the Navigator Window will change in size to continually identify which portion of the slide is being displayed.

There are three ways to zoom in and out on the slide:

- Select from the pre-defined resolutions (1.25x, 4x, 10x, 20x, and 40x), listed on the Navigation Toolbar at the lower left of your screen. (See Figure 3.5)



Figure 3.5. Pre-defined resolutions.

- Use the +/- buttons on the Navigation Toolbar.
- Use your mouse wheel to zoom in and out on the slide. When you use the Navigation Toolbar to zoom, the zoom is centered on your field of view. When you use the mouse wheel, the zoom is centered on your mouse cursor. At all times the text to the right of the 40x button will update to show the current resolution.

At any time, you can select Fit (also located on the Navigation Toolbar), which will fit the entire scanned image to the application window.

NOTE: No matter what resolution a slide has been scanned at, when you load a slide, it will display to “fit” to your screen size.

3.2 MAGNIFIER

The Magnifier is the window in the upper left corner of your screen. (See Figure 3.6 and Figure 3.7.)

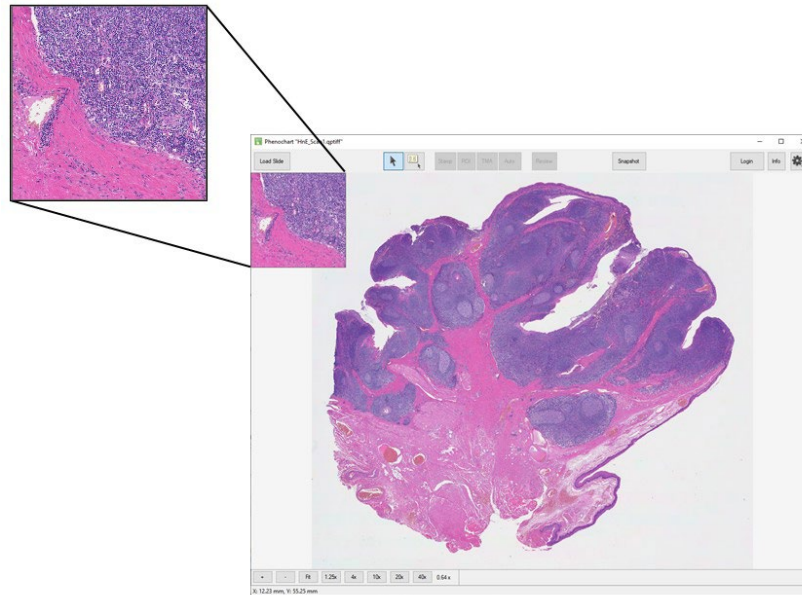


Figure 3.6. Magnifier window.

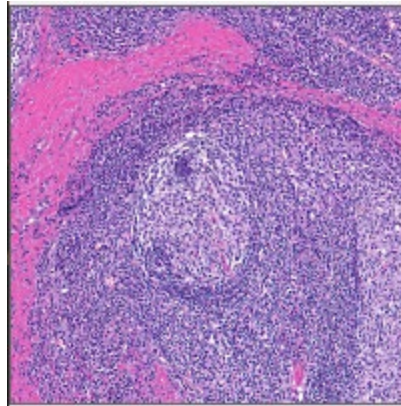


Figure 3.7. Magnifier window inset.

The Magnifier shows a magnified (zoomed in) view of the slide centered on your cursor. This allows for quick inspection of individual regions in more detail without changing the overall zoom.

The Magnifier window is displayed on your screen by default. You can hide this window by de-selecting Show Magnifier in the Gear menu at the top right of your screen. (See Figure 3.8.)

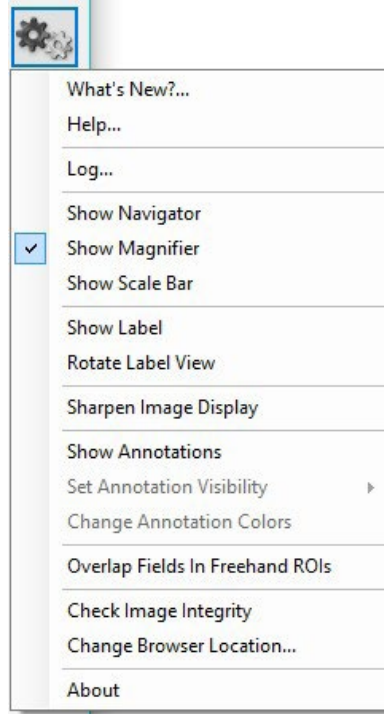


Figure 3.8. Gear menu > Show Magnifier.

3.3 SCALE BAR

The Scale Bar is located at the lower left corner of your screen. (See Figure 3.9. and Figure 3.10.)

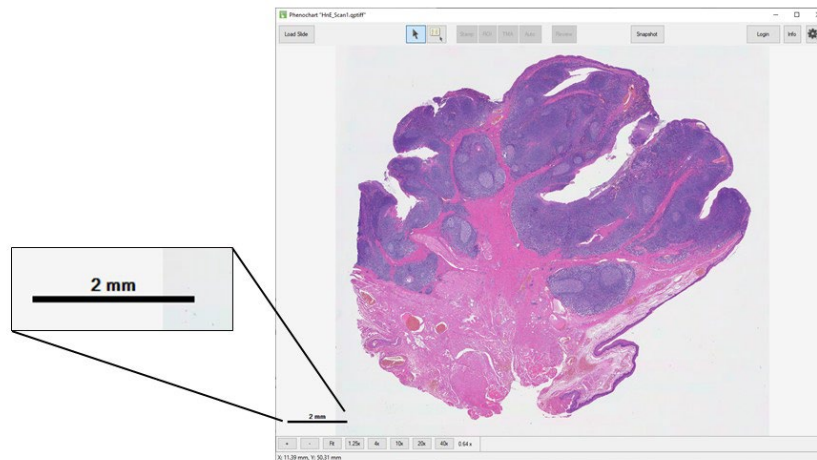


Figure 3.9. Image with Scale Bar.



Figure 3.10. Scale Bar inset.

The Scale Bar is a reference scale showing the actual size of tissue features in the image. The length of the bar will change as you zoom in and out on your slide and the units will change from microns to millimeters depending on your magnification.

The Scale Bar is displayed on your screen by default. You can hide it by deselecting Show Scale Bar in the Gear menu at the top right of your screen. (See Figure 3.11.)

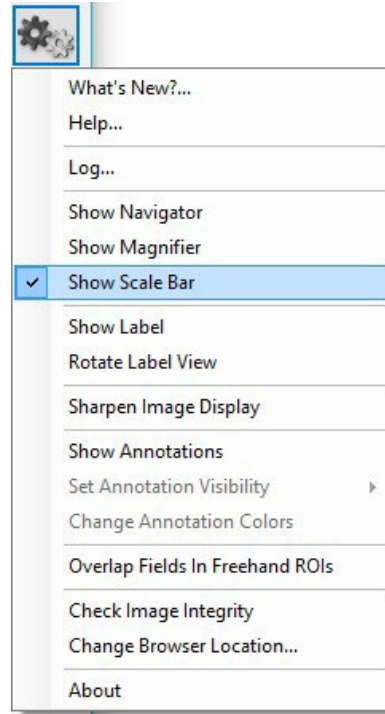


Figure 3.11. Gear menu > Show Scale Bar.

3.4 LABEL

The Label view is located at the lower right corner of your screen. (See Figure 3.12 and Figure 3.13.)

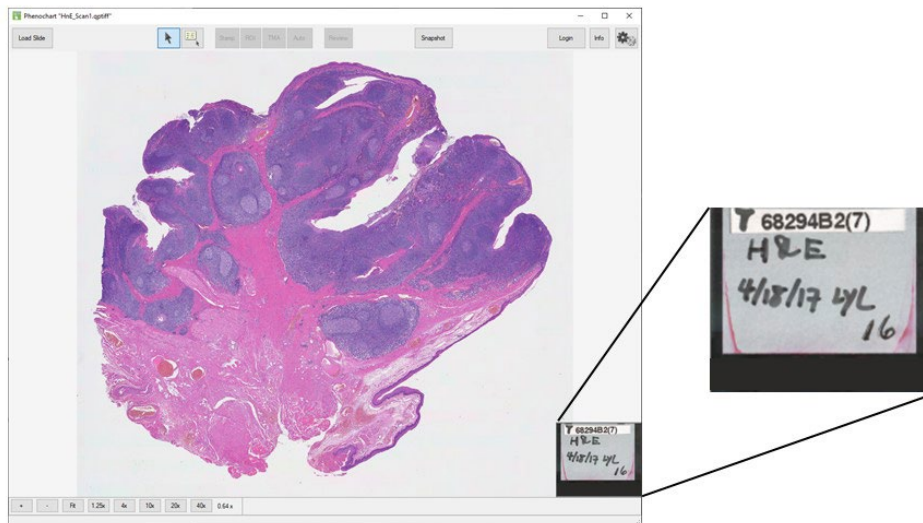


Figure 3.12. Label view.

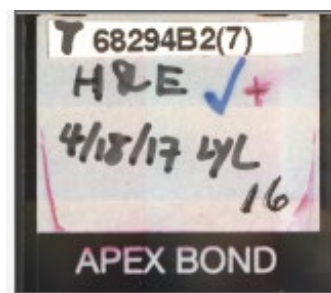


Figure 3.13. Label view inset.

For PhenImager HT and Fusion imagery, the Label view displays an image of the slide's label. If necessary, rotate the image by clicking on it or by selecting Rotate Label View in the Gear menu. (See Figure 3.14.)

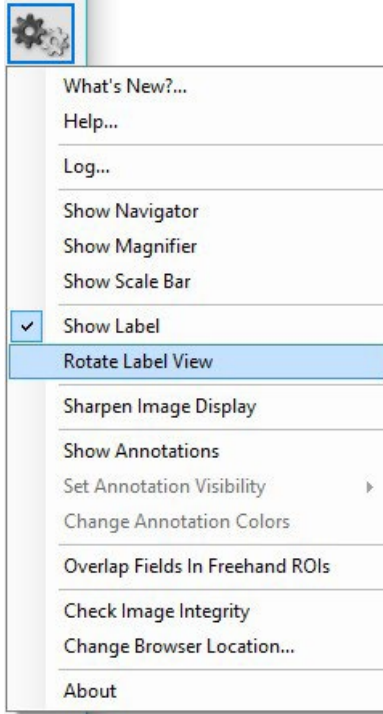


Figure 3.14. Gear menu > Rotate Label View.

The Label view is displayed on your screen by default. You can hide it at any time by de-selecting Show Label in the Gear Menu at the top right of your screen. (See Figure 3.15.)

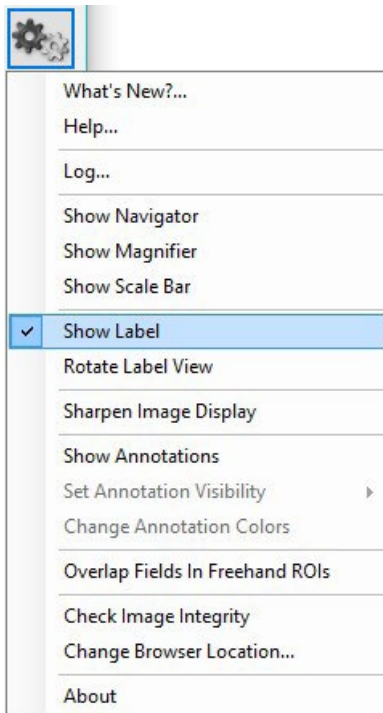


Figure 3.15. Gear menu > Show Label.

3.5 STATUS BAR

The Status Bar is located at the bottom of your screen. (See Figure 3.16 and Figure 3.17.)

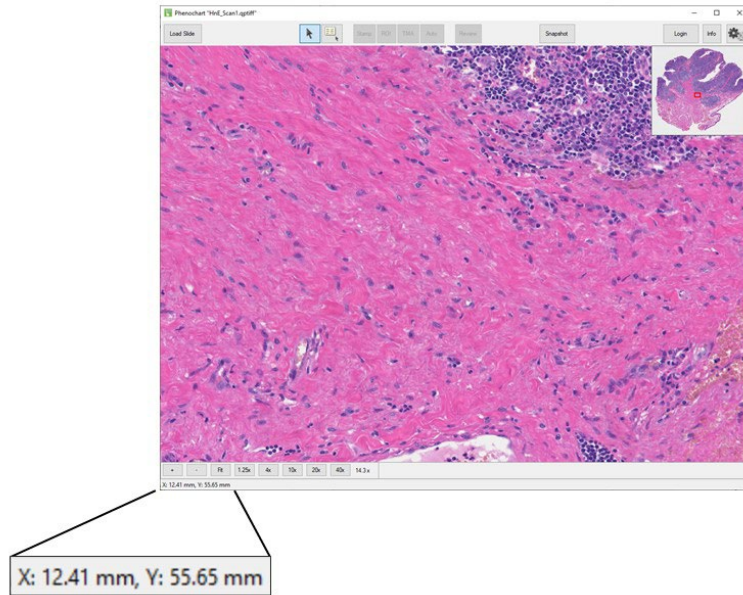


Figure 3.16. Image with Status Bar.



Figure 3.17. Status Bar inset.

The Status Bar shows the current position of your cursor on the slide scan.

The coordinates are shown in millimeters and are updated in real time as you move your cursor on the screen. The origin is the upper left-hand corner of the slide as it sits on the stage. This physical location will differ depending on your system hardware.

3.6 INTENSITY CURSOR

The Intensity Cursor enables you to see coordinate and intensity values in a panel at the location of the mouse cursor. (See Figure 3.18.)

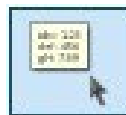


Figure 3.18. Intensity Cursor.

Intensity Values Displays

- **Brightfield imagery:** the cursor shows standard RGB values ranging from 0-255.

- **Fluorescence Phenolmager HT or Fusion imagery** (without unmixing): the cursor shows one value for each filter band. The value is in the range from 0-255 for standard 8-bit images. For Phenolmager HT images in Extended Range format, the reported values are equivalent to what a standard image would show, but can exceed 255 to take advantage of the extended range. When this occurs, an “above target” message will display at the bottom of the cursor and a “+” will display next to the channel with counts above 255. If the pixel at the current position of your cursor is saturated, in the panel the text of the component name and its value will display red followed by a red asterisk.
- **Unmixed Fluorescence imagery**: the cursor shows normalized counts for each unmixed component. The normalization takes into account exposure times. This means that the values are comparable between scans acquired with different exposure times. The same normalization is performed by default in inForm software. [See Chapter 3.13 Whole Slide Multispectral Unmixing](#) for more information. Note that the “above target” and “saturated” messages shown for images without unmixing are not shown for all unmixed image formats. To check for saturation, review the images without unmixing.

3.7 SLIDE INFO

You can get more information about your slide by clicking the Info button. The Info button is located in the toolbar at the top of the screen. (See Figure 3.19.)

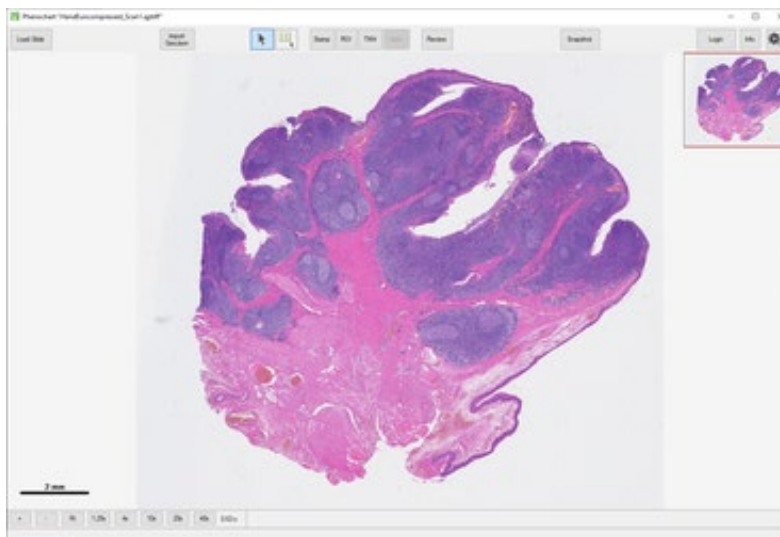


Figure 3.19. Slide Info window.

Click the Info button to open the Slide Info dialog. This dialog shows detailed information about your slide, how it was scanned, and properties of the resulting scan file. It also shows a color overview image of the slide and the label, if available. (See Figure 3.20.)

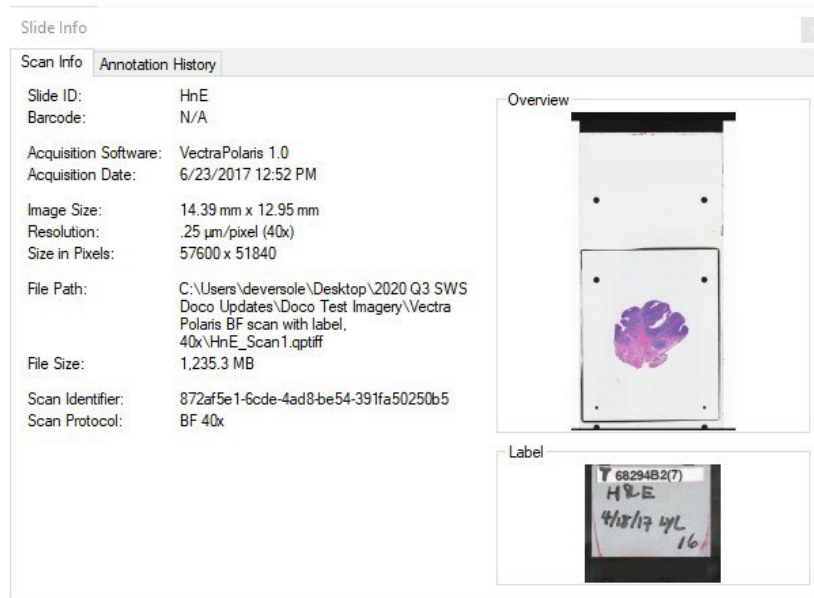


Figure 3.20. Slide Info – Scan Info tab.

Fluorescence imagery detailed information also includes filter names and corresponding exposure times.

NOTE: For information about Annotation History [see Chapter 4 Annotations](#).

3.8 IMAGE SHARPENING

Brightfield QPTIFF files acquired with PhenoImager HT or Fusion may be JPEG compressed to reduce storage size. It is possible to visually enhance these images with image sharpening. This setting only affects the display of the image; the data values remain unchanged.

Images are displayed with sharpening applied by default. You can turn this off by deselecting Sharpen Image Display in the Gear menu at the top-right of your screen. (See Figure 3.21.)

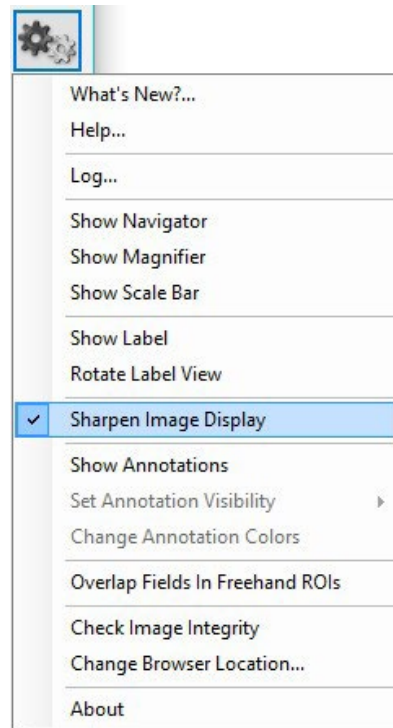


Figure 3.21. Gear menu > Sharpen Image Display.

3.9 IMAGE INTEGRITY

QTIFF files are tamper-evident. You can validate them to confirm that the image data and metadata have not been altered.

Select Check Image Integrity from the Gear menu at the top right of your screen. The software will perform the integrity check and report the results. (See Figure 3.22.)

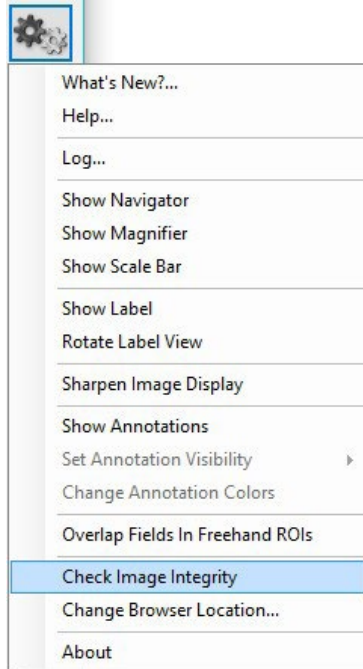


Figure 3.22. Gear menu > Check Image Integrity.

3.10 FLUORESCENCE LAYER TOOLBAR

The Fluorescence Layer Toolbar is located in the bottom-right of your screen. (See Figure 3.23 and Figure 3.24.)

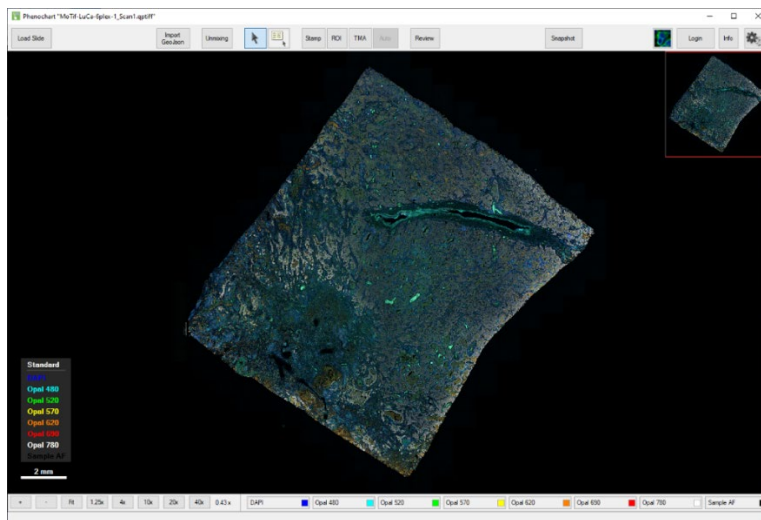


Figure 3.23. Fluorescence Layer toolbar.

The toolbar is only visible for fluorescent slides. The toolbar enables you to toggle between which fluorescence channels are shown and adjust their brightness or color.

For Fluorescent imagery, the Layer Toolbar will show one button for each filter used during the scan. (See Figure 3.24.)



Figure 3.24. Layer Toolbar filter buttons for fluorescent imagery.

Each Layer Button is labeled with the common name for that filter. Each layer’s color is determined by the color selected in the scan protocol. (See Figure 3.25.)

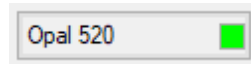


Figure 3.25. Layer Color.

You can turn the signal layer on and off by clicking the Layer Button. When the button is On, it shows the display color on your image. When the button is Off, it is drawn with a hatch pattern. (See Figure 3.26.)

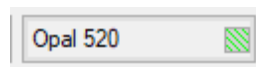


Figure 3.26. Hatch Pattern.

You can change the display color and signal intensity for any layer. Right-click on any Layer Button to open the Layer Editor. (See Figure 3.27.)

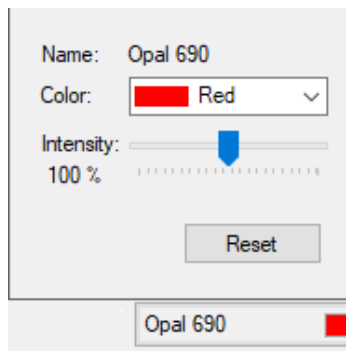


Figure 3.27. Layer Editor.

Change the color of the layer by selecting any of the preset colors or choose Custom to create a custom color.

You can also change the intensity, which will increase or decrease the apparent brightness for signals from that filter. The default intensity is 100%.

Click Reset at any time to return to the color and intensity default settings. The Layer Editor will close when you move your mouse off the editor.

3.11 FLUORESCENCE COMPOSITE VIEWS

Fluorescence Composite Views are intended to simplify visualization of fluorescence slides displaying large numbers of markers.

When an image is first opened, the scan will display the Standard view, which includes all components designated in the scan protocol.

3.11.1 EDITING COMPOSITE VIEWS

The Composite Views editor allows you to select which components you would like to display. You can edit existing views, create new views, and select which components to display per view. The Composite Views editor is only accessible for fluorescent slides.

To access the Composite Views Editor, click the Fluorescence mode button located in the upper right-hand corner of your screen (to the left of the Login button) and click Edit. (See Figure 3.28.)

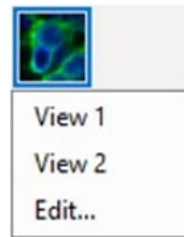


Figure 3.28. Composite View selection.

Select a composite view in the Views list on the left-hand side. Click Rename to change the name of this view.

To change which components are displayed you can select individual components from the Available Components list displayed on the right half of the window. Each view can display up to 8 components. Change the color of each component by selecting any of the preset colors or choose Custom to create a custom color. (See Figure 3.29.)

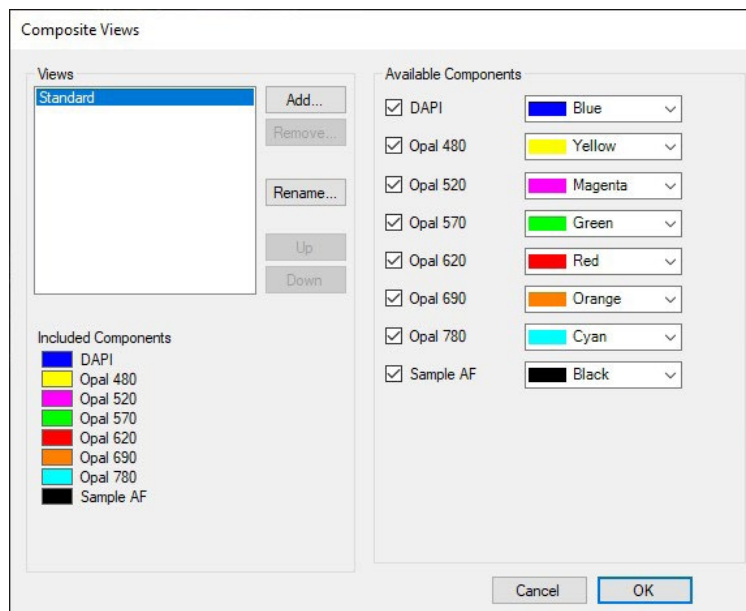


Figure 3.29. Composite Views Editor.

Click Add to create additional Views for display. You will first be prompted to name your view. After adding a name, select OK. You can change the view name later by selecting the view from the Views list and clicking Rename. The new view will automatically select the first 8

components. To change components, first click the checked boxes next to the name of the components you wish to remove and then select the new components from the Available Components list. Assign each component a desired color. There is no limit to the number of views that can be created.

The display order of all Views can be changed. To change the location of a view, select the desired view and click the Up and Down buttons.

To permanently remove a view, select the desired view and click the Remove button. To prevent accidental deletion of a view, you will be prompted to click the OK button in the Removing Composite View confirmation before a view is permanently deleted.

VIEWING COMPOSITE VIEWS

To toggle between Views, click the Fluorescence mode button located in the upper right-hand corner of your screen (left of the Login button). A drop-down list of all Views created will display. Select the view name you want to display. (See Figure 3.30.)

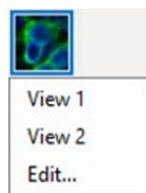


Figure 3.30. Composite View Selection.

You can also toggle between views by using the up and down keyboard arrows.

3.11.2 SAVING COMPOSITE VIEWS

You can save composite view settings when closing the current slide or loading another slide. When prompted, select the Yes button in the Saved Changed Views Window if you want to save the composite view setting. (See Figure 3.31.)

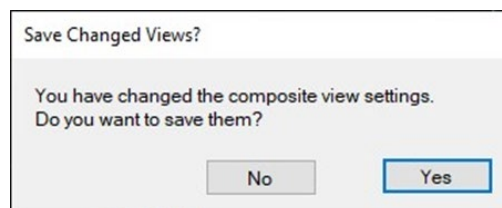


Figure 3.31. Save Changed Views.

3.12 LEGEND

The Legend is located at the lower left corner of your screen. (See Figure 3.32 and Figure 3.33.)

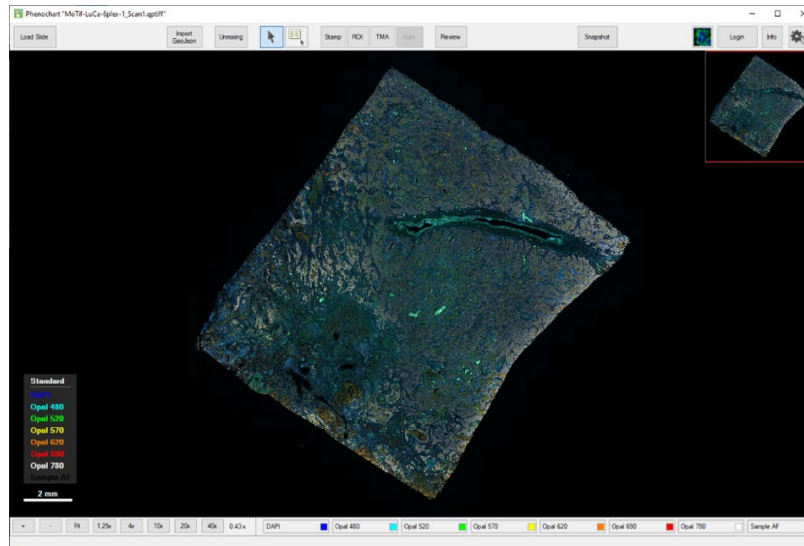


Figure 3.32. Image with legend.



Figure 3.33. Legend inset.

The Legend shows which components are included within the selected Composite View. The Legend is displayed on your screen by default. You can hide it at any time by de-selecting Show Legend in the Gear Menu at the top right of your screen. The Legend is only available when viewing in Fluorescence mode.

3.13 WHOLE SLIDE MULTISPECTRAL UNMIXING

Fluorescent scans acquired with a multispectral slide scan protocol on the Phenolmager HT or Fusion can be unmixed by Phenochart 1.0.10 or later and inForm 2.4.3 or later. The Unmixing button will only be displayed for these multispectral slide scans. (See Figure 3.34.)



Figure 3.34. Unmixing button (in red highlight box).

Phenolmager HT 2.0 and later also support writing files in an unmixed format, where unmixing is done as part of the scan process. There is no option to unmix these within Phenochart since they are already unmixed.

Click the Unmixing button to choose between two Unmixing Views:

- **None:** The image is shown without any unmixing applied. This is the default.
- **Opal + AF:** The image is shown as unmixed components using synthetic Opal spectra. By using a representative autofluorescence from an unstained human tonsil section, PhenoChart can provide a preview of signal and autofluorescence isolation. Unmixing does not alter the underlying data in any way. (See Figure 3.35.)

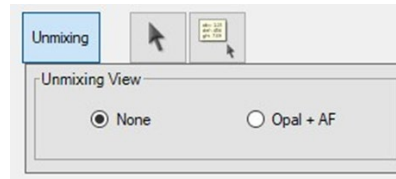


Figure 3.35. Unmixing View editor.

Multispectral scans that have been unmixed are displayed in Fluorescence mode against a black background.

After being opened, the Unmixing View Editor will remain open until you click another button on the toolbar. This allows you to toggle between the original and unmixed views as you navigate the slide.

3.14 SIMULATED BRIGHTFIELD VIEWS (PATHOLOGY VIEWS)

Simulated Brightfield mode (Pathology Views™) renders fluorescence images in a manner that is familiar to anyone trained in histopathology, displaying them as simulated hematoxylin and eosin (H&E) images or as simulated DAB/Hematoxylin images.

Simulated Brightfield mode is available for use with fluorescent scans acquired with a multispectral slide scan protocol on the PhenoImager HT or PhenoImager Fusion that have been unmixed or for PhenoCycler®-Fusion scans. (See Figure 3.36.)

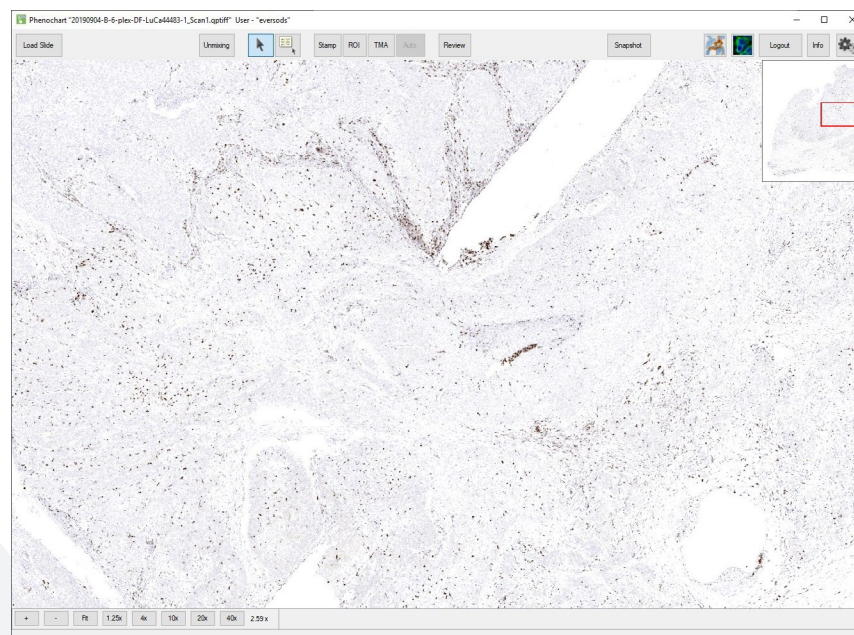


Figure 3.36. Simulated Brightfield Mode (Pathology Views).

Simulated H&E: The autofluorescence component is displayed as a simulated Eosin stain with Hematoxylin counterstain simulated from the DAPI signal against a white background.

Simulated DAB/Hematoxylin: Individual components are displayed as a simulated DAB stain with Hematoxylin counterstain simulated from the DAPI signal against a white background.

To toggle between components, click the Brightfield mode button located in the upper right-hand corner of your screen to the left of the Fluorescence mode button. A drop-down list of all available components will display. Select the view name you want to display. (See Figure 3.37.)

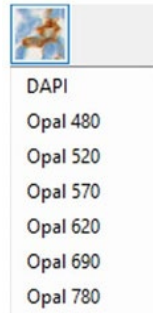


Figure 3.37. Component selection for Pathology Views.

You can also toggle from one component to the next by using the up and down keyboard arrows.

You can change the colors used in Brightfield mode by selecting Change Brightfield Colors in the Gear menu. (See Figure 3.38.)

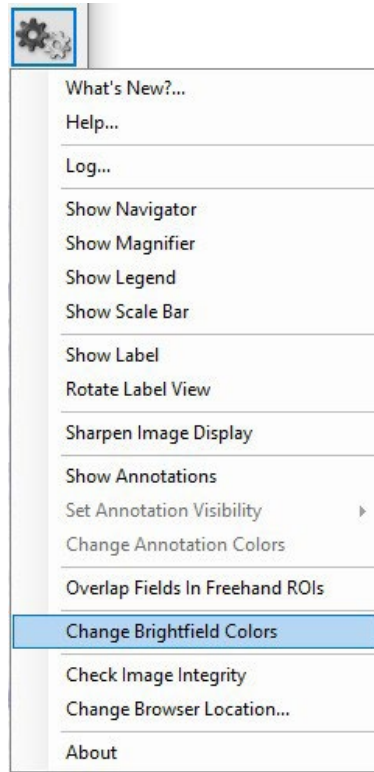


Figure 3.38. Gear menu > Change Brightfield Colors.

Select your desired colors in the Select Brightfield Rendering Colors dialog. Click Reset if you would like to set them back to the default. (See Figure 3.39.)



Figure 3.39. Select Brightfield Rendering Colors.

To toggle the display between fluorescence and brightfield modes, use the fluorescence and brightfield buttons located in the upper right-hand corner of your screen to the left of the Login button.

3.15 SNAPSHOTS

The Snapshot button allows you to save a picture of the main display area. You can use these images in publications or to share screenshots with colleagues.

Click the Snapshot button in the upper right corner of your application window to show the Snapshot Editor. (See Figure 3.40 and Figure 3.41.)

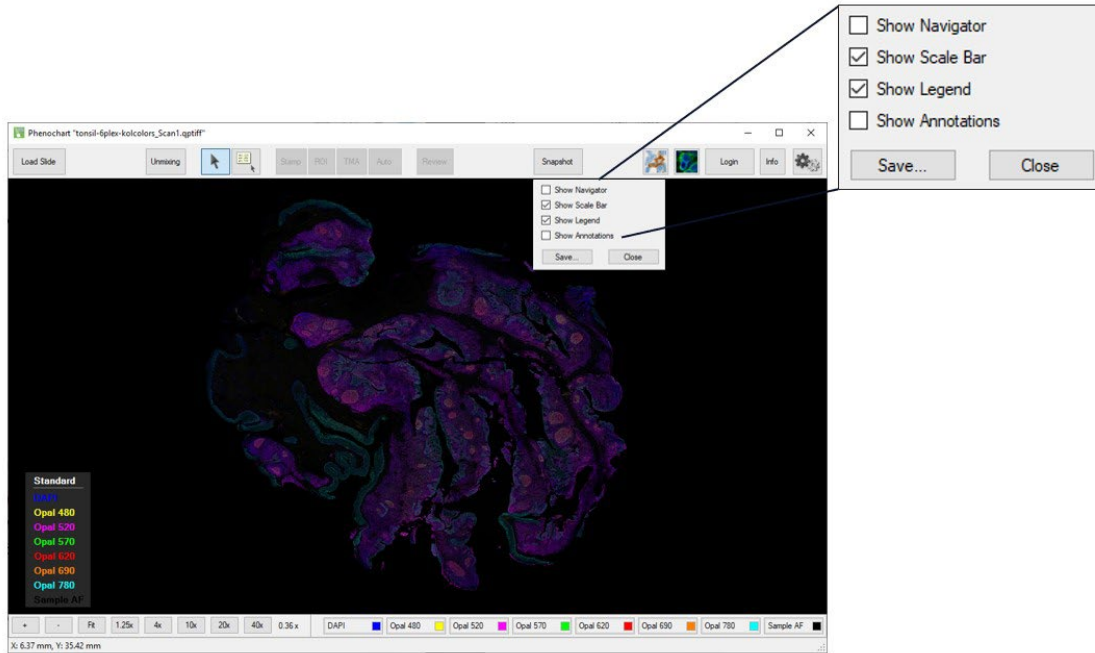


Figure 3.40. Snapshot Editor window.

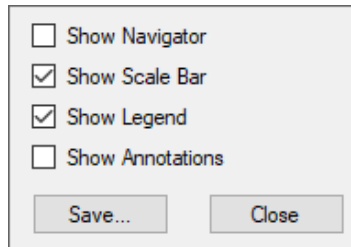


Figure 3.41. Snapshot Editor window inset.

Adjust the location and zoom for your slide using the navigation tools. Use the options in the Snapshot Editor to add or remove overlays of the Navigator Window, Scale Bar, Legend, or Annotations. These options affect only the saved Snapshot, not the main window settings.

NOTE: When you first launch PhenoChart, the Scale Bar overlay is turned on for snapshots. During your session, the software will retain the overlay selections from the last saved snapshot.

When your image is ready, click the Save button in the Snapshot Editor. You can navigate to different parts of the slide to take multiple snapshots. When you have saved all your snapshots, click the Close button to close the Snapshot Editor.

Chapter 4 Annotations

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An annotation is created when you mark an area of interest on a slide for further multispectral imaging by Phenolmager HT or for analysis in inForm.

Four ways to create annotations:

- Stamping fields.
- Drawing ROIs.
- Finding TMA cores.
- Automatically generating fields through inForm.

States within each type of annotation:

- Pending Acquisition - This annotation has been identified for multispectral field imaging but has not yet been acquired.
- Pending Review - A reviewer must accept this annotation before multispectral fields can be acquired.
- Acquired - Multispectral fields were taken for this annotation.
- Acquisition Failed - This annotation was slated for the acquisition of multispectral fields, but the acquisition failed to complete correctly.
- Rejected - A reviewer rejected this annotation; fields will not be acquired there.
- Ignored - This annotation was ignored. It will not be considered for multispectral field acquisition or direct analysis in inForm.
- Deleted - A user deleted this annotation. If there was multispectral field imagery associated with this annotation, it was also deleted.
- Selected For inForm Projects - Scan imagery from this annotation will be loaded into inForm when the qptiff is opened in inForm. This option is only available for slides scanned with Phenolmager HT or Fusion.
- Selected For inForm Batch - Scan imagery from this annotation will be loaded into inForm when the qptiff is selected for batch processing. This option is only available for slides scanned with Phenolmager HT or Fusion.
- Send to inForm - This annotation was sent to inForm for rudimentary analysis or inForm classifier training. This option is only available for slides scanned with the legacy Vectra 3 system.

Annotations are color coded according to their state. [See Annotation Colors](#) for how to change the colors. [See Annotation Visibility](#) for how to show/hide types of annotations.

To create or review annotations, you must log in. Click Login and enter a username. When you have completed your work, click the Logout button to log out. (See Figure 4.1.)



Figure 4.1. Login button.

When you are logged in, you can right-click on any annotation to ignore or delete it.

If multispectral fields have already been acquired at your annotation, double-click the annotation to open the acquired fields in inForm.

When the PhenoImager HT is acquiring multispectral fields for a slide, the annotations for the slide are locked. You will not be able to add or change the annotations until PhenoImager HT finishes acquiring fields.

Annotations slated for multispectral field acquisition that were not acquired correctly will be marked.

Acquisition Failed: To re-acquire an individual annotation, right-click on that field and select Re-acquire. This will set the state back to Acquire. You can also select Re-acquire All if there were multiple failed acquisitions. This will set all fields that failed back to the Acquire state so they can be re-acquired.

You can see the history of a scan's annotations in the Slide Info dialog. Click the Info button and click the Annotation History tab to view the log of annotations and actions. The log records each annotation action and the associated username. The annotation ID groups actions for each annotation together.

The annotation log includes a tamper-evident hash code. If a log file is altered, PhenoChart will give an error and refuse to open the file. (See Figure 4.2.)

Slide Info				
Scan Info		Annotation History		
ID	Type	Date	User	Action
1	Stamp	11/10/2015 9:48 AM	user	Created
1	Stamp	11/10/2015 9:48 AM	user	Pending Acquisition
2	Stamp	11/10/2015 9:48 AM	user	Created
2	Stamp	11/10/2015 9:48 AM	user	Pending Acquisition
3	Stamp	11/10/2015 9:48 AM	user	Created
3	Stamp	11/10/2015 9:48 AM	user	Pending Review
3	Stamp	11/10/2015 9:48 AM	user	Pending Acquisition
3	Stamp	11/10/2015 9:48 AM	anotherUser	Review Accepted
4	Stamp	11/10/2015 9:48 AM	user	Created
4	Stamp	11/10/2015 9:48 AM	user	Pending Review
4	Stamp	11/10/2015 9:48 AM	user	Pending Acquisition
4	Stamp	11/10/2015 9:48 AM	anotherUser	Rejected

Figure 4.2. Slide Info > Annotation History tab.

You can sort the columns by annotation, date, or user to see the actions taken for each annotation.

4.1 STAMPS

Stamp annotations allows you to select specific rectangular areas of interest. You must be logged in to create stamp annotations. Click the Stamp button to open the stamp editor. (See Figure 4.3.)

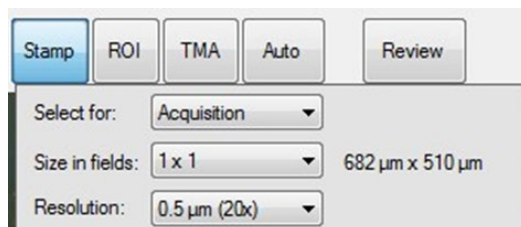


Figure 4.3. Stamp editor.

Five kinds of stamps:

- **Choose Acquisition** to take multispectral fields of the stamp area the next time this slide is on the Phenolmager HT.
- **Choose Review** to create stamps that must be reviewed before they can be acquired. Phenolmager HT will only acquire multispectral fields of accepted stamp annotations.
- **Choose Push to inForm** to launch the selected stamp area into inForm. This option will only allow stamps at the scan's native resolution and is only available for slides scanned with legacy Vectra 3 system, and when inForm 3.0.0 or higher is installed on your machine. [See Chapter 4.4.1 Training an inForm Classifier for Field Selection](#) for more information.
- **Choose inForm Projects** to select stamp areas for analysis in inForm. Load the qptiff into inForm 3.0 or later to analyze these fields. These stamps can only be made at native resolution. This option is available for slides scanned with Phenolmager HT or Fusion.
- **Choose inForm Batch** to select stamp areas for batch analysis in inForm. Batch process the qptiff in inForm 3.0 or later to analyze these fields. These stamps can only be made at native resolution. This option is available for slides scanned with Phenolmager HT or Fusion.

Select the stamp size in camera fields; this determines how many fields will be tiled together to make the image. The size of the resulting image will be shown in microns.

Select the resolution to use when acquiring the fields.

NOTE: For legacy Vectra 3 systems, you can only acquire 0.25 micron/pixel (40x) fields if you have a 40x objective installed.

Your cursor will show a rectangle which previews the stamp size based on your settings. Click on the slide to create a stamp at that location. You can create multiple stamps. The preview rectangle will snap to existing stamps when it is close to them. Zoom and pan normally with the mouse or zoom buttons to find areas of interest.

All pending annotations (acquisition or review) must be the same resolution. If you have selected a resolution in error, you will need to ignore or delete the pending fields. If you wish to acquire fields at multiple resolutions, stamp and acquire the fields at one resolution first. You may then return to stamp and acquire fields at other resolutions.

If you create an incorrect annotation, you can quickly delete it by holding down the Ctrl key and clicking on the annotation. The cursor will change to a trashcan with a red X when an annotation can be deleted.

4.2 REGION OF INTEREST

The Region of Interest (ROI) tool allows you to draw a region of interest to:

- Acquire multispectral images on a Phenolmager HT.
- Batch analyze fields using inForm.

The ROI tool creates stamp annotations corresponding to the region you draw. Use the ROI Editor to label the stamp annotations for Acquisition, Review, or inForm Batch.

- Choose Acquisition to take multispectral fields of the stamp(s) the next time this slide is on the Phenolmager HT.
- Choose Review to create stamps that must be reviewed before they can be acquired. Phenolmager HT will only acquire multispectral fields of accepted stamps.
- Choose inForm Batch to create stamps for batch analysis in inForm. Batch process the qptiff in inForm 3.0 or later to analyze these fields. These stamps can only be made at native resolution. This option is available for slides scanned with Phenolmager HT or Fusion.

You can also use the ROI Editor to select your resolution. (See Figure 4.4.)



Figure 4.4. ROI Editor.

When you have made your selections, draw a region of interest on the slide by clicking and dragging an outline. If you don't close the area, it will be closed for you when you release the mouse button. The software will then create tiled stamp annotations that contain the area you have drawn. (See Figure 4.5.)

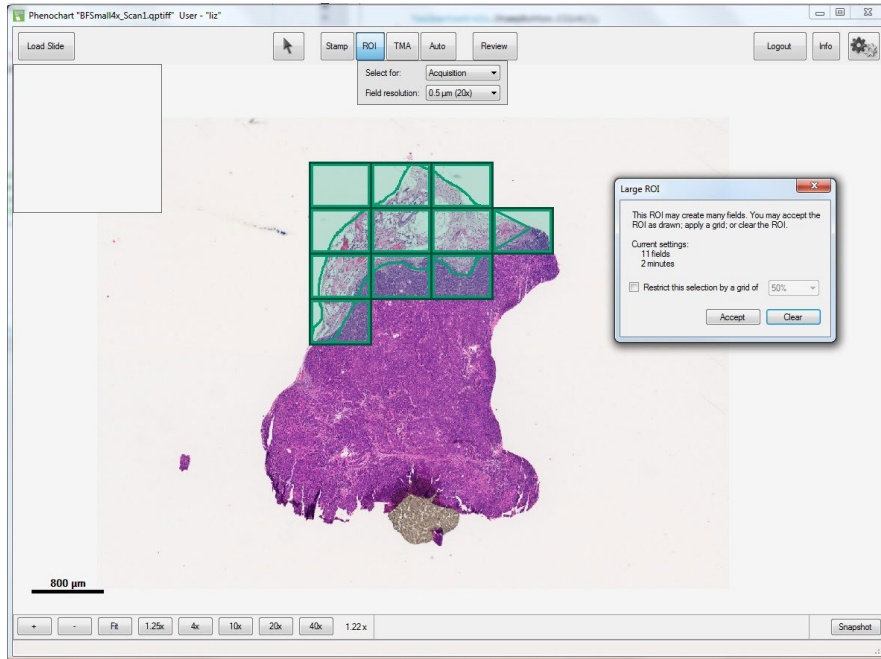


Figure 4.5. Tiled Stamp Annotations.

If your region is small enough to fit within a 3x3 stamp, it will be included in a single stamp annotation.

For larger regions, the system will break the region up into separate stamps. If you are drawing regions for Acquisition or Review, you will see a Confirmation Dialog that will tell you how many fields you are about to take. It will also give you an approximate time for the acquisition. You may want to change your region based on how long it will take to image. (See Figure 4.6.)

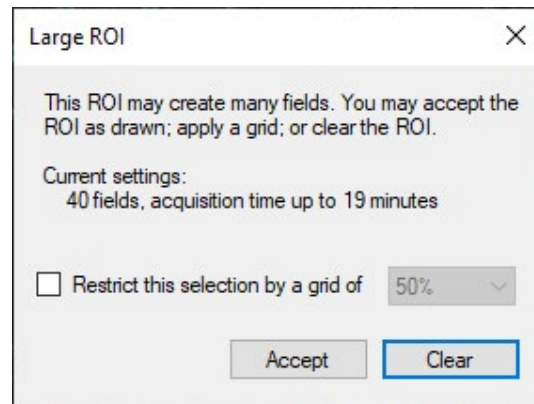


Figure 4.6. Large ROI window.

To reduce the number of fields within your region, you can apply a grid. Use the grid drop-down list to choose the percentage of fields for a gridded sample of stamp annotations. As you change the percentage, the overlay will update to show which stamps will be generated. In addition, the number of fields and approximate time will update accordingly.

By default, fields will abut with no overlap. If you would like the fields to overlap by a small percentage, select **Overlap Fields in Freehand ROIs** from the Gear menu. (See Figure 4.7.)

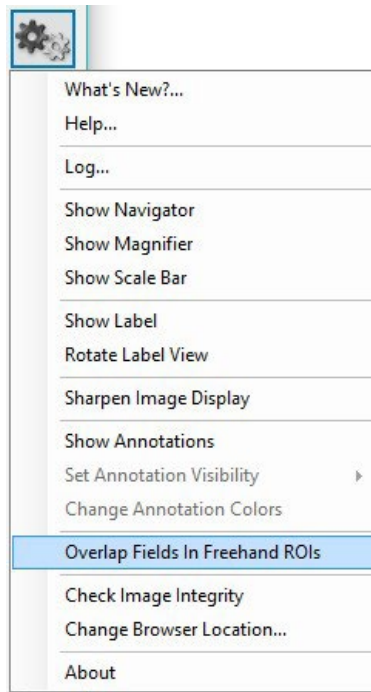


Figure 4.7. Gear menu > Overlap Fields in Freehand ROIs.

Click the **Accept** button to accept the ROI as shown, and the system will add all of the stamps to the list of pending annotations (acquisition or review).

Click the **Clear** button to remove the ROI, and no fields will be added. (See Figure 4.8.)

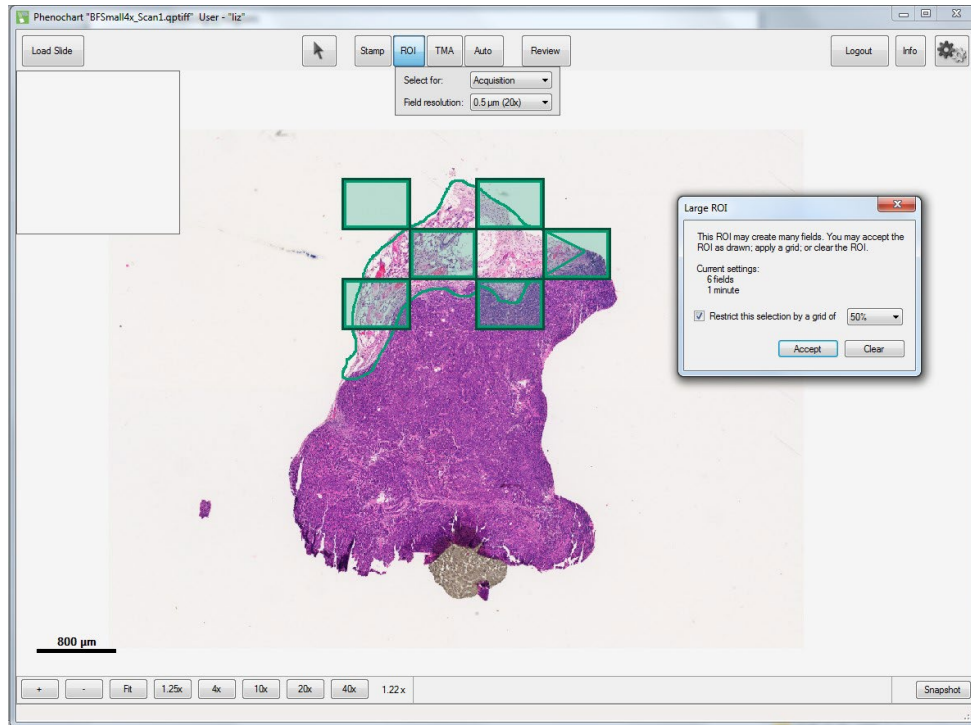


Figure 4.8. Accept ROI.

All pending annotations (acquisition or review) must be the same resolution. If you have selected a resolution in error, you will need to ignore or delete the pending stamp annotations. If you wish to acquire stamps at multiple resolutions, draw a region of interest and acquire the annotations at one resolution first. You may then return to draw regions and acquire annotations at other resolutions.

If you need to navigate to bring your region of interest in to view, hold the Ctrl key down and use your mouse to zoom and pan.

If you create an incorrect ROI, then right-click on any stamp in the region. If you then click Delete Region, all the stamps in that region will be deleted. If you click Delete, only the stamp you clicked on will be deleted. (See Figure 4.9.)

Additionally, you can delete individual stamps within an ROI by holding down the Ctrl key and clicking on the annotation. The cursor will change to a trashcan with a red X when a stamp can be deleted.

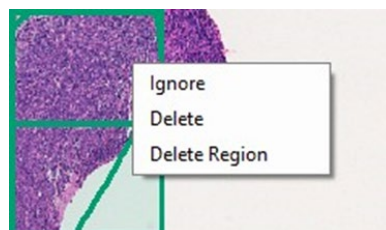


Figure 4.9. ROI right-click menu.

4.3 TMA

Tissue Micro-Array (TMA) annotations let you locate TMA cores within whole-slide images and analyze or acquire multispectral fields of each TMA core. You can use sector labels to identify different sections of cores, as appropriate, and use the row and column labels to match the TMA's numbering scheme. Correct labeling will make it possible to match downstream analysis with the original core information.

TMA Editor

Click the TMA button on the toolbar to open the TMA editor. (See Figure 4.10.)

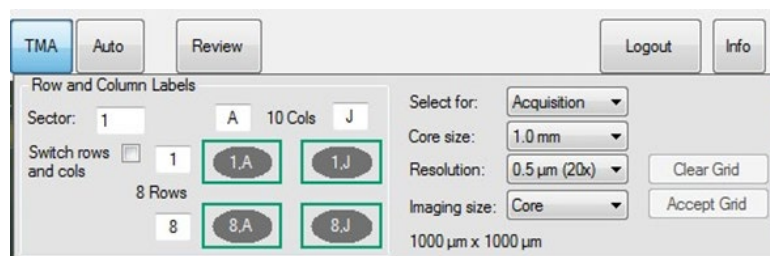


Figure 4.10. TMA Editor.

For best results, select the core finding parameters in the editor before drawing your sector.

Row and Column Labels

Select the Sector number for your TMA sector. Then choose the appropriate labels for your sector's rows and columns. Row and column labels can be either numbers or letters and can go in increasing or decreasing order. The grid will show you the labels of the sector's four corners.

PhenoChart displays labels as row, column, where rows go across and columns go up and down in the slide view. Check the Switch rows and cols checkbox if you have columns that go across and rows that go up and down.

Core Settings

After the labels are correct, select the desired core finding parameters to the right of the labels.

Identify if the TMA cores are for acquisition, review or inForm Batch. If you select cores for review, only the accepted cores will be acquired. If you select cores for inForm Batch, only the scan's native resolution can be used. inForm Batch is only available for slides scanned with Phenomager HT and Fusion.

Choose the size of your TMA cores. If the exact core size isn't listed, choose the next larger one.

Choose the resolution to use when imaging your cores. If you plan to image multiple sectors at the same time, the resolution must be the same for all sectors. To choose another resolution, you must first acquire, delete, or ignore your pending cores.

Choose an imaging size for your cores:

- **Core:** takes a square image of exactly Core Size x Core Size microns.
- **Core + 10%** and **Core + 20%:** gives square images with a margin to account for slight variations in core size.
- **NxN:** fields are rectangular fields as described. This option enables you to take imagery consistent with early versions of PhenoImager HT.

NOTE: These fields may take significant extra area around the core and may include neighboring cores.

Drawing the Sector

Finding Cores

Once you have set your parameters, click-and-drag to draw a rectangle around the entire sector. If you need to navigate to bring your entire sector in to view, hold the Ctrl key down and use your mouse to zoom and pan.

An example display is illustrated below. (See Figure 4.11.)

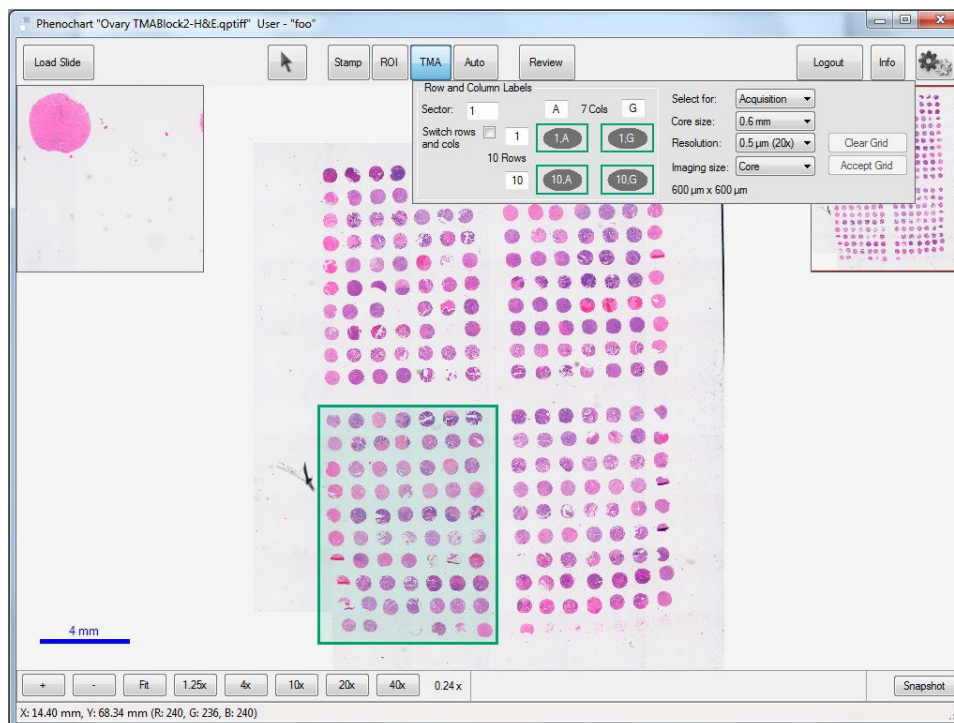


Figure 4.11. Drawing the sector display.

When you release your mouse, PhenoChart will find the cores within the rectangle. Each core will be labeled. If the labels aren't visible, zoom in to see them. (See Figure 4.12.)

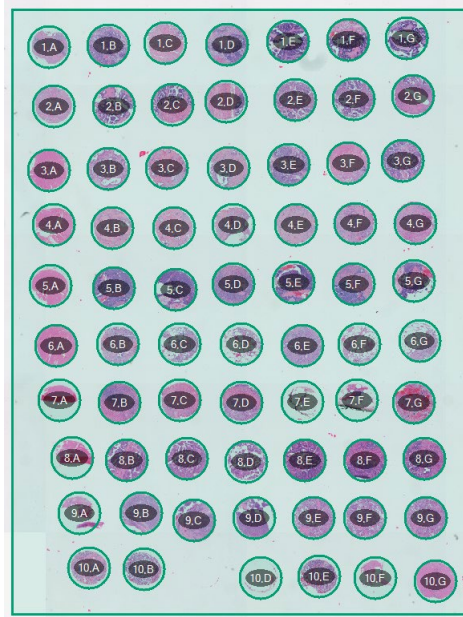


Figure 4.12. Labeled cores.

Adjusting the Cores

You can adjust your settings after the cores are found. The software will update immediately to the new settings.

If the core finding missed a core, you can right-click on the missing core location and click Add Missing Core. Select the missing core label from the drop-down list and click the OK button to add the core. (See Figure 4.13.)

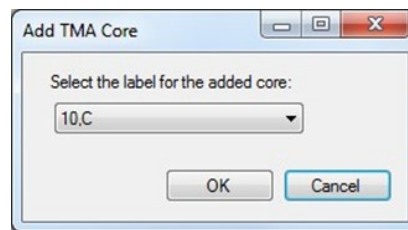


Figure 4.13. Add TMA Cores window.

There may be cases where you need to further refine the results of the core finding. To tweak the position of a single core annotation, hold the Ctrl key + click-and-drag on the annotation to move it. Be sure your settings are correct before adjusting cores. You will lose the individual core tweaks when you change your settings, and the system relocates the cores.

Right-click to ignore a core or to bring back an ignored core. You can also delete a core or delete the entire sector if necessary. (See Figure 4.14.)

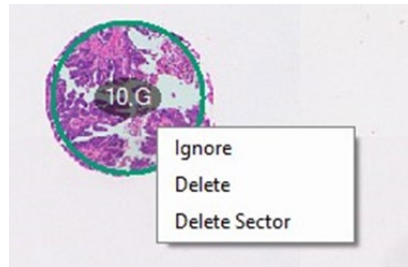


Figure 4.14. TMA right-click menu.

Accepting the Grid

Click Accept Grid to complete your TMA annotation. After this, no further changes can be made.

Click Clear Grid to reset the grid and start over by drawing a new sector rectangle.

You can continue to add additional sectors if desired/necessary.

Common Issues and Resolutions

Numbering

If the software finds more or fewer cores than you've drawn, you will see a warning in red. You may need to change your row and column labels so the number of cores found matches the number you selected.

If the sector, row, and column label of a new core is the same as that of another pending core, or if the software finds more or fewer cores than you have selected, you will see a warning in red. Change the sector, row, or column labels to correct the conflict.

Core Size

If the core finder provides drastically incorrect results, it usually means the core size is not set correctly. Try adjusting the Core Size so the size of the circles matches the size of your cores.

Fluorescence Layers

In Fluorescence, only visible layers will be used to find the cores. Turn off any layers that seem to be preventing the core finder from identifying the cores.

4.4 AUTOMATICALLY GENERATED

PhenoChart allows you to automatically select fields of interest using a trained inForm classifier. This will only be available on computers with an activated version of inForm Tissue Finder Version 2.2 or higher.

You can use inForm to add fields for acquisition or review.

Click the Auto button to open the editor. (See Figure 4.15.)

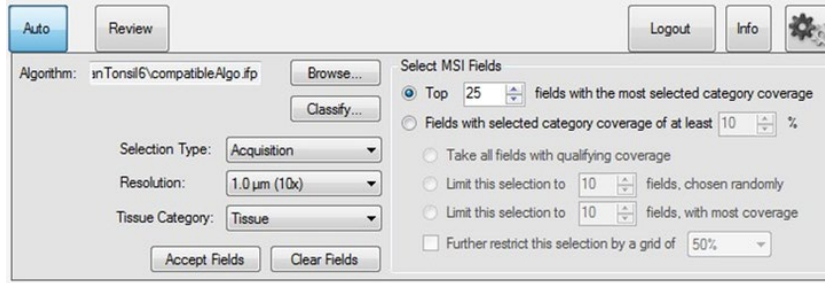


Figure 4.15. Auto Editor window.

Click the Browse... button to load an inForm algorithm or project that you've trained for this type of slide. If you haven't created an algorithm yet, [see Chapter 4.4.1 Training an inForm Classifier for Field Selection](#).

Phenochart will confirm that the algorithm is valid for your slide. This takes a few seconds.

Click Classify to classify the slide once the algorithm is validated. Depending on your algorithm settings and the size of your slide, this can take anywhere from a few to upwards of ten minutes.

Adjust the selection settings when the slide has been classified to see which fields will be chosen. (See Figure 4.16.)

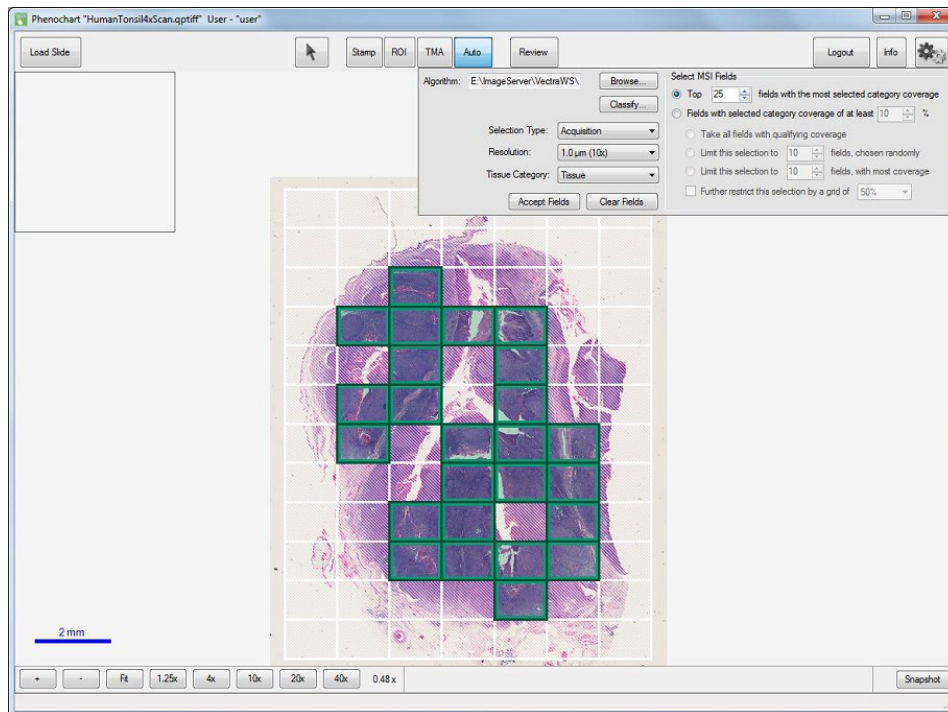


Figure 4.16. Classified Slide.

The fields on the screen update immediately as you change the selection settings.

Use Selection Type to identify whether the fields are for Acquisition or Review. Fields marked for review must be reviewed and accepted before they can be acquired by Phenolmager HT.

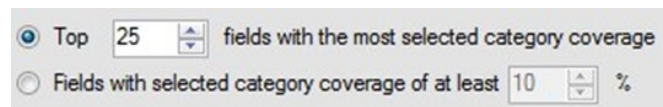
Choose the Resolution to be used when acquiring your fields.

Select the Tissue Category of interest from the Tissue Classifier in your inForm algorithm.

You will need to determine an MSI (multispectral imaging) Strategy to determine 1) how many fields you will take, and 2) how strong they are in the category of interest. This allows you to get the fields you are most interested in, with the most efficient use of time and disk space.

Two types of strategies are illustrated in Figure 4.17.

- Choose the top fields with the most selected category coverage.
- Choose fields with selected coverage of a set percentage.



The image shows a user interface for selecting MSI strategies. It features two radio button options. The first option, 'Top 25 fields with the most selected category coverage', is selected with a blue radio button. The number '25' is displayed in a small input field next to the text. The second option, 'Fields with selected category coverage of at least 10 %', is unselected with a grey radio button. The number '10' is displayed in a small input field next to the text, followed by a percentage sign.

Figure 4.17. Select MSI fields – MSI Strategies.

You can choose the top fields in your Tissue Category of interest. If you choose this, inForm will select the fields with the greatest coverage of your Category of Interest.

You can also choose from any fields that have at least some coverage of your Tissue Category. If you choose this, inForm will limit its selection of fields. Only fields that cover the required percentage (or greater) of the Tissue Category of interest will be considered.

If you choose the Fields with selected category coverage of at least X% option, you will need to choose what you want to take. (See Figure 4.18.)

- All the fields that meet the threshold.
- A selected number of randomly chosen fields that meet the coverage threshold.

NOTE: With this option, you may get fewer fields than requested.

- A selected number of fields with the most coverage of the Tissue Category of Interest. These fields must meet the minimum coverage threshold.

NOTE: With this option, you may get fewer fields than requested.

- Using a minimum threshold can result in a significant number of fields. You can further limit the selected fields to a grid by selecting the grid coverage.

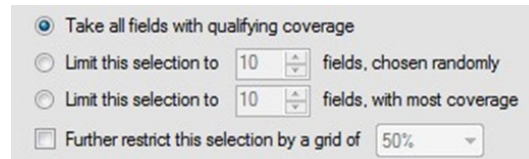


Figure 4.18. Coverage of % options.

As you adjust the settings listed above, your fields will update in real time to show you which fields meet your selection criteria. This is useful for classifier experimentation and testing for fully automated protocols on the legacy Vectra 3 system.

PhenoChart will show the fields that inForm has selected as well as the fields that were ignored (because they did not meet the selection criteria). If you would like to override the inForm decision, you can do so by using the right-click menu. The right-click menu gives you the percentage of your class of interest. Toggle to ignore or acquire/review that field.

When you are satisfied with the selected fields, click Accept Fields to make the selection permanent. Click Clear Fields to clear the selection and start over. The right-click menu will also have entries to accept fields and clear fields.

4.4.1 TRAINING AN INFORM CLASSIFIER FOR FIELD SELECTION

inForm Tissue Finder may be used from PhenoChart to automatically select Multispectral fields for acquisition by Phenolmager HT 2.0 including an optional review step.

PhenoChart is used to select fields for inForm training, and to validate the tissue classifier during development. inForm Tissue Finder version 3.0.0 or higher is required to train classifiers for use with PhenoChart.

To train a classifier in inForm, select the fields for training on your slide using PhenoChart's stamp tool. Within the Stamp tool, select Push to inForm, and select the fields that represent all tissue categories that you need. inForm will launch automatically and load the fields. You can load additional slides and stamp additional fields within PhenoChart.

When you have finished selecting fields within PhenoChart, switch to inForm to train your classifier:

1. Configure your project to have a Trainable Tissue Finding Step.
2. It is often beneficial to downsample your imagery. This makes the classification much faster and for 10x scans it can improve the results as well. Within the Prepare Images step, start with a downsampling of 4:1. If the classifier is not performing well, reduce the downsampling to 2:1. (See Figure 4.19.)

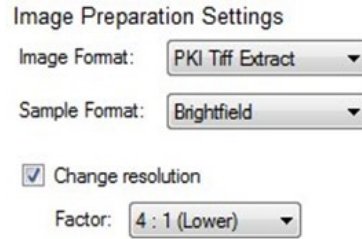


Figure 4.19. Prepare Images downsample.

3. In the Segment Tissue step, select your tissue categories and train your classifier.
4. In the Export step, check the Segmentation Maps (multi-image TIFF). (See Figure 4.20.)

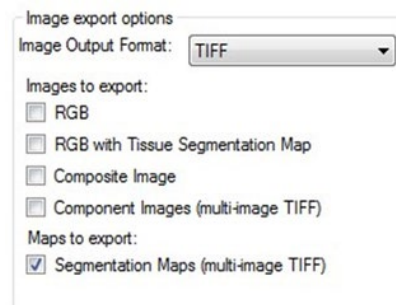


Figure 4.20. Maps to export Segmentation Maps.

5. Save your project. Saving the project within the study folder will make it easy to locate.

Return to PhenoChart if you would like to test out your classifier. Click Auto to open the automatic field editor, load your project, and click Classify. If you feel that the classifier needs more training, you can push additional fields into inForm and continue to train and test until you get satisfactory results.

4.5 ANNOTATION MENU OPTIONS

Use the Gear Menu to adjust the visibility and color of your annotations. (See Figure 4.21.)

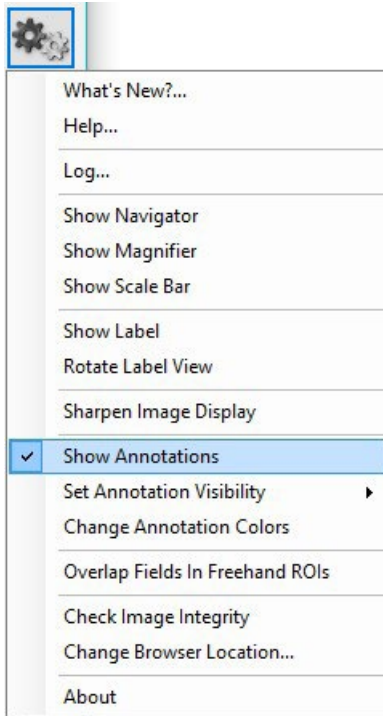


Figure 4.21. Gear menu > Show Annotations.

Click Show Annotations to see all annotations you have selected to be visible. (Note: See below for how to customize your visibility settings). Uncheck Show Annotations to hide all annotations on your slide.

Annotation Visibility

Use the Set Annotation Visibility submenu to customize visibility settings for each annotation type. (See Figure 4.22.)

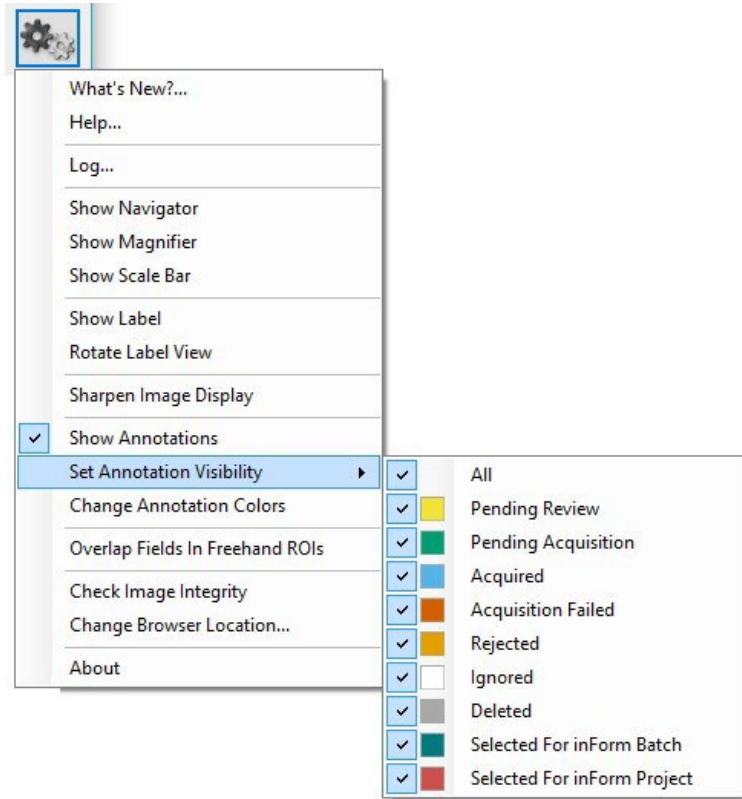


Figure 4.22. Gear menu > Set Annotation Visibility.

Check an annotation type to make it visible whenever annotations are shown. Uncheck it to hide all annotations of that type. Check All to turn all annotations on.

NOTE: When you first start PhenoChart, Ignored and Deleted annotations are hidden by default. Any changes that you make to the type of visibility will be remembered for the next session.

Annotation Colors

The default annotation colors are chosen to be distinguishable, even by most color-blind people. You can change the colors as needed. Select Change Annotation Colors from the Gear Menu to bring up the color changing dialog. (See Figure 4.23.)

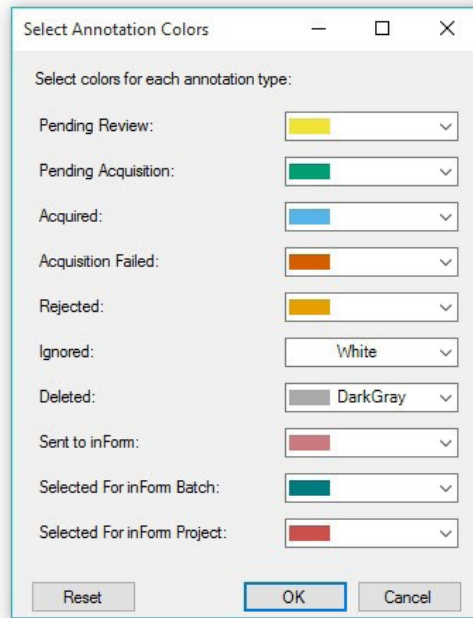


Figure 4.23. Select Annotation Color window.

Change the color by selecting any of the preset colors or choose Custom to create a custom color. Click Reset to restore the factory color-blind-safe defaults. Click OK to save your changes. Cancel will exit without saving your changes. Your color settings will be saved across sessions.

4.6 REVIEW

Annotations that have been selected for Review must be accepted before they will be acquired by Phenolmager HT. Click the Review button to open the review editor. (See Figure 4.24.)

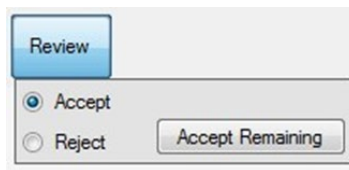


Figure 4.24. Review window.

Choose Accept to accept annotated fields or cores. When you move your mouse over an annotation awaiting review, the cursor will change to a green checkmark. (See Figure 4.25.)



Figure 4.25. Green checkmark.

Click the annotation to accept it. The annotation is now ready for acquisition.

Choose Reject to reject annotations. When you move your mouse over an annotation awaiting review, the cursor will change to a red x. (See Figure 4.26.)



Figure 4.26. Red x symbol.

Click the field to reject it. It will not be acquired.

Click Accept Remaining to accept all remaining un-reviewed fields. You will get a confirmation dialog telling you how many fields you are about to accept.

You can use the right-click menu to Accept or Reject fields and to Accept Remaining. You can also use the right-click menu to un-review a field or change your review of a field.

Appendix A: Support

Log File

When contacting Akoya Biosciences Customer Care about a PhenoChart issue, you will be asked for the log file. Including the log file with your initial report will aid in solving the problem.

1. Click the View tab.
2. Select Show hidden files, folders, or drives and then click the OK button.

If the issue has just occurred, select Log... from the Gear menu to obtain the current log file. (See Figure A.1.)

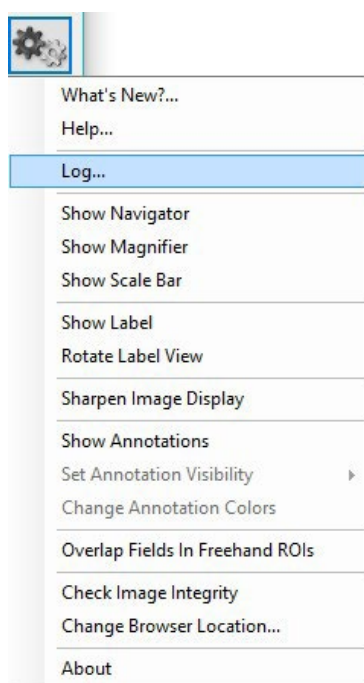


Figure A.1. Gear menu > Log...

If the issue occurred on a previous day, go to C:\Users\Public\Akoya\PhenoChart to find the appropriate log file in. Log files are named by date.

You can either attach the log file or cut and paste the log file contents into your correspondence.

Config File

In some cases, you may be asked for the PhenoChart.config file. You can find this file in C:\ProgramData\Akoya\PhenoChart\.

By default, this directory is hidden from view. Use your Windows browser to view this folder:

1. Select Organize and then Folder and search options. (See Figure A.2.)

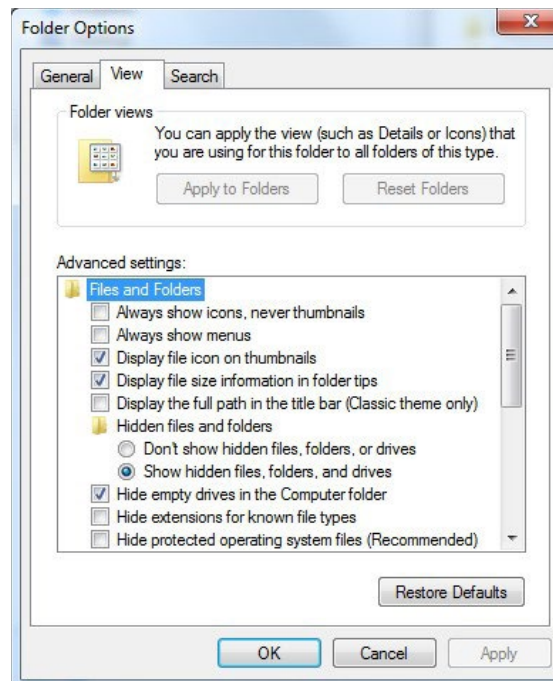


Figure A.2. Folder Options > View > Files and Folders.

Appendix B: Akoya QTIFF Specification

Background

This describes a TIFF format that Akoya Biosciences uses for its tissue images. The imagery may be a simple RGB image, a set of components extracted from a field or region, or a whole-slide scan. In the latter case, it may be a brightfield (BF) color RGB image or a multiband fluorescence (FL) image.

The goal is to use the same syntax and metadata for all these kinds of images and minimize the semantic distinctions where possible. Specifically, an extracted component represents signal attributed to a stain or fluorescent dye in a sample. The signal values incorporate image preprocessing such as normalization for exposure time and spectral unmixing when that technique is used.

Data format

The files are TIFF or BigTIFF images, depending on image size, with multiple images per file.

For images larger than about 2K x 2K pixels, tiled format is used, and the image is provided in several resolutions (pyramidal tiled images). Tile size is 512 x 512 pixels. Images smaller than 2K x 2K are stored in strips rather than in tiles.

The highest resolution (baseline) image(s) appear first in the file. For each resolution there are N baseline images where N depends on the contents. For BF images, N=1 and each image is an RGB image. For FL images or unmixed component images, N = number of bands, which is usually > 1, and each image is a grayscale image. The sample format for grayscale raw images can be unsigned 8-bit or unsigned 16-bit. For grayscale unmixed images, the sample format can be unsigned 16-bit, signed 16-bit, or 32-bit float.

A thumbnail RGB image is provided, and this is a good image to use as an icon in graphical image lists. Note that the thumbnail can be used for qualitative sample identification, but should not be used to assess relative brightness of different images. This comes after the baseline images, meaning it is the second image in BF (RGB) images, and the (N+1)st image for FL images or unmixed component images.

Next come the reduced-resolution images (if present). The pyramid contains enough levels that the image size is no larger than 2K x 2K at the coarsest resolution.

For whole-slide scans, there are two more non-tiled images after these: an optional RGB image of the label, and a macro (low-resolution) RGB image of the whole slide.

Overall, the arrangement is:

Table B.1. Images

DESCRIPTION	RGB / MONO	TILE / STRIP	RESOLUTION	NOTES
Baseline image	Varies	Varies	Full	Tiled if > 2K x 2K. RGB for BF, else mono.
More full-resolution images	Mono	Varies	Full	If N > 1.
Thumbnail	RGB	Strip	~500 x 500	
Half-resolution images	Varies	Varies	Half	Only if baseline is tiled.
Quarter, eighth, etc.	Varies	Varies	Quarter, eighth, etc.	Continues until 2K x 2K or smaller.
Macro (overview) image of whole slide	RGB	Strip	~2000 x 4000	Required for whole-slide scans. Optional for simple RGB images and extracted components.
Label image	RGB	Strip	~500 x 500	Optional, whole-slide scans.

Detection

Readers can recognize Akoya tissue images via the contents of the “Software” TIFF tag (see below). The file suffix is .qptiff for whole slide scans. Some TIFF tags contain “PerkinElmer” for historical reasons; they remain to retain 3rd party reader compatibility.

Metadata

Metadata is contained in two locations: within standard TIFF tags as listed in Table B.2. below, and within the ImageDescription string, using a set of XML tags described below. These are provided for each image (IFD) in the file and describe that image rather than the baseline image or the scan as a whole. The ScanProfile tag is only provided on the first, baseline image as it may be large.

Table B.2. TIFF tags

TIFF TAG	OPTIONAL?	DESCRIPTION OF CONTENTS
Software	No	Starts with PerkinElmer-QPI.
ImageDescription	No	Further metadata in XML format (see next section).
ImageWidth	No	Width of the image in pixels.
ImageLength	No	Height of the image in pixels.
ResolutionUnit	No	Unit used for resolution and position (see below).

TIFF TAG	OPTIONAL?	DESCRIPTION OF CONTENTS
XResolution	No	Pixel X resolution (see below).
YResolution	No	Pixel Y resolution (see below).
XPosition	Yes	Sample X location in ResolutionUnits. This is ULHC location except for Macro image which reports its image center.
YPosition	Yes	Sample Y location in ResolutionUnits. This is ULHC location except for Macro image which reports its image center.
SampleFormat	No	Integer (1) for BF, FL; or float (3) for extracted components.
SMinSampleValue	No	Minimum signal value in the image.
SMaxSampleValue	No	Maximum signal value in the image.
BitsPerSample	No	8 (FL); 8, 8, 8 (RGB); or 32 (unmixed component).
SamplesPerPixel	No	1 (FL or unmixed component) or 3 (RGB).
NewSubfileType	No	0 for full-resolution images, 1 for reduced res images.
TileWidth	Yes	Tile width (512) if tiled format is used.
TileLength	Yes	Tile height (512) if tiled format is used.
TileOffsets	Yes	List of tile offsets, if tiled format is used.
TileByteCounts	Yes	Size of each (compressed) tile, if tiled format is used.
StripOffsets	Yes	List of strip offsets, if tiled format is not used.
RowsPerStrip	Yes	Number of rows per strip, if tiled format is not used.
StripByteCounts	Yes	Size of each (compressed) strip, if tiled format is not used.
PlanarConfiguration	No	1 (chunky) for RGB images, 2 (planar) otherwise.
PhotometricInterpretation	No	2 (RGB) for RGB images, 1 (BlackIsZero) otherwise.
DateTime	No	Acquisition time.
Compression	No	May be None, CCITT Group 3, PackBits, LZW, or JPEG.
JPEG fields	Yes	JPEG fields are defined when JPEG compression is used.

ResolutionUnit, XResolution and YResolution are required fields in a valid TIFF file. When the true resolution of the image is known, ResolutionUnit will be 3 (cm) and XResolution and YResolution will be pixels/cm. When the true resolution is not known, ResolutionUnit

will be 2 (inch) and XResolution and YResolution will be 96 (pixels/inch). Pixels from Akoya Biosciences instruments are always square so XResolution and YResolution will always have the same value.

The TIFF spec is not explicit about the data type and value for SMinSampleValue and SMaxSampleValue; the writer uses the same data type as the image pixels (byte or float).

Image Description contents

The ImageDescription tag contains a string in XML format. The string contains a top-level < PerkinElmer-QPI-ImageDescription> element. Nested within this element are child elements with the tag names and values as listed in the table below. Elements appear in the order listed. Values are stored as text content of the element. Elements are required unless otherwise specified. See Table B.3.

Table B.3. Image Description tags

TAG	OPTIONAL?	CONTENTS
DescriptionVersion	No	Version of the image description field itself, a single number. This document describes version 5 of the field.
AcquisitionSoftware	No	Software used to acquire the image.
Identifier	No	GUID in string format. This is an identifier for the image file itself.
SlideID	Yes	ID of the slide that this image was taken from.
SampleDescription	Yes	Text description of the sample.
OperatorName	Yes	Name of the operator as reported by the operating system running the acquisition software.
Barcode	Yes	Barcode text of the slide this image was taken from.
ComputerName	Yes	Name of the computer on which the slide was scanned.
ImageType	No	A string identifying the type of image within the file (Table B.1) with the following values: <ul style="list-style-type: none"> • FullResolution • ReducedResolution • Thumbnail • Overview • Label

TAG	OPTIONAL?	CONTENTS
IsUnmixedComponent	No	True for unmixed multispectral images, otherwise False.
ExposureTime	No	Exposure time as an integer number of microseconds. For unmixed images, this is the exposure time for the dominant wavelength band for the component (FL); or the brightest wavelength in the cube (BF).
SignalUnits	No	A byte <code>www ttt</code> where the <code>ttt</code> nibble indicates the signal unit type from the following: <ul style="list-style-type: none"> raw counts normalized (counts/second/gain/full-scale/binning) OD (optical density) dark-corrected counts and the <code>www</code> nibble indicates how the signal is weighted across the spectral bands (or colors): <ul style="list-style-type: none"> average across all bands total summed signal across all bands peak signal in highest-valued band Thus, for example, a value of 68 (hex 44) encodes OD units with peak-signal weighting.
Name	Yes	Band name for FL whole slide scans, component name for extracted components. Not present for RGB images.
Color	Yes	Color to use when rendering this band, as decimal R, G, B byte triplet. Present for FL whole slide scans or extracted components. Not present for RGB images.
Responsivity	Yes	Instrument responsivity, if available, for FL whole-slide and unmixed images. See below for details.

TAG	OPTIONAL?	CONTENTS
Objective	Yes	Objective name, if known, otherwise not present. This does not imply a specific pixel resolution – see the XResolution and YResolution standard TIFF tags instead.
ScanProfile	Yes	Element containing scan and/or and unmix parameters. It is valid XML whose contents are opaque to most readers. It is only provided on the first (baseline) image and is omitted from all other IFDs.
ProtocolName	Yes	Name of the acquisition protocol.
ValidationCode	No	Used for internal data integrity checks – readers can ignore this.
ImagingCycle	Yes	Zero-based acquisition cycle for CO-DEX imagery.
Biomarker	Yes	String indicating target such as CD8, Ki67, etc.
ScaleFactor	Yes	After allowing for bit-depth conversion, pixel values in this dataset have been scaled by this factor. For example, if a 12-bit image is stored in an 8-bit image, and counts were scaled from 4095 to 255, this would be 1; if counts were scaled from 3102 to 255, ScaleFactor would be 1.3201. Default is 1.

TAG	OPTIONAL?	CONTENTS
NormalizedUnitsFactor	Yes	<p>For unmixed data, multiplying the pixel value by this factor will yield component signal in units of normalized counts.</p> <p>Normalized counts are defined by:</p> <p>Where:</p> <ul style="list-style-type: none"> • FSC is the camera full-scale value in counts • B is the binning • G is the camera gain • E is the exposure time, in seconds • WDF is a well-depth factor for the sensor used • SW is a spectral weighting factor that accounts for the total energy associated with that component's spectrum across all bands.
AutofluorescenceSubtracted	Yes	<p>String containing 'true' or 'false', indicating whether this dataset has been subtraction-corrected by a background (autofluorescence) image. Treated as 'false' by default.</p>
LampType	Yes	<p>String with the FL lamp type:</p> <ul style="list-style-type: none"> • Unknown (default) • PriorLumen200 • PriorLumen220 • XCiteMultiBandLed • PriorMimicLed • SolaLed • Colibri7
InstrumentType	Yes	<p>String with the instrument type, one of the following:</p> <ul style="list-style-type: none"> • Unknown (default) • Keyence700 • Keyence800 • AxioObserver • Dmi8 • PhenoImager HT

TAG	OPTIONAL?	CONTENTS
CameraType	Yes	String with the camera sensor type, one of the following: <ul style="list-style-type: none"> • SonyICX285AL • HamamatsuC11440 • IMX265 • IMX421
ExcitationFilter	Yes	Description of excitation filter with sub-elements listed below. If present, all its sub-elements must be provided.
Name	No	String with the filter public name.
Manufacturer	No	String with the manufacturer or OEM supplier.
PartNo	No	String with the manufacturer part number.
Bands	No	Collection of bands each as described below.
Band	No	Description of a band with child tags noted below.
Name	No	String with the band name.
Cuton	No	Cuton wavelength of this band in nm.
Cutoff	No	Cutoff wavelength of this band in nm.
Active	No	True if this band is excited, False if not.
EmissionFilter	Yes	Description of emission filter with sub-elements listed below. If present, all its sub-elements must be provided.
Name	No	String with the filter public name.
Manufacturer	No	String with the manufacturer or OEM supplier.
PartNo	No	String with the manufacturer's part number.
Bands	No	Collection of bands, each described by sub-elements below.
Band	No	Description of a band with sub-elements noted below.
Cuton	No	Cut-on wavelength of this band in nm.

TAG	OPTIONAL?	CONTENTS
Cutoff	No	Cut-off wavelength of this band in nm.
CameraSettings	Yes	Description of the camera settings used for this image with sub-elements listed below. If present, all its sub-elements must be provided.
Gain	No	Gain factor, such as 1, 3, etc.
Binning	No	Binning extent, such as 1 for full-resolution, 2 for 2x2 binning.
OffsetCounts	No	Offset count setting for the camera.
BitDepth	No	Bit resolution used to read out the camera. This may differ from the resolution used to store this image (for example, it may be acquired with 12-bit resolution but saved in a QTIFF file with 8-bit resolution).
Orientation	No	String containing one of the following values: <ul style="list-style-type: none"> • 'Normal' (default) • 'Rotate180' • 'MirrorV' • 'MirrorH'
ROI	Yes	Description of the sensor ROI contributing to the image. Coordinates are sensor pixels before binning. Even if the CameraSettings are provided, this field is optional.
X	No	X origin of ROI. Left-most pixel in sensor array is 0.
Y	No	Y origin of ROI. Top-most pixel in sensor array is 0.
Width	No	Width of ROI.
Height	No	Height of ROI.

For whole slide images (BF and FL), SignalUnits will be 64 (hex 40) (raw counts, peak signal). For unmixed images, SignalUnits will reflect the unmix settings.

Instrument Responsivity

The <Responsivity> tag is a container for a list of normalized instrument response values. This tag is present for whole-slide FL images from the Mantra, legacy Vectra 3 system,

Phenolmager HT, or Fusion and unmixed FL multispectral images originating from the legacy Vectra 3 system and Phenolmager HT.

For whole-slide images, the <Responsivity> tag will contain one <Filter> tag. The < Filter> tag contents will be different for each image within the TIFF file, reflecting the filter used to take the image.

For unmixed component images, the <Responsivity> tag will contain one <Band> tag for each band in the original image file. The <Band> tags are repeated for each unmixed component image.

The overview, thumbnail and label images do not have <Responsivity> tags.

Each <Filter> or <Band> tag describes the instrument responsivity for acquisitions using that filter or band. Their contents are described in Table B.4.

Table B.4. Contents of <Filter> or <Band> tags within <Responsivity> tag

TAG	CONTENTS
Name	The name of the filter (whole-slide image) or band (component image).
Response	The instrument response to the reference artifact, normalized for exposure. This is raw counts / (2 ^{bit depth} × exposure time × gain × binning area), where bit depth is the bit depth of the imagery, exposure time is in seconds, gain is the gain setting of the camera, and binning area is 1 for 1×1, 4 for 2×2, etc.
Date	The date and time of the reference image in UTC, ISO 8601 format.
FilterID	Detailed description of the acquisition filter, as {ExcitationFilter band name}_ {ExcitationFilter manufacturer}:{ExcitationFilter part number} / {EmissionFilter manufacturer}:{EmissionFilter part number} The {ExcitationFilter band name}_ prefix may be omitted if there is only a single excitation band.

Sample ImageDescription

Sample ImageDescription for the DAPI band of a FL whole-slide image, containing a single <Filter> tag:

```
<?xml version="1.0" encoding="utf-8"?>
<PerkinElmer-QPI-ImageDescription >
  <DescriptionVersion>1</DescriptionVersion>
  <AcquisitionSoftware>VectraScan 1.0.0</AcquisitionSoftware>
  <ImageType>FullResolution</ImageType>
```

```

<Identifier>AABED946-BB58-44FB-95B3-48E177E3BB83</Identifier>
<IsUnmixedComponent>False</IsUnmixedComponent>
<ExposureTime>50</ExposureTime>
<SignalUnits>64</SignalUnits>
<Name>DAPI</Name>
<Color>0,0,255</Color>
<Responsivity>
  <Filter>
    <Name>DAPI</Name>
    <Response>30.7</Response>
    <Date>2015-10-22T13:10:18.0618849Z</Date>
    <FilterID>Semrock:FF02-409/LP-25 Emission / Semrock:FF01-377/50-25
Excitation</FilterID>
  </Filter>
</Responsivity>
<Objective>4x</Objective>
<ScanProfile><!-- this will be a serialized scan protocol. It is valid
XML but otherwise opaque -->
</ScanProfile>
<ValidationCode>4281ff86778db65892c05151d5de738d</ValidationCode>
</PerkinElmer-QPI-ImageDescription >

```

Appendix C: Akoya Software Version Compatibility

Some files are read by multiple Akoya acquisition and analysis software programs to facilitate unmixing and analysis workflows. Due to major updates in the combined release of Phenomager HT 2.0.0, Phenochart 2.0.0, and inForm 3.0.0 software, outputs from these versions are not backward compatible with previous versions of software. However, files from previous versions of software are forward compatible. These compatibility considerations are detailed in Table C.1.

Table C.1. Akoya software compatibility considerations

	VectraPolaris 1.x.x	Phenomager HT 2.x.x	Phenochart 1.x.x	Phenochart 2.x.x	inForm 2.x.x	inForm 3.x.x
Scanning protocols created in VectraPolaris 1.x.x can be opened by	Yes	Yes	N/A	N/A	N/A	N/A
Scanning protocols created in Phenomager HT 2.x.x can be opened by	No	Yes	N/A	N/A	N/A	N/A
QPTIFF images acquired with VectraPolaris 1.x.x can be opened by	N/A	N/A	Yes	Yes	Yes	Yes
QPTIFF images acquired with Phenomager HT 2.x.x can be opened by	N/A	N/A	Some *	Yes	No	Yes
IM3 images acquired with VectraPolaris 1.x.x can be opened by	N/A	N/A	N/A	N/A	Yes	Yes
IM3 images acquired with Phenomager HT 2.x.x can be opened by	N/A	N/A	N/A	N/A	Yes	Yes
Annotations created by Phenochart 1.x.x can be opened by	Yes	Yes	Yes	Yes	Yes	Yes
Annotations created by Phenochart 2.x.x can be opened by	No	Yes	No	Yes	No	Yes

Algorithms created by inForm 2.x.x can be opened by	N/A	N/A	N/A	N/A	Yes	Yes
Algorithms created by inForm 3.x.x can be opened by	N/A	N/A	N/A	N/A	No	Yes
Projects created by inForm 2.x.x can be opened by	N/A	N/A	N/A	N/A	Yes	Yes **
Projects created by inForm 3.x.x can be opened by	N/A	N/A	N/A	N/A	No	Yes
Spectra extracted in inForm 2.x.x can be exported as an unmixing library for HT 2.x.x by	N/A	N/A	N/A	N/A	No	Yes
Spectra extracted in inForm 3.x.x can be exported as an unmixing library for HT 2.x.x by	N/A	N/A	N/A	N/A	No	Yes

* Standard 8-bit QTIFFs can be opened for viewing, but not all annotation features are available. Extended Range or Unmixed QTIFFs cannot be opened.

** Two considerations:

1. Due to slight changes to the assignment of cell IDs, phenotyping training sets from inForm 2.x.x cannot be used for re-training phenotyping classifiers in inForm 3.x.x. In this case, inForm 3.x.x will post a message that you can run the algorithm's phenotype step with its current training, or use inForm 2.8 or earlier to add training cells or revise phenotyping.
2. For projects created in inForm 2.8 with imagery that has 10+ channels, phenotyping computation may become unusably slow in inForm 3.x.x. It is recommended that inForm 2.8 projects with imagery that has 10+ channels are only run in inForm 2.8. New projects created in inForm 3.0 do not have this issue.

Appendix D: Phenochart EULA

The following is an agreement (the "Agreement") between you and Akoya Biosciences, Inc., 100 Campus Drive, 6th Floor, Marlborough, MA 01752 ("Akoya") for software known as Research Use Only Phenochart, including any updates, upgrades or enhancements provided by Akoya and any and all accompanying documentation (collectively, the "Software").

AKOYA IS WILLING TO LICENSE THE SOFTWARE TO YOU ONLY UPON THE CONDITION THAT YOU ACCEPT ALL OF THE TERMS CONTAINED IN THIS AGREEMENT. PLEASE READ THE TERMS CAREFULLY BEFORE USING THE SOFTWARE, AS INSTALLING AND/OR USING THE SOFTWARE WILL INDICATE YOUR AGREEMENT TO THEM. IF YOU DO NOT AGREE TO ALL OF THE TERMS AND CONDITIONS IN THIS AGREEMENT, AKOYA IS NOT WILLING TO LICENSE THE SOFTWARE TO YOU, AND YOU (1) MAY NOT USE THE SOFTWARE AND (2) MUST CONTACT AKOYA WITHIN THREE (3) DAYS OF RECEIPT TO ARRANGE FOR THE RETURN OF THE SOFTWARE. BY INSTALLING AND/OR USING THE SOFTWARE, YOU ALSO REPRESENT AND WARRANT THAT YOU HAVE THE AUTHORITY TO AGREE TO AND BE BOUND BY THE TERMS OF THE AGREEMENT.

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other proprietary rights notice in or on the Software. This Agreement may not be assigned in whole or in part, except that either party may assign this Agreement without the other party's prior written consent in the event of a merger with an affiliate or other reorganization with an affiliate. Any attempted assignment contrary to the terms of this Agreement shall be void.

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As between the parties, Akoya owns all right, title and interest, including all existing or future copyrights, trademarks, tools, know-how, and processes, in and to the Software and any copies, modifications, alterations or derivative works thereof. You shall have no title to or ownership of the Software. As between the parties, Akoya shall retain all rights to market, license and/or sell the Software. The Software (including accompanying documentation) constitutes the unpublished confidential information and trade secrets of Akoya. You (i) acknowledge, and agree not to contest, Akoya's rights in the Software; and (ii) agree not to disclose and to take reasonable steps to protect any and all confidential information of Akoya regarding the Software or that is otherwise disclosed to you in connection with this Agreement. You agree that you are responsible for ensuring your employees and agents are prohibited from taking any action otherwise prohibited to you under this Agreement and to notify Akoya promptly and in writing of the circumstances surrounding any possession, use or knowledge of the Software or any part thereof known to you which is prohibited hereunder. You recognize that the covenants contained in this Agreement are reasonable and necessary to protect the legitimate interests of Akoya, that Akoya would not have entered into this Agreement in the absence of such covenants, and that your breach or threatened breach of such covenants shall cause Akoya irreparable harm and significant injury, the amount of which shall be extremely difficult to estimate and ascertain, thus making any remedy at law or in damages inadequate. Therefore, you agree that Akoya shall be entitled, without the necessity of posting any bond or security, to the issuance of injunctive relief by any court of competent jurisdiction enjoining any breach or threatened breach of such covenants and for any other relief such court deems appropriate. This right shall be in addition to any other remedy available to Akoya at law or in equity.

4. Termination.

The license granted in Section 1 above is effective until terminated. This Agreement is conditioned upon your continued compliance with the terms and conditions hereof and will terminate automatically without notice from Akoya if you fail to comply with any term or condition of this Agreement. Furthermore, Akoya may terminate this Agreement at any time upon thirty (30) days' notice. Upon termination of this Agreement, your license to use the Software or any part thereof shall immediately terminate, and you shall immediately destroy all copies of the Software (including all accompanying documentation) and any other confidential and proprietary information you have received during or in connection with this Agreement. Termination of this Agreement by Akoya shall not entitle you to a refund of any fees paid for the license of the Software.

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