AKOYA BIOSCIENCES®
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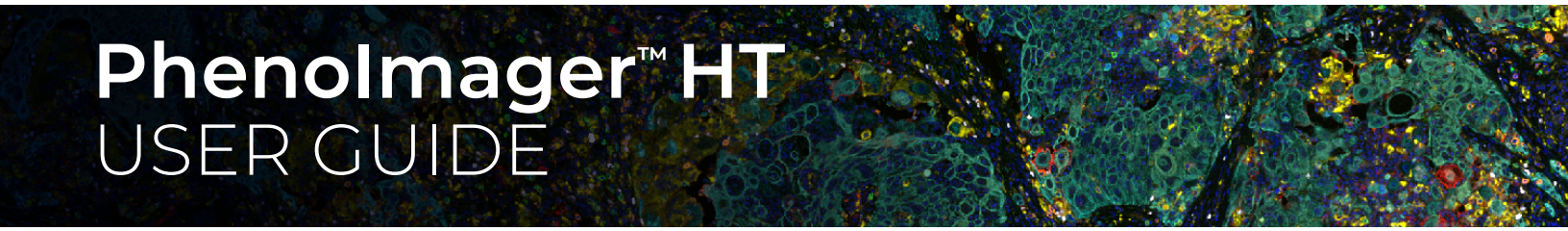
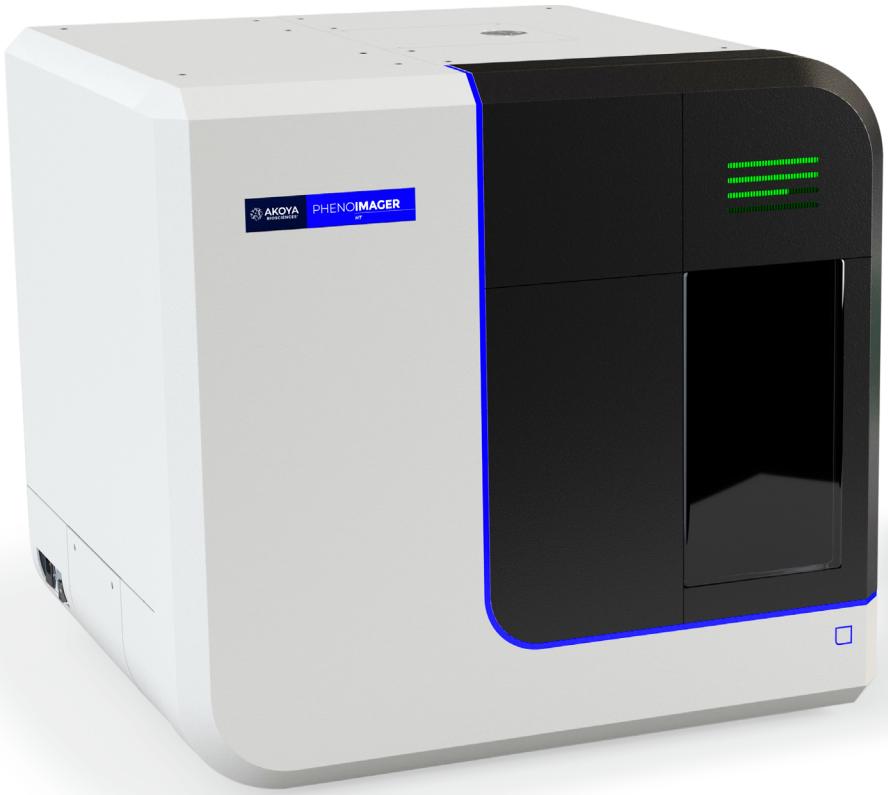
PhenoImager™ HT
USER GUIDE





An Automated Quantitative Pathology Imaging System

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PHENOIMAGER HT 1.0.13 | 2022

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This manual describes how to use PhenoImager HT (formerly Vectra Polaris) software.

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1 Preface

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Content

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WARNINGS:

- To reduce the risk of electric shock, do not remove the instrument panels. No user serviceable parts are inside. Refer to qualified service personnel if help is required.
- Use this product only in the manner described in this manual. If the equipment is used in a manner not specified by the manufacturer, the protection provided by the equipment may be impaired.

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If you have a question about a product that is not answered in this manual or online help, or if you need assistance regarding this product, or for information about training for this product, please contact Akoya Customer Care:

Phone: +1 855-896-8401

Fax: +1 855-404-0061

Email: CustomerCare@akoyabio.com

Email (outside the US): CustomerCareEMEA@akoyabio.com

Website: www.akoyabio.com

Before you call, have the following information available for the technical representative:

- Product serial number
- Software version (found by choosing About from the main Help menu)

Product Service and Customer Support Plans

Akoya offers a full range of services to ensure your success. From our original factory warranty through a comprehensive line of customer support plans, Akoya offers you Field Service Engineers and in-house Specialists who are dedicated to supporting your hardware, software, and application development needs.

Our programs can include such useful services as:

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- Diagnostic servicing performed on-site by Akoya field service engineers or remotely via Technical Support
- Validation performed on-site by Akoya field service engineers
- Extended use of the Akoya Technical Support Center
- Software updates
- Parts, labor, and travel expense coverage
- Other customized services upon request

CE

This device complies with all CE rules and requirements.

NOTE: Changes or modifications to this equipment not expressly approved by the party responsible for compliance could void the user's authority to operate the equipment.

REMARQUE: Tout changement ou modification apporté à cet instrument non expressément approuvé par l'entité responsable de la conformité peut annuler l'autorisation d'opérer l'appareil accordée à l'utilisateur.

FCC

RADIO AND TELEVISION INTERFERENCE
NOTE: This equipment has been tested and found to comply with the limits for a Class A digital device, pursuant to Part 15 of the FCC rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment. This equipment generates, uses and can radiate radio frequency energy and, if not installed and used in accordance with the instruction manual, may cause harmful interference to radio communications. Operation of this equipment in a residential area is likely to cause harmful interference in which case the user will be required to correct the interference at his own expense.
In order to maintain compliance with FCC regulations shielded cables must be used with this equipment. Operation with non-approved equipment or unshielded cables is likely to result in interference to radio & television reception.

KC

This device complies with MSIP (Ministry of Science, ICT, and Future Planning) EMC Registration requirements. This instrument is registered as a Class A instrument for business use only. Product seller and user should notice that this equipment is not for household use.

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이 기기는 업무용으로 전자파적합등록을 한 기기이오니
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잘못판매 또는 구입하였을 때에는 가정용으로 교환하시기
바랍니다.

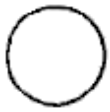
Table of Symbols

Table 1 7 contains symbols that identify particularly important information and alert you to the presence of hazards. These symbols may appear in this manual and/or on the product it describes.

Table 1. Important Symbols

Symbol **Symbole**	**Description** **Description**
	**DANGER:** An imminently hazardous situation, which, if not avoided, will result in death or serious injury. **DANGER:** Situation présentant un danger imminent qui, s'il n'est pas éliminé, peut entraîner des blessures graves, voire la mort.
	**WARNING:** Caution. Refer to the User's documentation. (ISO 7000-0434B) **AVERTISSEMENT:** Attention. Se reporter à la documentation de l'utilisateur.
	**NOTE:** A cautionary statement; an operating tip or maintenance suggestion; may result in instrument

	damage if not followed. **REMARQUE:** Énoncé indiquant une précaution à prendre, un conseil de fonctionnement ou une suggestion d'entretien; son non-respect peut provoquer des dommages à l'instrument.
	Hazardous voltage; risk of electric shock. (IEC 60417-6042) Tension dangereuse; risque de blessure par électrocution.
	Crush hazard. Risk of body parts, hair, jewelry, or clothing getting caught in a moving part. (ISO 3864) Danger d'écrasement. Faire attention que les parties corporelles, les cheveux, les bijoux ou les vêtements ne soient pas pris dans une pièce mobile.
	Risk of fire. (ISO 3864) Risque d'incendie.
	Risk of explosion. (ISO 3864) Risque d'explosion.
	Lifting hazard. May result in injury. (ISO 3864) Levage dangereux. Peut entraîner des blessures.
	Protective ground symbol. (IEC 60417-5019) Symbole de terre de protection.
	Ground symbol. (IEC 60417-5017) Symbole de terre.
	Fuse. (IEC 60417-5016) Fusible.
	Alternating current. (IEC 60417-5032) Courant alternatif.
	On (power). |(IEC 60417-5007) Marche (alimentation).

	Off (power). (IEC 60417-5008) Arrêt (alimentation).
	CE compliance mark. Marque de conformité CE.
	Serial Number (ISO 7000-2498) Numéro de série.
	Date of Manufacture. (ISO 7000-2497) Date de fabrication.
	WEEE symbol (EN50419:2005). Do not dispose of as unsorted municipal waste.
	Unified Korea Certification Mark (KC Mark)
	Signifies that the unit has passed safety tests for grounding, power line transience, and current leakage. Signifie que l'appareil a réussi les tests de sécurité pour la mise à la terre, le courant transitoire de ligne d'alimentation et la perte de courant.
	Input. Entrée.
	Output. Sortie.
Equipment labels are color coded: Les étiquettes de l'appareil sont codées couleur:	Yellow Caution, risk of danger Red Stop Blue Mandatory action Green Safe condition or information Jaune Attention, danger potentiel Rouge Arrêter Bleu Intervention obligatoire Vert Condition sûre ou informations de sécurité

2 Instrument Safety

The following safety information about the PhenoImager HT is included in this documentation. Read and review all safety information before operating the PhenoImager HT.

2.1 Required Training

Ensure that all personnel involved with the operation of the instrument have:

- Received instruction in general safety practices for laboratories.
- Received instruction in specific safety practices for the instrument.

WARNING

Use this product only in the manner described in this manual. If the equipment is used in a manner not specified by the manufacturer, the protection provided by the equipment may be impaired.

2.2 Electrical Safety

The PhenoImager HT is powered by a 100-120VAC/200-240VAC, 50-60Hz (±10%) input power supply.

The wall outlet or the power cable connector on the left side of the instrument should be accessible after the system's installation, to enable trained service personnel to safely disconnect power from the system during servicing.

WARNING

Do not operate the system in an environment with explosive or flammable gases.

WARNING

- *DO NOT remove instrument covers. There are no user serviceable parts inside. The covers are intended to be removed only by qualified Akoya Biosciences or PerkinElmer service personnel; they are not intended to be removed during operation or for maintenance by users. Contact Akoya Biosciences technical support if help is required (see Contact Us 6).*
- *Do not operate the system if there has been a malfunction of the system door or slide loading components. Contact Akoya Biosciences technical support if help is required (see Contact Us 6).*
- *Do not operate the system in places where it may be splashed with liquid.*

For further electrical safety information, refer to the following sections:

- Power Cord Selection 12
- Fuses 12
- Cables and Adapters 13

2.2.1 Power Cord Selection

Contact Akoya Biosciences Technical Support (see Contact Us 6) to order replacement power cords.

WARNING

- *Use only the power supply cord set provided with the PhenoImager HT system. If the correct cord set for the location was not provided, contact Akoya Biosciences Technical Support (see Contact Us 6) for a replacement. Do not use power supply cords with inadequate ratings.*
- *Use only a properly grounded power outlet when connecting the system to power.*

2.2.2 Fuses

Contact Akoya Biosciences Technical Support (see Contact Us 6) to order replacement fuses.

WARNING

The fuses in this instrument are only replaceable by trained Akoya Biosciences or PerkinElmer personnel.

2.2.3 Cables and Adapters

Some cables and adapters supplied with the system have proprietary specifications.

WARNING

Do not connect components supplied by Akoya Biosciences using unqualified cables or adapters. Contact Akoya Biosciences technical support (see Contact Us 6) to order replacement cables and adapters.

2.3 Mechanical Safety

WARNING

Instrument components may move during operation. Always keep body parts, hair, jewelry, and clothing away from the instrument during operation.

WARNING

Procedures which could result in injury may be performed only by operators who have been warned of the potential hazards and have received adequate training in performing the procedures in the safest possible manner.

2.4 Weight Warning

WARNING

LIFTING HAZARD. The PhenoImager HT instrument weighs 185 lbs. (84 kg). Do not move the PhenoImager HT instrument. Installing, servicing, and moving the PhenoImager HT instrument should be performed only by qualified Akoya Biosciences or PerkinElmer service personnel. Contact Akoya Biosciences technical support if help is required (see Contact Us 6).

2.5 Bright Light

WARNING

BRIGHT LIGHT HAZARD. The interior of the PhenoImager HT system includes a barcode reader with a Class 2 LED Light. Do not look into the bright light to avoid eye injury.

CAUTION - CLASS 2 LASER RADIATION WHEN OPEN
DO NOT STARE DIRECTLY INTO THE BEAM

3 Introduction

This manual describes the use and functionality of the PhenoImager HT Automated Quantitative Pathology Imaging System. It includes operating instructions, functional descriptions, troubleshooting, illustrations, and other relevant information.

This section of the manual contains the following topics:

3.1 Intended Use

The PhenoImager HT is a multimodal digital pathology instrument that integrates both multispectral analysis and automated slide scanning that allows researchers to visualize, analyze, quantify and phenotype immune cells in situ in FFPE tissue sections and TMAs.

NOTE: *Akoya's PhenoImager HT Imaging System is for research use only. Not for use in diagnostic procedures.*

3.2 Principles of Operation

Akoya's PhenoImager HT is an automated imaging system for performing whole slide scans of tissue sections and microarrays (TMAs), and for acquiring multispectral (MSI) regions of interest. The system has been optimized to image samples stained with Akoya's Opal™ multiplexed fluorescent immunohistochemistry reagent kits and is also compatible with typical brightfield staining reagents.

The PhenoImager HT is configured to store and inventory up to 20 slide carriers, each holding up to 4 tissue slides, for a total of 80 slides.

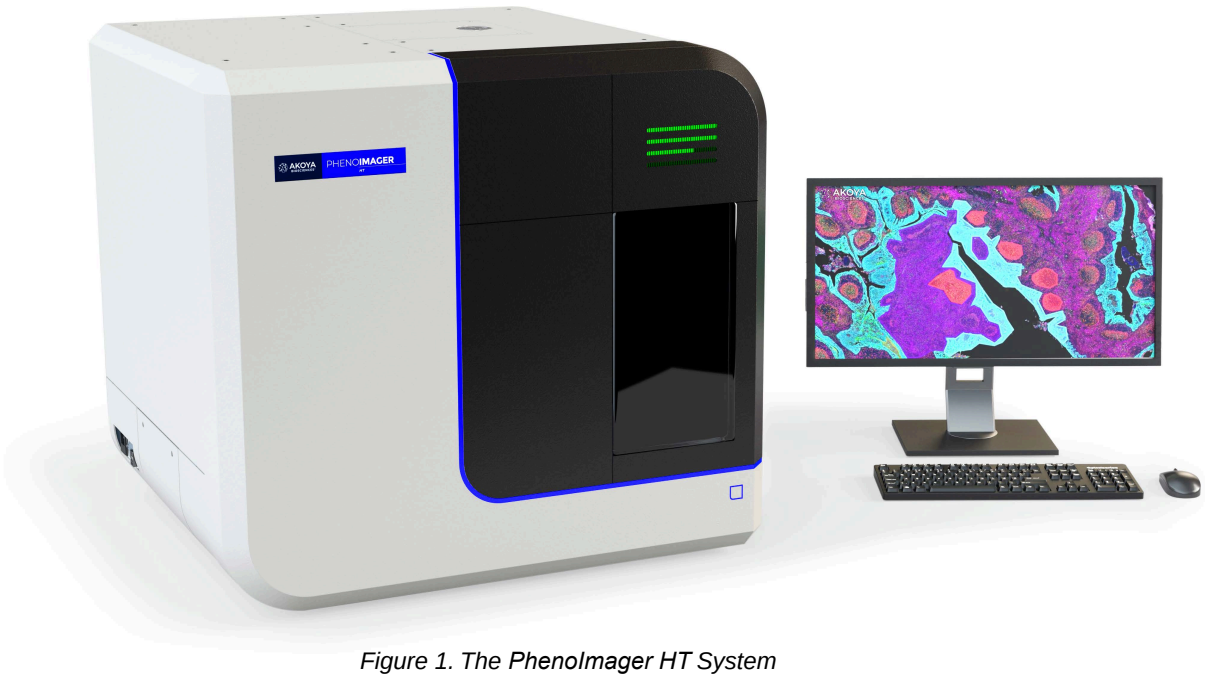


Figure 1. The PhenoImager HT System

The PhenoImager HT has been designed to expand and support a high-throughput workflow and includes:

- True whole slide scanning of slides at 1.0 um/pix, 0.5 um/pix, and 0.25um/pix
- Review and annotation of whole slides scans for multispectral field acquisition
- Acquisition of multispectral fields
- Analysis of multispectral whole-slide regions and fields including protein expression and phenotyping

To implement the full capabilities of the instrument and workflow, the PhenoImager HT system includes the following Akoya software:

- PhenoImager HT: Operator-centric software for performing whole slide scans and acquiring multispectral fields of interest. The PhenoImager HT software runs on the workstation connected to the PhenoImager HT instrument.
- Phenochart™: Whole-slide viewer and annotator of fluorescent and brightfield scans acquired by the PhenoImager HT. Phenochart allows the user to view the whole slide (zoom, pan, etc.), and make decisions (annotations) on next steps for the sample. Annotations in Phenochart are also used to record the workflow actions for each slide scan. Annotations include reviewer requested multispectral fields; automated (inForm) field requests; and reviewer edits, approvals, and rejections. The annotation file is a fully auditable transaction log. Phenochart is freely distributed and can be used by multiple users who wish to view or review slide scans taken by the PhenoImager HT.
- inForm® Tissue Finder: Software typically used for the analysis of MSI images. inForm supports features such as tissue classification and training, cell phenotyping, protein expression measurements, and data export. It can be run on the PhenoImager HT computer and other Microsoft® Windows® 10 computers. Additional inForm software seats beyond those that come with each PhenoImager HT system are available for purchase.

PhenoImager HT workflows range from simple two-step procedures (e.g., acquire whole slide scan and review) to automated acquisition of regions of interest selected by the user or the PhenoImager HT itself. An example fluorescence workflow might include the following steps:

1. Stain tissue with Akoya Biosciences Opal™ fluorescent IHC reagents.
2. Acquire whole slide fluorescent imagery using the PhenoImager HT.
3. Review the whole slide imagery with Phenochart and annotate regions of interest for MSI analysis.
4. Acquire the MSI regions with PhenoImager HT.
5. Use inForm to phenotype cells and measure protein expression levels in the acquired MSI regions.

For example applications for the PhenoImager HT, see Example Applications 17.

3.2.1 Example Applications

Some examples of PhenoImager HT applications include:

- Whole slide scanning and multispectral interrogation of tissue samples and microarrays stained with Opal™ reagent kits
- Whole slide scanning of tissue samples stained with H&E and conventional IHC stains
- Phenotypic analysis and protein expression of immune and cancer cells in the context of the tumor microenvironment.

3.3 Theory of Imaging

This section introduces some important concepts used by Akoya's PhenoImager HT imaging systems, including:

- Light 18
- Human Perception of Light Intensity and Color 18
- Light Absorbance and Reflection 19
- Fluorescence 19
- Photobleaching 20
- Filter Sets for Conventional vs. Multispectral Imaging 21
- Multispectral Analysis 22

3.3.1 Light

For purposes of this discussion, light refers to the part of the electromagnetic spectrum that can be seen by the human eye and the nearby ultraviolet and infrared wavelengths. While the physical description of light can be highly complex, we will focus on these wavelengths of light and how they interact with physical and biological materials.

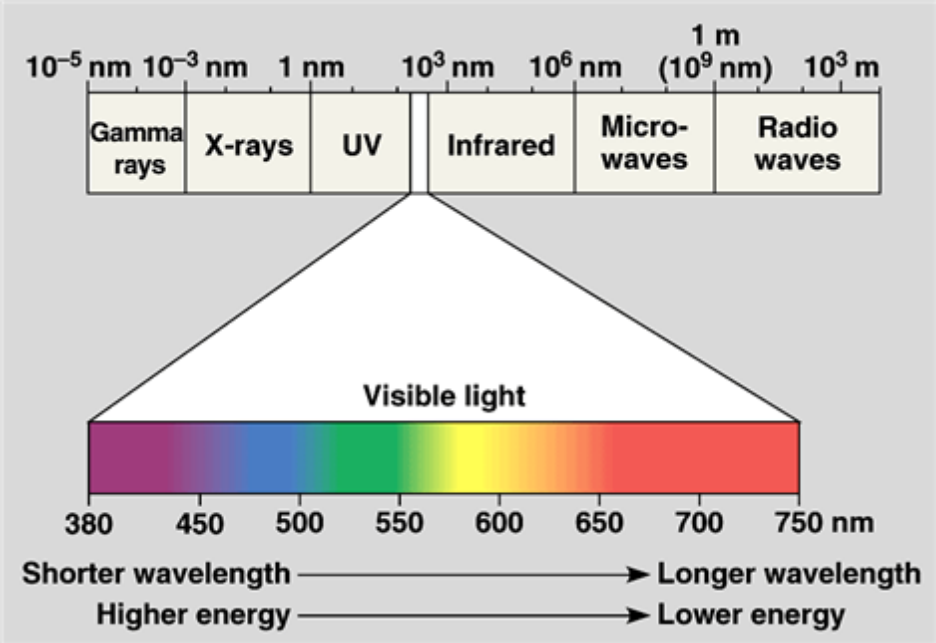


Figure 2. The Electromagnetic Spectrum

3.3.2 Human Perception of Light Intensity and Color

Response to Illumination

The human eye is a highly adaptive light detector. It is significantly more sensitive in low light than in bright light. When light levels change, it takes some time for the eye to fully adjust. This is the reason people need to “dark adapt” in a darkened room before they are able to observe weak fluorescence through a microscope.

Humans can see both in very dark and very bright settings. Because the eye is so adaptable to various lighting conditions, humans are unable to quantify absolute levels of light. In any given situation, the eye has a limited ability to discriminate levels of illumination. US Department of Defense research indicates that most humans can only distinguish approximately 30 to 35 levels of gray, ranging from black to white.

The eye's response to illumination is not linear. It more closely approximates a logarithmic function. The result is that the human eye cannot see small proportional changes in brightness.

Contrasting the eye with conventional microscope imagery, any sensor that has 8-bit resolution can detect 256 levels of gray. As the number of bits of resolution increase, the number of gray levels also increases. A 12-bit sensor yields 4096 levels of gray. Digital electronic sensors are linear in response to light levels.

Ability to Distinguish Colors

While the eye is relatively poor at discriminating intensity, it is very good at distinguishing colors. Most individuals are able to discriminate thousands of colors. However, no two individuals see a given color in exactly the same way. The eye contains three different types of color sensors, also known as cone cells. While the arrangement of cone cells is generally standard from person to person, the ratio of each type of cone cell varies, as does their actual physical arrangement within the eye. These minor variations (along with the brain's interpretation of the color) lead to the differences in perceived color between individuals.

3.3.3 Light Absorbance and Reflection

We perceive objects based on the way they transmit, absorb, and/or reflect light.

Absorbance and **reflection** work in tandem. **Absorbance** refers to the wavelengths of light that are 'taken in by' the objects. This means that an object that we perceive as red has absorbed all visible wavelengths of light except red. The red wavelengths are **reflected** back to the eye of the observer.

Transmittance refers to light emitting objects such as light sources, and fluorescing or phosphorescing objects. An object we would perceive as red in transmission is one that transmits primarily red wavelengths, while absorbing or reflecting other wavelengths.

In brightfield light microscopy we observe light that passes through a specimen. Except for a few pigments and inclusions, biological specimens are essentially invisible. To impart contrast, we employ some absorbing dye, or specific optical arrangement. It is this need for contrast that led to the initial development of biological stains and stain protocols and subsequently to phase contrast and other optical contrast enhancing techniques.

Optical Density (OD) is used to measure the interaction of light with absorbing materials. The science of absorbing spectroscopy is based on the Beer-Lambert law. When absorbing images collected in brightfield are converted to OD images, the information contained in each pixel is quantitative, as to the amount of absorbing material present. Akoya's brightfield multispectral imagery is automatically converted to optical density at acquisition time, enabling quantitative analysis.

3.3.4 Fluorescence

Many biological and natural materials give off light of a particular color when exposed to light of another color. This property is a type of luminescence. There are two types of **luminescence**:

- **Fluorescence** refers to luminescence that occurs when the light is emitted rapidly after illumination (around one-millionth of a second).
- If the light emission takes longer than one-millionth of a second, the luminescence is called **phosphorescence**.

Materials that exhibit fluorescence have proven extremely useful as labels or indicators in many biological systems.

Fluorescence light emission is different than light absorption. Each fluorescent molecule generates light. Fluorescent light can be measured quantitatively because it does not interact with other

materials. While it would seem that fluorescence could be measured more accurately than absorbed light, there are a number of factors that complicate such measurements. For example, light scatters, it is affected by the local environment (such as pH), and the measurements can be affected by surrounding molecules.

Stokes Shift

When you excite a specimen with a particular (shorter) wavelength (such as blue), the specimen then shines in a different (longer) wavelength (such as red, orange, or yellow). The difference between the wavelength of the (shorter) exciting light and the wavelength of the (longer) emitted light is called the **Stokes Shift**, which is based on **Stokes Law.**

The wavelength or color you use to excite the specimen (i.e. the 'excitation light') and the color the specimen glows (i.e. the 'emission light') depend on the dye involved. For any given fluorescence dye, there will be a range of excitation wavelengths that will excite fluorescence. This range of excitation wavelengths is known as the absorption spectrum. Each dye also emits across a range of wavelengths, known as the emission spectrum. Figure 3 contains an example of excitation and emission spectra, showing Stokes Shift and the overlap of the spectra.

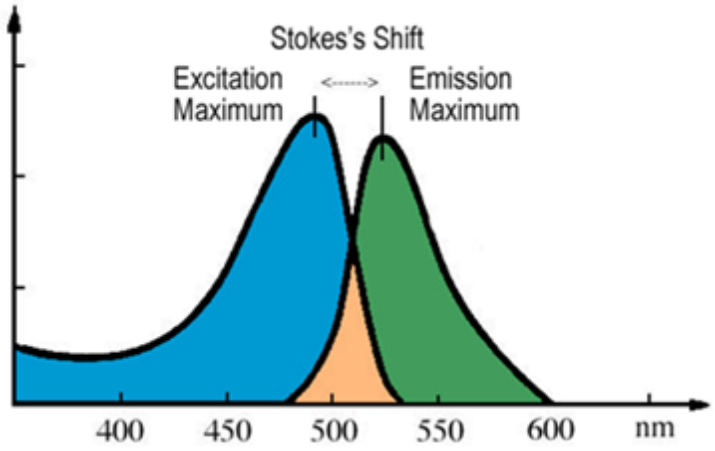


Figure 3. Stokes Shift

In addition, many biological materials are naturally fluorescent. This is known as autofluorescence. In particular, many vitamins, some hormones, and a variety of biological enzymes and structural proteins are naturally fluorescent. These materials often fluoresce strongly enough to interfere with specific fluorescence labeling studies.

3.3.5 Photobleaching

Because dyes can be damaged by intense light, reducing the emission signal (‘photobleaching’), it is important to limit the time they are exposed to excitation light or to bright light during routine handling. Usually, blue or UV light is the most damaging. The PhenoImager HT uses an electronically-gated excitation source synchronized with its camera so the sample is only exposed to light while the camera is taking an image. Also, the PhenoImager HT front door is made of a translucent plastic that absorbs harsh blue and UV light.

When using PhenoImager HT, avoid spending a long time in the Protocol Exposures Editor while fixed on any one spot of the sample, since it takes a live image stream.

These steps enable repeated measurements with minimal effect on the sample.

3.3.6 Filter Sets for Conventional vs. Multispectral Imaging

Filters used for conventional fluorescence imaging are often designed so they only transmit a very narrow range of wavelengths of light. Limiting the measurement to wavelength bands where the dye is inherently most responsive helps distinguish the desired dye from other dyes or background signals in the sample. In this way, it is possible to image several dyes, provided that their spectra are separated rather than overlapping. Based on the properties of common dyes, this puts an upper limit of ~4 on how many dyes can be imaged in any one sample.

An alternative approach is to image the sample multispectrally. In this case, a broad emission filter is used, and a tunable filter is engaged in the imaging path. The instrument takes pictures at several wavelengths within the emission band, so maps out the full shape of the dye response. This enables analysis software to identify what dye(s) are present, in what amounts, in each pixel, by spectral decomposition ("unmixing"). It also enables identifying, and removing, contributions from autofluorescence.

These two approaches are illustrated in Figure 4 and Figure 5

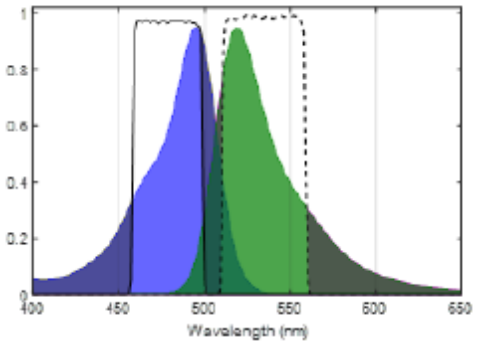


Figure 4. Narrowband excitation (solid line) and emission (dashed line) filter for conventional imaging.

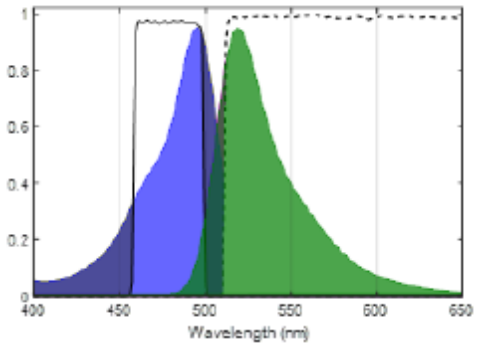


Figure 5. Narrowband excitation (solid line) and long-pass emission filter (dashed line) for multispectral imaging.

Ideally, the excitation filter would match the excitation maximum of the fluorescence label being used, and the emission filter would include the emission maximum. In practical terms, the filter maxima may be slightly different from the ideal case, due to limitations of filter manufacturing and because for many dyes the Stokes shift is small, so the maxima are quite close to one another.

To image multiple dyes conventionally, one selects dyes that have very distinct excitation and/or emission response, and selects filters that are narrow enough to predominantly transmit the signal of only one dye at a time (Figure 6 and Figure 7).

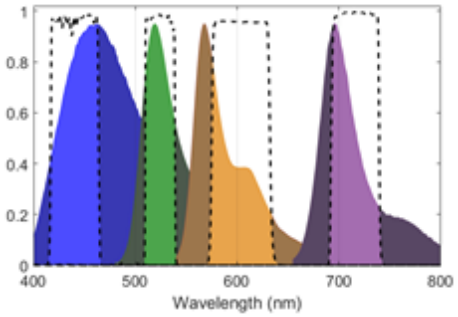


Figure 6. Imaging four dyes by conventional fluorescence methods.

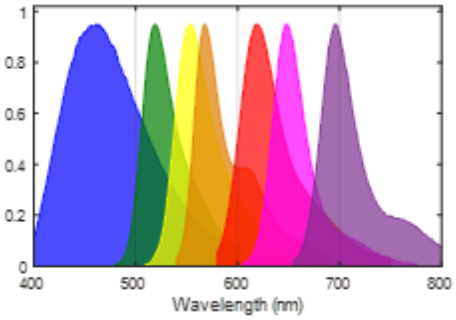


Figure 7. Seven dyes is too many to image by conventional methods.

This shows the emission spectra for four dyes (DAPI, FITC, Cy3, and Cy5.5) that are spectrally fairly distinct, and can be separated by conventional imaging. This works because there is only a little overlap between adjacent dyes. Excitation filters and dye response are omitted for clarity, but are similarly separable.

This approach breaks down when more dyes are present, or when it is important to account for the effects of autofluorescence. Figure 7 shows the spectra of 7 dyes, and with this many dyes there is no way to isolate their signals using conventional imaging techniques, or to account for autofluorescence.

3.3.7 Multispectral Analysis

The PhenoImager HT imaging system offers a unique solution to the problem of separating the signals from highly multiplexed samples. Multispectral analysis is based on the fact that all fluorescent materials produce a unique spectral emission. If you excite a material and examine the emitted fluorescence over a range of wavelengths, the resulting emission intensities can generate an “emission spectrum”. This spectrum is different for each specific fluorescent material. For many fluorescent labels of biological interest, the emission spectra overlap, and may be further obscured by autofluorescence from the specimen. Multispectral imaging provides a way to distinguish between many overlapping emission spectra within the same area, overcoming the limitations of conventional filter-based imaging. With the additional information provided by the LCTF during multispectral field imaging the system can distinguish between dyes with fully overlapping spectra within a single channel (Figure 8).

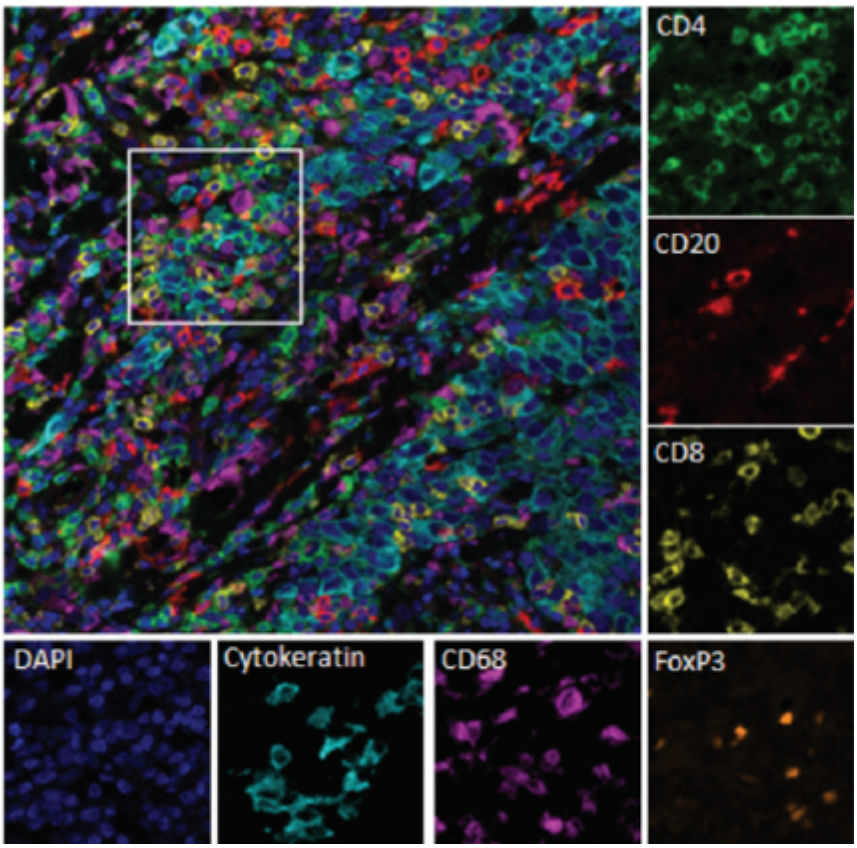


Figure 8. Unmixed multispectral image of human breast cancer tissue stained against CD4 (Green), CD20 (Red), CD8 (Yellow), FoxP3 (Orange), CD68 (Purple), Cytokeratin (Light Blue), and DAPI (Dark Blue) using Opal reagents.

In general, multispectral analysis generates the spectral curves for the various fluorescent dyes or materials in a specimen. In addition, it generates a spectral curve for the autofluorescence that almost always is present to some degree. Using spectral analysis algorithms, the contribution of the individual fluorescence spectra are separated. The result is a set of images representing each spectrum that contributes to the final image (Figure 9).

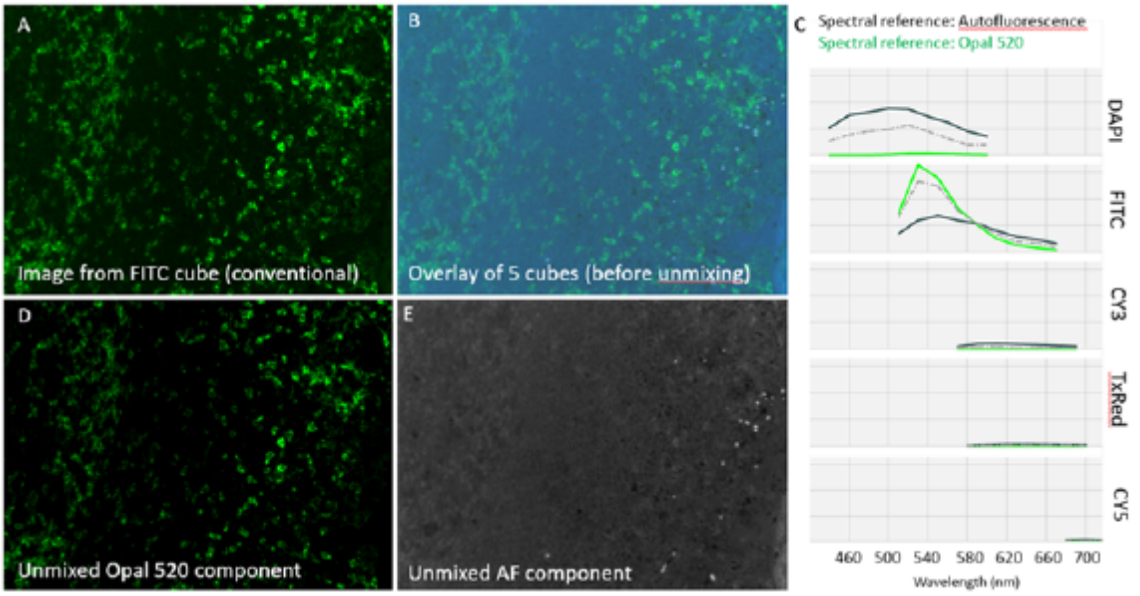


Figure 9. Removing auto-fluorescence with multispectral imaging. A) Conventional fluorescence image of tonsil tissue stained against CD4 with Opal 520 dye. B) Overlay of all 35 layers of a multispectral image acquired using five filter cubes. C) Emission spectra of pure autofluorescence (black line), pure Opal 520 (green line), and the mixture observed in 'B' (gray dashed line). The spectral references are used to 'unmix' the contribution of autofluorescence and Opal 520 at each pixel. D) Image of unmixed Opal 520 signal extracted from 'B' with > 10-fold higher signal-to-background compared to 'A' because the autofluorescence contribution has been separated into the component image in 'E'.

3.4 Whole Slide Scanning

PhenoImager HT scans slides using the following process:

1. **Color Overview:**
   PhenoImager HT takes a low power color overview of all four slides in each carrier, including the label for each slide. This initial step is performed regardless of whether you are using a fluorescence or brightfield protocol and is used to identify the presence of slides in the carrier and capture their labels.

2. **Coverslip Finding:**
   For each slide, PhenoImager HT will then find the coverslip using this overview scan. The coverslip defines the potential scan area.
   For fluorescence protocols, you can further restrict this area by making a closed loop with a red, green, or blue Sharpie® marker. If closed-loop markup is present, the system will only scan within the loop. This is useful if your tissue is faintly stained, punctate, or if you have highly fluorescing non-tissue material (PAP pen, for example.)
   For brightfield protocols, the coverslip will define the potential scan area. Closed-loop markup is not available for brightfield scans.

3. **Slide Height Finding:**

PhenoImager HT engages specialized height-sensing optics to measure the top of the coverslip at up to 9 locations. This gives an initial focus estimate based on the expected coverslip thickness.

4. **Fluorescence Overview (Fluorescence protocols only):**

   If you are using a fluorescence protocol for this slide, PhenoImager HT will take another overview within the coverslip (or closed-loop markup), this time in fluorescence.

5. **Tissue Finding:**

   Using the corresponding overview, PhenoImager HT will automatically detect the sample on the slide. The resulting area will be scanned. If requested within the protocol, PhenoImager HT will scan the entire area within the coverslip (or closed-loop markup).

6. **Focus Finding:**

   PhenoImager HT will measure focus at multiple points on the tissue to determine the best focus. It uses the sample map from the previous step to choose the measurement grid, and continues until the grid is fully measured.

   If the measured tissue height is irregular, PhenoImager HT increases the grid density and takes more readings until it finds the readings are regular at the newly finer scale.

   All focus measurements include a dust-rejection algorithm, and an overall consistency check is applied as well, to further reduce the likelihood of dust-induced focus errors.

7. **Scanning:**

   PhenoImager HT then scans the slide.

   Brightfield scans are conventional color scans that have been color and background corrected.

   Fluorescence scans are multi-layered, with one layer for each filter you chose. To avoid photobleaching, the system uses a pulsed LED so the sample is only exposed to light during the time that the camera is taking a picture.

   When scans are complete, they can be opened in Phenochart, the whole slide viewing application.

   NOTE: *Some dyes narrowly express in a single filter. Other dyes may express in multiple filters and may appear in more than one layer in a PhenoImager HT fluorescence scan. For example, Opal 570 will have signal in both Cy3 and TexasRed filters. If your sample is highly multiplexed, multiple dyes may appear in the same channel.*

3.5 Multispectral Field Imaging

After the PhenoImager HT has completed a whole slide scan of the tissue, individual fields can be selected for multispectral imaging.

This section contains:

3.5.1 Configuring the Multispectral Fields

Multispectral imagery is acquired using the following process:

1. **Selection of Multispectral Fields:**

Fields for multispectral imaging are selected on a previously scanned slide. Using Phenochart, you can select individual fields or regions of interest. If desired, you can also select fields using a trained inForm algorithm. See the Phenochart documentation for more information.

3.5.2 Processing the Multispectral Fields

After you configure the slide for field acquisition, PhenoImager HT will perform the following actions:

1. **Color Overview:**
   PhenoImager HT takes a low power color overview of all four slides in each carrier, including the label for each slide. This initial step is performed regardless of whether you are using a fluorescence or brightfield protocol and is used to identify the presence of slides in the carrier.
2. **Slide Registration:**
   Using the color overview along with the slide's original overview, PhenoImager HT accounts for any shift or rotation of the slide to ensure that the multispectral region locations are accurate. The slide edges are used to account for any rotation or horizontal shift. The coverslip edges to account for vertical shifting.
3. **Slide Height Finding:**
   PhenoImager HT engages specialized height-sensing optics to measure the top of the coverslip at up to 9 locations. This gives an initial focus estimate based on the expected coverslip thickness.
4. **Acquisition of Multispectral Fields:**
   PhenoImager HT will then travel to each multispectral region site, autofocus, correct for exposure if requested, and acquire the multispectral image.
   Multispectral imagery can then be viewed and analyzed in inForm.

4 Hardware Reference

This section identifies and describes the PhenoImager HT system hardware. It also lists the PhenoImager HT technical specifications.

WARNING:

Lifting Hazard. Do not move the PhenoImager HT instrument. Installing, servicing, and moving the PhenoImager HT instrument should be performed only by qualified Akoya Biosciences or PerkinElmer service personnel. Contact Akoya Biosciences technical support if help is required (see Contact Us 6 *).*

This section contains the following information:

4.1 Front View

Figure 10 shows the front view of the PhenoImager HT instrument with the system door *open*. The parts identified are described in Table 1.

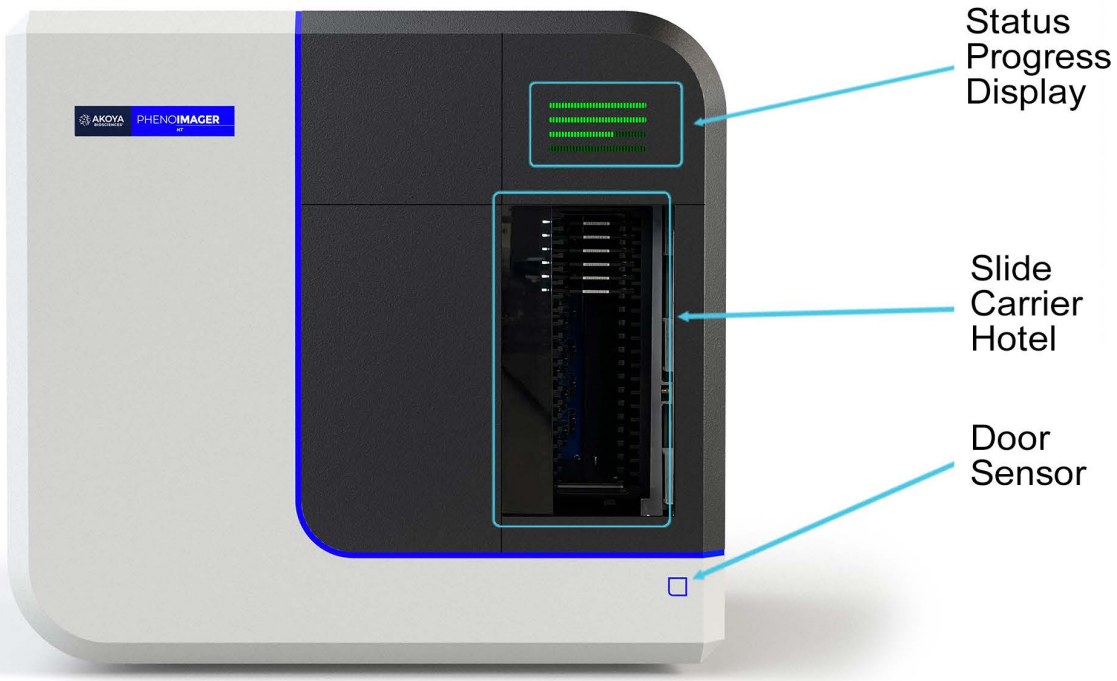


Figure 10. PhenoImager HT Front View - System Door Open

Table 1. Front View - Hardware Components	
Part	**Description**
Status Progress Display	Each light represents an individual slide and indicates the progress of the slide currently being scanned.
Slide Carrier Hotel	Houses up to 20 slide carriers. See Slide Carrier Hotel 32 for more detailed information.
Door Sensor	Opens or closes the System Door 29 when a hand is placed in front of the sensor.

Figure 11 shows the front view of the PhenoImager HT instrument with the system door *closed*. The parts identified are described in Table 2.

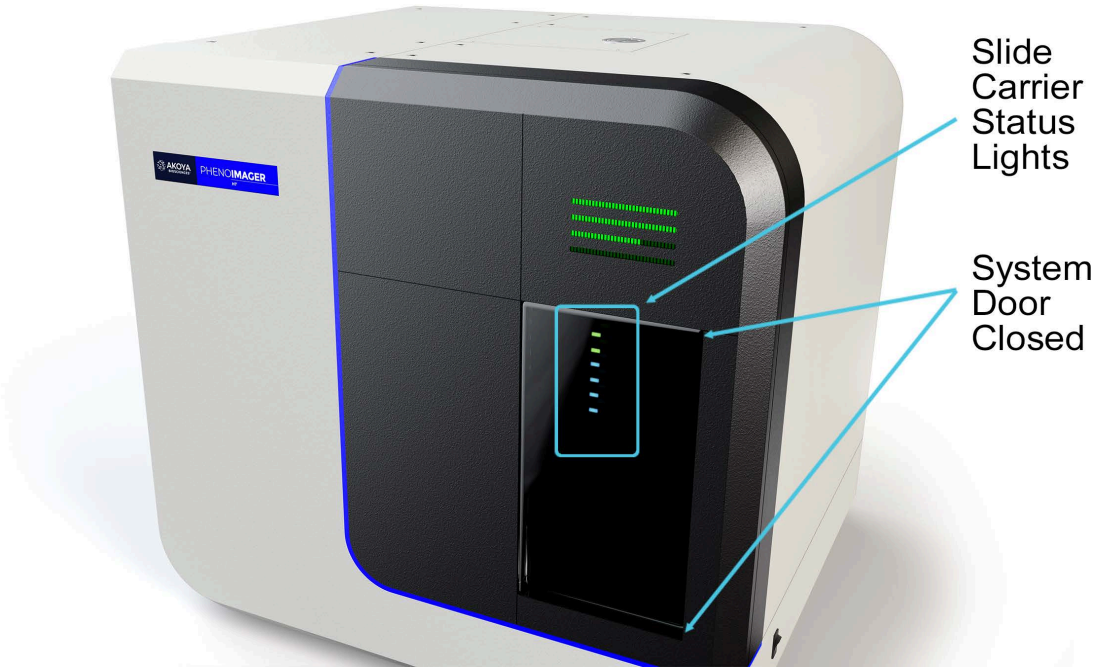


Figure 11. PhenoImager HT Front View - System Door Closed

Table 2. Front View - System Door Closed- Hardware Components

Part	Description
Slide Carrier Status Lights	Each Slide Carrier Status Light 32 represents an individual slide carrier and indicates the processing status of each Slide Carrier 33. See Table 7 32 for the color codes of the slide carrier status lights.
System Door	When closed, covers the Slide Carrier Hotel 32 and slide processing can occur. When open, reveals the Slide Carrier Hotel 32.

WARNING:

Do not operate the system if there has been a malfunction of the system door or slide loading components. Contact Akoya Biosciences technical support if help is required (see Contact Us 6).

4.2 Top View

Figure 12 shows the top view of the PhenoImager HT instrument. The part identified is described in Table 3

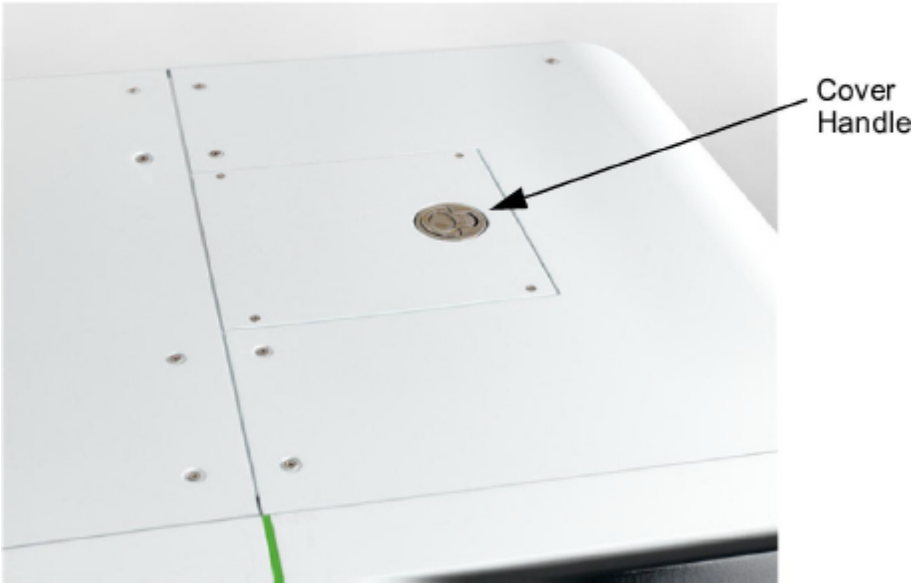


Figure 12. PhenoImager HT Top View

Table 3. Top View - Hardware Components

Part	Description
Cover Handle	Used by only service personnel to remove instrument covers during service and installation.

WARNING: *DO NOT remove instrument covers. There are no user serviceable parts inside. The covers are intended to be removed by qualified service personnel only; they are not intended to be removed during operation or for maintenance by users. Contact Akoya Biosciences Technical Support if help is required (see* *Contact Us* 6 *).*

4.3 Right-Side View

Figure 13 shows the right-side of the PhenoImager HT instrument. The part identified is described in Table 4

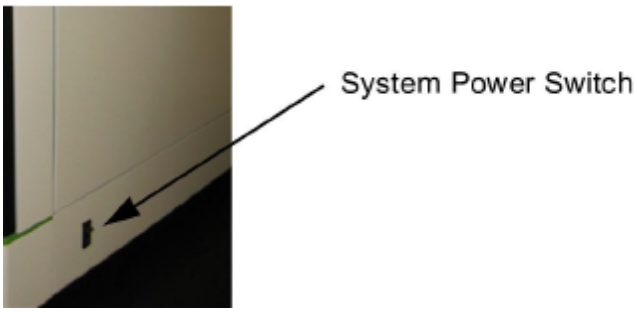


Figure 13. PhenoImager HT Right-Side View

Table 4. Right-Side View- Hardware Components	
Part	**Description**
System Power Switch	Turns the PhenoImager HT instrument ON | or OFF ◯

4.4 Left-Side Connectors

Figure 14 identifies the connectors on the bottom left-side of the PhenoImager HT instrument. The connectors identified are described in Table 5

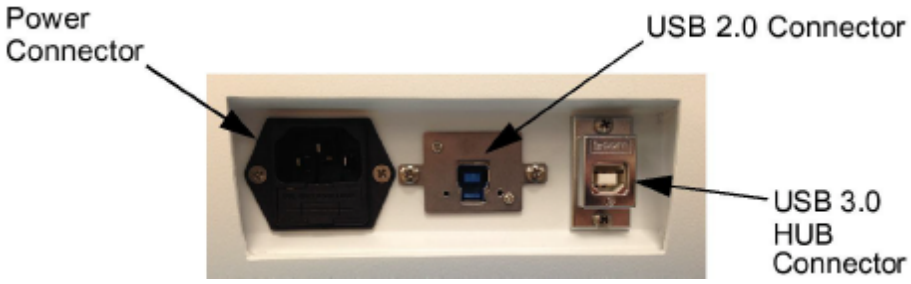


Figure 14. PhenoImager HT Left-Side Connectors

WARNING:

- Appliance inlet is a disconnecting device. Place device or equipment in a manner so that disconnecting device is accessible at all times.
- Use only the power supply cord set provided with the PhenoImager HT system. If the correct cord set for the location was not provided, contact Akoya Biosciences Technical Support (see Contact Us 6) for a replacement.
- Use only a properly grounded power outlet when connecting the system to power.

Table 5. Left-Side View - Connection Components	
Part	**Description**
Power Connector	Connects to the power cord to provide power to the PhenoImager HT instrument.
USB 2.0 Connector	Connects a USB 2.0 cable to a USB 2.0 port on the System Computer Connectors 36.
USB 3.0 Hub Connector	Connects a USB 3.0 HUB to a USB 3.0 port on the System Computer Connectors 36.

4.5 Slide Carrier Hotel

The Slide Carrier Hotel houses up to 20 Slide Carriers 33 and is visible when the System Door 29 opens. Figure 15 shows a closeup of the Slide Carrier Hotel and identifies its components. The components are described in Table 6.

WARNING: Do not operate the system if there has been a malfunction of the system door or slide loading components. Contact Akoya Biosciences technical support if help is required (see Contact Us 6).

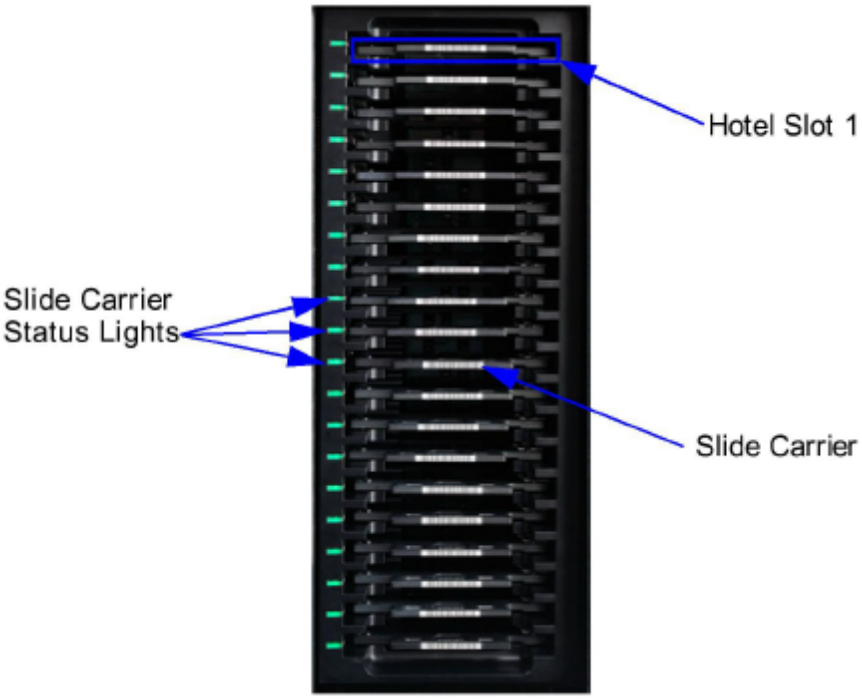


Figure 15. Slide Carrier Hotel

Table 6. Slide Carrier Hotel - Hardware Components

Part	Description
Slide Carrier Status Lights	Indicates the processing status of each slide carrier. Table 7 32 lists the color codes associated with the slide carrier status lights.
Hotel Slot	Each hotel slot holds one Slide Carrier 33. The hotel slots are numbered 1-20, starting at the top of the hotel.
Slide Carrier	Houses up to four slides. See Slide Carrier 33 for more detailed information.

Table 7. Slide Carrier Status Lights - Color Codes

Color	Status

None	Slide carrier hotel is empty.
White	Initial state of slot after slide carrier is inserted and no rules have been applied via software.
Solid Red	Hotel slot is malfunctioning.
Blinking Red	Slide carrier is not completely inserted.
Blue	Awaiting Processing.
Blinking Blue	Prioritized and awaiting processing.
Yellow	Processing instructions are either incomplete or invalid.
Blinking Green	Processing.
Solid Green	Processing Complete.
Orange	Processing complete but a processing error occurred.

4.6 Slide Carrier

Slide Carriers are an integral component of PhenoImager HT. The Slide Carrier holds up to four microscope slides and helps protect slides from damage. The microscope slide positions are labeled 1 to 4 and correspond to slide identification in the PhenoImager HT software.

16 shows a closeup of a Slide Carrier and identifies its components. The identified components are described in Table 8

Figure 16. PhenoImager HT Slide Carrier

Table 8. PhenoImager HT Slide Carrier - Hardware Components	
Part	**Description**
Slide Position	Each slide position is populated with one slide. Slide position 1 is on the far left; slide position 4 is on the far right.
Slide	Each microscopic slide is manually loaded into the slide positions. Table 9 34 identifies the details of the slide format.
Insert Indicator	An icon that indicates the side of the slide carrier to be inserted into a Hotel Slot 32.
Tab Cover	Holds the spring-loaded tabs in place.
Spring-Loaded Tab	When a slide is inserted into a slide slot, it is gently placed up against a spring-loaded tab.
Carrier Handle	The side of the slide carrier to hold when inserting and removing a slide carrier from a Hotel Slot 32. A unique number is printed on top of the handle for slide carrier identification purposes.

Table 9. Details of Slide Formats			
Slide Type	Width	Height	Thickness
Metric	25.0 ± 1.0mm	75.0 ± 1.0mm	1.00 ± 0.10mm

English	25.4mm	76.2mm	1.00 ± 0.10mm

4.7 Barcode Reader

The PhenoImager HT is equipped with an internal Barcode Reader. The Barcode Reader reads the barcode on the slide labels.

WARNING

BRIGHT LIGHT HAZARD. The interior of the PhenoImager HT system includes a barcode reader with a Class 2 LED Light. Do not look into the bright light to avoid eye injury.

CAUTION - CLASS 2 LASER RADIATION WHEN OPEN
DO NOT STARE DIRECTLY INTO THE BEAM

4.8 System Computer and Monitor

The PhenoImager HT system includes a widescreen monitor and a computer pre-installed with PhenoImager HT, Phenochart, and inForm software. A wireless keyboard and mouse are also included. See below for a description of the computer connectors.

Figure 17 shows the PhenoImager HT computer connectors. The connectors identified are described in Table 20

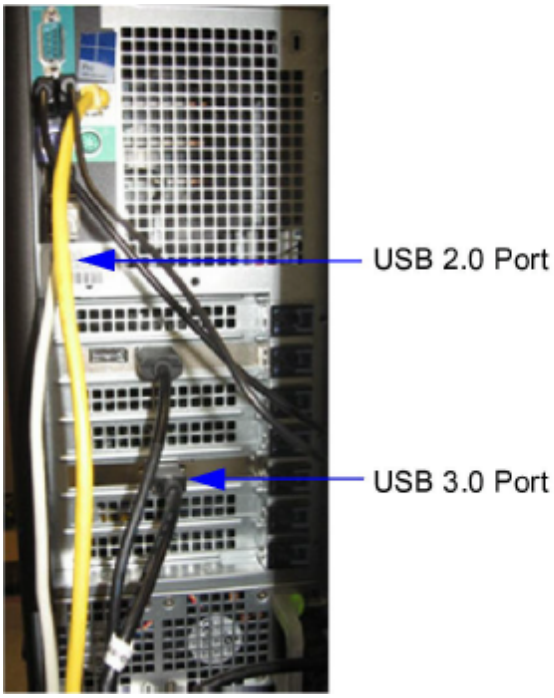


Figure 17. System Computer Connectors

Table 10. System Computer Connections	
Part	**Description**
USB 2.0 Port	Connects a USB 2.0 cable to the USB 2.0 connector on the instrument's Left-Side Connectors 31.
USB 3.0 Port	Connects a USB 3.0 HUB to the USB 3.0 HUB connector on the instrument's Left-Side Connectors 31.

4.9 Specifications

This section lists the technical specifications for the PhenoImager HT instrument. Technical specifications are subject to change without notice.

NOTE: *PhenoImager HT is for research use only. Not for use in diagnostic procedures.*

System

Size (H x W x L)	28" (72 cm) x 30" (77 cm) x 27.25" (69.2 5cm)
Weight	185 lbs. (84 kg)
Spectral Range	440 nm to 780 nm
Objectives	4x, 10x, and 20x
Pixel Resolution	0.25 um, 0.5 um, or 1.0 um
File Format	Akoya Biosciences .qptiff format for whole slide scans, proprietary .im3 file format for multispectral data; 24- bit Windows-compatible bitmap for RGB/Mono imagery
Operating System	Microsoft® Windows 10, 64-bit
RAM	16GB

Environmental

Operating Temperature	59°F to 83°F (18°C to 28°C)
Operating Humidity	30% - 50% non-condensing
Storage Temperature	59°F to 86°F (15°C to 30°C)
Storage Humidity	0% - 80% relative humidity, non-condensing
Altitude	Up to 2000m (6560ft.)

Shipping Temperature (up to 72 hours max)	14°F to 113°F (-10°C to +45°C)
Pollution Degree	2
Indoor Use Only	

Electrical

One properly grounded AC power outlet for the computer, monitor, and instrument must be located within 6 feet (1.8 m) of the location.

Input Voltage	100 to 230 VAC (±10%), 500 W, 50/60 Hz System does not have transient overvoltage protection.
Computer Interface	USB 2.0, USB 3.0
Fuse	4A Littelfuse® 250V, 5mm x20mm

Barcode Reader Light Source (White laser diode)

Wavelength	400 nm to 750
Power Output	70mAmps

5 Operation

This section describes how to operate the PhenoImager HT hardware. It is important to read and understand Instrument Safety 11 before using the system. If you are not familiar with the PhenoImager HT system hardware, Hardware Reference 27 provides a hardware component description of each Reference in the PhenoImager HT system.

This section includes the following procedures:

- System Startup 38
- System Shutdown 39
- Inspecting Slides and Slide Carriers 39
- Loading Slides into the Slide Carriers 40
- Loading Slide Carriers into the Slide Carrier Hotel 41
- Removing Slide Carriers from the Slide Carrier Hotel 43
- Removing Slides from the Slide Carriers 43

5.1 System Startup

This section describes the procedures needed to start the PhenoImager HT system:

- Turn on the PhenoImager HT Instrument 38
- Launch the PhenoImager HT Software 39

5.1.1 Turn on the PhenoImager HT Instrument

To start the PhenoImager HT instrument:

1. If necessary, plug the PhenoImager HT power cord into an appropriate power outlet.

   WARNING:

   - Use only the power supply cord set provided with the PhenoImager HT system. If the correct cord set for the location was not provided, contact Akoya Biosciences Technical Support (see Contact Us 6) for a replacement. Do not use power supply cords with inadequate ratings.
   - Use only a properly grounded power outlet when connecting the system to power.
   - Appliance inlet is a disconnecting device. Place device or equipment in a manner so that disconnecting device is accessible at all times.

2. Turn on the computer and allow Windows® 10 to start.

3. Switch the System Power Switch 31 to the ON (I) position. The Status Progress Display 28 flash green to indicate a quiescent state. The Door Sensor 28 is responsive and functional.

5.1.2 Launch the PhenoImager HT Software

To launch the PhenoImager HT software:

1. Double-click the PhenoImager HT icon on the Windows® 10 desktop.
2. The PhenoImager HT home page opens (see Software Overview 44).

5.2 System Shutdown

This section describes how to shut down the PhenoImager HT

system. To shutdown the PhenoImager HT system:

1. Exit the PhenoImager HT software. If open, the System Door 28 closes.

2. Select Shut down from the Windows® Start Menu to power down the computer.

3. Switch the System Power Switch 31 on the right side of the instrument to the OFF (O) position. The status lights turn off and the system shuts down.

5.3 Inspecting Slides and Slide Carriers

Before inserting slides into slide carriers, both should be inspected for potential defects.

This section describes the steps needed to properly inspect the slides and slide carriers.

Inspecting Slides:

- Verify the slides meet the required formats and dimensions (see Table 9 34).
- Do not use broken or damaged slides, or slides with broken or damaged coverslips.
- Use only slides that are free of debris, fingerprints, and dust.

Inspecting Carriers:

- Verify the slide Carrier Tab Cover is secure.
- Do not use slide carriers that are warped or bent.

- If any sticky residue is on the carrier handle or outer surfaces, clean before use (see Cleaning the Slide Carriers 88).

5.4 Loading Slides into the Slide Carriers

After the Slides and Slide Carriers have been successfully inspected, slides can be loaded into the Slide Carriers.

To load a slide into a slide carrier:

1. Place the slide carrier onto a flat surface.
2. Hold the microscope slide by the label end with the coverslip side up.
3. Gently push the opposite end of the slide into the desired Slide Position 34 until the slide is up against the Spring-Loaded Tab 34.

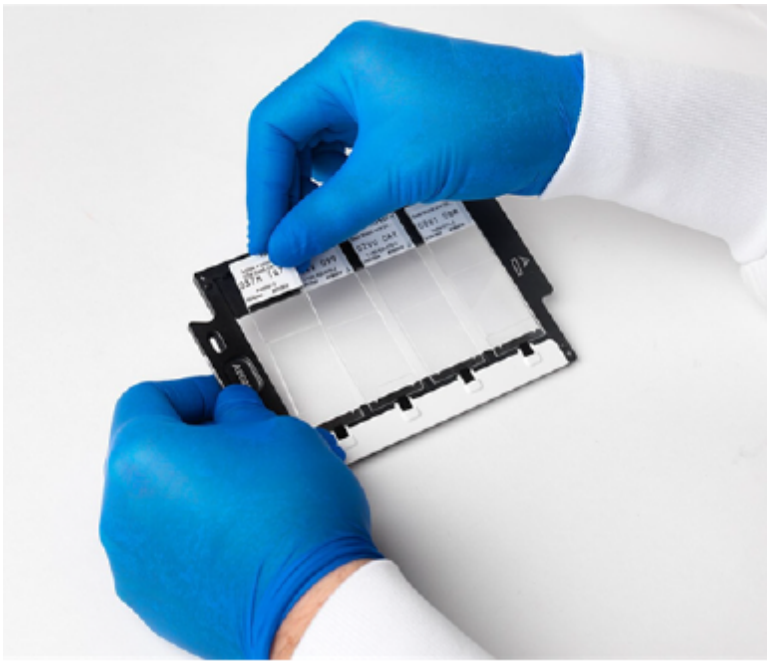


Figure 18. Insert Slide into Slide Carrier

4. Gently lay the label end of the slide completely into the slide position. The tab should push the slide against the opposite wall of the slide position.
5. Load up to four slides into each slide carrier. Slides need not be contiguous.
6. Click the **Enter Slide IDs** button (see Slide IDs 78) in the PhenoImager HT software. The Enter Slide IDs dialog opens.
7. Type the applicable slide IDs into the text boxes and click the **OK** button.

8. To load additional slides into another slide carrier, repeat steps 1 40 to 7 40 above. Slide carriers may be stacked on top of one another for easy handling and storage (see Figure 19).

Figure 19. Slide Carrier Stack

5.5 Loading Slide Carriers into the Slide Carrier Hotel

The Slide Carrier Hotel stores Slide Carriers before and after microscope slide scanning. The slide carrier hotel can store up to 20 slide carriers for a total of 80 slides. The Hotel Slots are numbered 1-20, starting at the top of the hotel.

NOTE: Slide carriers can be loaded into the slide carrier hotel before or after launching the software.

To load a slide carrier into the slide carrier hotel:

1. Inspect the slide carrier (see Inspecting Slide Carriers 39).
2. Verify the PhenoImager HT System is on (see System Start-up 38).
3. If the System Door is closed, place your hand in front of the Door Sensor to open it. The system doors slides to the left to reveal the slide carrier hotel.
4. Hold the slide carrier by the Carrier Handle with the slide labels facing upwards.

5. Gently insert the end of the slide carrier with the Insert Indicator  into a Hotel Slot.

Figure 20. Ends of a Slide Carrier

6. When the Slide Carrier is halfway into the Hotel Slot, the carrier engages a roller and creates a small amount of friction. Push the carrier further into the slot until it is fully seated.

Figure 21. Push the Slide Carrier until Fully Seated

- If the carrier is inserted correctly, a click noise indicates that the carrier has been seated correctly into the slot. The Slide Carrier Status Light (Slide Carrier Status Lights 32) next to the associated Hotel Slot turns white.
- If the carrier is not inserted correctly, the Slide Carrier Status Light next to the Hotel Slot blinks red until the slide carrier is removed from the slot or is positioned correctly.

7. Continue to load up to 20 slide carriers into the slots.
8. When done, place your hand in front of the Door Sensor to close the System Door.

5.6 Removing Slide Carriers from the Slide Carrier Hotel

When slide scanning is complete, the Slide Carriers 33 can be removed from the Slide Carrier Hotel 32.

To remove a slide carrier from the hotel:

1. Pull the Carrier Handle 34 until the slide carrier is free from its Hotel Slot 32. The Slide Carrier Status Lights 32 next to the Hotel Slot turns off, indicating no slide carrier is inside the Hotel Slot.
2. Lay the slide carrier onto a flat surface.

5.7 Removing Slides from the Slide Carriers

After Slide Carriers 33 are removed from the Slide Carrier Hotel 32, the microscopic slides can be removed from the Slide Carriers.

To remove a slide from a slide carrier:

1. Gently push against the slide label end of the microscope slide using your index finger, compressing the tab on the far end of the slide.
2. Using the same finger, lift the label end of the microscope slide from the slot.
3. Grab the label end of the slide with your thumb and index finger.
4. Remove the remaining end of the slide from the slide carrier.

6 Software Operation

This section describes how to operate the PhenoImager HT software.

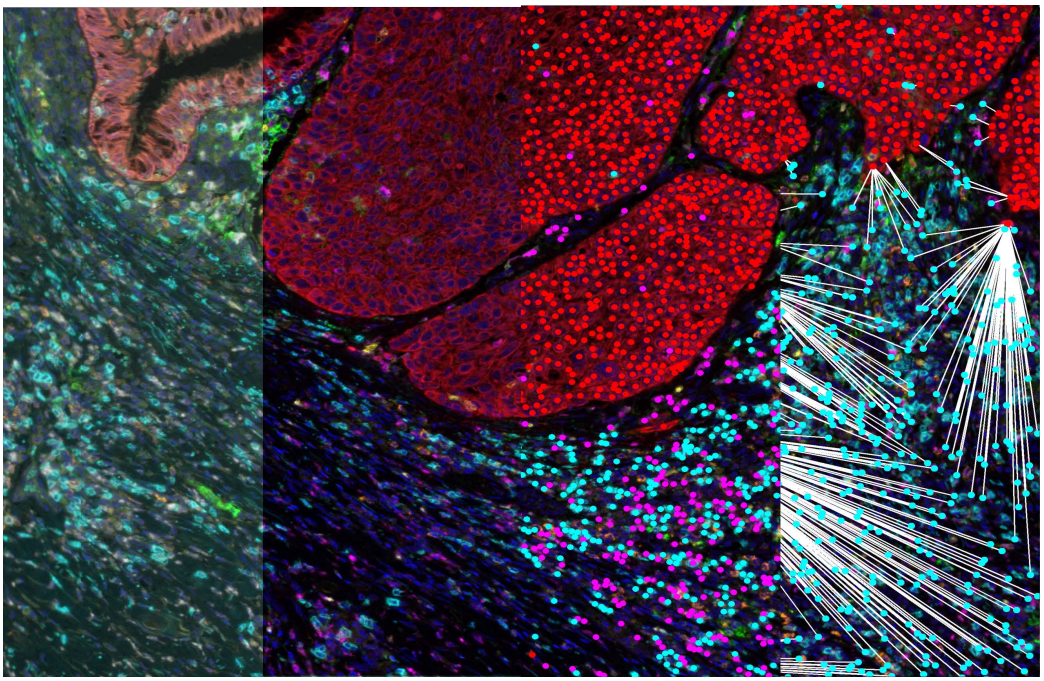


Figure 22. PhenoImager HT Software

This section includes the following topics:

6.1 Software Overview

The Home Page links to the pages needed to maintain and run the PhenoImager HT.

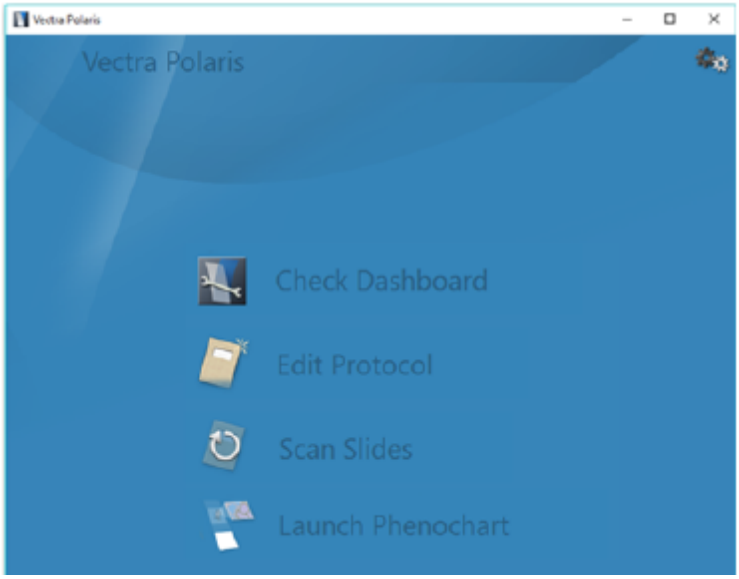


Figure 23. PhenoImager HT Home Page

The Home Page buttons link to the following pages:

Check Dashboard 46 - Check your remaining disk space and acquire references.

Edit Protocol 46 - Create or edit brightfield and fluorescent protocols and studies.

Scan Slides 46 - Select the rules to scan your slides and scan your slides based on those rules.

Launch Phenochart 46 - Launch the Phenochart viewing software.

You can also get to those pages by using the Gear Menu 46 (in the upper right):

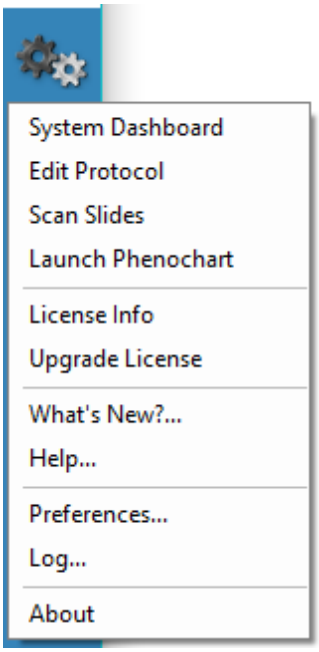


Figure 24. Gear Menu

Additionally, you can use the Gear Menu 46 to check your version number or perform other functions, and launching the online help.

Below is an overview of each page.

Dashboard:

Use the Dashboard to check the status of your system prior to imaging. It includes the following:

- Disk space - checks the available space on the disk drive where images will be stored.
- Acquire References - views and acquires brightfield references and fluorescent references.

For detailed information on how to use the System Dashboard, see System Dashboard 47.

Edit Protocol:

Use the Edit Protocol page to define protocols and take snapshots.

A protocol describes how a sample is to be imaged, including the imaging mode (brightfield or fluorescence), and the spatial resolution (magnification) for the whole slide scan and for multispectral regions of interest. For fluorescent imaging, it also describes the exposure times and what bands to use for focusing and imaging.

For detailed information on how to create and edit protocols and studies (see Creating and Editing Protocols 53).

Scan Slides:

Use the Scan Slides page declare how slides should be imaged. Slide scanning can be started and stopped from this page.

For detailed information on how to perform whole slide scans (see Scanning Slides 75).

Launch Phenochart:

Use this button to launch the Phenochart program.

For detailed information on how to use Phenochart, see the Phenochart User's Manual or online Help from within the Phenochart program.

6.1.1 Gear Menu

Use the Gear menu (in the upper right hand corner of the Home Page) to:

- Link to the following pages:
    - Check Dashboard 47

    - Edit Protocol 53
    - Scan Slides 75
    - Launch Phenochart 46
- Find out What's New in the current installed version
- Launch the online Help system
- Open the Preferences dialog
- Open the PhenoImager HT software Log for reference or troubleshooting
- Open the About window to view the current software version
- View contact information for Akoya Biosciences technical support

6.2 System Dashboard

Use the System Dashboard to:

- See the available Disk Space (see Disk Space 48)
- View and acquire Brightfield References (see Brightfield References 48)
- View and acquire Fluorescence References (see Fluorescence References 50)

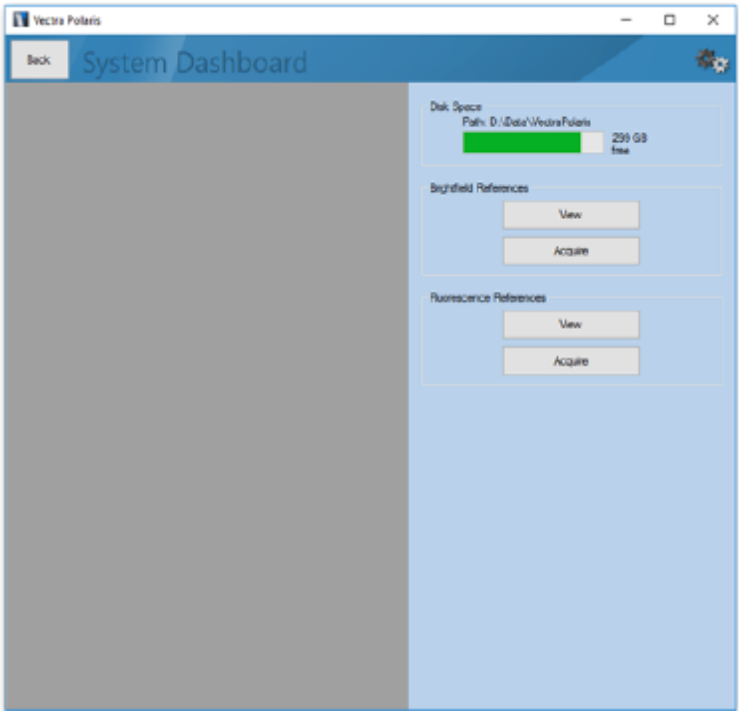


Figure 25. System Dashboard Window

6.2.1 Disk Space

The disk space bar shows where your data is stored and how much space is available on that drive.

6.2.2 Brightfield References

Viewing Brightfield References

Click **View** to see reference imagery and information for the label image, brightfield overview, color image and multispectral image.

- Export For Diagnostics will save this image to aid in technical support.
- Show raw images will display the images without scaling, which is useful for technical support.
- Compensation Information 52 describes how these reference images are applied.

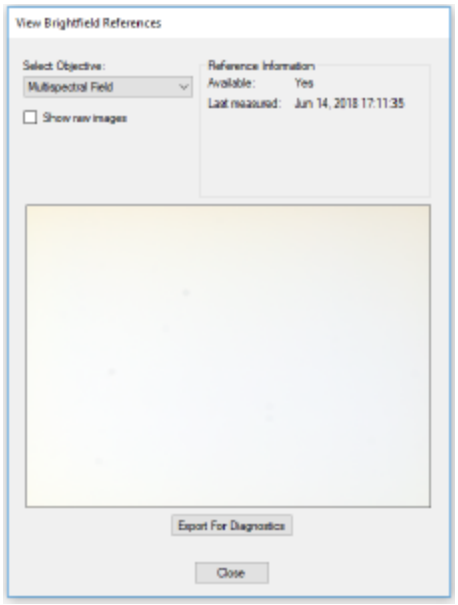


Figure 26. View Brightfield References Window

Acquiring Brightfield References

Click **Acquire** to take new brightfield references. Use the stage control to:

- Load the Reference Carrier onto the slide.
- Move around to a clean area in the live view (no coverslip lines, tissue, or label) using the stage navigation tool.
- Click within the slide to change positions. You can refine the position by using arrow keys for small movements, and control+arrow for slightly larger movements.
- Click **OK** to take references.

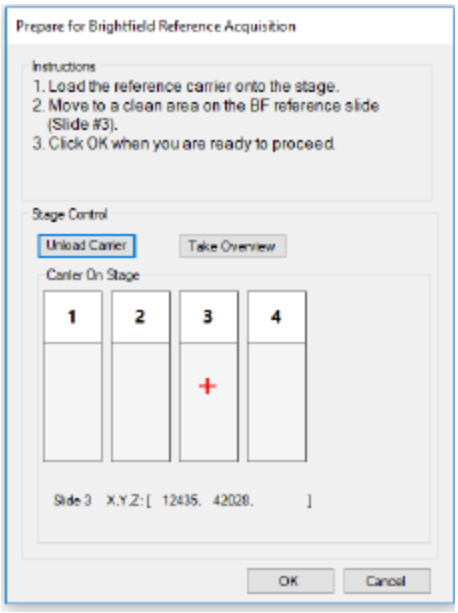


Figure 27. Prepare for Brightfield Reference Acquisition

6.2.3 Fluorescence References

Viewing Fluorescence References

Click **View** to see reference imagery and information for the overview, 20x and 40x resolution references for each filter.

- Export For Diagnostics will save this image to aid in technical support.
- Compensation Information 52 describes how these reference images are applied.

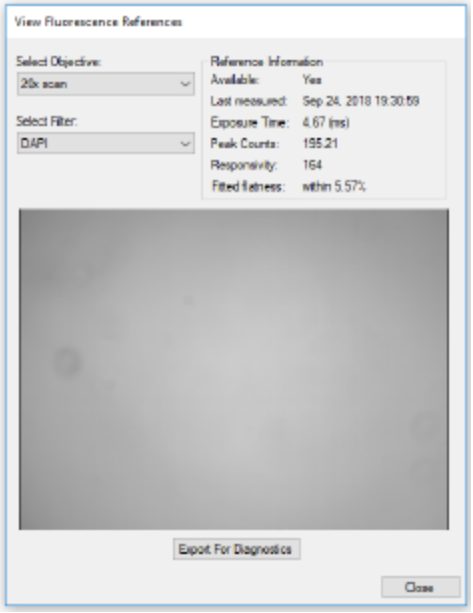


Figure 28. View Fluorescence References Window

Acquiring Fluorescence References

Click **Acquire** to take new fluorescent references. We recommend you take references for all filters at once, but you can take references for a single filter if necessary. Acquiring references for all filters can take over 45 minutes, and the system can be left unattended during this time.

Use the stage control to:

- Load the Reference Carrier onto the stage.
- Move to a tissue-filled location on the fluorescent reference slide using the stage navigation tool.
- Click within the slide to change positions. You can refine the position by using arrow keys for small movements, and control+arrow for slightly larger movements.
- Click **OK** to take references.

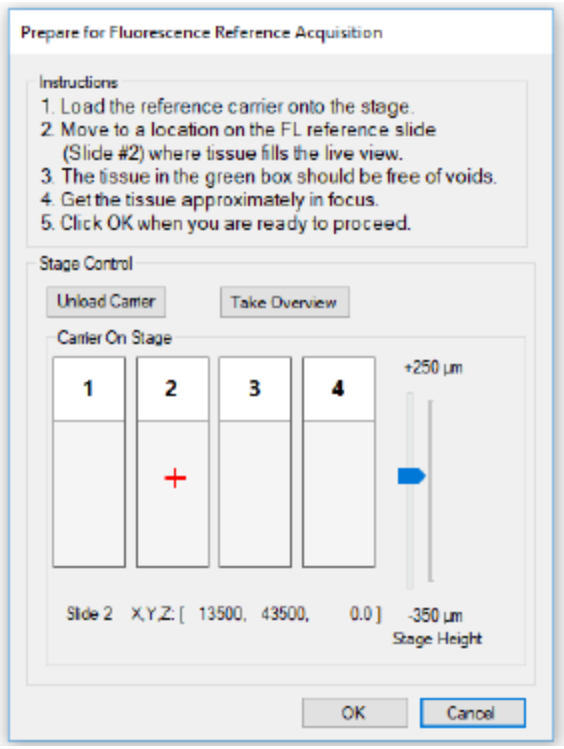


Figure 29. Acquire Fluorescence References Window

6.2.4 Compensation Information

Brightfield

Acquired images of a sample are normalized (divided) by the reference images on a pixel-by-pixel, wavelength-by-wavelength basis. This yields the sample transmission T, which is in the range 0 - 1. This transmission (T) is then mapped:

- For multispectral images, the Optical Density is calculated - this is based on Log10(T).
- For simple color images, like a whole slide scan, the transmission is mapped from 0 - 255.

Fluorescence Protocols

Acquired images are normalized by a shading pattern derived from the reference images. The shading pattern, which is the reference image divided by the mean intensity in the center, is applied on a pixel-by-pixel basis, with one pattern per epi-filter. Here, center means the central 1/16th of the image area. While the exact shape of the shading pattern varies per instrument, the overall effect is to increase the signal near the image edges, and to do little or nothing to the signal from the center of the image.

6.3 Creating and Editing Protocols

This section contains the following topics:

6.3.1 Studies

PhenoImager HT stores scan data by Study.

A study is a group of slides that belong together. This could be an experimental study (e.g. Ki67 markers in breast cancer tissue), all slides from one source, or other groupings. Each study contains one or more slides. Each slide may be scanned more than once, if needed.

- The default location for a study is D:\Data\VectraPolaris\[Study] (where [Study] is the name of the study).
- Whole-slide scans and supporting imagery acquired from specific slides are saved to slide-specific subfolders in the main study folder. See the section on Scanning Slides for more details about imagery.
- Multispectral Fields acquired from a particular slide scan will be stored in an MSI folder within the scan folder.

A protocol defines the set of rules to be used during whole slide and multispectral region acquisition, including imaging mode, pixel resolution, filter cubes, exposure times, and other parameters.

- Protocols have the file extension ".ppr" and are saved in D:\Data\VectraPolaris\[Study].

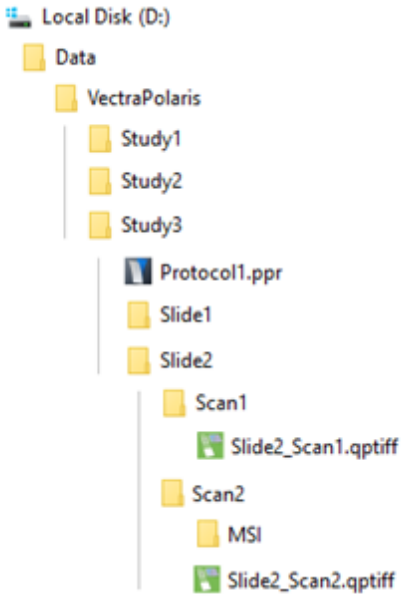


Figure 30. Study structure

6.3.2 Creating Protocols

Before you create a protocol, it is helpful to know how the slide was stained.

- Brightfield protocols are used to acquire imagery from slides stained with H&E or conventional chromogenic IHC methods.
- Fluorescence protocols are used with Akoya Biosciences Opal and other fluorescent dyes.

To create a new protocol:

- Select Edit Protocol from the PhenoImager HT Home Page.
- Click the **New** button. The Create New Protocol window opens.

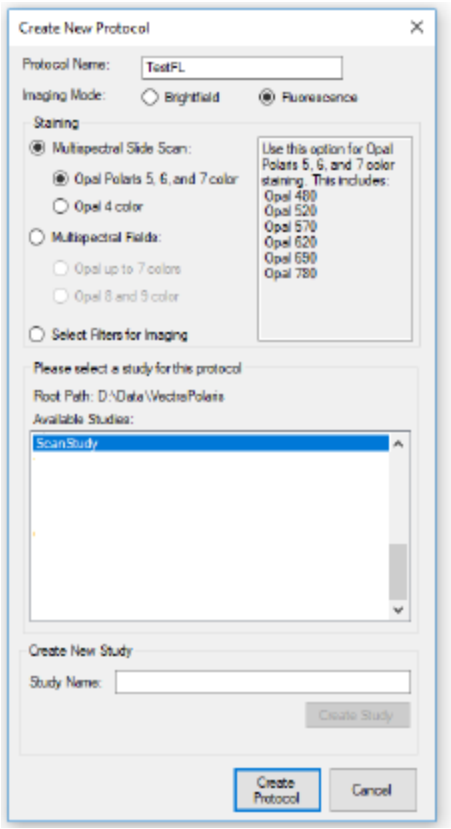


Figure 31. Create New Protocol Window

- Enter a Protocol name.
- Select Brightfield or Fluorescence under Imaging Mode.
- If you have upgraded your system for multispectral whole slide scans, choose the type of staining kit used for this protocol, or choose to select your own filters for imaging.
- Select a previously created Study or create a new Study.
    - To select a previously created study, click on the study in the Available Studies list. This will highlight the study.
    - To create a new study, enter the **Study Name** in the text box and click the **Create Study** button. The new study will be added to the Available Studies list where you can select the study.
- Click the **Create Protocol** button or the Select Scan Bands button to create the protocol in the selected study.
- If you chose to select your own filters, the Select Scan Bands window opens. Select the bands to use for imaging and click the **OK** button.

To load an existing protocol, click **Load** and select the protocol from the study folder.

6.3.3 Editing Protocols

After you have created your brightfield or fluorescence protocol and assigned it to a study, use the Edit Protocol screen to add specific details to the protocol. The next sections are organized by two different types of protocols: brightfield and fluorescence. The sections appear as follows:

Brightfield Protocols

After you have created your brightfield protocol and assigned it to a study, the Edit Protocol window (for brightfield protocols) opens.

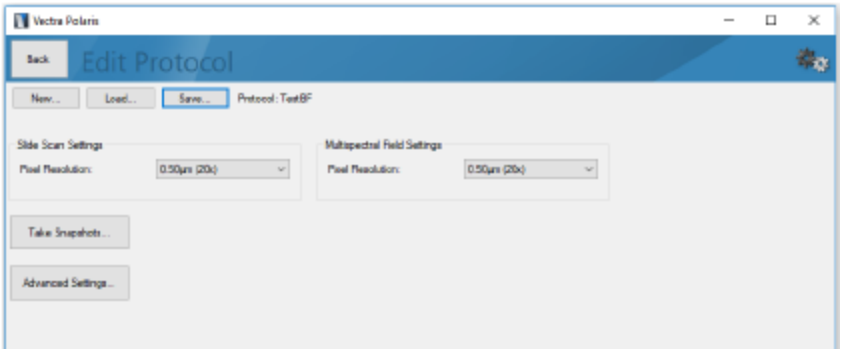


Figure 32. Edit Protocol Window

Under Slide Scan Settings, select the **Pixel Resolution** in the drop-down list to select the resolution to apply to the whole slide. You can choose 1 um per pixel (nominally 10x), 0.5 um per pixel (nominally 20x), or 0.25 um per pixel (nominally 40x).

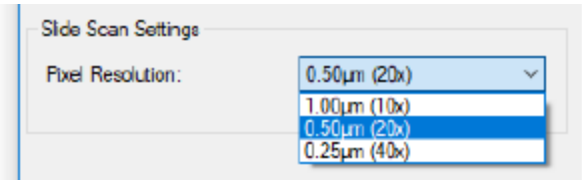


Figure 33. Slide Scan Settings- Pixel Resolution Drop-down List

If you plan to take Multispectral Fields, select the Pixel Resolution in the drop-down list to use for imaging. Again, available resolutions are 1 um per pixel (nominally 10x), 0.5 um per pixel (nominally 20x), or 0.25 um per pixel (nominally 40x).

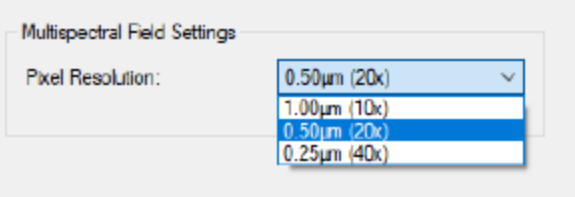


Figure 34. Multispectral Field Settings - Pixel Resolution Drop-down List

Advanced Settings

There may be situations when you need to fine-tune how PhenoImager HT scans the slides. Click the **Advanced Settings** button to adjust the following functions:

- **Coverslip Thickness** - PhenoImager HT is designed for use with standard #1.5 coverslips, where the distance between the tissue and the top of the sample is approximately 170 microns. While this is the optimal setup, PhenoImager HT can handle other scenarios.
    - o If your lab uses thinner coverslips (#0), select “100 um (#0 or similar)” for a Coverslip Thickness.
    - o If your lab uses thicker coverslips, or has a significant amount of mounting media between the sample and coverslip, select the option that best matches your slides.

  NOTE: PhenoImager HT is capable of handling slides with up to 420 microns of combined coverslip or mounting medium above the sample, though imagery at these extremes may be sub-optimal.

- **Scan within the entire coverslip region** - If PhenoImager HT is having difficulty finding your entire tissue sample, check the Scan within the entire coverslip region checkbox. This will increase scan time and file size, but will enable you to complete scanning of difficult samples.
- **Sample is a TMA** - PhenoImager HT has a specialized algorithm for finding TMA samples. If your samples are TMAs, select Sample is a TMA.
- **Image Compression** - By default, brightfield whole slide scans in PhenoImager HT are .jpg compressed in order to save disk space. You can adjust the image quality as needed; higher quality will result in larger files. If you would like use lossless compression instead, select LZW rather than JPEG.
- **Label Barcode Reading** - If you would like PhenoImager HT to decode the barcode on your slide label and save it as part of the scan file, select the type of barcode you are using. By default, PhenoImager HT will not attempt to decode a barcode unless you specifically select this option.
    - o If you have only 1D barcodes, select All 1D barcodes (autodetect type).
    - o If your lab uses a variety of 2D barcodes, select All 2D barcodes (autodetect type).

  NOTE: If your lab only uses a specific type of 2D barcodes, you can decrease the decoding time by selecting that specific type. PhenoImager HT supports decoding of Data Matrix, PDF 417, QR Code, Aztec, and Maxicode.

    - o If your lab is using a combination of 1D and 2D barcodes, select **All barcodes (autodetect type)**. This option will increase scan time and file size, and should only be used if necessary.

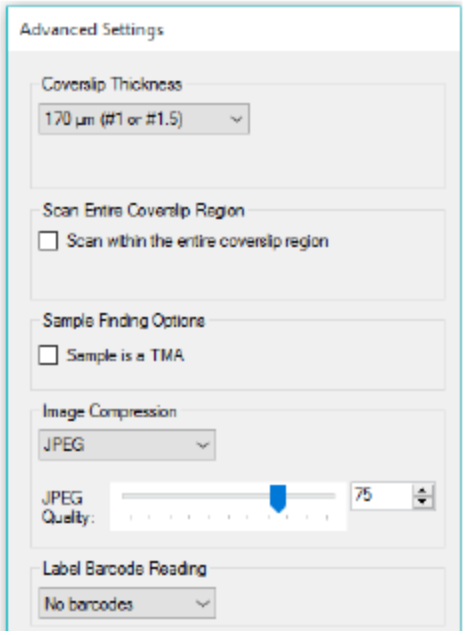


Figure 35. Advanced Settings Window

Click **Save** to save the protocol. The study you previously chose will be automatically selected. You can change the protocol name or study if needed.

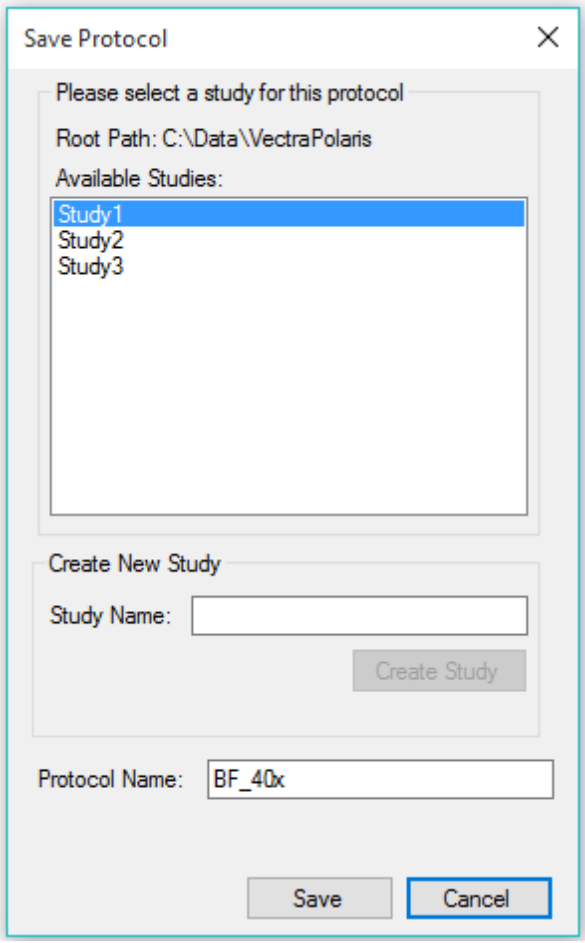


Figure 36. Save Protocol Window

Brightfield Snapshots

Once you have selected your scan and multispectral field resolutions, you can take example snapshots of your slides.

Click **Take Snapshots** to load the Brightfield Snapshots editor.

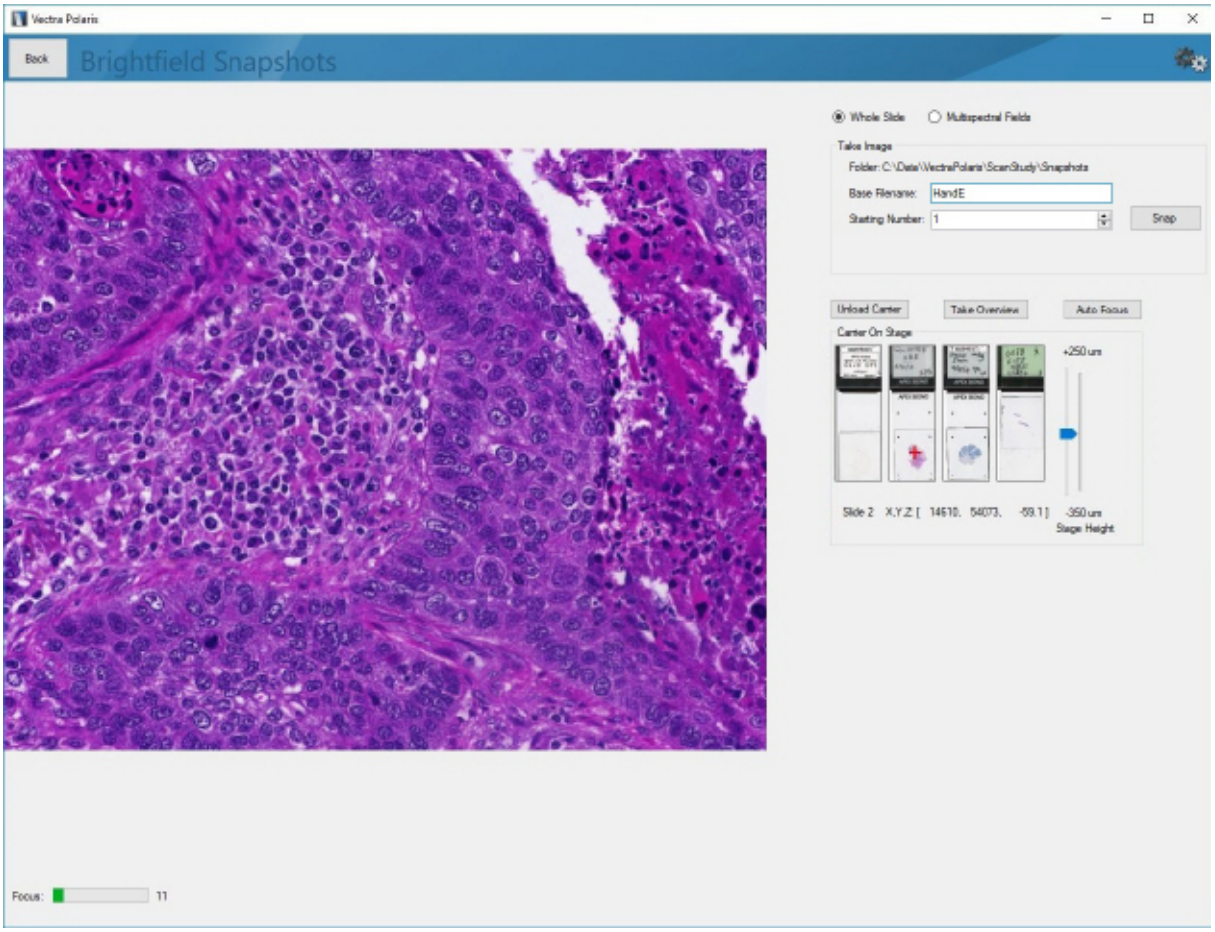


Figure 37. Brightfield Snapshots

If a carrier is not already on stage, click **Load Carrier** to select a carrier that contains the slides you wish to image.

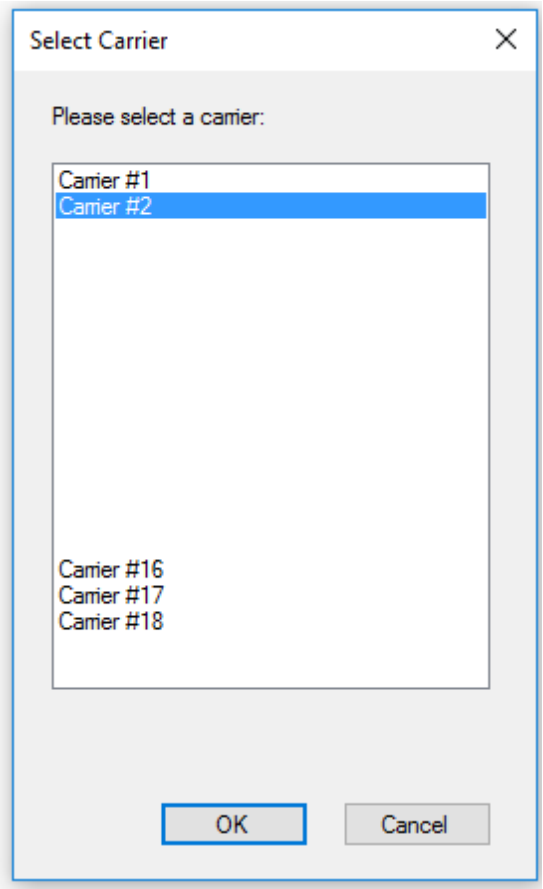


Figure 38. Select Carrier

Use the navigation tool to select an area on the slide. Click on the slide to change positions.

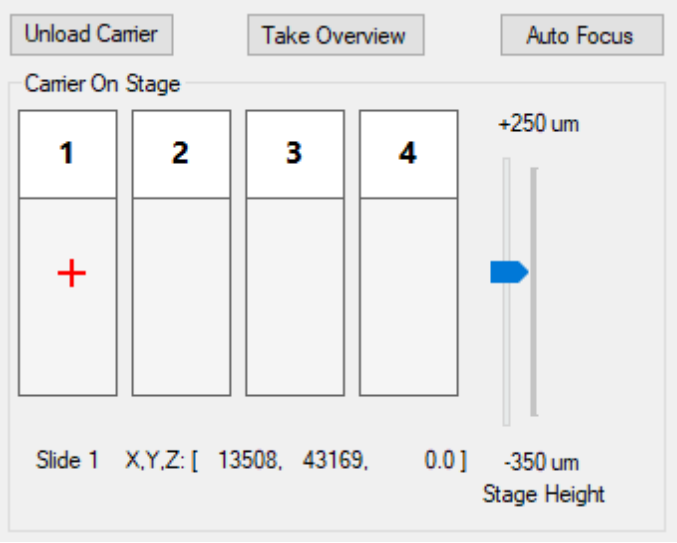


Figure 39. Navigation Tool

For easier navigation, click **Take Overview** to acquire imagery of all slides in your carrier. You can also select that option in the Preferences dialog in the Gear Menu. PhenoImager HT will then automatically acquire overview imagery whenever a carrier is loaded.

Click on the tissue to change positions.

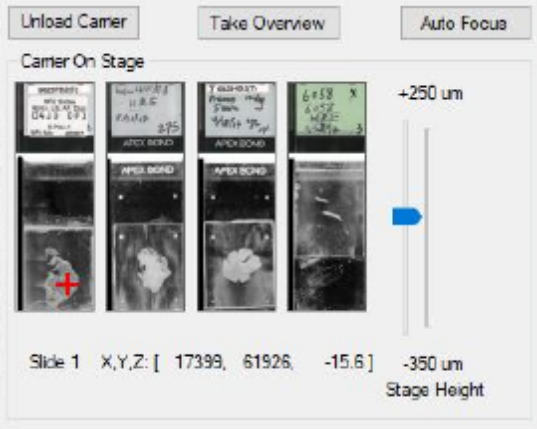


Figure 40. Navigation tool with overview

You can refine the position by using arrow keys (for small movements) or control+arrow (for slightly larger movements).

Click **Autofocus** or use the Stage Height slider to bring the live view into focus.

You can click on a feature within the live view to center on that location or click and drag within the live view to change locations.

To take a snapshot of your current live view, select either the whole slide or the MSI regions radio button, pick a base file name, and click **Snap**.

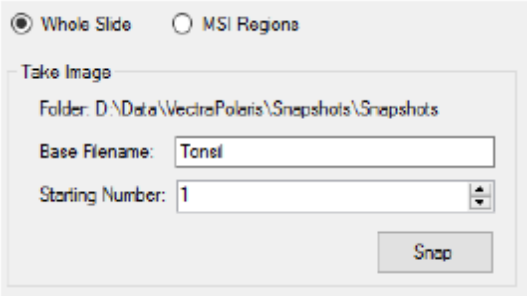


Figure 41. Whole Slide and MSI Regions Radio Buttons

You can navigate to new places, focus as needed, and take as many snapshots as you want. Snapshots will be numbered incrementally.

Click **Back** when you are ready to return to the Edit Protocol window.

Fluorescence Protocols

When creating a fluorescence protocol, you will need slides that include positive expression in all markers of interest in order to set suitable exposure times.

After you have created your fluorescence protocol and assigned it to a study, the Edit Protocol window (for fluorescence protocols) opens.

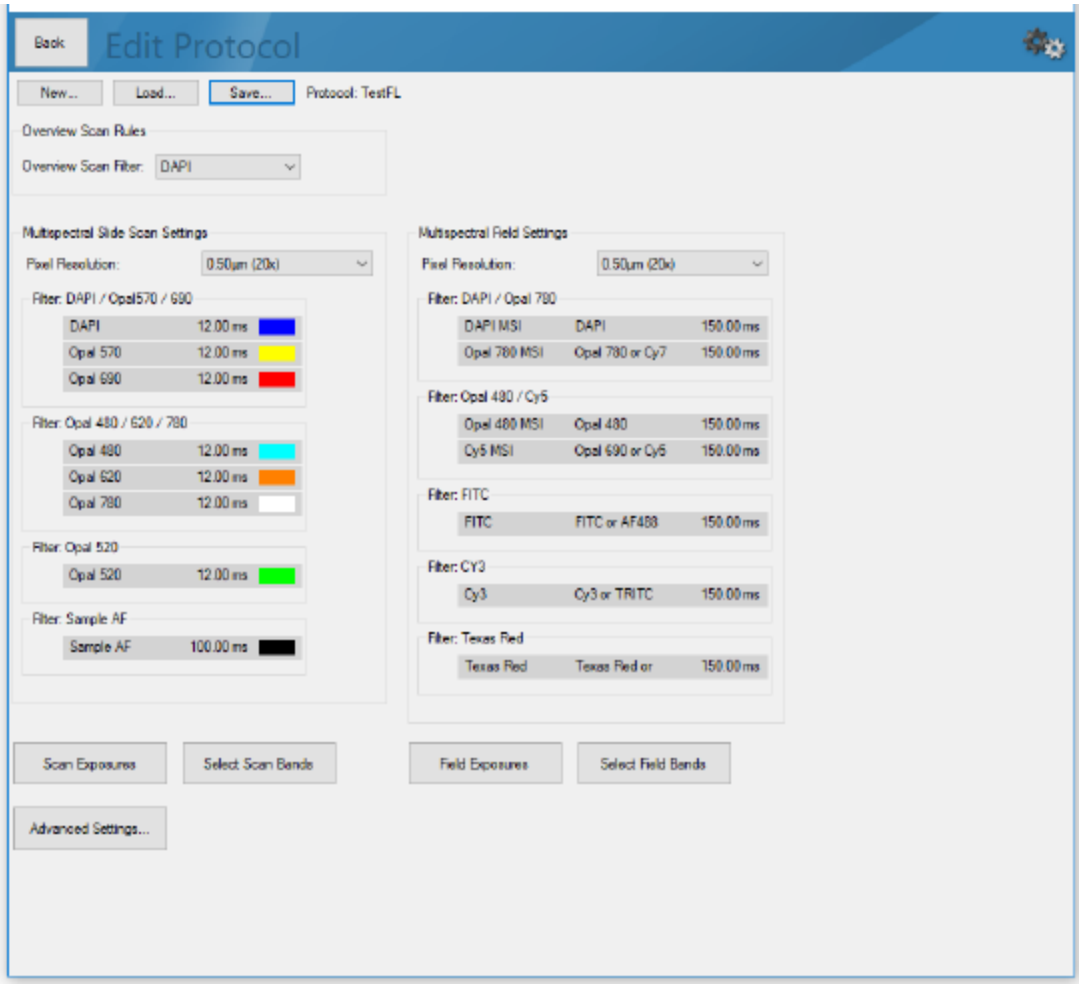


Figure 42. Fluorescence Protocol Editor

Under Overview Scan Rules, select the desired filter that will be used to help find tissue on the slide. This will typically be your DAPI counterstain. If there is no counterstain, then choose a filter that aligns with the expression of your most common stain or auto-fluorescence.

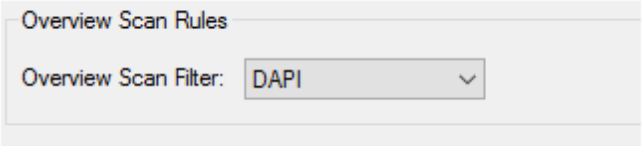


Figure 43. Overview Scan Rules

Under Slide Scan Settings, choose the Pixel Resolution to be used while imaging the slide (see Figure 43). Options include:

- 1.0 um per pixel (nominally 10x)
- 0.5 um per pixel, (nominally 20x)
- 0.5 um per pixel, (nominally 20x, binned from a 40x acquisition)
- 0.25 um per pixel (nominally 40x).

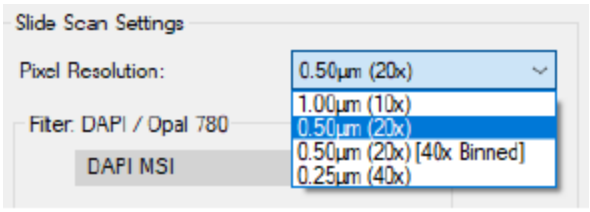


Figure 44. Slide Scan Settings

NOTE: Each time you change the pixel resolution, you will need to reset the exposure times.

Button	Description
Auto-Update	You can auto-update your exposures. This is recommended as a first step to give a close approximation of your exposure times. We then recommend you use the exposures editor to fine-tune your exposures.
Manual Update	You can manually update your exposures. In this case, the current exposures remain unchanged and you must use the exposures editor to obtain valid exposures.
Cancel	You can cancel. This will leave the pixel resolution unchanged.

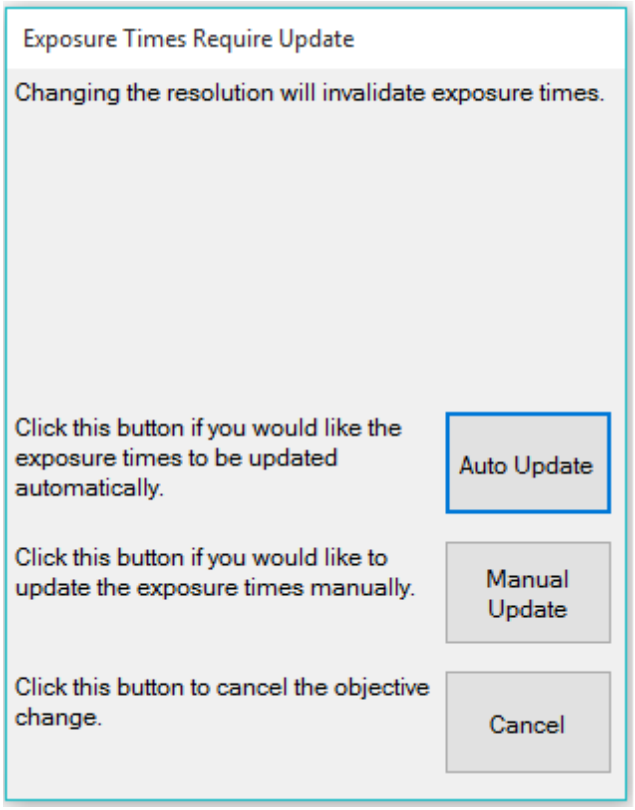


Figure 45. Exposure Times Require Update Window

If you plan to take Multispectral Fields, choose the Pixel Resolution that you want to use for fields imaging. Available resolutions are 10x, 20x, 20x binned, or 40x.

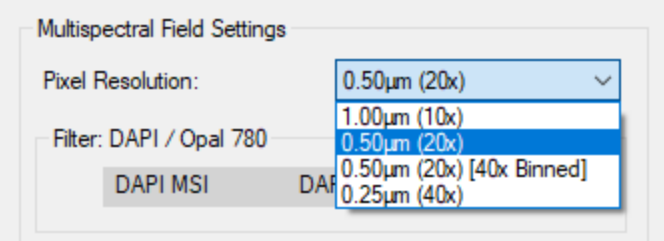


Figure 46. Multispectral Field Settings

The Select Scan Bands button allows you to choose which bands you use to take your slide scan. Imagery for each band will be taken in the order it is shown. By default, PhenoImager HT will take a slide scan using the best selection available for your staining kit, or the bands you selected for a custom scan.

If you would like to remove bands where fluorescent signals are not expressing, uncheck the band. Drag and drop to change the order in which they are acquired. You can change the colors associated with each band; these are the colors that Phenochart will use to display the scan.

NOTE: If you remove the Overview or Focus band, PhenoImager HT will select new bands for you. You may want to visit Advanced Settings to confirm these selections.

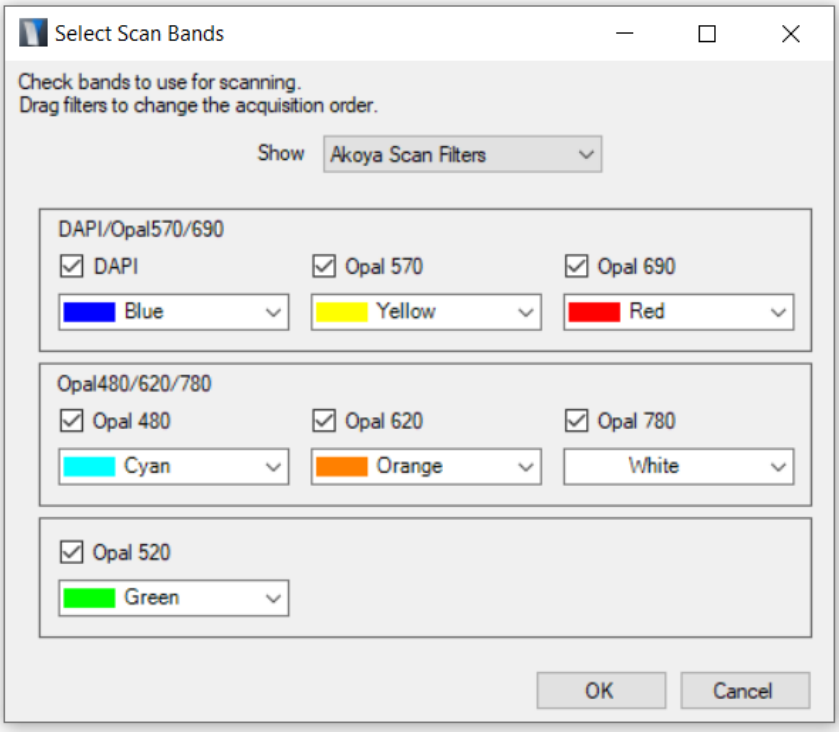


Figure 47. Select Scan Bands

You can limit the filters displayed in the Select Scan Bands dialog.

- Show Akoya Scan Filters to limit the available filters to installed Akoya filters that are appropriate for slide scanning.
- Show All Akoya Filters to display all installed Akoya filters, both for scanning and taking multispectral fields.

- Show All Filters to display all installed filters, including custom filters that you may have installed.

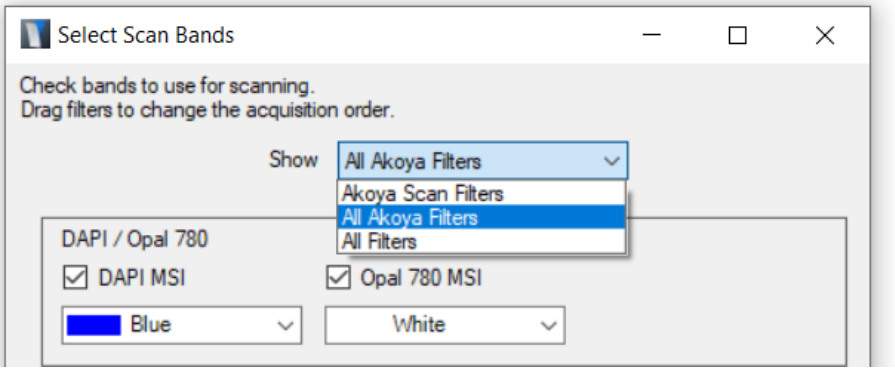


Figure 48. Select Scan Bands - Show Drop-down List

The Select Field Bands button allows you to choose which bands you use to take your multispectral fields. We strongly recommend that you maintain the default band configuration. Non-signal bands are integral to multispectral stain extraction and unmixing.

If you would like to add or remove bands, select or de-select the filter. If there is more than one band available for a filter, you will need to select the band from the drop-down list. Drag and drop to change the order in which they are acquired.

NOTE: If you remove the Focus band, PhenoImager HT will select a new band for you. You may want to visit Advanced Settings to confirm this selection.

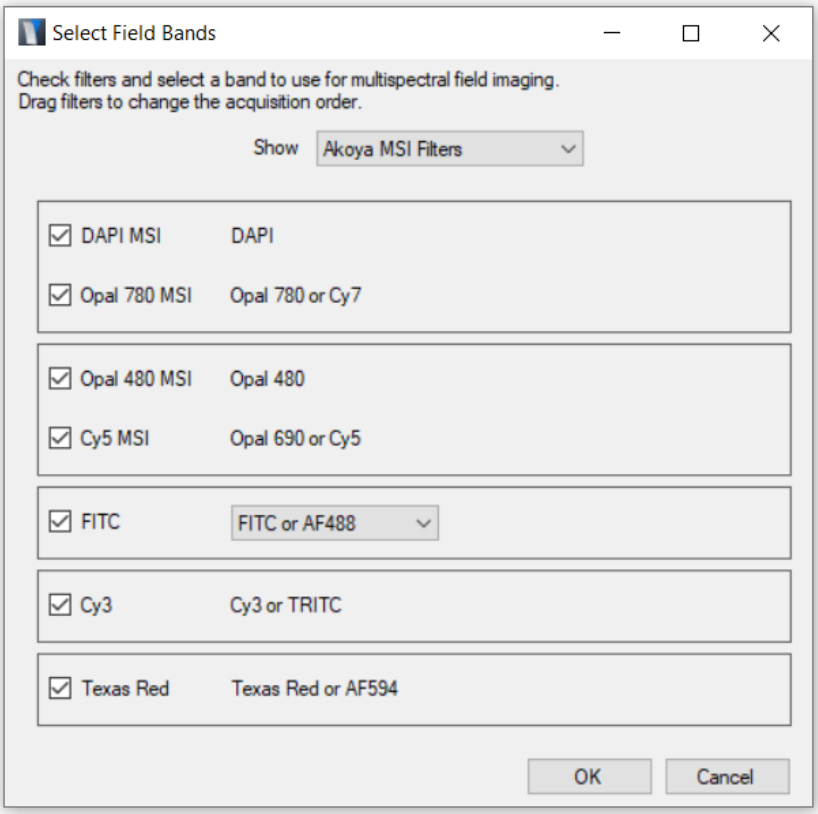


Figure 49. Select Field Bands - Band Selection

You can limit the filters displayed in the Select Field Bands dialog.

- Show Akoya MSI Filters to limit the available filters to installed Akoya filters that are appropriate for multispectral fields.
- Show All Akoya Filters to display all installed Akoya filters, both for scanning and taking multispectral fields.
- Show All Filters to display all installed filters, including custom filters that you may have installed.

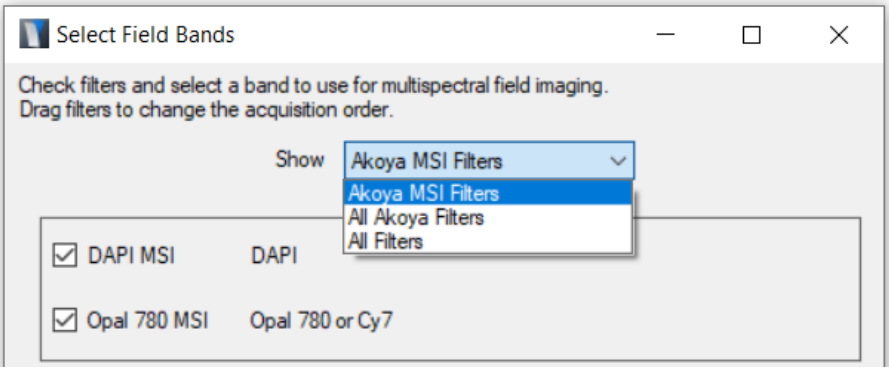


Figure 50. Select Field Bands - MSI Filters

Advanced Settings

There may be situations when you need to fine-tune how PhenoImager HT scans the tissue. Click the **Advanced Settings** button to adjust the following functions:

- PhenoImager HT is designed for use with standard #1.5 coverslips, where the distance between the tissue and the top of the sample is approximately 170 microns. While this is the optimal setup, PhenoImager HT can handle other scenarios.
    - o If your lab uses thinner coverslips (#0), select “100 um (#0 or similar)” for a Coverslip Thickness.
    - o If your lab uses thicker coverslips, or has a significant amount of mounting media between the sample and coverslip, select the option that best matches your slides.

  NOTE: *PhenoImager HT is capable of handling slides with up to 420 microns of combined coverslip or mounting medium above the sample, though imagery at these extremes may be sub-optimal.*

- You can specify which filter will be used for setting focus during whole slide scans and for multispectral field imaging. Choose the filters where your counterstain expresses. This is normally DAPI.
- Saturation protection prevents you from overexposure when acquiring multispectral field imagery. We highly discourage deactivating the Use Saturation Protection setting.
- If PhenoImager HT is having difficulty finding your entire tissue sample, check the 'Scan within the entire coverslip region' checkbox. This will increase scan time and file size, but will enable you to complete scanning of difficult samples.

  NOTE: *If there is Sharpie® markup on your fluorescent slide, PhenoImager HT will use that as the region instead of the coverslip.*

- PhenoImager HT has a specialized algorithm for finding TMA samples. If your samples are TMAs, select Sample is a TMA.
- **Label Barcode Reading** If you would like PhenoImager HT to decode the barcode on your slide label and save it as part of the scan file, select the type of barcode you are using. By default, PhenoImager HT will not attempt to decode a barcode unless you specifically select this option.
    - o If you have only 1D barcodes, select All 1D barcodes (autodetect type).
    - o If your lab uses a variety of 2D barcodes, select All 2D barcodes (autodetect type).

  NOTE: If your lab only uses a specific type of 2D barcodes, you can decrease the decoding time by selecting that specific type. PhenoImager HT supports decoding of Data Matrix, PDF 417, QR Code, Aztec, and Maxicode.

    - o If your lab is using a combination of 1D and 2D barcodes, select All barcodes (autodetect type). This option will increase scan time and file size, and should only be used if necessary.

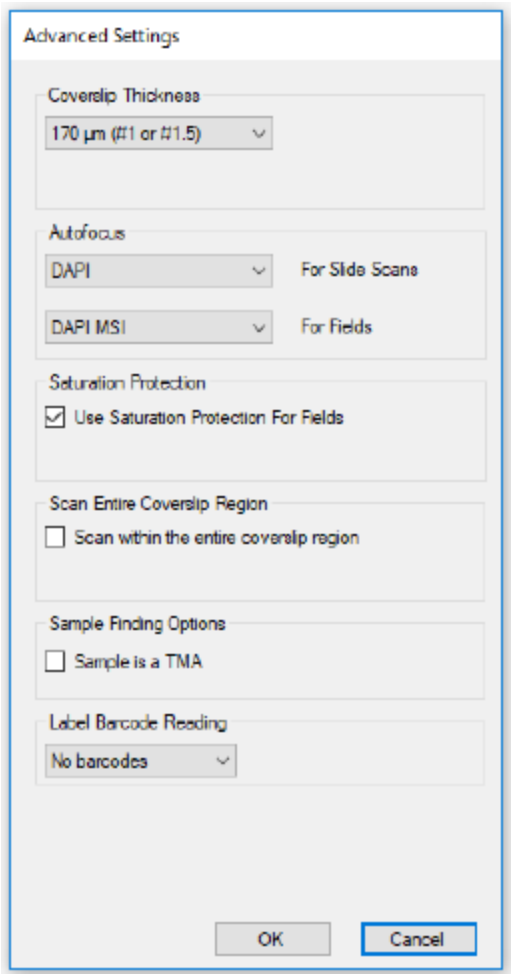


Figure 51. Fluorescence - Advanced Settings

After you have selected your settings, use the Exposures Editor to set exposures 69 for your protocol.

6.3.4 Setting Exposures

After you have selected your scan and multispectral field resolutions, filters, and bands, you need to set exposures. Click **Set Scan Exposures** or **Set Field Exposures** in the Fluorescence Protocol Editor to set exposure times for scanning and field acquisition.

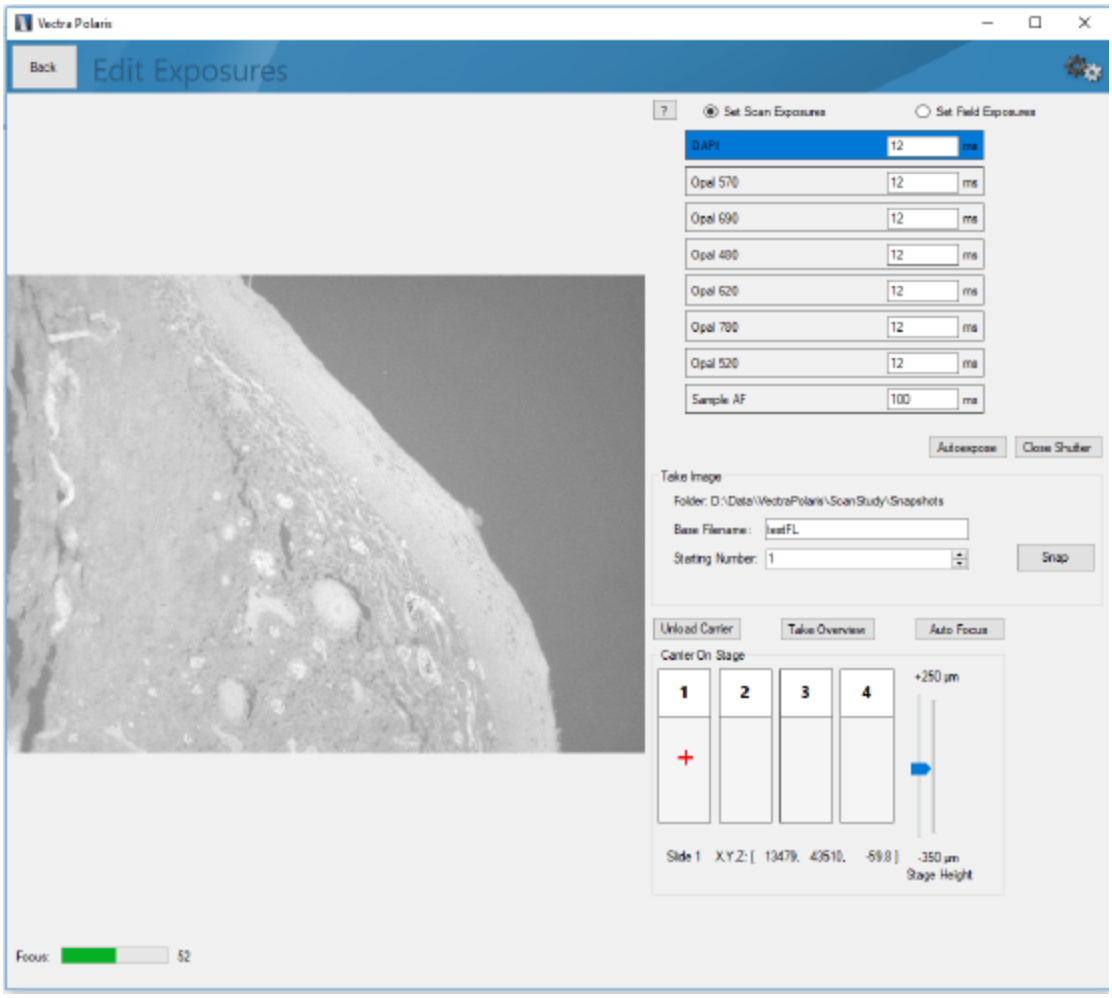


Figure 52. Edit Exposures

If a carrier is not already on stage, click **Load Carrier** to select a carrier that contains representative slides. Select your carrier from the list and load it to the stage.

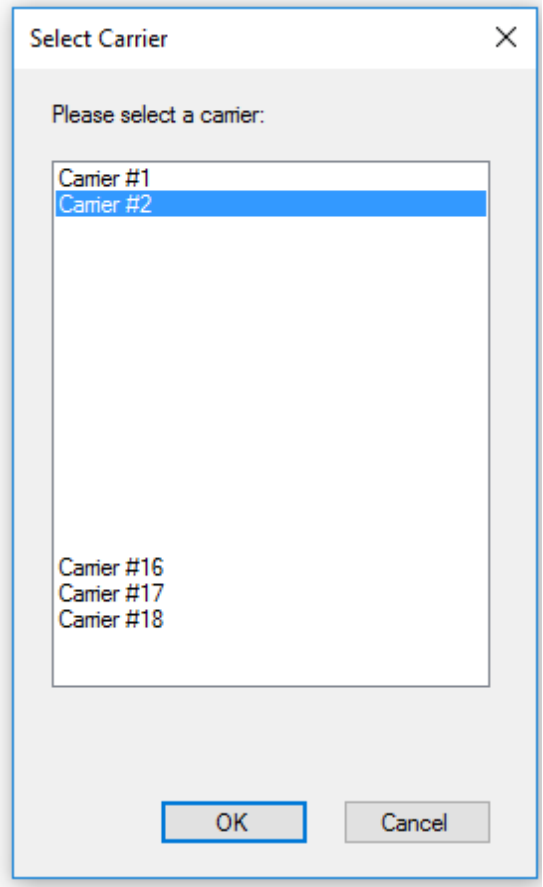


Figure 53. Select Carrier

Use the navigation tool to select a slide. You will see a live view of your first filter for your imaging rules. In most cases, this will be DAPI. The highlighted entry in the table identifies which filter or band is currently shown.

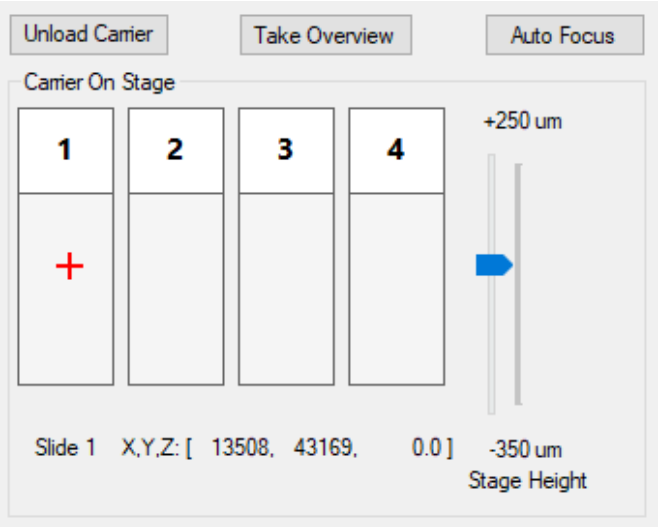


Figure 54. Navigation tool

For easier navigation, click **Take Overview** to acquire digitally enhanced imagery of all slides in your carrier. You can also select that option in the Preferences dialog in the Gear Menu. PhenoImager HT will then automatically acquire overview imagery whenever a carrier is loaded.

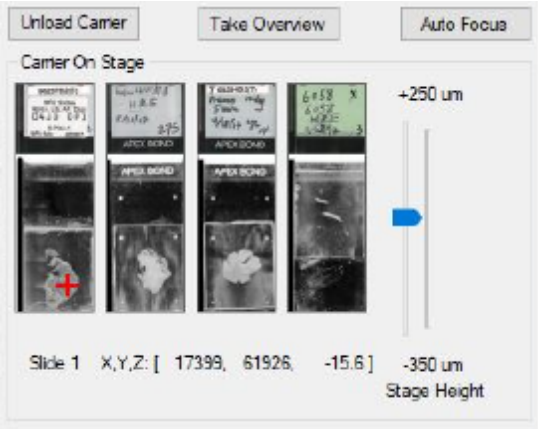


Figure 55. Navigation tool with overview

Click on the tissue to change positions.

You can refine the position by using arrow keys (for small movements) or Ctrl+arrow (for slightly larger movements).

You can click on a feature within the live view to center on that location, or click and drag within the live view to change locations.

Click **Autofocus** or use the **Stage Height slider** to bring the live view into focus. Click **Autoexpose** to have the system find the best exposure for that filter/band.

- After auto-exposing, you may want to auto-focus and auto-expose again to refine your focus and exposure estimates.
- You can override the auto-exposure value by typing a value in the highlighted cell. Values must be between 0.1 and 2000 ms.
- If you would like to turn off the fluorescence illumination and live view, click **Close Shutter**. You will need to re-open the shutter to see a live view and set exposures.

If you plan to use this protocol for multispectral field acquisition, you should set the multispectral field exposures at this time. In most cases, you can use the same area of the slide to set your multispectral field exposures. Select the **Set Field Exposures** radio button to set exposures for multispectral fields. Focus and click **Autoexpose**.

- As with whole slide scan exposures, after auto-exposing, you may want to auto-focus and auto-expose again to refine your focus and exposure estimates.
- You can override the auto-exposure value by typing a value in the highlighted cell. Values must be between 0.1 and 2000 ms.
- If you would like to turn off the fluorescence illumination and live view, click **Close Shutter**. You will need to re-open the shutter to see a live view and set exposures.

Repeat the steps above for all filters and bands in your protocol. You may need to change locations and/or slides to find the best signals for setting your exposures.

Filter Cube Recommendations when using Akoya Biosciences Opal™ Reagents

If you are using your PhenoImager HT system with Akoya's Opal™ multiplex IHC detection kits or with individual Opal fluorophores, please refer to Table 11 and Table 12 for exposure time recommendations for acquiring spectral library images.

When acquiring multispectral slide scans with dyes in Opal 4-color / 3-plex or Opal 7-color / 6-plex kits:

Table 11. Multispectral Slide Scan Band

	DAPI	Opal 480	Opal 520	Opal 570	Opal 620	Opal 690	Opal 780	Sample AF
DAPI								
Opal 480								
Opal 520								
Opal 570								
Opal 620								
Opal 690								
Opal 780								

When acquiring multispectral fields:

Table 12. Multispectral Field Band

	DAPI	Opal 480	FITC	Cy3	Texas Red	Cy5	Opal 780
DAPI							
Opal 480							
Opal 520							
Opal 540							
Opal 570							
Opal 620							
Opal 650							

Opal 670							
Opal 690							
Opal 780							

Key:

- Colors are signal bands: autoexpose or manually set an appropriate exposure per sample.
- Dark Gray is an autofluorescence band: autoexpose or manually set an appropriate exposure per sample.
- Light Gray is a non-signal band: manually set exposure according to Table 13.

Table 13. Exposure Times for Non-signal Bands

	Resolution			
	10x	**20x**	**20x (40x binned)**	**40x**
Multispectral Slide Scans	6 ms	25 ms	2.5 ms	8 ms
Multispectral Fields	35ms	150 ms	15 ms	50 ms

Snapshots

When you have set your exposures, you can take snapshots to see sample imagery of how your exposures perform on a given area of the slide. Select either the **Set Scan Exposures** or the **Set Field Exposures** radio button, pick a base file name, and click the **Snap** button. You can navigate to new places and take as many snapshots as you want. They will be numbered incrementally.

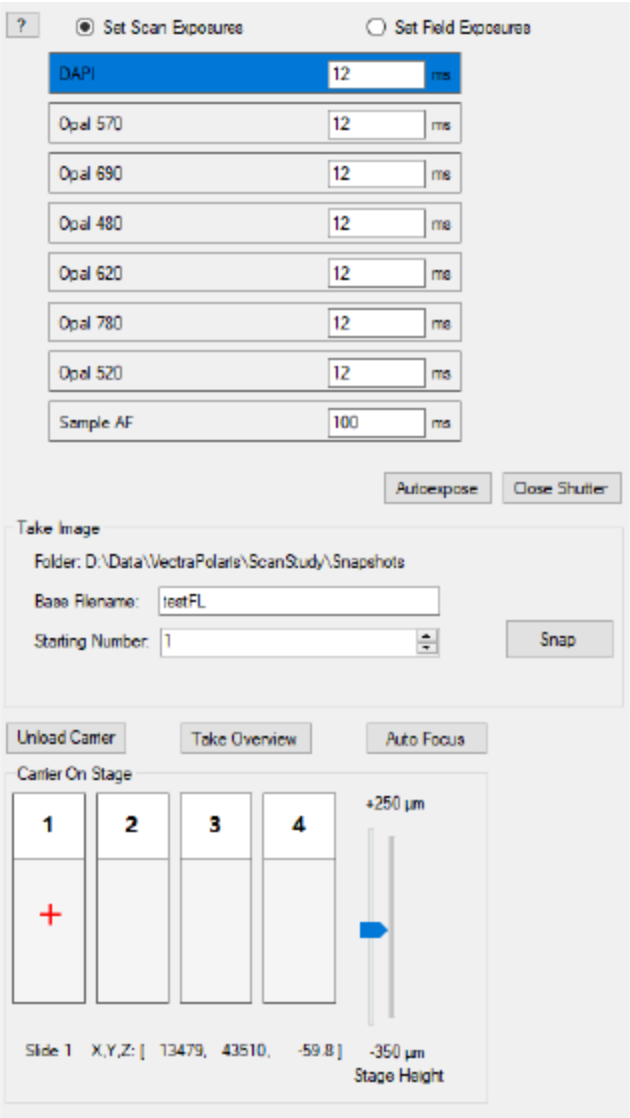


Figure 56. FL Exposures Panel

Click the **Back** button when you are ready to return to the Edit Protocol window.

NOTE: The exposures you just set are only valid for the resolutions you picked. If you change your scan or multispectral field resolution, you will need to revisit the exposures editor to update your exposures.

6.4 Scanning Slides

The section describes the procedures involved when scanning slides:

- The Carrier 76
- Carrier Status 76
- Slide Status 78

- Setting Up Scan Rules 78

6.4.1 The Carrier

Slides are loaded into PhenoImager HT via carriers, which are stacked into the Slide Carrier Hotel.

Figure 57. Slide Carrier

The hotel can hold up to 20 carriers, and each carrier can hold up to 4 slides. This means PhenoImager HT can be loaded with up to 80 slides at any given time. Those 80 slides can all be scanned based on the same set of rules, or you can tailor your scan rules to each slide and/or each carrier as needed.

6.4.2 Carrier Status

Within the software, each hotel slot is identified as 'Slot N,' where N is 1-20 running top to bottom. When a slide carrier is loaded into the hotel, an LED light changes color to identify the status of that carrier.

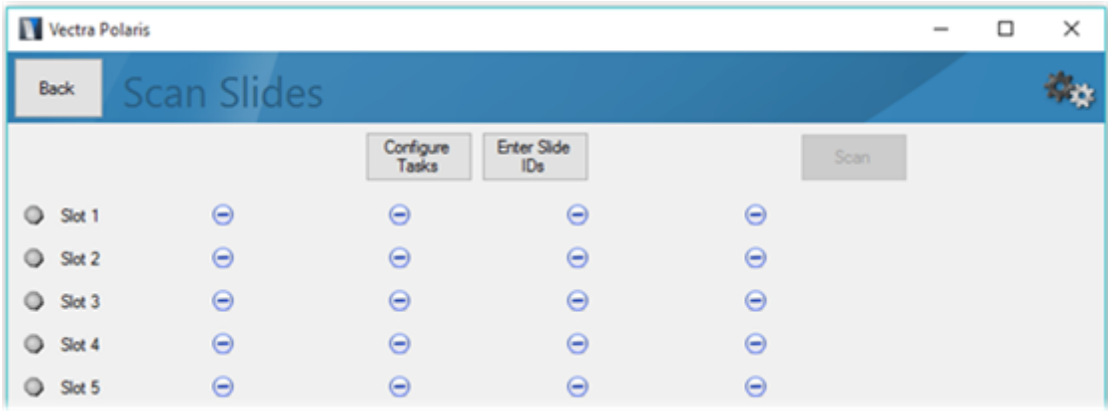


Figure 58. Scan Slides - Carrier Status

The User Interface will reflect the current state of each slot in the hotel. If there is no carrier present, you will see:

Each carrier present in the hotel will be represented as follows:

Each carrier is represented by a carrier status icon, four slide status icons, and four slide IDs.

The carrier status icon corresponds to the hotel LED for that particular carrier. Each possible status is represented by a different color.

When you insert the carrier into the hotel, a white icon indicates that the carrier is present but has no rules to use for slide scanning. If the icon is blinking red, the carrier has not been completely inserted. Try pushing it in further.

Once the carrier has been inserted in the hotel, you can define rules for your scan. The icon will change to blue when there is at least one slide ready to be scanned. Carriers that have been prioritized will be blinking blue.

If no slides on the carrier can be scanned with the current rules, the icon will change to yellow. This carrier will be ignored until the problem is resolved.

When scanning has started, the icon for the carrier on stage will be blinking green. When the carrier is returned to the hotel, the icon will either be green (for a successful scan) or orange (if there was a problem with the scan).

If the icon is solid red, that hotel slot's sensor is malfunctioning. Do not use this slot.

6.4.3 Slide Status

Each carrier also has a status icon for the 4 slides it contains.

means the slide has complete rules and is ready to be scanned.

(blinking) means the slide is currently being scanned.

means the slide was scanned successfully.

Other options include , which means the slide has rules, but they cannot be used. (They may be incomplete, in which case you will need to edit the rules for that slide). indicates that the slide failed to scan correctly.

Finally, will indicate that the slide is being ignored. This is useful if the carrier has fewer than four slides or if you don't want to process that slide at this time.

6.4.4 Setting Up Scan Rules

Scan rules must be defined for each slide you would like to process. Scan rules are complete when a study, protocol, task, and slide ID have been assigned.

This section contains:

Slide IDs

You must manually enter the ID for each slide in the carrier. You can enter IDs before the carrier is placed into the hotel, or you can enter IDs after the carrier has been placed into the hotel.

If you would like to enter the IDs while the carrier is still in your hand, click **Enter Slide IDs**.

Figure 59. Enter Slide IDs Button

It will bring up this dialog:

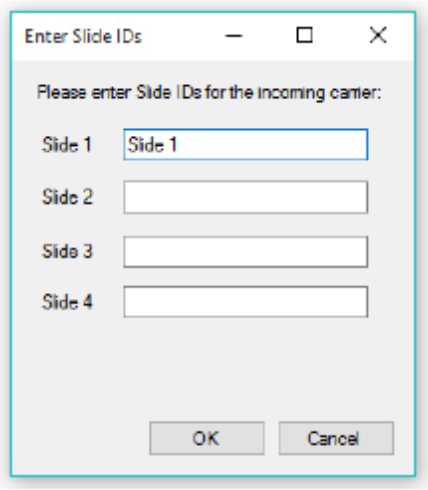


Figure 60. Enter Slide IDs Window

You can then enter between 1-4 slide IDs for your slides and click **OK**. Immediately put the carrier in the hotel, and PhenoImager HT will assign the slide IDs you entered to that carrier.

If your carrier is already present in the hotel, you can edit the carrier to add the slide IDs along with the other scanning rules.

Editing a Single Slide

To edit the scanning rules for a single slide, click the **status icon** for that slide. It will bring up this editor:

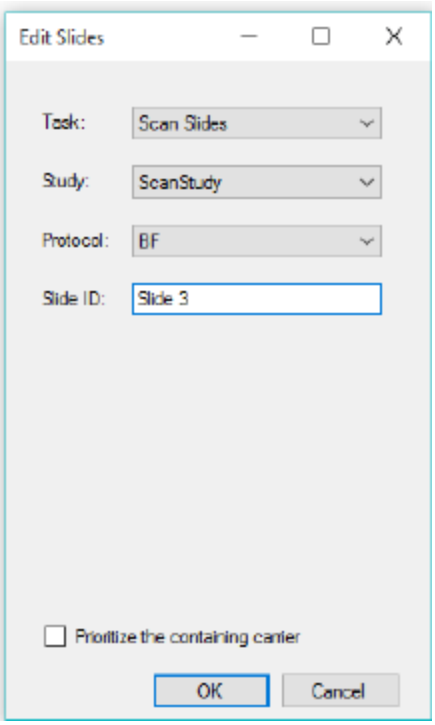


Figure 61. Edit Slides

For this slide, enter:

- **Task** - Choose **Scan Slides** or **Acquire Fields**.

  NOTE: Select Ignore if you do not wish to process this particular slide.

- **Study** - Select a study.
- **Protocol** - Select any protocol you have saved within the study.
- **Slide ID** - Add your slide ID, if necessary.

Check Prioritize the containing carrier if you want this slide's carrier to be scanned at the front of the line.

Editing All Slides Within a Single Carrier

To edit the scanning rules for a single carrier, click the **status icon** for that carrier. It will bring up this editor:

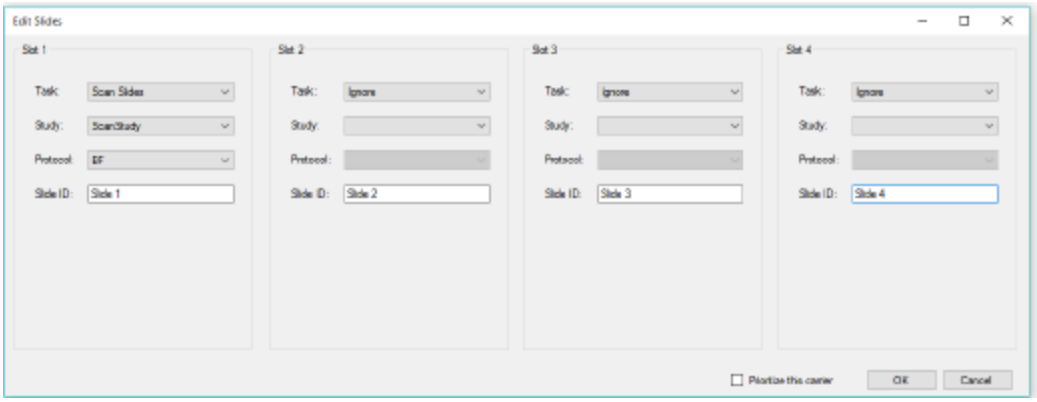


Figure 62. Edit Slides

For each slide, you can enter:

- **Task** - Choose **Scan Slides**, **Acquire Fields** or **Ignore**. Select **Ignore** if there is no slide in the slot.
- **Study** - Select a study.
- **Protocol** - Select any protocol you have saved within the study.
- **Slide ID** - Add your slide ID, if necessary.

Check Prioritize this carrier if you want this carrier to be scanned at the front of the line.

Editing Multiple Carriers

To edit multiple carriers, Click **Configure Tasks**.

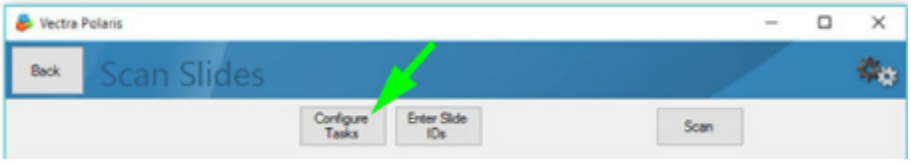


Figure 63. Configure Tasks Button

Select the carriers you want to edit. Multiple selection is available.

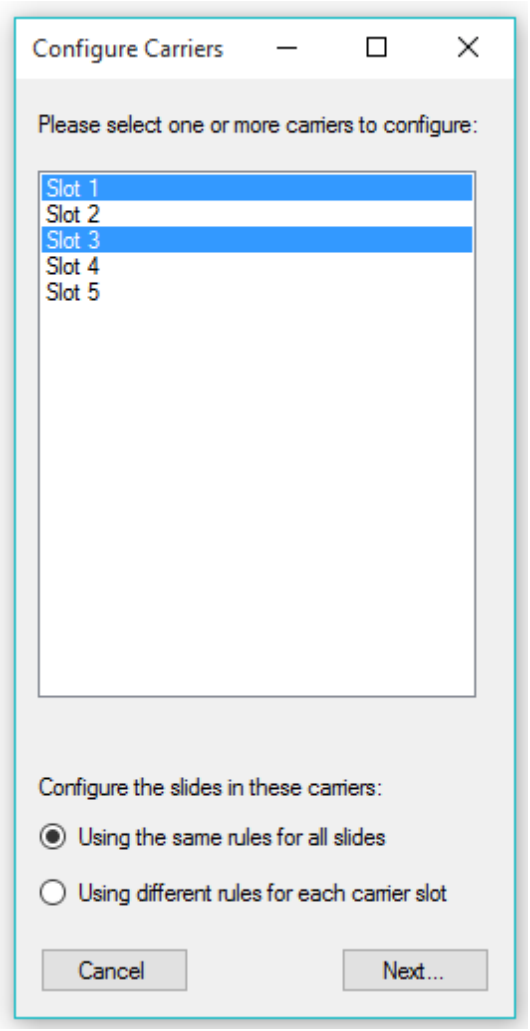


Figure 64. Configure Carriers

You will also need to select how you want to process your slides.

To process all slides using the same rules, choose Using the same rules for all slides and click **Next**... The Edit Slides window opens.

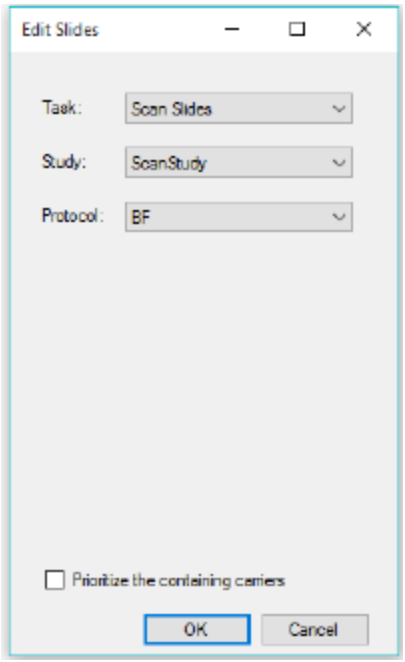


Figure 65. Edit Slides

- Select your task, study, and protocol.
- Check **Prioritize the containing carriers** if you want the selected carriers to be scanned at the front of the line.

NOTE: You will not be able to edit the slide ID, because you are applying rules across multiple slides. If you need to enter slide IDs, you can edit the individual carriers.

To use different processing rules for each carrier position, choose **Using different rules for each carrier slot** and click **Next**... For each position in the carrier, select the task, study, and protocol. Prioritize if desired. Again, you will not be able to edit the slide ID, because you are applying rules across multiple slides.

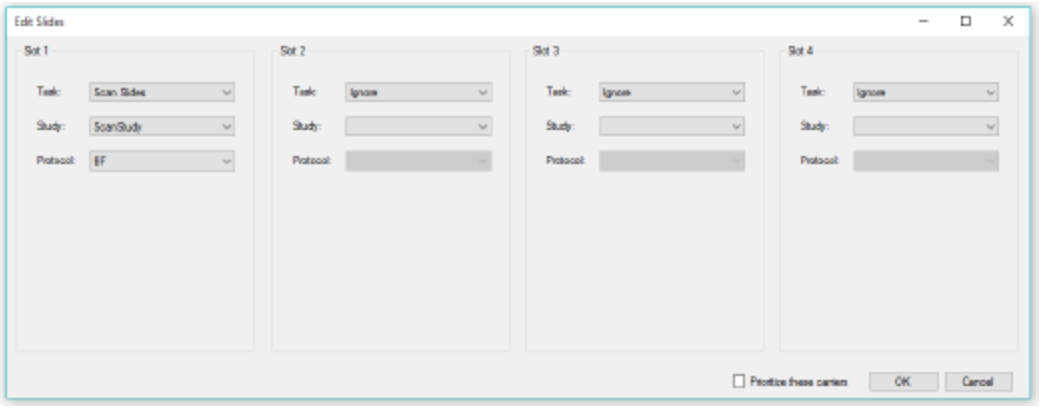


Figure 66. Edit Slides

Saving and Restoring Scan Setup

After setting up all the details for your slides, you may want to save your work for later use. This may be useful if the scanning process is interrupted before the task is finished or if you need to return later to re-scan certain slides.

You can save your slide IDs, studies, protocols, and tasks by clicking the **Save Setup** button. PhenoImager HT will also prompt you to save before scanning (when you click **Scan**). At this point, you can enter a name and some notes about this scan setup.

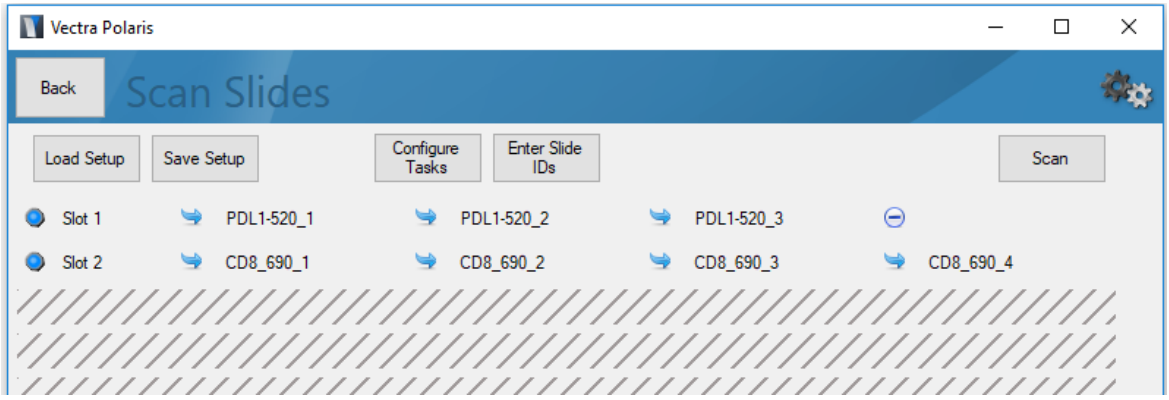


Figure 67. Scan Slides

Saving a setup is not required, but it can help you locate the information later if needed. By default, this information will be deleted after 30 days, but you can override the default and opt to keep the information until you manually delete it.

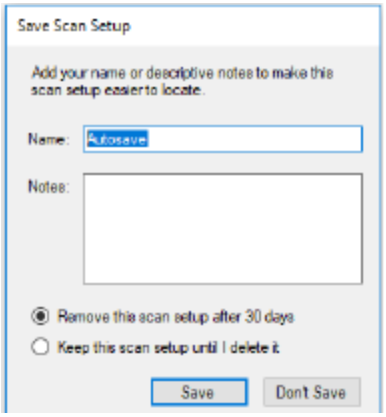


Figure 68. Save Scan Setup

To restore an earlier scan setup, click **Load Setup**.

All available setups will be shown. You can filter by date to show setups only from a specific time period. Click the headers to sort by date, name, notes, studies, etc.

NOTE: You can also delete entries that are no longer needed by selecting those items and clicking **Delete**.

To use a setup, select it from the dialog and click **Load**.

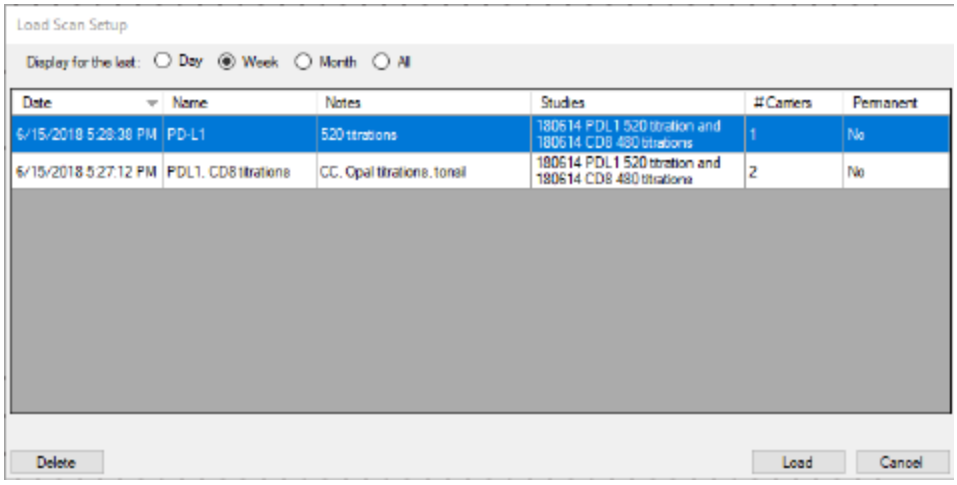


Figure 69. Load Scan Setup

After you have selected a setup, you need to configure the carriers in PhenoImager HT to match the information in the setup.

If the carriers and slides are the same location as when you saved the setup, you only need to click **OK**, because everything is in the right place.

In other cases (for example: when you need to put your slides back in), this dialog will help you figure out where your slides belong.

1. When scan information is available and a carrier is present in the hotel, the system will show the slide IDs (slot 1 in Figure 70).

2. When scan information is available but there is no carrier present in the hotel slot, the slot will be labeled as Missing in red (see slot 5 in Figure 71). The slide IDs for that carrier are listed, so you can locate the correct slides, place them in the carrier, and insert the carrier into the hotel.

3. When no information is available for a carrier that is present in the hotel, the system will show italicized Slide N text for the slide IDs (slots 7, 9, etc). Slides in these carriers were not present in the original setup.

For every item that the scan setup had - meaning, anywhere the system has information on a carrier that should be present (items 1 and 2) - you need to specify what to do before the carrier can be loaded. There are 3 options:

1. **Use as saved:** The system will load all slide information including the scan task.

2. **Ignore task:** The system will load all slide information except for the scan task. The scan task will be set to ignore. This is useful if you are loading the setup so you can rescan a few select items, or if you know that you're going to be changing the task from how it was originally saved.

3. **Don't restore:** No information for this carrier will be reloaded. This is useful if you do not have or do not need the carrier.

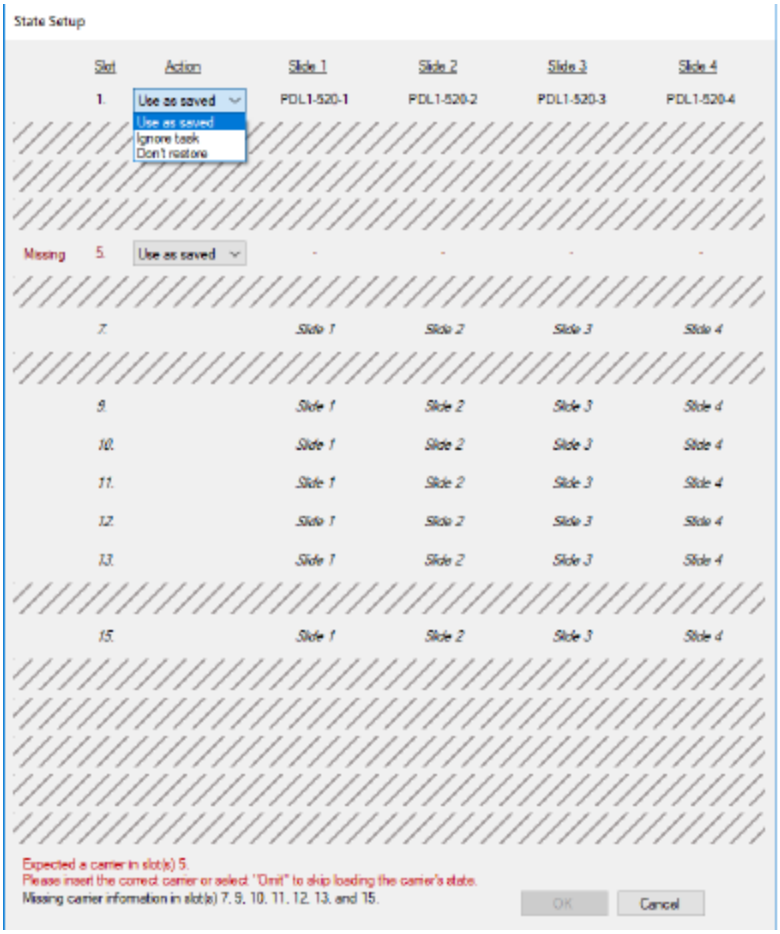


Figure 70. Slide Setup

The red text at the bottom of the screen will list any issues that need to be fixed before scanning.

Click **OK** when you have finalized your selections. The scan slides editor will be cleared and replaced with your selected scan information.

Scanning

The Scan button is enabled when at least one carrier is ready to be scanned. Click **Scan** to start scanning. If any carriers have been prioritized, these carriers will be processed first.

PhenoImager HT will report scanning progress for each slide on the progress dialog and on the front panel LEDs.

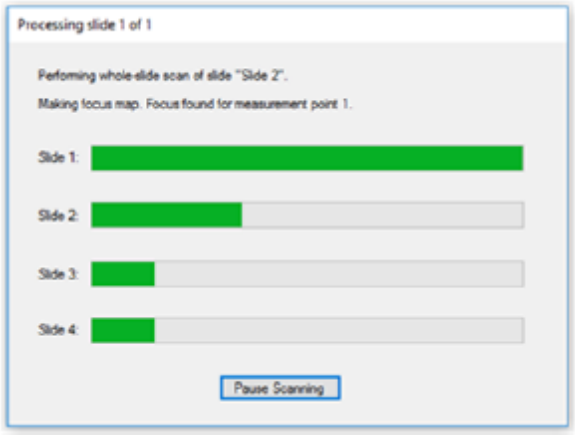


Figure 71. Processing Slides Window

Click **Pause Scanning** to remove completed carriers and add new carriers to be scanned. When you pause a scan, PhenoImager HT will return the current carrier to the hotel. It will then be safe to open the door. You can remove carriers that are completed and replace them with unscanned carriers. Edit those carriers to set them up for scanning, and reprioritize carriers if needed. When you're ready, click **Scan** to resume scanning.

7 Maintenance

This section includes procedures that are to be performed periodically, either to make the PhenoImager HT run better, or to protect its components from damage.

7.1 Cleaning the Instrument Exterior

Clean the PhenoImager HT instrument exterior as necessary.

The non-electrical exterior parts of the PhenoImager HT can be wiped down with a soft cloth using standard laboratory grade cleaning solutions including:

- 70% ethanol
- 10% bleach
- Clidox®
- Sporicidin®

WARNING:

DO NOT spray cleaning solutions directly onto the PhenoImager HT instrument. Sprays and liquids that come into contact with the PhenoImager HT instrument may result in damage to the system or electric shock. Always spray the cleaning solution onto a cloth and then wipe the instrument exterior with the cloth.

7.2 Cleaning the Monitor

Clean the monitor as necessary with a soft, lint-free cloth. If needed, dampen the cloth with water or an eyeglass cleaner.

WARNING:

DO NOT spray cleaning solutions directly onto the monitor screen. Sprays and liquids that come into contact with the screen may result in damage to the system or electric shock. Always spray the cleaning solution onto a cloth and then wipe the screens with the cloth.

7.3 Cleaning the Power and Communication Ports

Dust can be removed from the power and communication ports with a gentle air stream from compressed air cans. DO NOT shake the can before use. Hold can at a distance from the ports to prevent condensation.

WARNING:

Turn off the electrical power to the PhenoImager HT system by shutting down the system (see System Shutdown 39) before cleaning any part of the instrument where electrical or fiber optic cables make connections.

7.4 Cleaning the Slide Carriers

It is important to keep carriers free from sticky debris caused by slide labels, tape, or mounting media.

Slide carriers can be cleaned using soap and water with a soft cloth, or using an ultrasonic bath.

7.5 Replacing the Fuses

For blown fuses and to order replacement fuses, contact Akoya Biosciences Technical Support (see Contact Us 6).

WARNING:

Electric Shock Hazard. DO NOT attempt to replace the fuses. Only qualified Akoya Biosciences or PerkinElmer service personnel can replace the fuses.

8 Appendix A: Akoya TIFF 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 use stripped format.

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.

A thumbnail RGB image is provided, and this is a good image to use as an icon in graphical image lists. 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 1. Images

Description	RGB/mono	Title/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	Stripped	~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	Stripped	~2000 x 4000	Required for whole-slide scans Optional for simple RGB images and extracted components
Label image	RGB	Stripped	~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 the table 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 2. TIFF tags

TIFF Tag	Optional?	Description of contents
Software		Starts with "PerkinElmer-QPI"
ImageDescription		Further metadata in XML format (see next section)
ImageWidth		Width of the image in pixels
ImageLength		Height of the image in pixels
ResolutionUnit		Unit used for resolution and position (see below)
XResolution		Pixel X resolution (see below)
YResolution		Pixel Y resolution (see below)
XPosition	Y	Sample X location in ResolutionUnits. This is ULHC location except for Macro image which reports its image center.
YPosition	Y	Sample Y location in ResolutionUnits. This is ULHC location except for Macro image which reports its image center.
SampleFormat		Integer (1) for BF, FL; or float (3) for extracted components
SMinSampleValue		Minimum signal value in the image
SMaxSampleValue		Maximum signal value in the image
BitsPerSample		8 (FL); 8, 8, 8 (RGB); or 32 (unmixed component)
SamplesPerPixel		1 (FL or unmixed component) or 3 (RGB)
NewSubfileType		0 for full-resolution images, 1 for reduced res images
TileWidth	Y	Tile width (512) if tiled format is used

TileLength	Y	Tile height (512) if tiled format is used
TileOffsets	Y	List of tile offsets, if tiled format is used
TileByteCounts	Y	Size of each (compressed) tile, if tiled format is used
StripOffsets	Y	List of strip offsets, if tiled format is not used
RowsPerStrip	Y	Number of rows per strip, if tiled format is not used
StripByteCounts	Y	Size of each (compressed) strip, if tiled format is not used
PlanarConfiguration		1 (chunky) for RGB images, 2 (planar) otherwise
PhotometricInterpretation		2 (RGB) for RGB images, 1 (BlackIsZero) otherwise
DateTime		Acquisition time
Compression		May be None, CCITT Group 3, PackBits, LZW, or JPEG
JPEG fields	Y	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 the example below.

Table 3. Image Description tags

Tag	Optional?	Contents
DescriptionVersion		Version of the image description field itself, a single number. This document describes version 2 of the field.
AcquisitionSoftware		Software used to acquire the image
Identifier		GUID in string format. This is an identifier for the image file itself.
SlideID	Y	ID of the slide that this image was taken from.
Barcode	Y	Barcode text of the slide this image was taken from.
ComputerName	Y	Name of the computer on which the slide was scanned.
ImageType		A string identifying the type of image within the file (Table 1), with the following values: • FullResolution • ReducedResolution • Thumbnail

		• Overview • Label
IsUnmixedComponent		"True" for unmixed multispectral images, otherwise "False".
ExposureTime		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		A byte *wwww tttt* where the *tttt* nibble indicates the signal unit type from the following: • – raw counts • – normalized (counts/second/gain/full-scale/binning) • – OD (optical density) • – dark-corrected counts and the *wwww* nibble indicates how the signal is weighted across the spectral bands (or colors): • – 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	Y	Band name for FL whole slide scans, component name for extracted components. Not present for RGB images
Color	Y	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	Y	Instrument responsivity, if available, for FL whole-slide and unmixed images. See below for details.
Objective	Y	Objective name, if known, otherwise not present.
ScanProfile	Y	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	Y	Name of the acquisition protocol.
ValidationCode		Used for internal data integrity checks – readers can ignore this.
ImagingCycle	Y	Zero-based acquisition cycle for CODEX imagery
Biomarker	Y	String indicating target such as CD8, Ki67, etc.
ScaleFactor	Y	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.
AutofluorescenceSubtracted	Y	String containing 'true' or 'false', indicating whether this dataset has been subtraction-corrected by a background (autofluorescence) image. Treated as 'false' by default.
LampType	Y	String with the FL lamp type • Unknown (default) • PriorLumen200 • PriorLumen220 • XCiteMultiBandLed

		• PriorMimicLed • SolaLed • Colibri7
InstrumentType	Y	String with the instrument type, one of the following: • Unknown (default) • Keyence700 • Keyence800 • AxioObserver • Dmi8 • Polaris • Vectra3
CameraType	Y	String with the camera sensor type, one of the following: • SonyICX285AL • HamamatsuC11440 • IMX265 • IMX421
ExcitationFilter	Y	Description of excitation filter with sub-elements listed below If present, all its sub-elements must be provided.
Name		String with the filter public name
Manufacturer		String with the manufacturer or OEM supplier
PartNo		String with the manufacturer part number
Bands		Collection of bands each as described by sub-elements below
Band		Description of a band with sub-elements noted below
Name		String with the band name
Cuton		Cuton wavelength of this band in nm
Cutoff		Cutoff wavelength of this band in nm
Active		True if this band is excited, False if not
EmissionFilter	Y	Description of emission filter with sub-elements listed below. If present, all its sub-elements must be provided.
Name		String with the filter public name
Manufacturer		String with the manufacturer or OEM supplier
PartNo		String with the manufacturer part number
Bands		Collection of bands each as described by sub-elements below
Band		Description of a band with sub-elements noted below
Name		String with the band name
Cuton		Cuton wavelength of this band in nm
Cutoff		Cutoff wavelength of this band in nm
CameraSettings	Y	Description of the camera settings used for this image with sub-elements listed below. If present, all its sub-elements must be provided
Gain		Gain factor, such as 1, 3, etc.
Binning		Binning extent, such as 1 for full-resolution, 2 for 2x2 binning
OffsetCounts		Offset count setting for the camera
BitDepth		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 QPTIFF file with 8-bit resolution)
Orientation		String containing one of the following values: • 'Normal' (default) • 'Rotate180' • 'MirrorV' • 'MirrorH'
ROI	Y	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		X origin of ROI. Left-most pixel in sensor array is 0.
Y		Y origin of ROI. Top-most pixel in sensor array is 0.
Width		Width of ROI
Height		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 PhenoImager HT and unmixed FL multispectral images originating from Vectra 3 and PhenoImager 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. The contents of the <Filter> and <Band> tags are described below.

Table 4: Contents of Filter and Band tags

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 / ($2^{\text{bit depth}}$ × 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 >
```

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