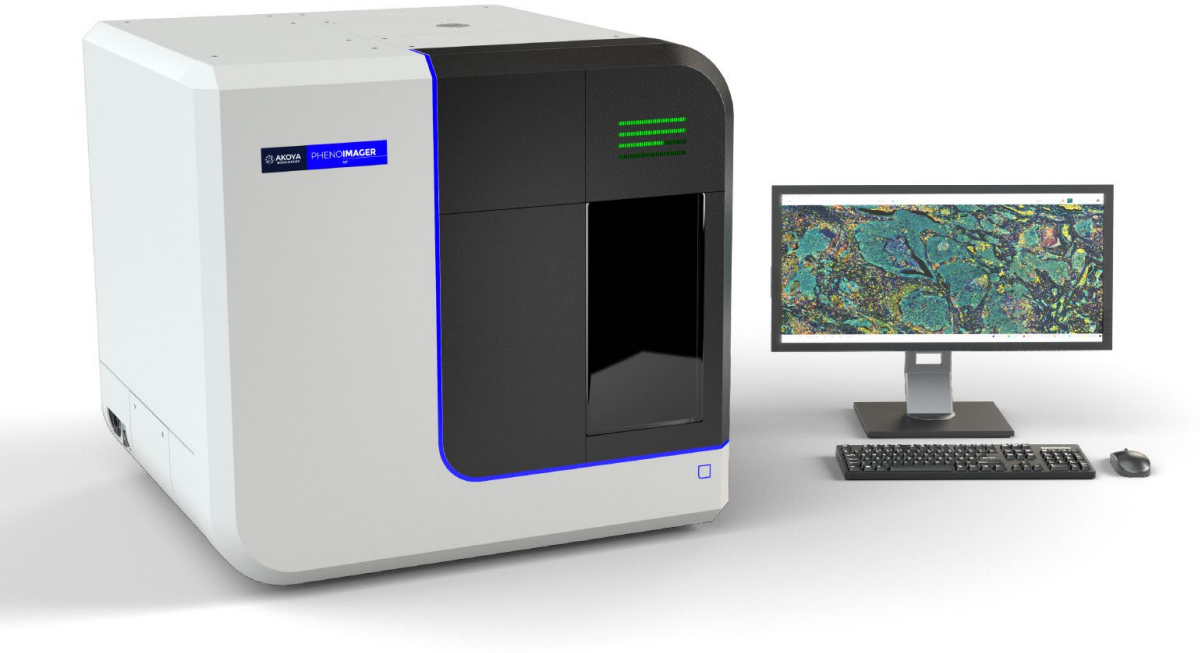
AKOYA BIOSCIENCES®
THE SPATIAL BIOLOGY COMPANY®

PhenoImager HT
USER GUIDE

Automated Quantitative Pathology Imaging System

2.1.0



PD-000010 Rev B

PhenoImager HT Version: 2.1.0 | Publication Date: 03.18.2024

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Contents

Preface

PROPER EQUIPMENT OPERATION

WARNING: 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.

AVERTISSEMENTS: Pour réduire le risque de choc électrique, ne pas retirer le couvercle. Ce produit ne contient aucune pièce pouvant être réparée par l'utilisateur. Au besoin, confier l'appareil à un réparateur qualifié.

Ce produit ne doit être utilisé que comme décrit dans ce manuel. Si cet appareil est utilisé d'une manière autre que celle spécifiée par le fabricant, la protection fournie par l'appareil peut être entravée.

CONTACT US

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 calling, 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 success. From the original factory warranty through a comprehensive line of customer support plans, Akoya offers Field Service Engineers and in-house Specialists who are dedicated to supporting hardware, software, and application development needs.

Programs can include such useful services as:

- Preventive maintenance.
- 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.

Guide Conventions

This guide contains the following information highlights and cross-references:

NOTE	*NOTE: Identifies important points, helpful hints, special circumstances, or alternative methods. (en français: remarque)*

CE

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'entitéresponsable de la conformité peut annuler l'autorisation d'opérer l'appareil accordée à l'utilisateur.***

KOREAN CERTIFICATIONS (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 sellers and users should notice that this equipment is not for household use.

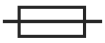
A급 기기 (업무용 정보통신기기)

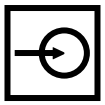
이 기기는 업무용으로 전자파적합등록을 한 기기이오니 판매자 또는 사용자는 이 점을 주의하시기 바라며, 만약 잘못판매 또는 구입하였을 때에는 가정용으로 교환하시기 바랍니다.

DEFINITION OF SYMBOLS

This section details the meaning, intent, and location of the labels (containing symbols) that appear on the PhenoImager® HT system and/or in the user documentation.

Symbol Symbole	**Contents** 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.*

Symbol Symbole	**Contents** Description
	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.*

Symbol Symbole	**Contents** Description
	Serial Number. (ISO 7000-2498) *Numéro de série.*
	Date of Manufacture. (ISO 7000-2497) *Date de fabrication.*
	WEEE symbol. (EN50419:2005).
	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 potential.* *Rouge: Arrêter.* *Bleu: Intervention obligatoire.* *Vert: Condition sûre ou informations de sécurité.*

SAFETY CONSIDERATIONS

Safety information for the PhenoImager HT is included in this guide. Read and review all safety information before operating the PhenoImager HT instrument.

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.

DANGER, WARNINGS, AND CAUTION SIGNS

	*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.*

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. This enables 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 service personnel; they are not intended to be removed during operation or for maintenance by users. Contact Technical Support at support@akoyabio.com.* *DO NOT operate the system if there has been a malfunction of the system door or slide loading components. Contact Technical Support at support@akoyabio.com if help is required.* *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

Contact Technical Support at support@akoyabio.com to order replacement power cords.

	*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 Technical Support at support@akoyabio.com for a replacement. Do not use power supply cords with inadequate ratings.*
	*Warning: Use only a properly grounded power outlet when connecting the system to power.*

Fuses

Contact Technical Support at support@akoyabio.com to order replacement fuses.

	*Warning: The fuses in this instrument are only replaceable by trained Akoya Biosciences personnel.*

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 at support@akoyabio.com to order replacement cables and adapters.*

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.*

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 service personnel. Contact Technical Support at support@akoyabio.com if help is required.*

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.*

Chapter 1: Introduction

Figure 1.1. The PhenoImager HT system.

1.1 INTENDED USE

This guide 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.

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 tissue microarrays (TMAs).

NOTE ***Akoya Biosciences PhenoImager HT Quantitative Pathology Imaging System is for research use only. Not for use in diagnostic procedures.***

1.2 PRINCIPLES OF OPERATION

Akoya Biosciences 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 Biosciences PhenoCode™ Signature Panels (formerly 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.

The PhenoImager HT has been designed to support a wide range of workflows:

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

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. This 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 automated (inForm) analysis field requests; reviewer-requested multispectral field acquisition; 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 want to view or review slide scans taken by the PhenoImager HT.

inForm® Tissue Finder

Tissue Finder: Software typically used for the analysis of multiplexed BF and FL 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 2-step procedures (e.g., acquire whole slide scan and review) to automated acquisition of regions of interest selected by the user. An additional option allows the user to go directly to whole-slide analysis using third-party software.

Example applications for the PhenoImager HT are described in the next section.

1.2.1 Example Applications

Some examples of PhenoImager HT applications include:

- Whole slide scanning of tissue samples and microarrays stained with PhenoCode Signature or Opal kits.
- Whole slide scanning of tissue samples stained with hematoxylin and eosin (H&E) and conventional immunohistochemistry (IHC) stains.
- Phenotypic analysis and protein expression of immune and cancer cells in the context of the tumor microenvironment.
- Field-based multispectral imaging (MSI) analysis of samples with up to 8 markers and counterstain with autofluorescence removal.

1.3 THEORY OF IMAGING

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

- Light
- Human perception of light intensity and color
- Light absorbance and reflection
- Fluorescence
- Photobleaching
- Dynamic range and saturation
- Multispectral Analysis
- Filter Sets for Conventional vs. Multispectral Imaging

1.3.1 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. (See Figure 1.2.)

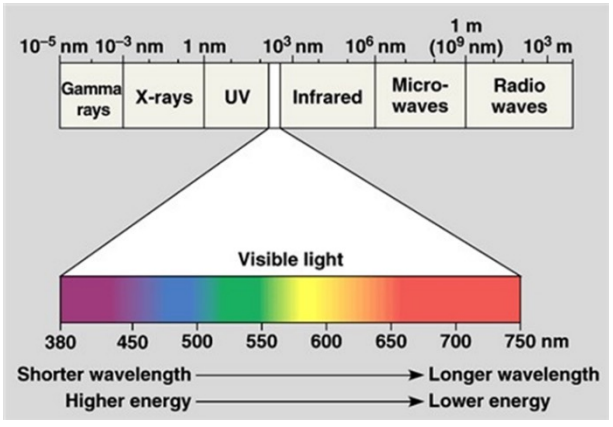


Figure 1.2. The electromagnetic spectrum.

1.3.2 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 can 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 color) lead to the differences in perceived color between individuals.

1.3.3 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 Biosciences' brightfield multispectral imagery is automatically converted to optical density at acquisition time, enabling quantitative analysis.

1.3.4 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 fluorescence could be measured more accurately than absorbed light, a number of factors could 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 a specimen is excited 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 used 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 1.3 contains an example of excitation and emission spectra, showing Stokes Shift and the overlap of the spectra.

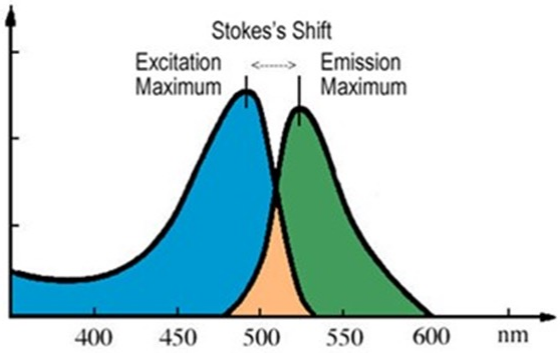


Figure 1.3. Stokes Shift.

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.

1.3.5 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.

NOTE ***When using PhenoImager HT, avoid spending prolonged periods in the Protocol Exposures Editor while fixed on any one spot of the sample, since live image streams are being acquired.***

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

1.3.6 Saturation and Dynamic Range

Fluorescent imaging involves measuring light emitted from a sample; the amount of light depends on the particular dye used, and the amount that is present. That, in turn, depends on the labeling and the markers involved.

In general, choose an exposure time that is long enough to measure the signals of interest without exceeding the maximum reading for the detector. Saturating the detector means the true signal level is unknown - all one can say is that it exceeded the upper measurement limit for those pixels. The exposure for each channel is chosen during assay development to balance the concerns of having good detection and avoiding saturation.

The PhenoImager HT can acquire images in either Standard or Extended dynamic range. In the latter case, readings up to 3x brighter than nominal full scale are processed without saturation. This is valuable because it allows for unexpectedly high expression in samples without any data loss.

Extended dynamic range images use more disk storage, however, because the full 12-bit camera signal is saved to disk rather than scaling it to an 8-bit data form as is done for Standard dynamic range scans.

1.3.7 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 a material is excited and the fluorescence emission examined over a range of wavelengths, then repeated for various combinations of excitation and emission wavelengths, the result is a "spectral fingerprint" which 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 method to distinguish between many overlapping emission spectra within the same area based on unique spectra, overcoming the limitations of conventional imaging. (See Figure 1.4.)

The result is that accurate estimates are obtained of each dye despite their overlapping spectra and the presence of background autofluorescence. The PhenoImager HT achieves

this through Akoya Biosciences' patented technologies for multispectral imaging of whole-slide scans or individual fields integrated with a simplified workflow. PhenoImager HT utilizes intelligent automation and patented technology for hands-off, error-free scanning and an optical configuration designed specifically for accurate signal measurements and unparalleled sample protection. The whole slide multispectral imaging capability creates the robust workflow since fields of view do not need to be selected. This eliminates selection bias and provides a whole-slide record for easy re-analysis of imagery with new understanding.

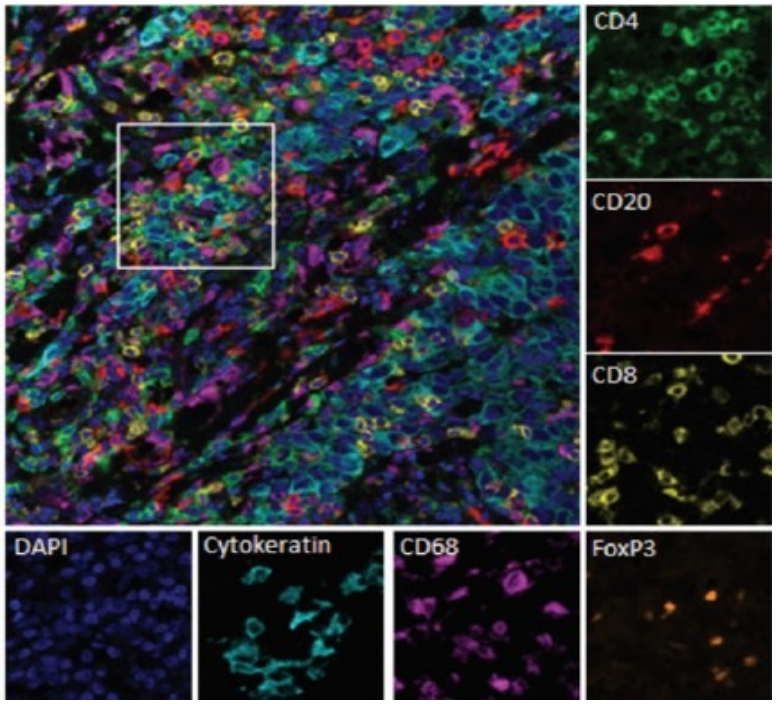


Figure 1.4. Unmixed multispectral image of human breast cancer tissue stained against CD4 (green), CD20 (red), CD8 (yellow), FoxP3 (orange), CD68 (purple), Cytokeratin (light blue), and DAPI (dark blue) using Opal reagents.

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 contributions of the individual fluorescence spectra are separated. The result is a set of images representing each spectrum that contributes to the final image. The spectral measurements consist of 8 carefully chosen filter bands, for multispectral whole slide scans; or a set of emission spectra taken with a liquid crystal tunable filter, for MSI fields. (See Figure 1.5.)

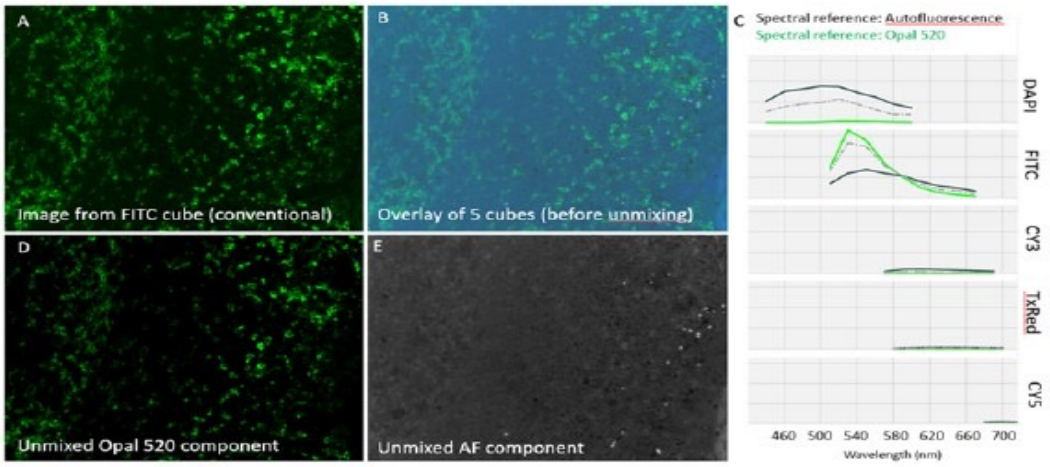


Figure 1.5. Removing auto-fluorescence with field-based multispectral imaging. A. Conventional fluorescence image of tonsil tissue stained against CD4 with Opal 520 dye. B. Overlay of all 35 layers of a multispectral image acquired using five filter cubes. C. Emission spectra of pure autofluorescence (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.

1.3.8 Filter Sets for Conventional vs. Multispectral Imaging

Filters used for conventional fluorescence imaging are often designed to image 3-4 widely separated dyes. Typically, their excitation and emission bands have moderate spectral width, chosen to match bands where the dye is inherently most responsive. There is no attempt to measure the sample autofluorescence, or to quantify and remove it.

The PhenoImager HT provides a filter set that is optimized for whole slide multispectral imaging, with a total of 8 paired excitation and emission bands.

An alternative approach for field-based multispectral imaging makes use of a liquid crystal tunable filter (LCTF). 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, to map 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 Figures 1.6 and 1.7.

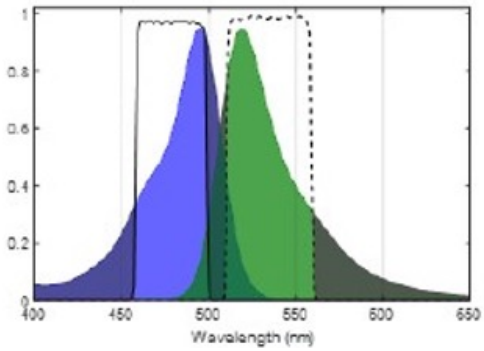


Figure 1.6. Narrowband excitation (solid line) and (dashed line) filter for conventional multispectral imaging.

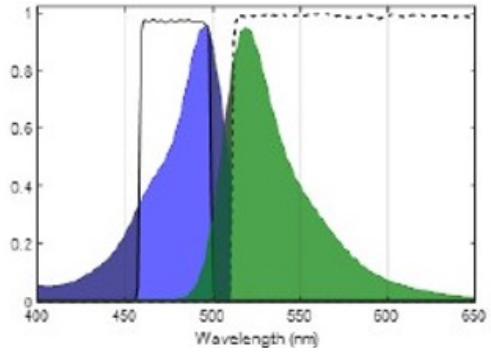


Figure 1.7. Narrowband excitation (solid line) and emission long-pass emission filter (dashed line) for imaging.

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 1 dye at a time. (See Figures 1.8 and 1.9.)

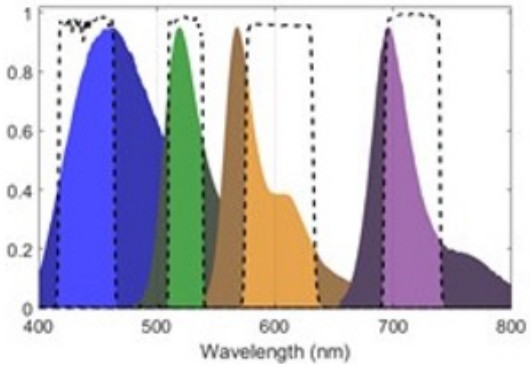


Figure 1.8. Four dyes imaged by conventional methods.

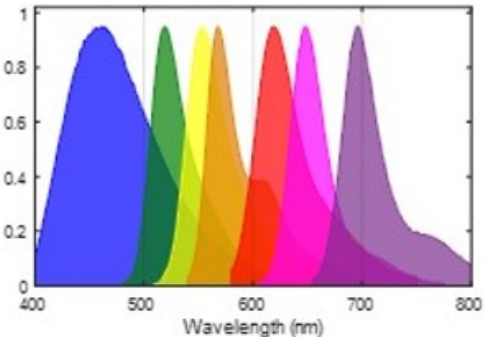


Figure 1.9. Seven dyes are too many to image fluorescence by conventional methods.

Figure 1.8 shows the emission spectra for 4 dyes (DAPI, FITC, Cy3, and Cy5.5) that are spectrally distinct and can be separated by conventional imaging. This works because there is 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 1.9 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.

1.4 WHOLE SLIDE SCANNING

PhenoImager HT scans slides using the following process:

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 a fluorescence or brightfield protocol is used and identifies the presence of slides in the carrier and capture their labels.

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, this area can be further restricted 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 the tissue is faintly stained, punctate, or is a 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.

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.

Fluorescence Overview (fluorescence protocols only)

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

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).

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 new, finer scale.

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

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 chosen. To avoid photobleaching, the system uses a pulsed LED so the sample is only exposed to light during the time the camera is taking a picture.

Completed scans can be opened in Phenochart, the whole slide viewing application.

NOTE ***Some dyes narrowly express in a single filter. Other times, dyes may express in multiple filters and can appear in more than one layer in a fluorescence scan. This can occur when using PhenoImager HT's broad filters like the Cy3 or Texas Red filters with Opal 570 dye, or when using non-Akoya dyes. If the sample is highly multiplexed, multiple dyes may appear in the same channel.***

Spectral Unmixing

When spectral unmixing is requested, PhenoImager HT unmixes the scan in the background, while it turns to the next slide. This produces a new scan file containing the unmixed imagery.

Unmixing is highly optimized, and typically completes while the next slide is still underway. Thus, the tasks are fully nested and overall system throughput is kept very high.

1.5 MULTISPECTRAL FIELD IMAGING

In some workflows, the PhenoImager HT is used to acquire multispectral fields of a sample, after it has completed a whole slide scan of the tissue.

1.5.1 Configuring the Multispectral Fields

Multispectral imagery is acquired using the following process:

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

1.5.2 Processing the Multispectral Fields

After configuring 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 a fluorescence or brightfield protocol is used and identifies 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 are used 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 then travels to each multispectral region site, autofocuses, corrects for exposure if requested, and acquires the multispectral image.

Multispectral imagery can then be viewed and analyzed using inForm.

Chapter 2: Hardware Reference

This chapter identifies and describes the PhenoImager HT system hardware and lists the 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 service personnel. Contact Technical Support at support@akoyabio.com if help is required.

2.1 FRONT VIEW

The front view of the PhenoImager HT instrument with the system door open. (See Figure 2.1.) The parts identified are described in Table 2.1.

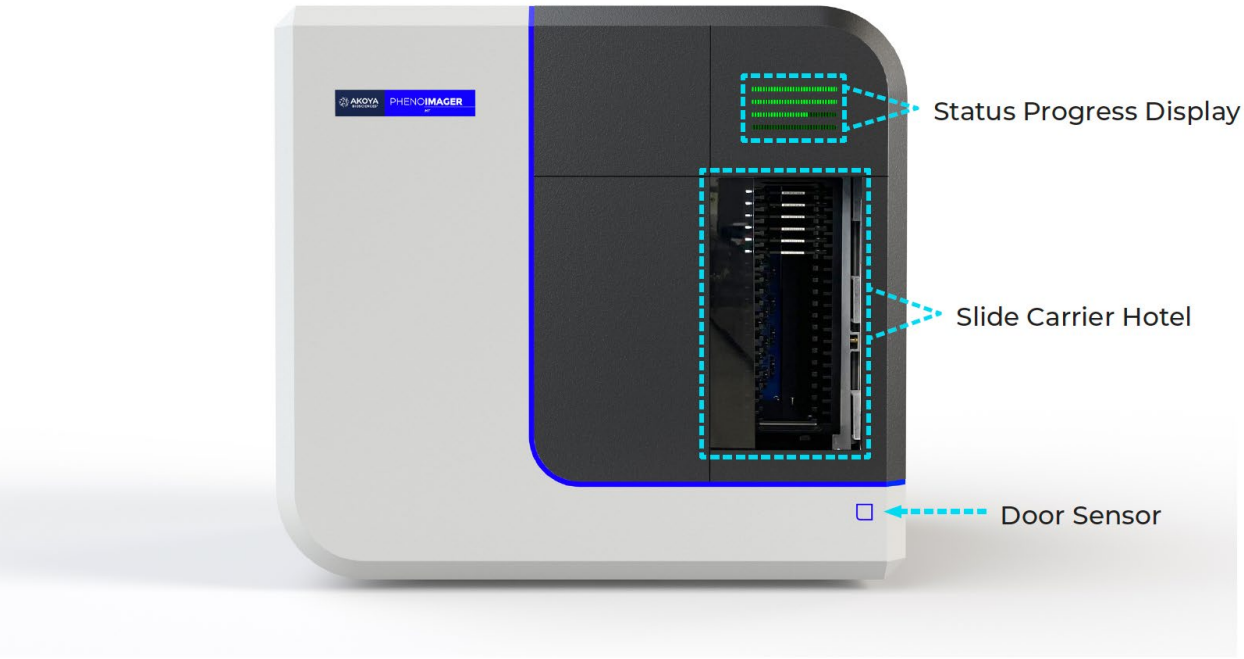


Figure 2.1. PhenoImager HT front view—system door open.

Table 2.1. 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 for more detailed information.
Door Sensor	Opens or closes the System Door when a hand is placed in front of the sensor.

The front view of the PhenoImager HT instrument with the system door closed. (See Figure 2.2.) The parts identified are described in Table 2.2.

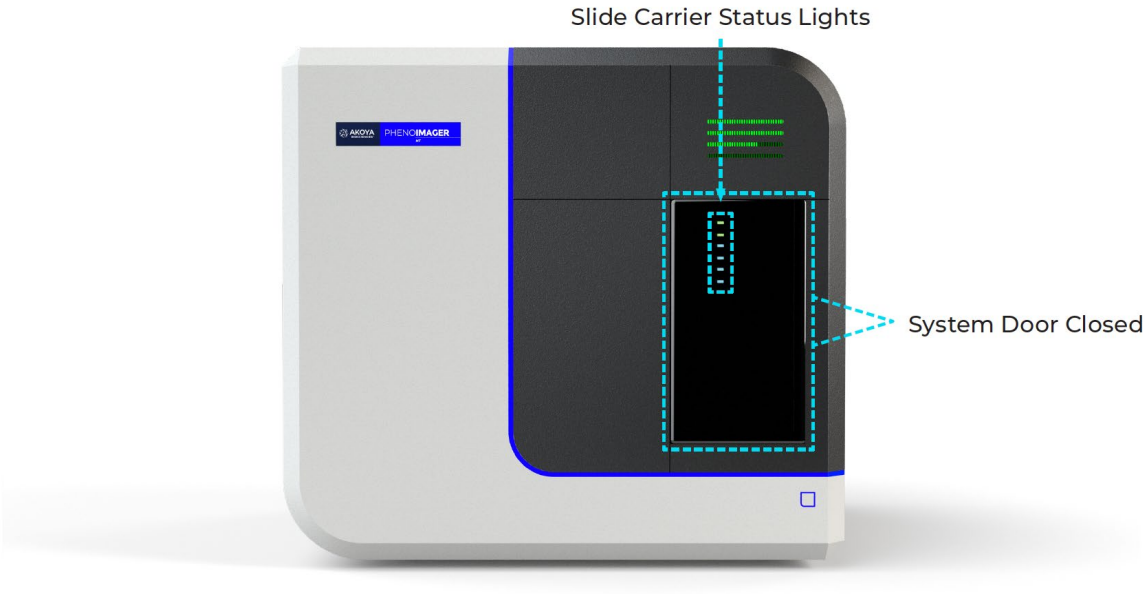


Figure 2.2. PhenoImager HT front view—system door closed.

Table 2.2. Front View (system door closed)—hardware components

Part	Description
Slide Carrier Status Lights	Each Slide Carrier Status Light represents an individual slide carrier and indicates the processing status of each Slide Carrier. See Table 2.7 for the color codes of the slide carrier status lights.
System Door	When closed, covers the Slide Carrier Hotel and slide processing can occur. When open, reveals the Slide Carrier Hotel.

⚠	*Warning: Do not operate the system if there has been a malfunction of the system door or slide loading components. Contact Technical Support at support@akoyabio.com if help is required.*

2.2 TOP VIEW

The top view of the PhenoImager HT instrument is seen in Figure 2.3 and the Cover Handle is described in Table 2.3.

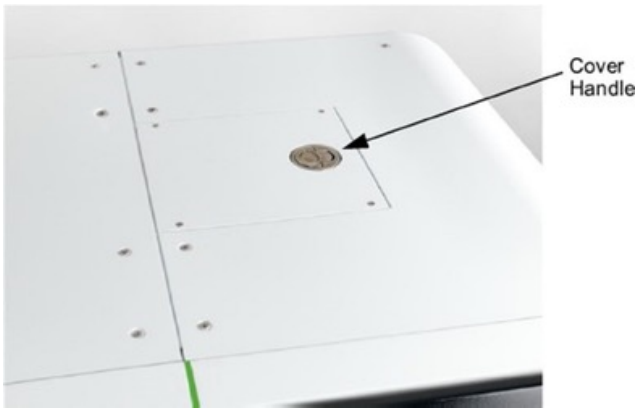


Figure 2.3. PhenoImager HT - top view.

Table 2.3. 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 Technical Support at support@akoyabio.com if help is required.*

2.3 RIGHT-SIDE VIEW

Figure 2.4 shows the right-side of the PhenoImager HT instrument. The System Power Switch identified is described in Table 2.4.

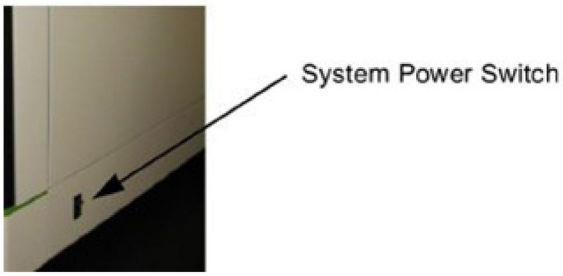


Figure 2.4. PhenoImager HT – right-side view.

Table 2.4. Right-Side View—hardware components

Part	Description
System Power Switch	Turns the PhenoImager HT instrument ON or OFF. | *ON* *or* ○ *OFF*

2.4 LEFT-SIDE CONNECTORS

Figure 2.5 identifies the connectors on the bottom left side of the PhenoImager HT instrument. The connectors identified are described in Table 2.5.

⚠	*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 at support@akoyabio.com for a replacement.* *Use only a properly grounded power outlet when connecting the system to power.*

Figure 2.5. PhenoImager HT left-side connectors.

Table 2.5. 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.
USB 3.0 Hub Connector	Connects a USB 3.0 HUB to a USB 3.0 port on the System Computer Connectors.

2.5 SLIDE CARRIER HOTEL

The Slide Carrier Hotel houses up to 20 Slide Carriers and is visible when the System Door opens. Figure 2.6 shows a closeup of the Slide Carrier Hotel and identifies its components. The hardware and color components are described in Table 2.6 and Table 2.7 respectively.

⚠	*Warning: Do not operate the system if there has been a malfunction of the system door or slide loading components. Contact Technical Support at support@akoyabio.com if help is required.*

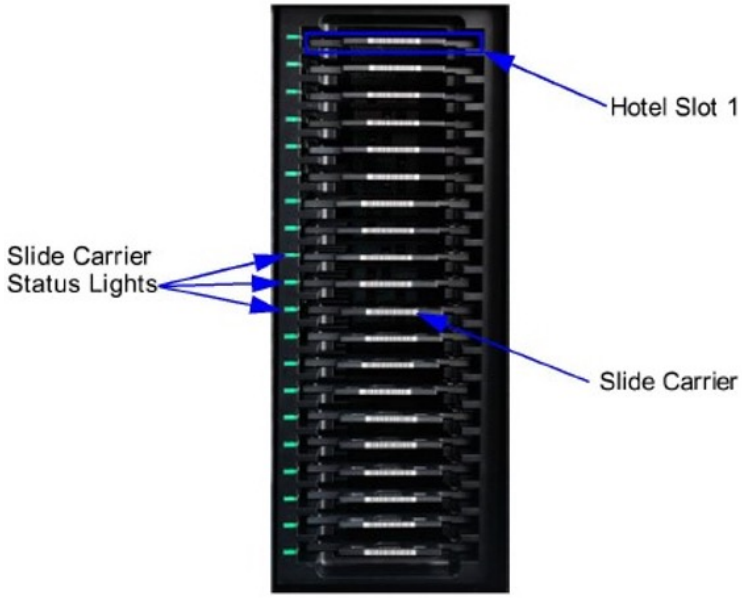


Figure 2.6. Slide Carrier Hotel.

Table 2.6. Slide Carrier Hotel—hardware components

Part	Description
Slide Carrier Status Lights	Indicates the processing status of each slide carrier. The color codes associated with the slide carrier status lights are listed in Table 2.7.
Hotel Slot	Each hotel slot holds one Slide Carrier. The hotel slots are numbered 1-20, starting at the top of the hotel.
Slide Carrier	Houses up to 4 slides. See the Slide Carrier section for more details.

Table 2.7. Slide Carrier Status Lights—color codes

Color		Status
—	None	Slide Carrier Hotel is empty.

Color		Status
	White	Initial state of slot after slide carrier is inserted and no rules have been applied via software.
	Solid	Hotel slot is malfunctioning.
	Blinking	Slide carrier is not completely inserted.
	Solid	Awaiting Processing.
	Blinking	Prioritized and awaiting processing.
	Solid Yellow	Processing instructions are either incomplete or invalid.
	Blinking	Processing.
	Solid Green	Processing Complete.
	Orange	Processing complete but a processing error occurred.

2.6 SLIDE CARRIER

Slide Carriers are an integral component of PhenoImager HT. The Slide Carrier holds up to 4 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.

Figure 2.7 shows a closeup of a Slide Carrier and identifies its components. The identified components are described in Table 2.8. Table 2.9 provides details of each slide format.

Figure 2.7. PhenoImager HT Slide Carrier.

Table 2.8. 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 microscope slide is manually loaded into the slide positions. Table 2.9 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.
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 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. A unique number is printed on top of the handle for slide carrier identification purposes.

Table 2.9. Details of slide formats

Part	Width	Height	Thickness
Metric	25.0 ± 1.0 mm	75.0 ± 1.0 mm	1.00 ± 0.10 mm
English	25.4 mm	76.2 mm	1.00 ± 0.10 mm

2.7 BARCODE READING

The PhenoImager HT reads barcodes on the slide labels by taking a picture of the label and then decoding it.

2.8 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.

The PhenoImager HT computer system connectors are seen in Figure 2.8. The connectors identified are described in Table 2.10.

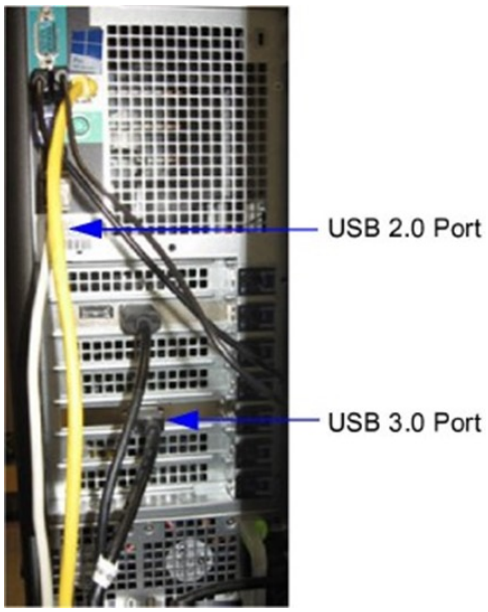


Figure 2.8. System Computer Connections.

Table 2.10. 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
USB 3.0 Port	Connects a USB 3.0 HUB to the USB 3.0 HUB connector on the instrument's Left-Side Connectors

2.9 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 5 cm)
Weight	185 lbs. (84 kg)
Spectral Range	440 nm to 780 nm
Objectives	4x, 10x, and 20x
Pixel Resolution	0.25 µm, 0.5 µm, or 1.0 µm
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	32 GB

ENVIRONMENTAL	
The location where the PhenoImager HT will be installed must meet the following specifications:	
Operating Temperature*	64°F - 83°F (18°C - 28°C)
Storage Temperature*	59°F - 86°F (15°C - 30°C)
Shipping Temperature** (up to 72 hours max)	14°F - 113°F (-10°C - +45°C)
Operating Humidity**	10% - 60% non-condensing
Storage Humidity**	10% - 60% relative humidity, non-condensing
Altitude	Up to 2000 meters (6561 feet)
Vibration	Up to 0.4mm/sec @ 8-80Hz
Pollution Degree	2

**Ambient temperature should not vary >2°C (4°F) over 24 hours.*

***Do not expose the instrument to humidity >80% or temperature outside of -10°C to 45°C (14°F to 113°F) for more than 72 hours.*

NOTE ***For indoor use only.***

- ***A clean, dust-free room is recommended.***
- ***Do not operate the system in an environment with explosive or flammable gases.***
- ***Do not operate the system in places where it may be splashed with liquid.***

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, 5 mm x 20 mm

BARCODE READER LIGHT SOURCE (white laser diode)	
Wavelength	400 nm to 750 nm
Power Output	70 mAmps

Chapter 3: Operation

This chapter describes how to operate the PhenoImager HT hardware. It is important to read and understand the Instrument Safety section before using the system. The Hardware Reference chapter provides a description of each hardware component in the PhenoImager HT system.

3.1 SYSTEM STARTUP

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

3.1.1 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 at support@akoyabio.com 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 to the ON (|) position. The Status Progress Display flashes green to indicate a quiescent state. The Door Sensor is responsive and functional.

3.1.2 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 4.1. Software Overview.)

3.2 SYSTEM SHUTDOWN

To shut down the PhenoImager HT system:

1. Exit the PhenoImager HT software. If open, the System Door closes.
2. Select Shut Down from the Windows Start Menu to power down the computer.
3. Switch the System Power Switch on the right side of the instrument to the OFF (O) position. The status lights turn off and the system shuts down.

NOTE ***For best performance, power cycle the PhenoImager HT system periodically (a few times per week) or if it has been idle for an extended period of time.***

3.3 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.

3.3.1 Inspecting Slides

- Verify the slides meet the required formats and dimensions. (See Table 2.9.)
- 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.

3.3.2 Inspecting Slide 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, then clean the handle or surfaces before use. (See 5.4. Cleaning the Slide Carriers.)

3.4 LOADING SLIDES INTO THE SLIDE CARRIERS

After inspecting the Slides and Slide Carriers, load the slides 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 facing up.
3. Gently push the opposite end of the slide into the preferred Slide Position until the slide is up against the Spring-Loaded Tab. (See Figure 3.1.)

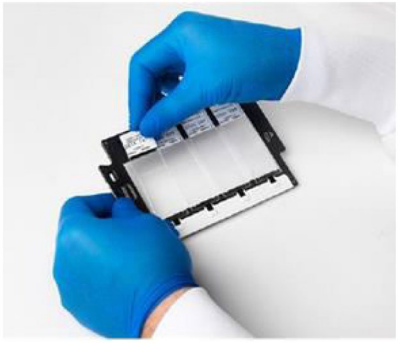


Figure 3.1. Loading a slide into a Slide Carrier.

4. Gently lay the label end of the slide completely into the slide position so the slide lays flat in the carrier. As the slide is released, the tab should push the slide against the top wall of the slide position.
5. After the slide is secured by the Spring-Loaded Tab, gently push the slide to the right so its long edge is flush with the right wall of the slide position.

NOTE ***Slide indexing is critical to the PhenoImager HT slide detection process. Best practice recommendation is to index slides in the Slide Carrier to the right to ensure proper detection and scanning of all loaded slides.***

6. Load up to four slides into each Slide Carrier. Slides need not be contiguous.
7. Click the Enter Slide IDs button. See Slide IDs in the PhenoImager HT software. The Enter Slide IDs dialog opens.
8. Type the applicable slide IDs into the text boxes and click the OK button.
9. To load additional slides into another Slide Carrier, repeat steps 1-8 above. Slide Carriers may be stacked on top of one another for easy handling and storage. (See Figure 3.2.)

Figure 3.2. Slide Carrier stack.

3.5 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 3.3.2. Inspecting Slide Carriers). Notice the ends of the Slide Carrier. (See Figure 3.3.)
2. Verify the PhenoImager HT System is on. (See 3.1. System Startup)
3. If the System Door is closed, place a hand in front of the Door Sensor to open it. The System Door will slide to the left to reveal the Slide Carrier Hotel.
4. Hold the Slide Carrier by the Carrier Handle with the slide labels facing upwards into a Hotel Slot.

5. Identify which end of the Slide Carrier is marked with the Insert Indicator. ( )
6. Gently insert the end of the Slide Carrier with the Insert Indicator into a Hotel slot.

Figure 3.3. Ends of a Slide Carrier.

7. 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. (See Figure 3.4.) Hold the Slide Carrier by the Carrier Handle with the slide labels facing upwards into a Hotel Slot.

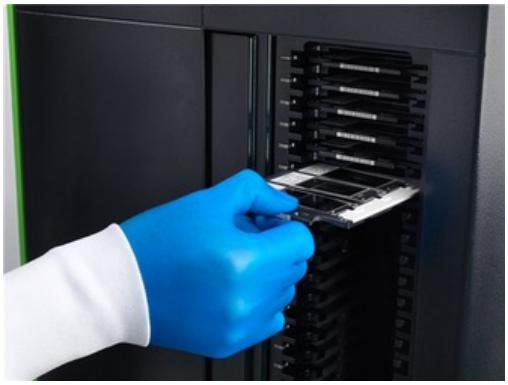


Figure 3.4. Pushing the Slide Carrier in until fully seated.

- 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 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.

8. Continue to load up to 20 Slide Carriers into the slots.
9. When completed, place a hand in front of the Door Sensor to close the System Door.

3.6 REMOVING SLIDE CARRIERS FROM THE SLIDE CARRIER HOTEL

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

To remove a Slide Carrier from the hotel:

1. Pull the Carrier Handle until the Slide Carrier is free from its Hotel Slot. The Slide Carrier Status Lights 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.

3.7 REMOVING SLIDES FROM THE SLIDE CARRIERS

After Slide Carriers are removed from the Slide Carrier Hotel, 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 an 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 thumb and index finger.
4. Remove the remaining end of the slide from the Slide Carrier.

Chapter 4: Software Operation

This chapter describes how to operate the PhenoImager HT software.

Figure 4.1. PhenoImager HT software.

4.1 SOFTWARE OVERVIEW

The Home Page links to the pages needed to maintain and run the PhenoImager HT. The Home Page is seen in Figure 4.2.

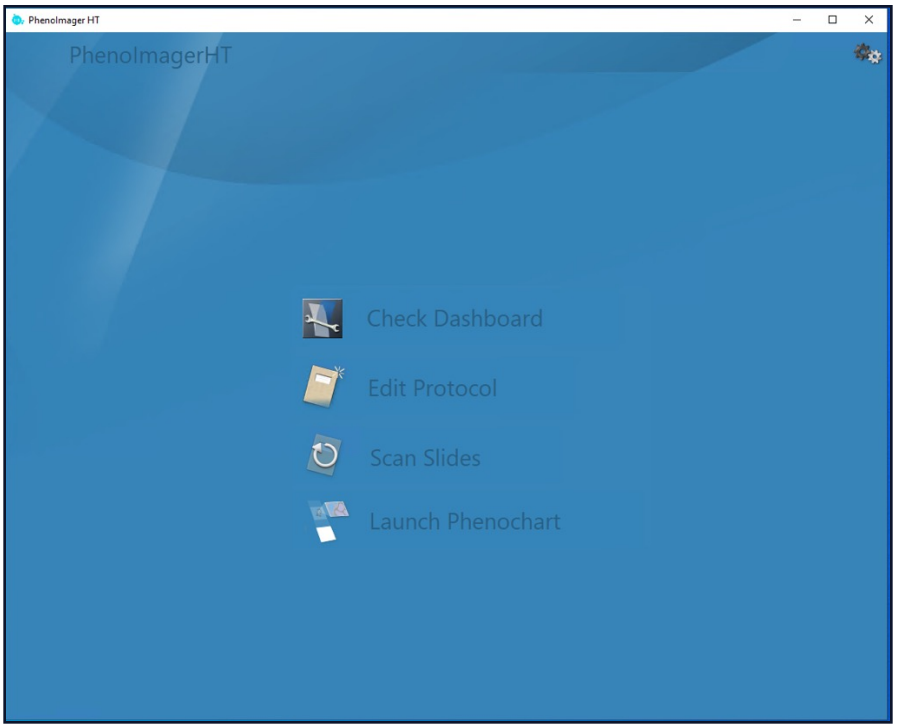


Figure 4.2. PhenoImager HT software home page.

The Home page buttons link to the following pages:

- **Check Dashboard** – Check the remaining disk space and acquire references.
- **Edit Protocol** – Create or edit brightfield and fluorescent protocols and studies.
- **Scan Slides** – Select the rules to scan the slides and scan the slides based on those rules.
- **Launch Phenochart** – Launch the Phenochart viewing software.

The Home pages can be accessed by using the Gear Menu located in the upper-right corner of the window. (See Figures 4.2 and 4.3.) See Section 4.1.1: Gear Menu for more information.

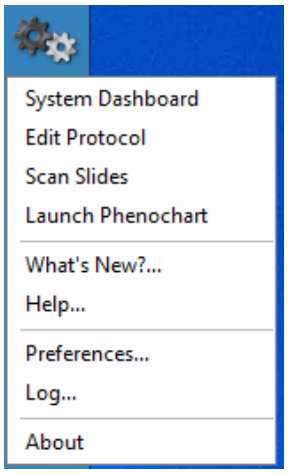


Figure 4.3. Gear menu.

Additionally, the Gear menu can be used to check the version number or perform other functions and launch the online help.

Below is an overview of each page.

Check Dashboard

Use the Dashboard to check the status of the 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 Section 4.2. System Dashboard.

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 Section 4.3 Creating and Editing Protocols.

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 Section 4.4 Scanning Slides.

Launch Phenochart

Use the Launch Phenochart to launch the Phenochart program.

For detailed information on how to use Phenochart, see the Phenochart User Guide and online help from within the Phenochart program.

4.1.1 Gear menu

Use the Gear menu in the upper right-hand corner of the Home page to:

- Link to the following page:
  - System Dashboard
  - Edit Protocol
  - Scan Slides
  - Launch Phenochart
- 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.

4.2 SYSTEM DASHBOARD

Use the System Dashboard shown in Figure 4.4 to:

- See the available Disk Space.
- View and acquire Brightfield References.
- View and acquire Fluorescence References.

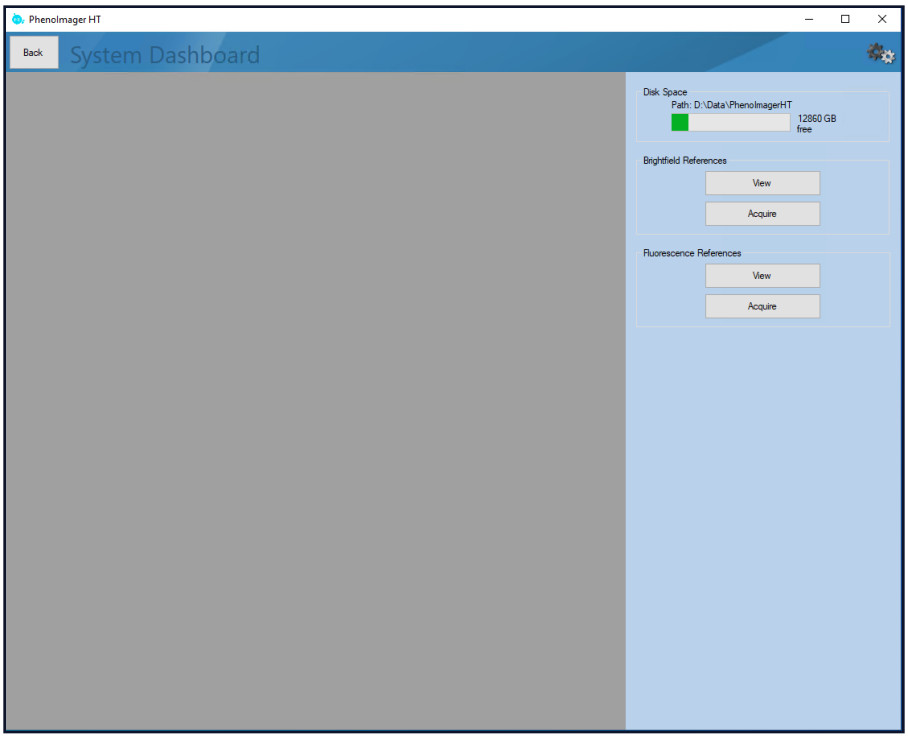


Figure 4.4. System Dashboard window.

4.2.1 Disk Space

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

4.2.2 Brightfield References

Viewing Brightfield References

Click View to see reference imagery and information for the label image, brightfield overview, color image and multispectral image. (See Figure 4.5.)

- 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 describes how these reference images are applied. (See Section 4.2.4. Compensation Information)

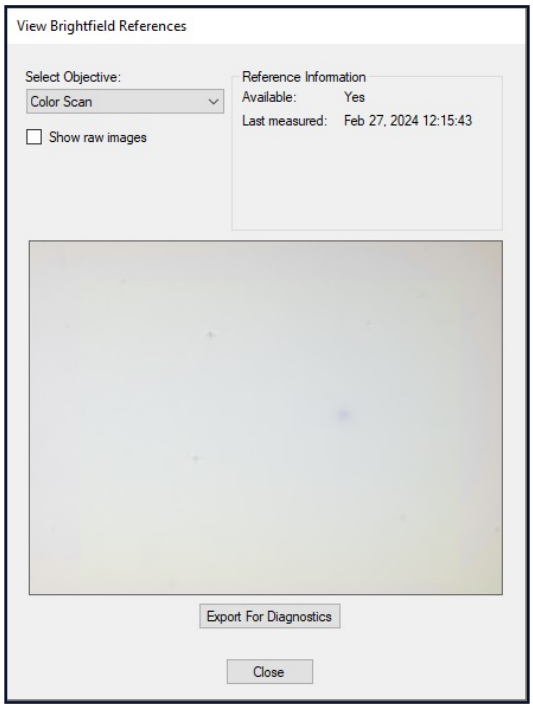


Figure 4.5. View Brightfield References window.

Acquiring Brightfield References

Click Acquire to take new brightfield references. (See Figure 4.6.) 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. Refine the position by using arrow keys for small movements, and control + arrow for slightly larger movements.
- Click OK to take references.

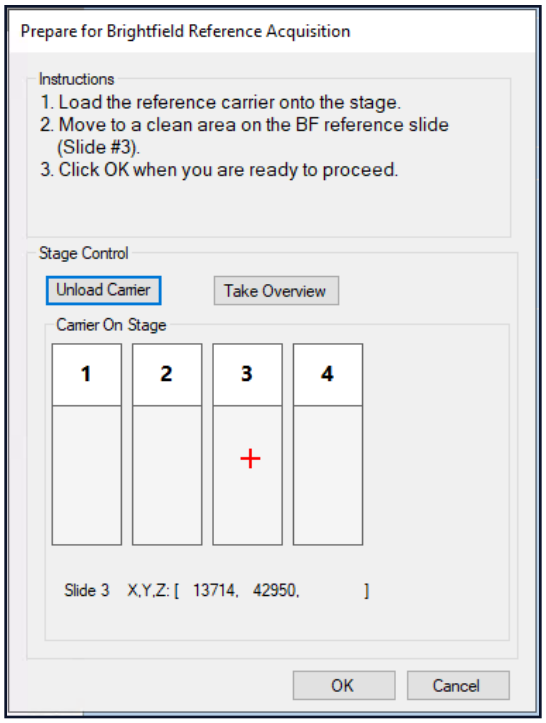


Figure 4.6. Prepare for Brightfield Reference Acquisition.

4.2.3 Fluorescence References

Viewing Fluorescence References

Click View to see reference imagery and information for the overview, 20X and 40X resolution references for each filter. (See Figure 4.7.)

- Export For Diagnostics will save this image to aid in technical support.
- Compensation Information describes how these reference images are applied and the Fitted Flatness assessment. (See 4.2.4. Compensation Information)
- Responsivity Information describes how the system response is measured.

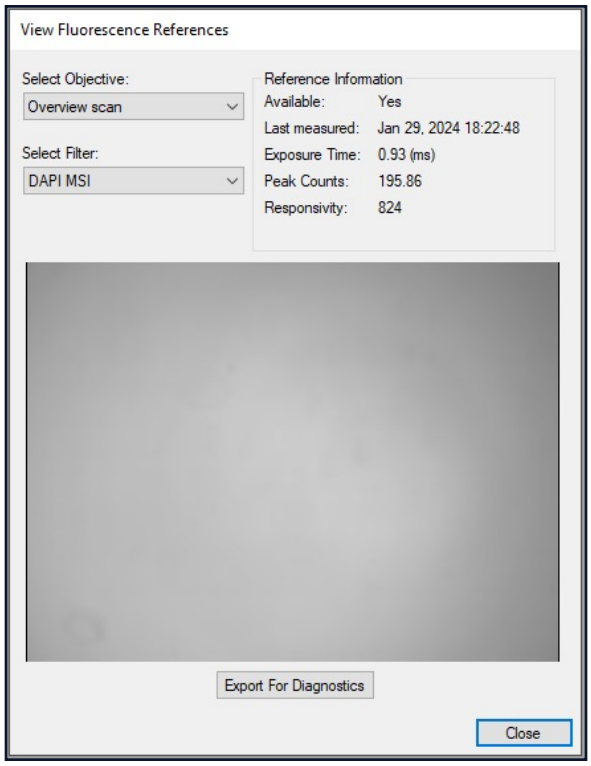


Figure 4.7. View Fluorescence References window.

Responsivity Information

Responsivity values recorded by PhenoImager allow the monitoring of the overall system response to a standard fluorescent artifact over time. Responsivity is affected by system components and alignments related to fluorescence illumination and detection, so a substantial decrease in Responsivity may indicate that the reference images need a closer look by the Akoya support team.

The measurements are made using acrylic fluorescent artifacts, which can vary in intensity between systems but do not vary significantly over time. Responsivity values are assessed for each combination of objective and filter band that can be used for fluorescence imaging each time Fluorescence References are acquired. For each band, several images are acquired of the fluorescent artifact, then a signal intensity is calculated by averaging the mean intensity within the central 1/16th area patch of the images. That signal intensity is then normalized by the exposure time and other camera settings to produce a Responsivity value with units of counts per second.

Acquiring Fluorescence References

Click Acquire to generate new fluorescent references. We recommend acquiring references for all filters at once but 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. The position can be refined by using arrow keys for small movements, and control + arrow for slightly larger movements.
- Click OK to acquire references.

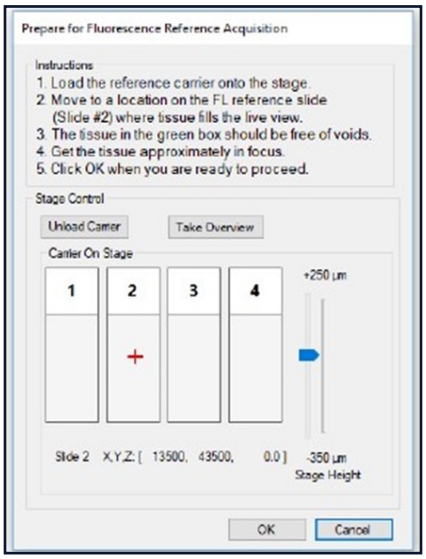


Figure 4.8. Acquire Fluorescence References window.

4.2.4 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 such as 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 to produce flat-fielded imagery. 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.

The PhenoImager provides a Fitted Flatness metric to estimate the quality of flat fielding after dividing by the reference image (metric shown in Figure 4.7). The Fitted Flatness metric is measured from overlapping images of the fluorescence reference slide acquired during fluorescence reference acquisition (Section 4.2.3), after applying shading compensation. The overlapping, compensated images are divided into small strips to compare the same area of tissue imaged in the center of the field vs the edges of the field. The Fitted Flatness metric reports the worst measured intensity difference observed when comparing the center of the field to any of the four edges of the field.

4.3 CREATING AND EDITING PROTOCOLS

4.3.1 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. (See Figure 4.9). Each slide may be scanned more than once, if needed.

- The default location for a study is D:\Data\PhenoImagerHT\[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\PhenoImagerHT\[Study]. Figure 4.10 shows the QPTIFF format for the slides.

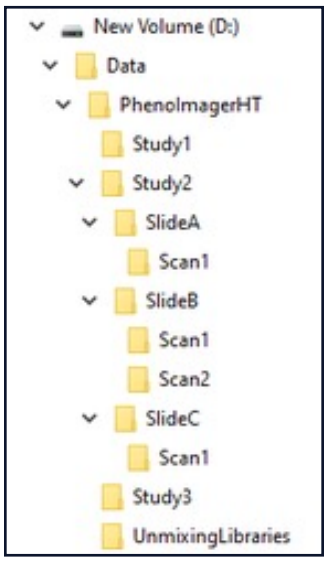


Figure 4.9. Study structure.

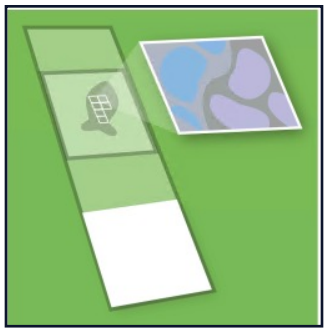


Figure 4.10. Slide QPTIFF.

4.3.2 Creating Protocols

Before creating 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 PhenoCode Signature panels, and its Opal dyes.

To create a new protocol:

1. Select Edit Protocol from the PhenoImager HT Home Page.
2. Click the New button. The Create New Protocol window opens. (See Figure 4.11.)

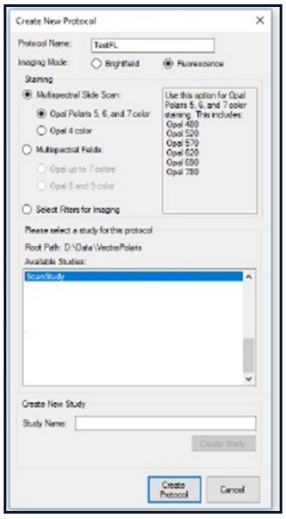


Figure 4.11. Create New Protocol window.

3. Enter a Protocol name.
4. Under Imaging Mode, select either Brightfield or Fluorescence.
5. If the system has been upgraded for multispectral whole slide scans, choose the type of staining kit used for this protocol, or choose to select custom filters for imaging.
6. 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 Create Study. The new study will be added to the Available Studies list where the study can be selected.

7. Click Create Protocol or Select Scan Bands to create the protocol in the selected study.
8. If choosing to select custom filters, the Select Scan Bands window opens. Select the bands to use for imaging and click OK.

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

4.3.3 Editing Protocols

After the brightfield or fluorescence protocol has been created and assigned 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.

Brightfield Protocols

After the brightfield protocol has been created and assigned to a study, the Edit Protocol window (for brightfield protocols) opens. (See Figure 4.12.)

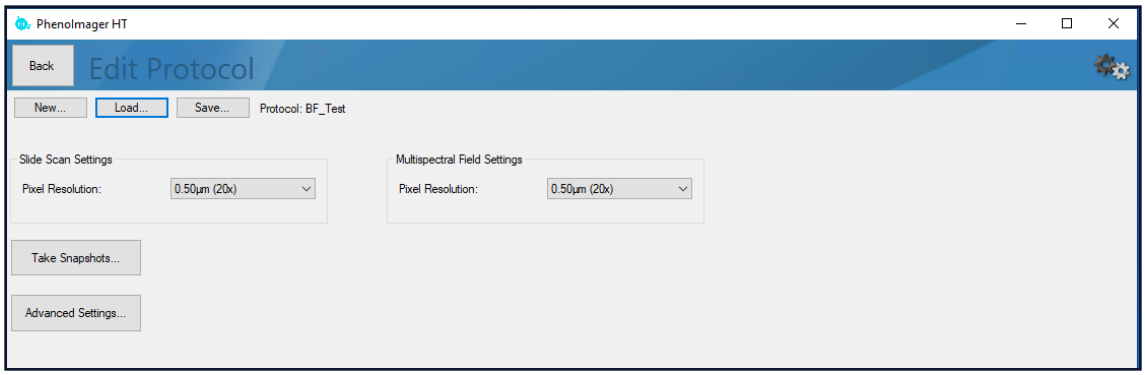


Figure 4.12. Edit Protocol Window.

Under Slide Scan Settings, select Pixel Resolution in the drop-down list to select the resolution to apply to the whole slide. (See Figure 4.13.) Choose 1 µm per pixel (nominally 10x), 0.5 µm per pixel (nominally 20x), or 0.25 µm per pixel (nominally 40x).

Figure 4.13. Slide Scan Settings - Pixel Resolution drop-down list.

Under Multispectral Fields, select the Pixel Resolution in the drop-down list to use for imaging. (See Figure 4.14.) Again, available resolutions are 1 µm per pixel (nominally 10x), 0.5 µm per pixel (nominally 20x), or 0.25 µm per pixel (nominally 40x).

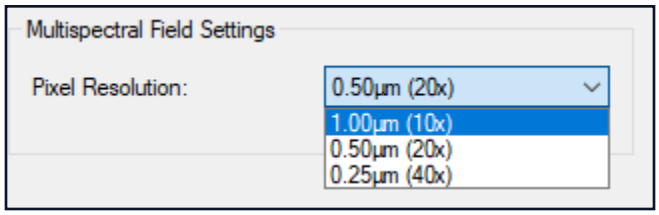


Figure 4.14. Multispectral Field Settings - Pixel Resolution drop-down list.

Advanced Settings

There may be situations when fine-tuning how PhenoImager HT scans the slides is necessary. Figure 4.15 showcases the Advanced Settings button. 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.
    - If using thinner coverslips (#0), select 100 µm (#0 or similar) for a Coverslip Thickness.
    - If using thicker coverslips or using a significant amount of mounting media between the sample and coverslip, select the option that best matches the 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 suboptimal.***

- **Scan within the entire coverslip region** - If PhenoImager HT cannot find the entire tissue sample, check the Scan within the entire coverslip region checkbox. This will increase scan time and file size but will enable complete scanning of difficult samples.
- **Sample is a TMA** - PhenoImager HT has a specialized algorithm for finding TMA samples. If samples are TMAs, select Sample is a TMA.
- **Image Compression** - By default, brightfield whole slide scans in PhenoImager HT are .jpg compressed to save disk space. Adjust the image quality as needed; higher quality will result in larger files. If less compression is preferred, select LZW rather than JPEG.
- **Label Barcode Reading** - PhenoImager HT can decode the barcode on the slide label and save it as part of the scan file. To do this, select the type of barcode being used. By default, PhenoImager HT will not attempt to decode a barcode unless this option is specifically selected.
    - If only 1D barcodes, select All 1D barcodes (autodetect type).
    - If there are a variety of 2D barcodes in use, select All 2D barcodes (autodetect type).

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

- If 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.

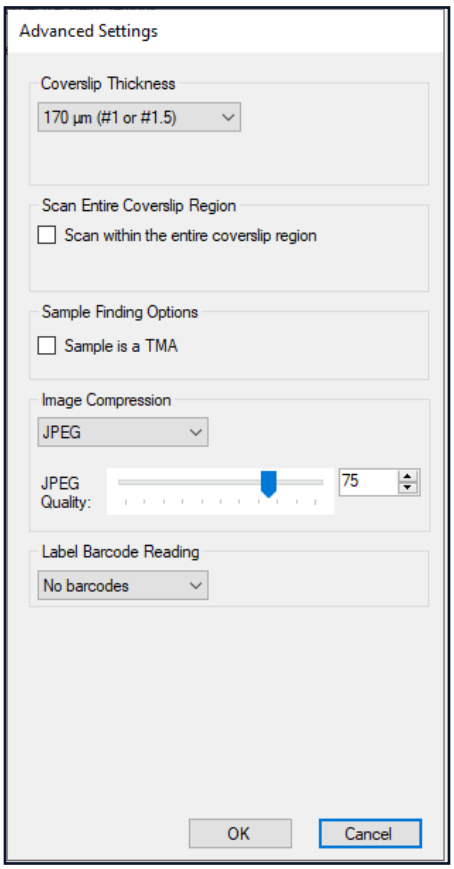


Figure 4.15. Advanced Settings window.

Click Save to save the protocol. (See Figure 4.16.) The study previously chosen will be automatically selected. The protocol name or study can be changed if needed.

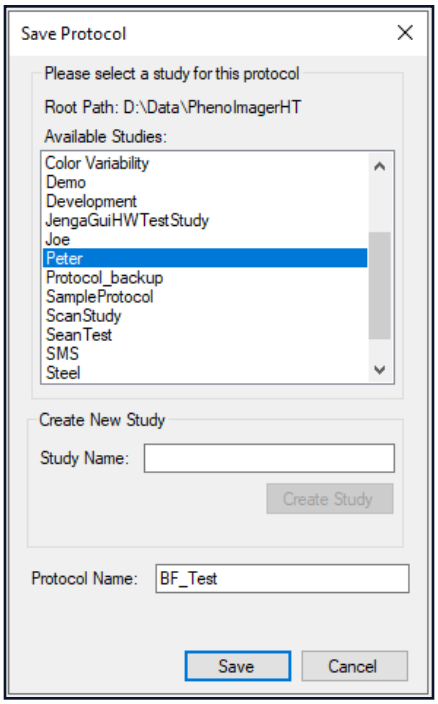


Figure 4.16. Save Protocol window

Brightfield Snapshots

Once the scan and multispectral field resolutions have been selected, example snapshots of the slides can be taken.

Click Take Snapshots to load the Brightfield Snapshots editor. (See Figure 4.17.)

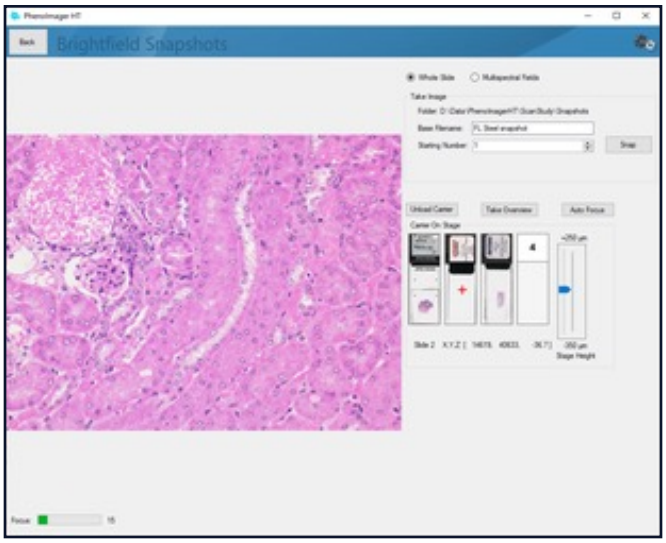


Figure 4.17. Brightfield Snapshots.

If a carrier is not already on stage, click Load Carrier to select a carrier that contains the slides to be imaged. (See Figure 4.18.)

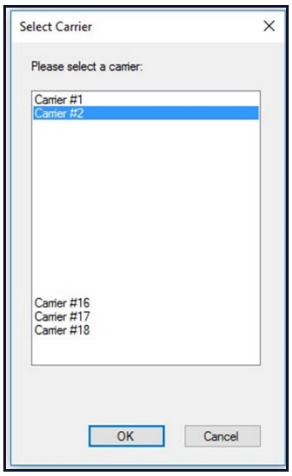


Figure 4.18. Select Carrier.

Use the navigation tool to select an area on the slide. (See Figure 4.19.) Click on the slide to change positions.

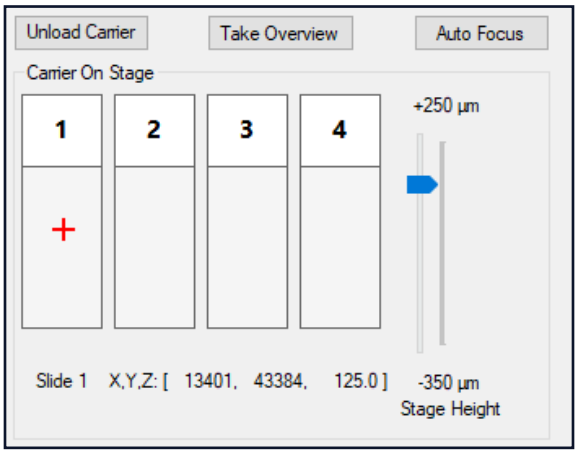


Figure 4.19. Navigation Tool.

For easier navigation, click Take Overview to acquire imagery of all slides in the carrier. An option to do this automatically can be selected 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.

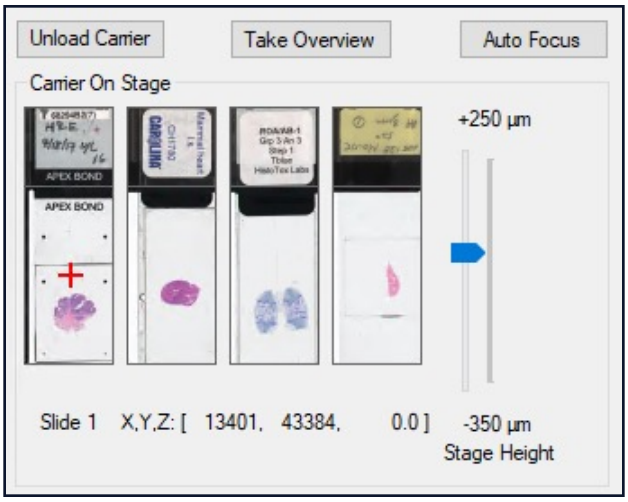


Figure 4.20. Navigation tool with Overview.

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.

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 the current live view, select either the whole slide or the MSI regions radio button (see Figure 4.21), pick a base file name, and click Snap.

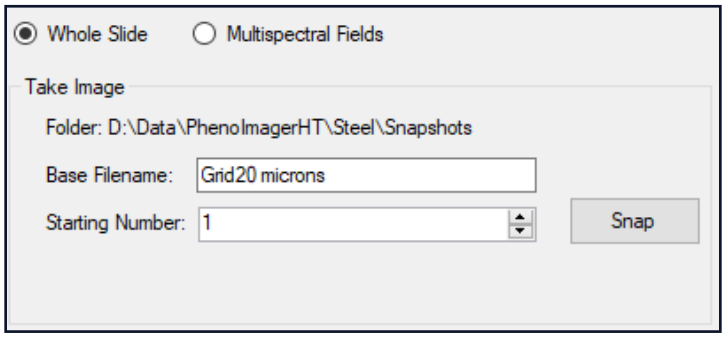


Figure 4.21. Whole Slide and MSI Regions radio buttons.

Navigate to new places, focus as needed, and take as many snapshots as required. Snapshots will be numbered incrementally.

Click Back when ready to return to the Edit Protocol window.

Fluorescence Protocols

When creating a fluorescence protocol, slides are required that include positive expression in all markers of interest to set suitable exposure times.

After creating the fluorescence protocol and assigning it to a study, the Edit Protocol window (for fluorescence protocols) opens. (See Figure 4.22.)

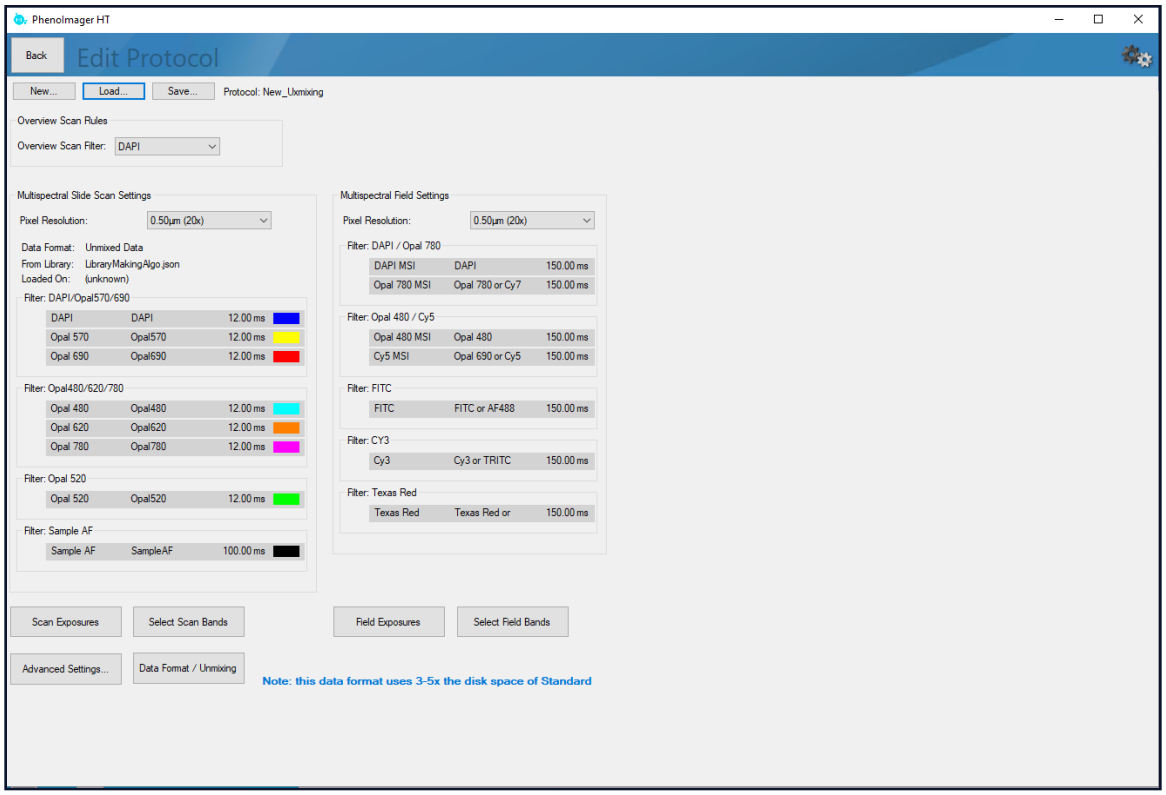


Figure 4.22. Fluorescence Protocol editor.

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

Figure 4.23. Overview Scan Rules.

Under Slide Scan Settings, choose the Pixel Resolution to be used while imaging the slide. (Figure 4.23). Options include:

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

Choosing higher resolution scans will result in larger file sizes and longer scan times.

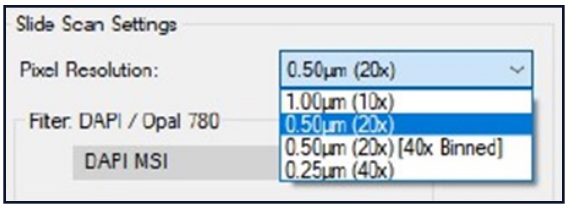


Figure 4.24. Slide Scan settings.

NOTE ***Each time the pixel resolution is changed the exposure times will need to be reset, as displayed in Figure 4.24.***

Exposure Times

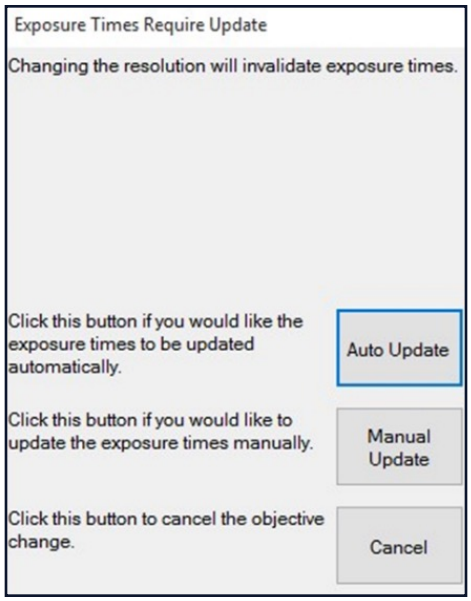


Figure 4.25. Exposure times require update window.

Functions on the Exposure Times window are described below. (See Table 4.1.)

Table 4.1. Exposure Times window functionality

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

If planning to take Multispectral Fields, choose the Pixel Resolution that should be used for fields imaging. Available resolutions are 10x, 20x, 20x binned, or 40x.

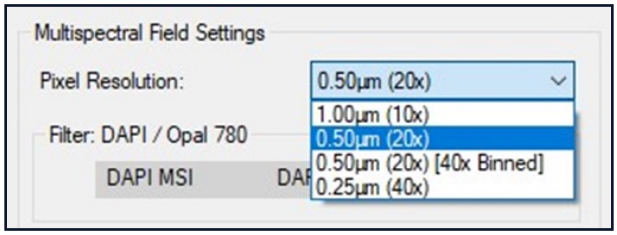


Figure 4.26. Multispectral Field Settings.

The Select Scan Bands button provides the option to choose which bands used to take the 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 the staining kit, or the bands selected for a custom scan.

To remove bands where fluorescent signals are not expressing, uncheck the band. Drag-and-drop to change the order in which they are acquired. The colors associated with each band can be changed; these are the colors that Phenochart will use to display the scan.

NOTE ***If the Overview or Focus bands are removed, PhenoImager HT will select new bands. Visit Advanced Settings to confirm these selections.***

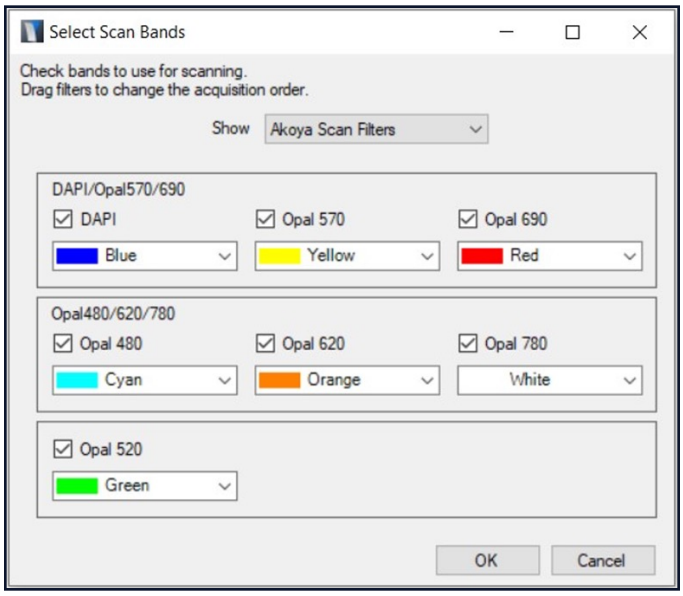


Figure 4.27. Select scan bands.

Limit the filters displayed in the Select Scan Bands dialog (Figure 4.27).

- 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 (Figure 4.28.)
- Show All Filters to display all installed filters, including custom filters that have been installed.

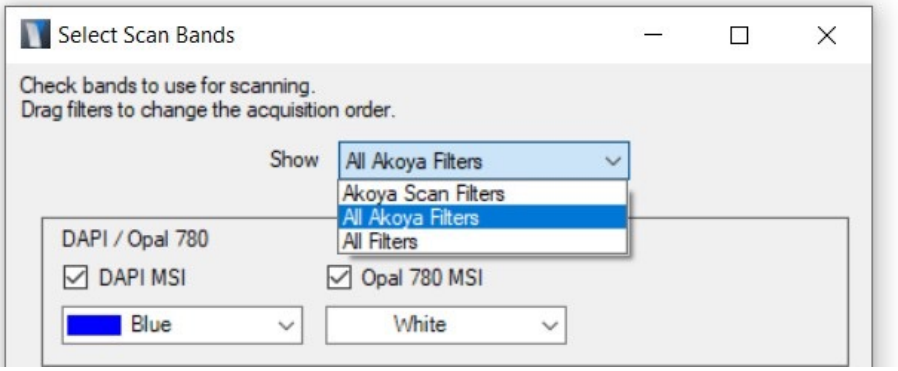


Figure 4.28. Select scan bands—show drop-down list.

The Select Field Bands button provides the option to choose which bands are used to take the multispectral fields. Akoya Biosciences strongly recommends maintaining the default band configuration. Non-signal bands are integral to multispectral stain extraction and unmixing.

To add or remove bands, select or de-select the filter. If there is more than one band available for a filter, select the band from the drop-down list. Drag-and-drop to change the order in which they are acquired. (See Figurer 4.29.)

NOTE ***If the Focus band is removed, PhenoImager HT will select a new band. Visit Advanced Settings to confirm this selection.***

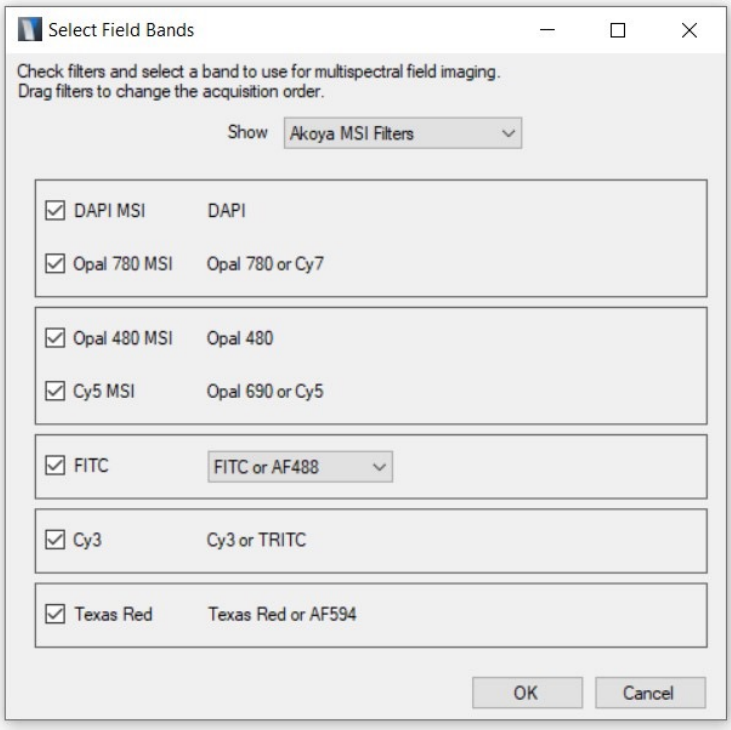


Figure 4.29. Select Field Bands – band selection.

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. (See Figure 4.30.)
- 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 have been installed.

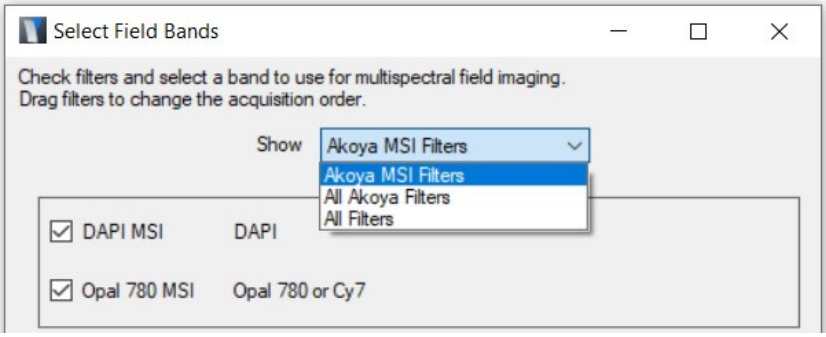


Figure 4.30. Select Field Bands – MSI filters.

Data Format and Unmixing

PhenoImager HT can save fluorescent scan data in several formats. By default, it uses "extended range" which provides a safety margin against accidental over-exposure. (See 1.3.6 Saturation and Dynamic Range.) The Standard format from Version 1 software may be used to save disk space. Finally, PhenoImager HT can spectrally unmix the data as part of the scan operation and record the marker names in the dataset.

To select between these modes, click the Data Format / Unmixing button on the Fluorescence Protocol Editor. This will bring up a dialog to select the data format, and an option to specify unmixing. (See Figure 4.31.)

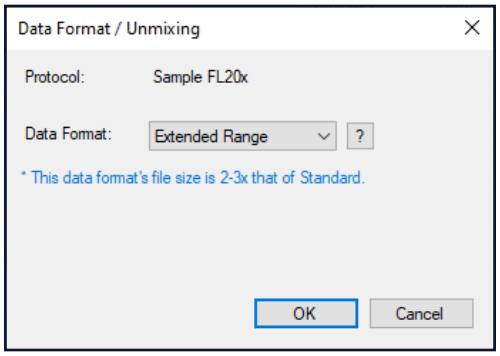


Figure 4.31. Extended Range selection.

This shows the default selection and posts a reminder that this format uses more disk space than Standard. To select between the data formats, use the drop-down tool. (See Figure 4.32.)

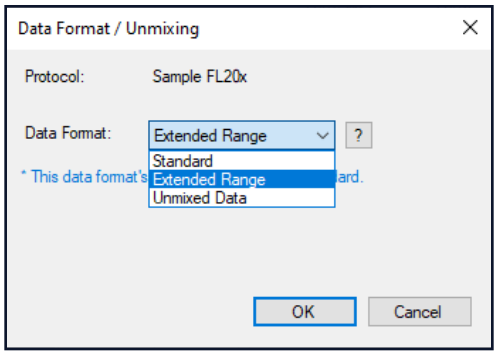


Figure 4.32. Select Data Format and Unmixing.

When Standard is selected, there is no reminder about disk space usage. (See Figure 4.33.)

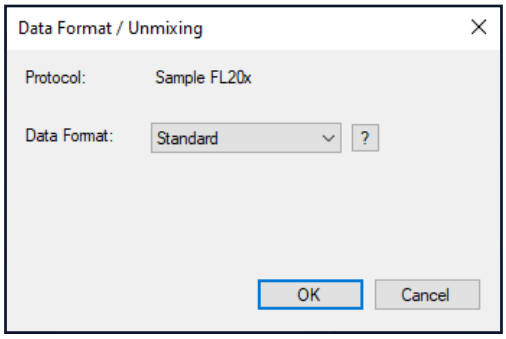


Figure 4.33. Standard Format Selection.

When Unmixed Data format is selected, a similar warning is shown, along with a notice to load an unmixing library. (Figure 4.34.) The unmixing library is contained in a .umxlib file that must be created in inForm and then exported for use by PhenoImager HT. It describes the properties of the dyes and sample autofluorescence that will be used to unmix the sample.

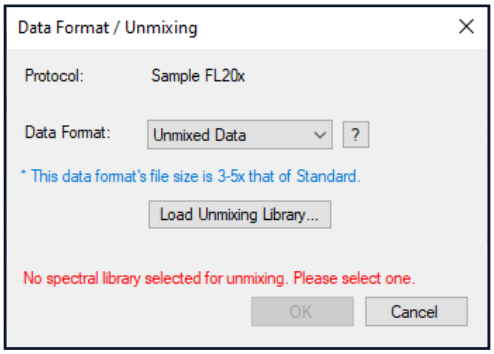


Figure 4.34. Unmixing Selection.

Click the Load Unmixing Library button and select a .umxlib file to use for the protocol. Once it has been loaded, there will be a prompt to fill in the names of the markers associated with each dye channel.

NOTE ***This enables the same .umxlib to be used for a variety of protocols, targeting different markers. (See Figure 4.35.)***

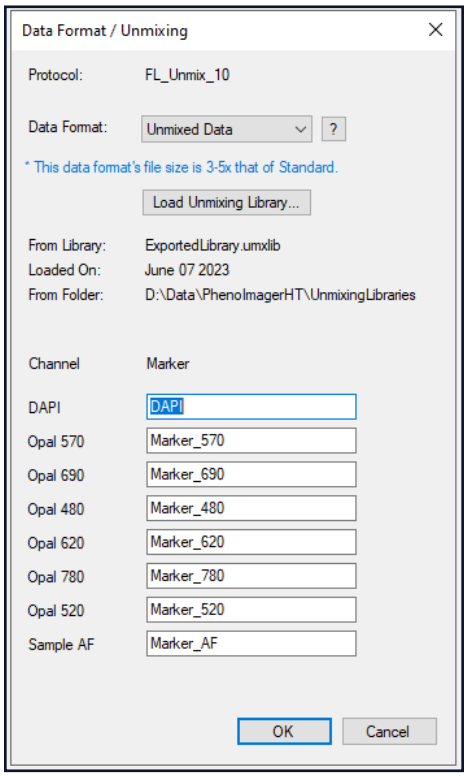


Figure 4.35. Band name assignment.

While the example above shows a protocol for imaging 6 markers plus DAPI and sample AF, the protocol may contain fewer markers if it has DAPI and sample AF and at least 1 marker channel; and the unmixing library has entries for all the channels used.

This enables the creation of an unmixing library once, with all channels, that can be used on a range of protocols with fewer channels when imaging samples with fewer markers.

The Fluorescent Protocol Editor shows the data format, with information about the unmixing library and marker names when unmixed data format is selected. (See Figure 4.36.)

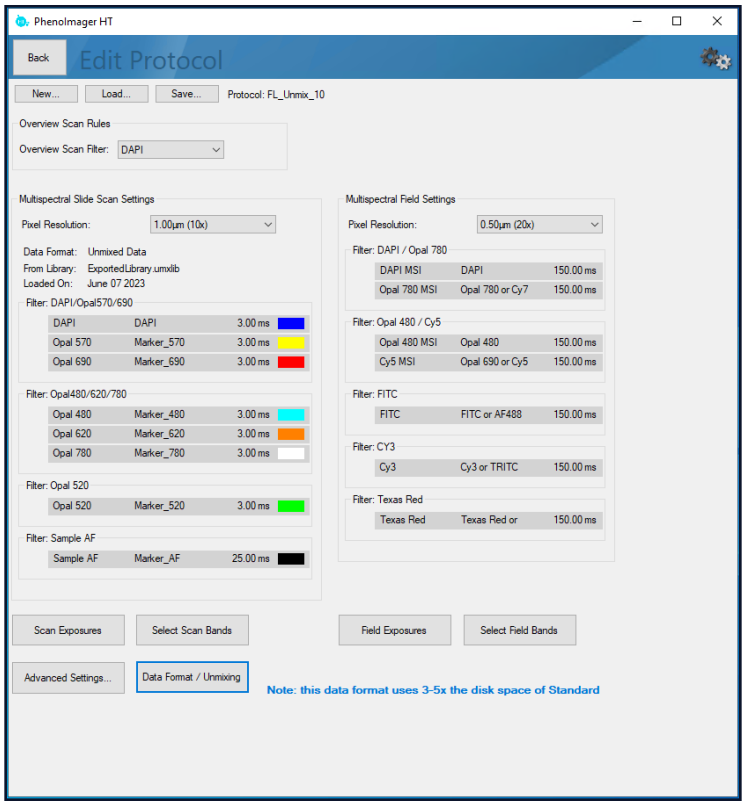


Figure 4.36. Fluorescent Protocol Editor.

Advanced Settings

There may be situations when fine-tuning how PhenoImager HT scans the tissue is necessary. 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.
    - If using thinner coverslips (#0), select “100 um (#0 or similar)” for a Coverslip Thickness.
    - If using thicker coverslips or have a significant amount of mounting media between the sample and coverslip, select the option that best matches the 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 suboptimal.***

- Specify which filter will be used for setting focus during whole slide scans and for multispectral field imaging. Choose the filters where the counterstain expresses. This is normally DAPI.
- Saturation protection prevents overexposure when acquiring multispectral field imagery. Akoya Biosciences highly discourages deactivating the Use Saturation Protection setting.

- If PhenoImager HT cannot find the entire tissue sample, check the Scan within the entire coverslip region checkbox. This will increase scan time and file size but will enable complete scanning of difficult samples.

NOTE ***If there is Sharpie markup on the 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 the samples are TMAs, select Sample is a TMA.
- Label Barcode Reading
  - PhenoImager HT can decode the barcode on the slide label and save it as part of the scan file, select the type of barcode being used. By default, PhenoImager HT will not attempt to decode a barcode unless this option is specifically selected.
  - If only 1D barcodes are used, select All 1D barcodes (autodetect type.)
  - If a variety of 2D barcodes is being used, select All 2D barcodes (autodetect type.)

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

  - If a combination of 1D and 2D barcodes is used, select All barcodes (autodetect type). This option will increase scan time and file size and should only be used if necessary. (See Figure 4.37.)

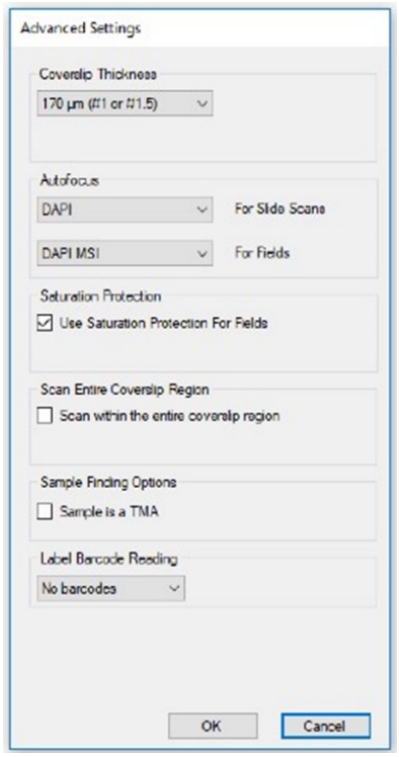


Figure 4.37. Fluorescence—Advanced Settings.

After selecting the settings, use the Exposures Editor to set exposures for the protocol.

4.3.4 Setting Exposures

After Selecting the scan and multispectral field resolutions, filters, and bands, exposures need to be set. Click Set Scan Exposures or Set Field Exposures in the Fluorescence Protocol Editor to set exposure times for scanning and field acquisition. Figure 4.38 shows the Edit Exposures window.

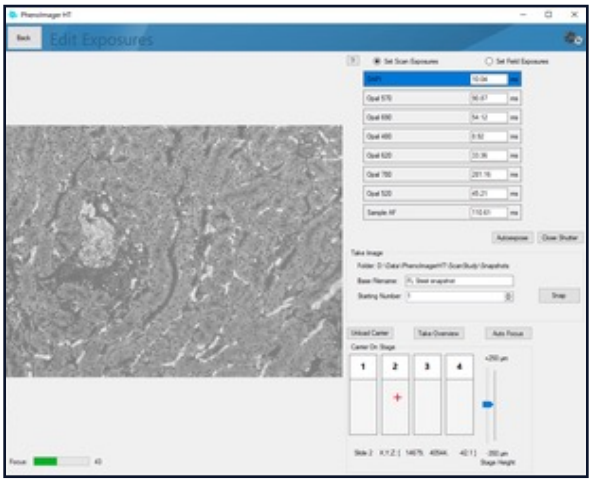


Figure 4.38. Edit Exposures.

If a carrier is not already on stage, click Load Carrier to select a carrier that contains representative slides. Select the carrier from the list and load it to the stage. (See Figure 4.39.)

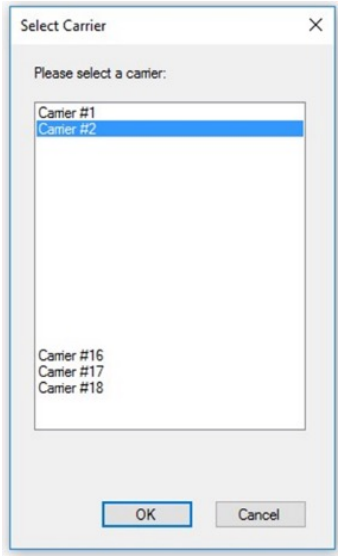


Figure 4.39. Select Carrier.

Use the navigation tool to select a slide. A live view of the first filter for the imaging rules will be shown. In most cases, this will be DAPI. The highlighted entry in the table identifies which filter or band is currently shown. (Figure 4.40.)

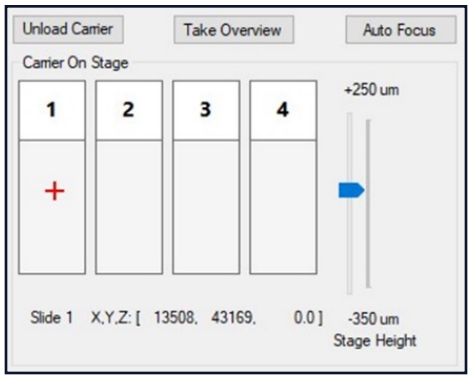


Figure 4.40. Navigation tool.

For easier navigation, click Take Overview to acquire digitally enhanced imagery of all slides in the carrier. (See Figure 4.41.) That option in the Preferences dialog can be selected in the Gear menu. PhenoImager HT will then automatically acquire overview imagery whenever a carrier is loaded.

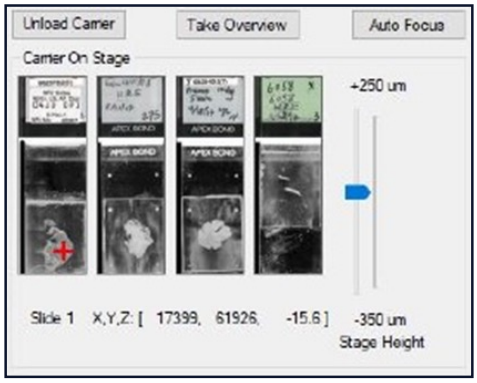


Figure 4.41. Navigation tool with overview.

Click on the tissue to change positions.

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

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, auto-focus and auto-expose again to refine the focus and exposure estimates.
- Override the auto-exposure value by typing a value in the highlighted cell. Values must be between 0.1 and 2000 ms.
- To turn off the fluorescence illumination and live view, click Close Shutter. Then re-open the shutter to see a live view and set exposures.

If this protocol is intended for multispectral field acquisition, set the multispectral field exposures at this time. In most cases, the same area of the slide can be used to set the 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, auto-focus and autoexpose again to refine the focus and exposure estimates.
- Override the auto-exposure value by typing a value in the highlighted cell. Values must be between 0.1 and 2000 ms.
- To turn off the fluorescence illumination and live view, click Close Shutter. Re-open the shutter to see a live view and set exposures.

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

Filter Cube Recommendations when using Akoya Biosciences Opal Reagents

If using the PhenoImager HT system with Akoya Biosciences' Opal fluorophores (either as part of PhenoCode Signature Panels or as individual fluorophores), please refer to Table 4.2 and Table 4.3 for exposure time recommendations for acquiring spectral library images.

Table 4.2. Multispectral Slide Scan Band
Columns represent filter bands; rows represent Opal fluorophores.

OPAL FLUOROPHORES	FILTER BANDS							
	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 4.3. Multispectral Field Band

Columns represent filter bands; rows represent Opal fluorophores.

OPAL FLUOROPHORES	FILTER BANDS						
	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							

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 4.4.

Table 4.4. 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	35 ms	150 ms	15 ms	50 ms

Snapshots

Once the exposures have been set, take snapshots to see sample imagery of how the 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. (See Figure 4.42). Navigate to new places and take as many snapshots as desired. They will be numbered incrementally.

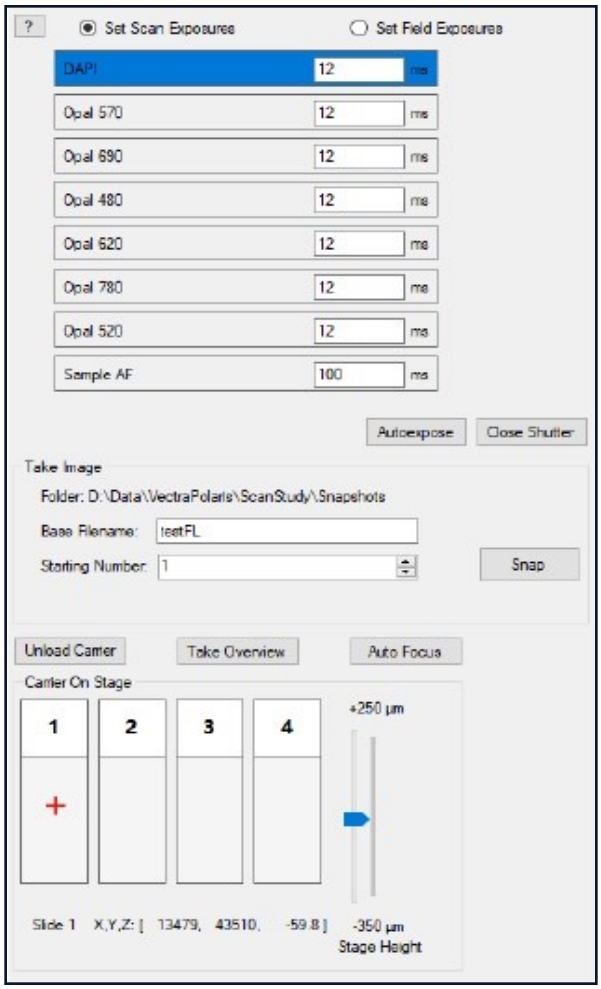


Figure 4.42. FL exposures panel.

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

NOTE ***The exposures just set are only valid for the resolutions that were selected. If the scan or multispectral field resolution is changed, then revisit the exposures editor to update the exposures.***

4.4 SCANNING SLIDES

4.4.1 The Carrier

Slides are loaded into PhenoImager HT via carriers, which are stacked into the Slide Carrier Hotel. (See Figure 4.43.)

Figure 4.43. 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 the scan rules can be tailored to each slide and/or each carrier as needed.

4.4.2 Carrier Status

Within the software, each hotel slot is identified as Slot N, where N is 1-20 running top to bottom (Figure 4.44). When a Slide Carrier is loaded into the hotel, an LED light changes color to identify the status of that carrier.

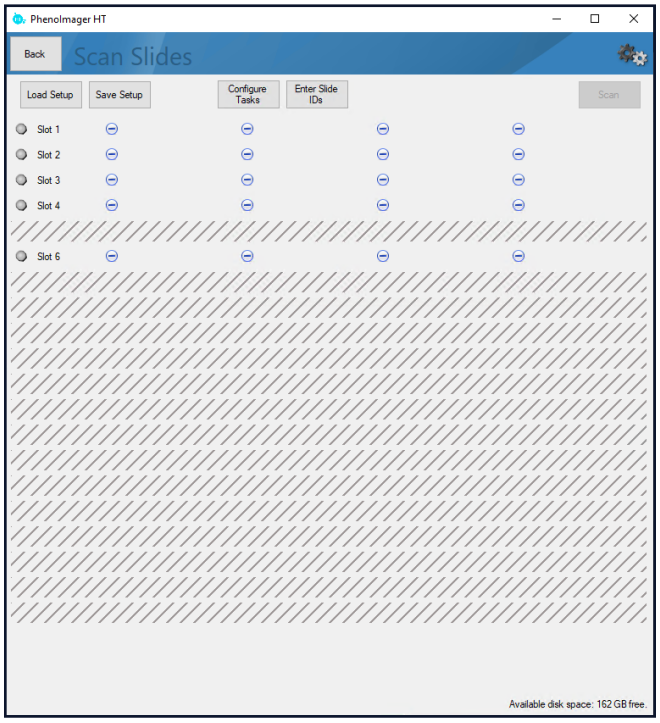


Figure 4.44. Scan slides—carrier status.

The User Interface will reflect the current state of each slot in the hotel. If there is no carrier present, a striped display will result. (See Figure 4.45.)

Figure 4.45. Striped display indicating no carrier.

Carrier presence in the hotel is represented as indicated below in Figure 4.46.

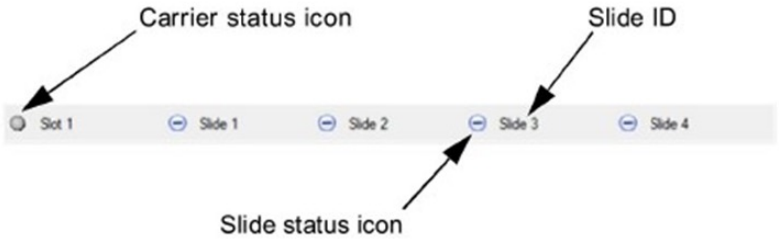


Figure 4.46. Slide carrier indicators in the hotel.

Each carrier is represented by a carrier status icon, 4 slide status icons, and 4 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 the carrier is inserted 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, define the rules for the 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.

4.4.3 Slide Status

Each carrier also has a status icon for the 4 slides it contains. Table 4.5 shows the status icons and their corresponding descriptions for each slide.

Table 4.5. Slide status icons and their meanings

Status Icon	Description
	The slide has passed the requirements and is ready to be scanned.
	The slide was previously skipped due to insufficient disk space and is ready to be scanned. Alternately, there may be a warning related to instrument configuration. Hover over the icon to view details.
	<Blinking> The slide is currently being scanned.
	The slide was scanned successfully.
	The slide has rules, but they cannot be used. (They may be incomplete, in which case the rules will need to be edited for that slide.)
	The slide failed to scan correctly. Hover over the icon to view details of the error.
	The slide is being ignored. This is useful if the carrier has <4 slides or if that slide will not be processed at the current time.

4.4.4 Setting Up Scan Rules

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

Slide IDs

The ID must be entered manually for each slide in the carrier. The IDs may be entered before the carrier is placed into the hotel or enter IDs after the carrier has been placed into the hotel.

To enter the IDs before the carrier is placed into the hotel, click Enter Slide IDs. (See Figure 4.47.)

Figure 4.47. Enter Slide IDs button.

An Enter Slide ID window appears. (See Figure 4.48.)

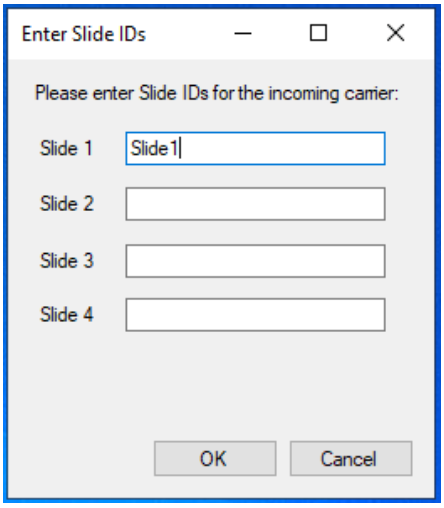


Figure 4.48. Enter Slide IDs window.

Enter between 1-4 slide IDs for the slides and click OK. Immediately put the carrier in the hotel, and PhenoImager HT will assign the slide IDs that were entered to that carrier.

If the carrier is already present in the hotel, 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 the Edit Slides dialog box. (See Figure 4.49.)

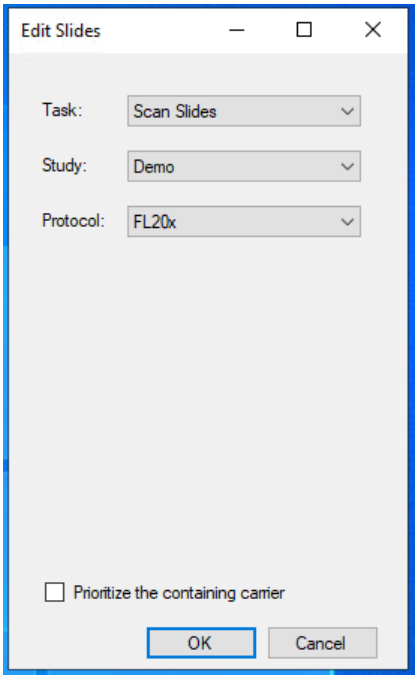


Figure 4.49. Edit Slides.

For this slide, enter:

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

NOTE ***Select Ignore to skip processing this particular slide.***

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

To prioritize this slide's carrier to be scanned at the front of the line, check Prioritize the Containing Carrier.

Editing All Slides Within a Single Carrier

To edit the scanning rules for a single carrier, click the status icon for that carrier. The Edit Slides editor window is seen in Figure 4.50.

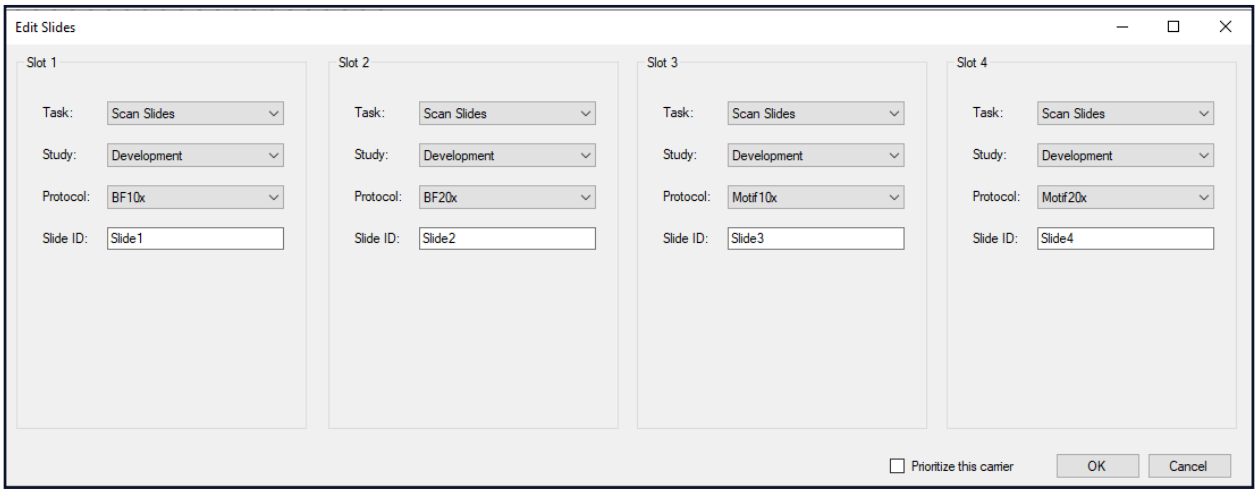


Figure 4.50. Edit Slides editor window.

For each slide, 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 saved within the study.
- **Slide ID** - Add the slide ID, if necessary.

Check Prioritize This Carrier if the carrier should be scanned first.

Editing Multiple Carriers

To edit multiple carriers, click Configure Tasks. (See Figure 4.51.)

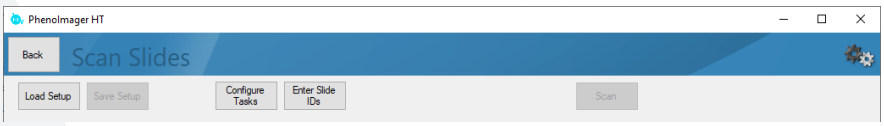


Figure 4.51. Configure Tasks button.

Select the carriers to be edited. Multiple selection is available. (See Figure 4.52.)

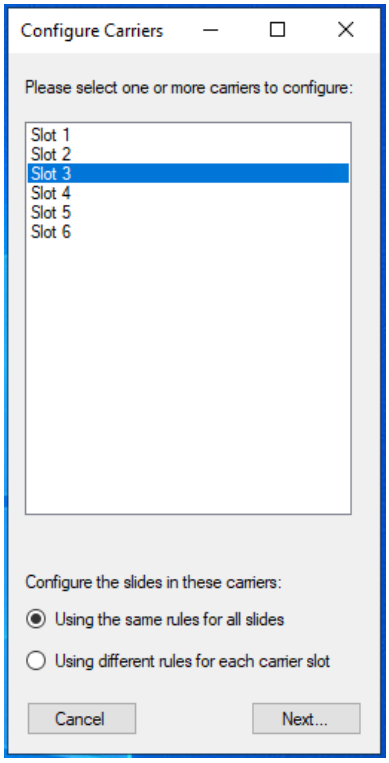


Figure 4.52. Configure Carriers.

Select how to process the 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 4.53.)

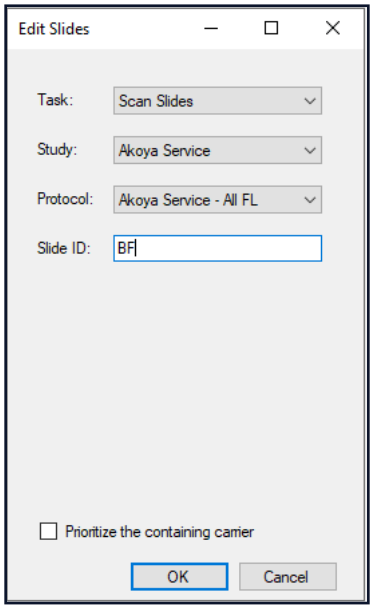


Figure 4.53. Edit Slides.

- Select the task, study, and protocol.
- To prioritize the selected carriers to be scanned at the front of the line, check Prioritize the Containing Carriers.

NOTE ***The Slide ID cannot be edited because rules are applied across multiple slides. If slide IDs must be entered, 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 needed. Again, the Slide IDs cannot be edited because rules are applied across multiple slides. (See Figure 4.54.)

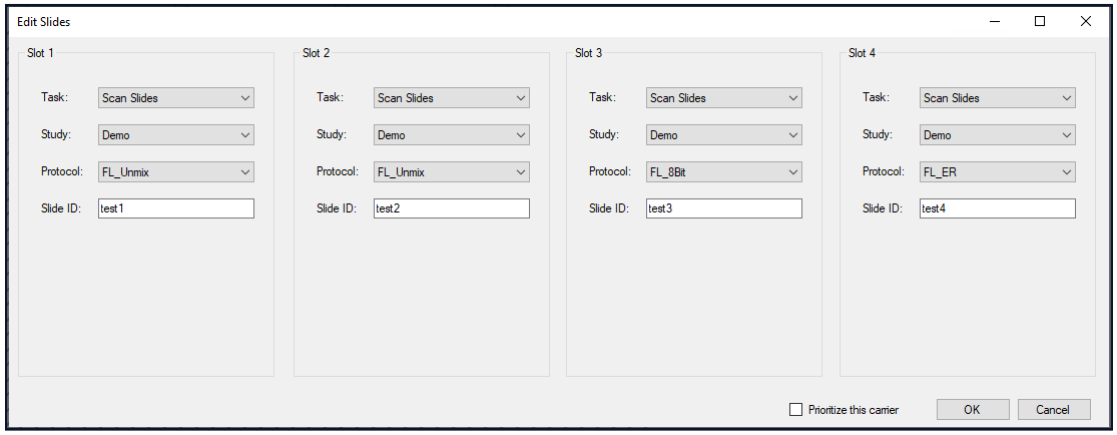


Figure 4.54. Edit Slides.

4.4.5 Assessing Disk Space Before Scanning

After setting up rules for all slides, assess whether any slides might be skipped due to insufficient disk space by viewing the disk space indicators at the bottom of the window. (See Figure 4.55.) Total available disk space is displayed at the bottom right. The bottom left text estimates how many slides will complete successfully given the available disk space.

Figure 4.55. Disk space indicators.

Since file sizes depend on the imaged area of each slide, which is not determined until a slide is loaded on the stage, predictions are shown for both typical scan areas (15 mm x 15 mm) and maximum scan areas (29 mm x 57 mm). If unsure of the scan areas, assume the maximum scan area to avoid skipped slides.

The text at the bottom of the screen will be red if any slides are predicted to be skipped even if all are typical size. The text will be blue if slides are only predicted to be skipped if all are maximum size. (See Figure 4.56.) Clear disk space to reduce the estimated number of skipped slides.

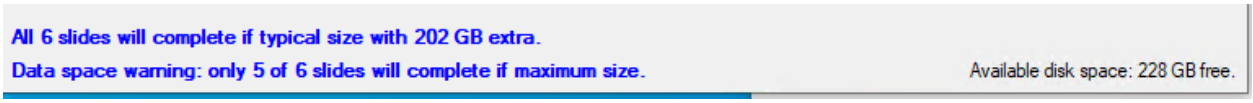


Figure 4.56. Blue text indicating slides are only predicted to be skipped if all are maximum size.

4.4.6 Saving and Restoring Scan Setup

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

Save the slide IDs, studies, protocols, and tasks by clicking the Save Setup button. PhenoImager HT will also prompt to save before scanning (after clicking Scan). At this point, enter a name and some notes about this scan setup. (See Figure 4.57).

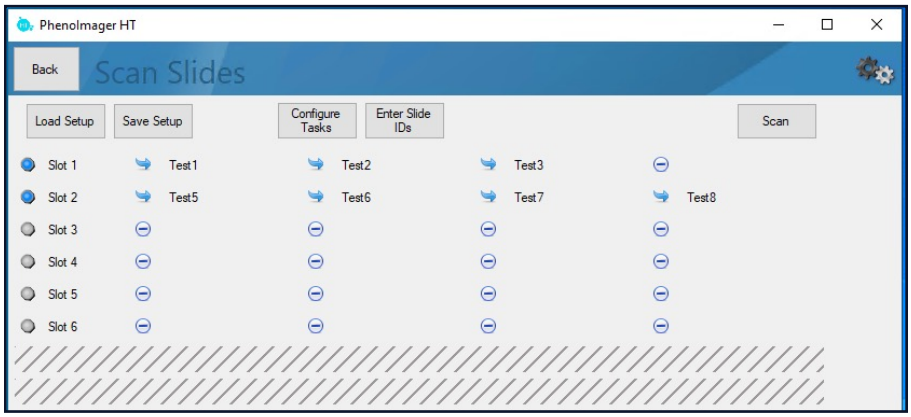


Figure 4.57. Scan Slides.

Saving a setup is not required, but it can help locate the information later if needed. (See Figure 4.58.)

- By default, this information will be deleted after 30 days, but can override the default and opt to keep the information until manually deleted.

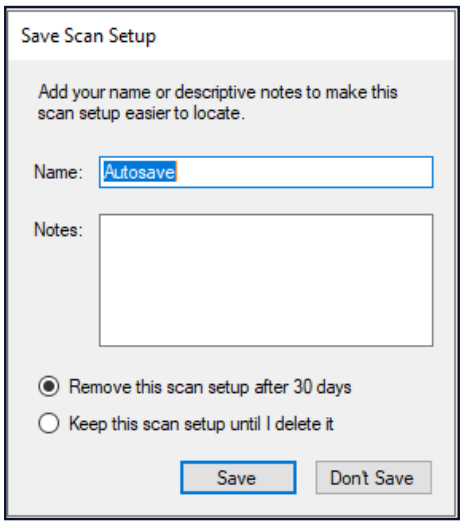


Figure 4.58. Save Scan setup.

To restore an earlier scan setup, click Load Setup.

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

NOTE ***Entries can be deleted that are no longer needed by selecting those items and clicking Delete.***

To use a setup, select it from the dialog and click Load. (See Figure 4.59.)

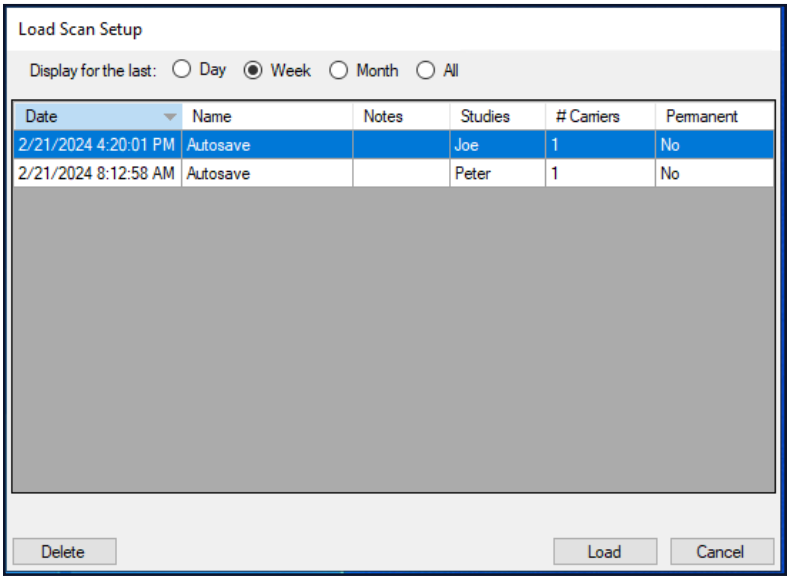


Figure 4.59. Load Scan setup.

After selecting a setup, configure the carriers in PhenoImager HT to match the information in the setup.

If the carriers and slides are the same location as what was saved during setup, then click OK because everything is in the right place.

In other cases (for example, when putting the slides back in), this dialog will assist in figuring out where the slides belong.

1. When scan information is available and a carrier is present in the hotel, the system will show the slide IDs. (See Figure 4.60, Slot 1.)
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 Figure 4.60, Slot 5.) The slide IDs for that carrier are listed, so one 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 (e.g., Slots 7 and 9). Slides in these carriers were not present in the original setup.

For every item in the scan setup (meaning anywhere the system has information on a carrier that should be present [Items 1 and 2]), before the carrier can be loaded, one of the following must be specified:

- **Use as saved:** The system will load all slide information including the scan task.
- **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 for loading the setup to rescan a few select items, or to change the task from how it was originally saved.
- **Don't restore:** No information for this carrier will be reloaded. This is useful if the carrier is not needed or is missing.

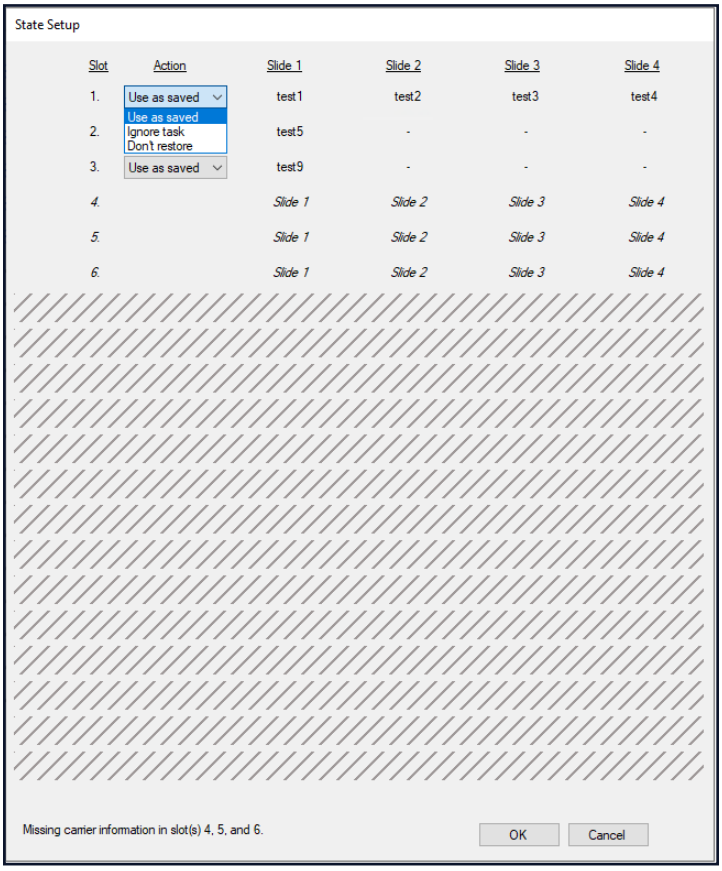


Figure 4.60. Slide setup.

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

Click OK when the selections have been finalized. The scan slides editor will be cleared and replaced with the selected scan information.

4.4.7 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. (See Figure 4.61.)

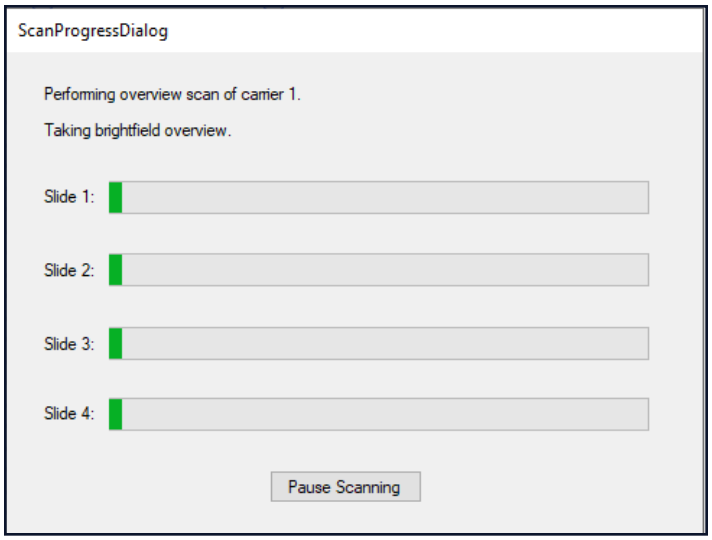


Figure 4.61. Processing Slides window.

Click Pause Scanning to remove completed carriers and add new carriers to be scanned. When a scan is paused, PhenoImager HT will return the current carrier to the hotel. It will then be safe to open the door. 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 ready, click Scan to resume scanning.

NOTE ***If scanning using the Unmixed Data format (see Section 4.3.3), the last unmixed slide may still be processing after the final carrier is unloaded. In this case, the progress dialog will indicate status as "Performing unmixing on the final scan." This step could take up to 120 minutes if using 40x scan resolution and large scan area.***

Chapter 5: Maintenance

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

5.1 POWER CYCLING THE INSTRUMENT

For best performance, power cycle the PhenoImager HT system periodically (a few times per week) or if it has been idle for an extended time. See section 3.1 and 3.2 for startup and shutdown procedures.

5.2 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.*

5.3 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.*

5.4 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 3.2. System Shutdown) before cleaning any part of the instrument where electrical or fiber optic cables make connections.*

5.5 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.

5.6 REPLACING THE FUSES

For blown fuses and to order replacement fuses, contact Technical Support at support@akoyabio.com.

	*Warning: Electric Shock Hazard. DO NOT attempt to replace the fuses. Only qualified Akoya Biosciences service personnel can replace the fuses.*

Appendix A: Akoya QPTIFF Specification

Background

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

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

Data Format

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

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

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

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

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

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

Table A.1. Images

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

Detection

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

Metadata

Metadata is contained in two locations: within standard TIFF tags as listed in 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 A.2. TIFF tags

TIFF Tag	Optional?	Description of Content
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)

TIFF Tag	Optional?	Description of Content
XPosition	Y	Sample X location in ResolutionUnits. This is ULHC loca-tion except for Macro image which reports its image center.
YPosition	Y	Sample Y location in ResolutionUnits. This is ULHC loca-tion 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.
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).

ImageDescription Contents

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

Table A.3. ImageDescription Tags

TIFF Tag	Optional?	Contents
DescriptionVersion		Version of the image description field itself, a single number. This document describes version 5 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.
SampleDescription	Y	Text description of the sample
OperatorName	Y	Name of the operator as reported by the operating system running the acquisition software.
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 B.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).
ExposureTimeArray	Y	Exposure times as an integer number of microseconds. Holds multiple values if there is more than one sample per pixel, as for RGB imagery; otherwise the array has a single element.

TIFF Tag	Optional?	Description of contents
Signal Units		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. This does not imply a specific pixel resolution – see the XResolution and YResolution standard TIFF tags instead.
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 ex-ample, 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, Scale-Factor would be 1.3201. Default is 1.

TIFF Tag	Optional?	Description of contents
NormalizedUnitsFactor	Y	For unmixed data, multiplying the pixel value by this factor will yield component signal in units of normalized counts. Normalized counts are defined by: $Normalized\ counts = \frac{counts}{[FSC * B^2 * G * E * WDF * SW]}$ Where: *FSC* is the camera full-scale value in counts *B* is the binning *G* is the camera gain *E* is the exposure time, in seconds *WDF* is a well-depth factor for the sensor used *SW* is a spectral weighting factor that accounts for the total energy associated with that component's spectrum across all bands.
AutofluorescenceSubtracted	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
BFLampType	Y	String with the BF lamp type – Unknown – FlashLamp – WhiteLED – TricolorLED
InstrumentType	Y	String with the instrument type, one of the following: – Unknown (default) – Keyence700 – Keyence800 – AxioObserver – Dmi8 – Polaris – Vectra3

TIFF Tag	Optional?	Description of contents
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 below
Band		Description of a band with child tags 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 EOM supplier
PartNo		String with the manufacturer's part number
Bands		Collection of bands, each described by sub-elements below
Band		Description of a band with sub-elements noted below
Cuton		Cut-on wavelength of this band in nm
Cutoff		Cut-off 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

TIFF Tag	Optional?	Description of contents
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 Vectra Polaris and unmixed FL multispectral images originating from Vectra 3 and Vectra Polaris.

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

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

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

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

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

Tag	Contents
Name	The name of the filter (whole-slide image) or band (component image).
Response	The instrument response to the reference artifact, normalized for exposure. This is raw counts/ ($2^{\text{bit depth}}$ × 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 >
```

Appendix B: Akoya Software & Spectra Compatibility

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

Table B.1. Akoya Software Compatibility Considerations

	VectraPolaris 1.x.x	PhenoImager HT 2.x.x	Phenochart 1.x.x	Phenochart 2.x.x	inForm 2.x.x	inForm 3.x.x
Scanning protocols created in VectraPolaris 1.x.x can be opened by	Yes	Yes	N/A	N/A	N/A	N/A
Scanning protocols created in PhenoImager HT 2.x.x can be opened by	No	Yes	N/A	N/A	N/A	N/A
Imagery acquired with VectraPolaris 1.x.x can be opened by	N/A	N/A	Yes	Yes	Yes	Yes
Imagery acquired with PhenoImager HT 2.x.x can be opened by	N/A	N/A	Some*	Yes	No	Yes
Annotations created by Phenochart 1.x.x can be opened by	Yes	Yes	Yes	Yes	Yes	Yes
Annotations created by Phenochart 2.x.x can be opened by	No	Yes	No	Yes	No	Yes
Algorithms created by inForm 2.x.x can be opened by	N/A	N/A	N/A	N/A	Yes	Yes
Algorithms created by inForm 3.x.x can be opened by	N/A	N/A	N/A	N/A	No	Yes
Projects created by inForm 2.x.x can be opened by	N/A	N/A	N/A	N/A	Yes	Yes**
Projects created by inForm 3.x.x can be opened by	N/A	N/A	N/A	N/A	No	Yes

	VectraPolaris 1.x.x	PhenoImager HT 2.x.x	Phenochart 1.x.x	Phenochart 2.x.x	inForm 2.x.x	inForm 3.x.x
Spectra extracted in inForm 2.x.x can be exported as an un-mixing library for HT 2.x.x by	N/A	N/A	N/A	N/A	**No**	**Yes**
Spectra extracted in inForm 3.x.x can be exported as an un-mixing library for HT 2.x.x by	N/A	N/A	N/A	N/A	**No**	**Yes**

*Can be opened, but will not allow for stamping for inForm.

**Background on Projects created by inForm 2.x opening in inForm 3.x. Two considerations:

1. Beginning with inForm 3.0, there are slight changes to cell splitting or segmentation due to the different numerical routines used. Also, the cell numbering can be assigned differently even for the same segmentation results. These do not affect the results in a significant way for normal use, but as a result, the assignment of cell ID #s to cells can differ between 2.x and 3.x. This means that phenotyping training sets from inForm 2.x projects cannot be used for training phenotyping classifiers in 3.x because the training sets consist of tables of phenotypes keyed by cell ID. If the cell ID is wrong, the training data is erroneous so the resulting classifier will not be properly trained. For this reason, inForm 3.x will not bring in training cell ID information for projects from inForm 2.x. It will post this message when this happens: “[Project] was created with inForm 2.8 or earlier, and phenotype training data from 2.8 cannot be imported into inForm 3.0. All cell phenotype training data has been discarded for that reason. The algorithm's phenotype step can be run with its current training, or use inForm 2.8 or earlier to add training cells or revise phenotyping."
2. For projects created in inForm 2.8 with imagery that has 10+ channels (i.e., PhenoCycler imagery), phenotyping computation may become unusably slow in inForm 3.x. It is recommended that inForm 2.8 projects with imagery that has 10+ channels are only run in inForm 2.8. New projects created in inForm 3.0 do not have this issue.

Considerations for Side-by-Side Installations

- Vectra Polaris 1.x and PhenoImager HT 2.x software can be installed side-by-side. If needed, Vectra Polaris 1.x may continue to be used for some ongoing studies that use inForm 2.x algorithms.
- Discerning which version is launching will be easy due to differing program names. Phenochart 1.x and 2.x can be installed side-by-side.
- Users often launch Phenochart by double-clicking a QPTIFF. In this case, the launched version might not be obvious but will be easily confirmed via About Box. inForm 2.x and 3.x can be installed side-by-side.
- Users sometimes launch inForm by double-clicking on a project. In this case, the launched version might not be obvious but will be easily confirmed via About Box.

Spectra compatibility considerations are detailed in Table B.2.

Table B.2. Spectra compatibility and incompatibility considerations

	Unmix 8-bit QPTIFFs in inForm	Unmix Extended-bit QPTIFFs in inForm	Unmix in HT via .umxlib
Synthetic Opal & AP spectra can be applied to	YES	YES	YES
Opal spectra extracted from 8-bit QPTIFFs can be applied to	YES	YES	YES
AF spectra extracted from 8-bit QPTIFFs can be applied to	YES	NO	YES
Opal spectra extracted from Extended-bit QPTIFFs can be applied to	YES	YES	YES
AF spectra extracted from Extended-bit QPTIFFs can be applied to	NO	YES	YES

Appendix C: PhenoImager HT EULA

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