AKOYA BIOSCIENCES®
THE SPATIAL BIOLOGY COMPANY®

PhenoImager Fusion
USER GUIDE

For use with PhenoCycler-Fusion 1-Flow Cell Configuration and Fusion 4-Slide Configuration

PD-000001 Rev L

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Contents

Preface

Welcome to the Akoya Biosciences PhenoImager™ Fusion User Guide.

INTENDED AUDIENCE FOR THIS DOCUMENT

This document is intended for laboratory personnel who have received instruction in:

- General safety practices for laboratories.
- Specific safety practices for the PhenoCycler™ and PhenoImager Fusion instruments.

ASSOCIATED DOCUMENTS

- PhenoCycler®-Fusion User Guide
- Opal® Multiplex Assay Development Guide
- Phenochart™ Whole Slide Context Viewer for Annotation and the Review User Guide
- inForm® Advanced Image Analysis Software User Guide

GUIDE CONVENTIONS

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

	*DANGER: Warns the user of an imminently hazardous situation, which if not avoided, will result in death or serious injury.*
	*Warning: Warns the user regarding actions that may result in physical damage to the system or personal injury.*
	*Caution: Cautions the user regarding actions that may result in operational issues or data loss.*
NOTE	*NOTE: Identifies important points, helpful hints, special circumstances, or alternative methods.*
CRITICAL	*CRITICAL: Identifies areas where the user should pay close attention to the instructions to avoid possible physical damage to the system or personal injury.*

PRODUCT SERVICE AND CUSTOMER SUPPORT PLANS

Akoya offers a full range of services to ensure success. From our original factory warranty to a comprehensive line of customer support plans, Akoya provides Field Service Engineers and internal specialists who are dedicated to supporting hardware, software, and application development needs.

Our 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.

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.***

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.

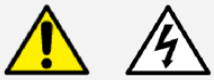
KOREAN CERTIFICATIONS (KC)

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.

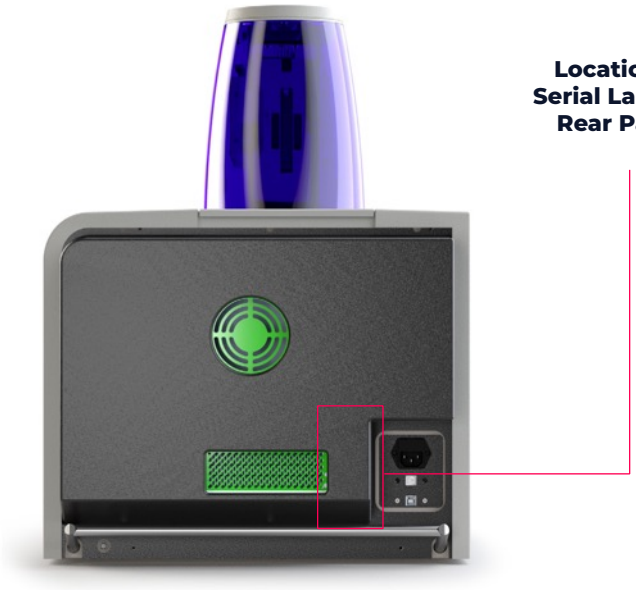
DEFINITION OF SYMBOLS (LABELING OF EQUIPMENT AND LOCATION)

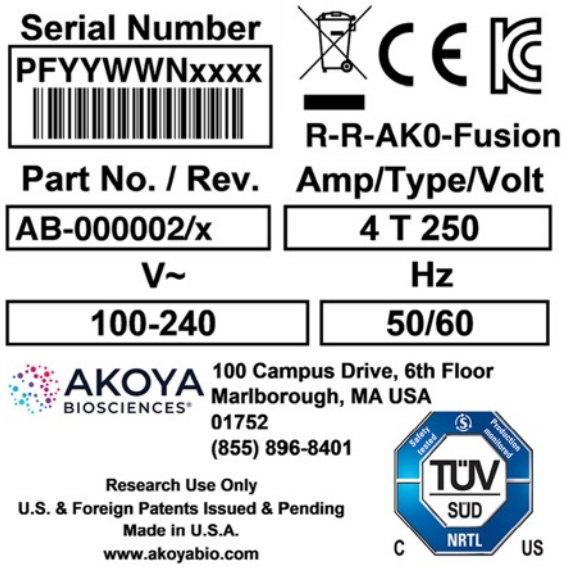
This section details the meaning, intent, and location of the labels (containing symbols) that appear on the PhenoImager Fusion system and/or in the user documentation.

The figures below this table show the location of the labels on the instrument.

Symbol	Description	Symbol	Description
	This symbol indicates danger. It indicates an imminently hazardous situation, which, if not avoided, will result in death or serious injury.		Lifting Hazard. May result in injury. (ISO 3864)
	This symbol warns the user regarding actions that may result in physical damage to the system or personal injury.		Risk of Explosion. (ISO 3864)
	This symbol indicates caution. Cautions the user regarding actions that may result in operational issues or data loss.		Bright Lights
	This symbol is a Note. A note is a cautionary statement, an operating tip, or maintenance suggestion. Ignoring the note may result in instrument damage if not followed.		Protective ground symbol. (IEC 60417-5019)
	This symbol represents hazardous voltage. Hazardous voltage causes risk of electric shock, per IEC 60417-6042.		Ground Symbol. (IEC 60417-5017)
	Crush Hazard. Risk of body parts, hair, jewelry, or clothing getting caught in a moving part. (ISO 3864)		Fuse. (IEC 60417-5016)
	Risk of Fire. (ISO 3864)		Alternating Current. (IEC 60417-5032)

PhenoImager Nameplate





Serial Label

SAFETY CONSIDERATIONS

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

	*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.*

DANGER, WARNINGS, AND CAUTION SIGNS

Observe the following dangers, warnings, and cautions when using the PhenoImager Fusion system.

GENERAL HAZARDS

None.

ELECTRICAL SAFETY

The PhenoImager Fusion 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 Akoya Biosciences Technical Support* support@akoyabio.com.
	*Warning: Turn off the electrical power to the PhenoImager Fusion system by shutting down the system before cleaning any part of the instrument where electrical or fiber optic cables connect.*
	*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 screen with the cloth.*

POWER CORD SELECTION

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

	*Warning: Use only the power supply cord set provided with the PhenoImager Fusion system. If the correct cord 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.*
	*Warning: Use only a properly grounded power outlet when connecting the system to power.*
	*Warning: The appliance inlet is a disconnecting device. Place the device or equipment where disconnecting the device is always accessible.*

FUSES

Contact Akoya Biosciences 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: 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.*

WEIGHT WARNING

	*Warning: LIFTING HAZARD. The PhenoImager Fusion instrument weighs ~120 lbs. (~54 kg). Do not move the PhenoImager Fusion instrument. Installing, servicing, and moving the PhenoImager Fusion instrument should be performed only by qualified Akoya Biosciences service personnel. Contact Akoya Biosciences technical support at support@akoyabio.com if help is required.*

BRIGHT LIGHT

	*Warning: BRIGHT LIGHT HAZARD. The interior of the PhenoImager Fusion system includes a Class 2 LED Light. Do not look into the bright light to avoid an eye injury.* *Caution: Do not stare directly into the beam.*

Chapter 1: Introducing the PhenoImager Fusion

INTENDED USE

- The PhenoImager™ Fusion is a multimodal digital pathology instrument that integrates both multispectral analysis and automated slide scanning.
- This technology allows researchers to visualize, analyze, qualify, and phenotype immune cells in situ with tissue sections and tissue microarrays (TMAs).
- The PhenoImager Fusion should only be used by appropriately trained operators. Akoya highly recommends reading this manual prior to operating the system.
- Failure to comply with the instructions in this manual may pose a danger to the operator.

NOTE ***Akoya's PhenoImager Fusion system is for research use only. Not for use in diagnostic procedures.***

PRINCIPLES OF OPERATION

- Akoya's PhenoImager Fusion is an automated imaging system that has been
- optimized to image samples stained with Akoya's Opal® multiplexed fluorescent immunohistochemistry (IHC) reagent kits.
- The system is also compatible with typical Brightfield staining reagents and compatible immunofluorescence/fluorescence staining reagents.
- The PhenoImager Fusion is configured to visualize up to 6-plex Opal staining on a Slide Carrier that can carry up to 4 slides.
- The PhenoImager Fusion paired with PhenoCycler™ also enables imaging of 40+ biomarkers per sample using the PhenoCycler®-Fusion workflow.
- The PhenoImager Fusion has been designed to expand and support the PhenoCycler workflow, defined in true whole slide scanning of slides at 0.5 µm/pix and 0.25 µm/pix.
- The analysis of multispectral whole-slide scans includes protein expression and spatial phenotyping.

AKOYA SOFTWARE

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

PhenoImager Fusion

PhenoImager Fusion is an operator-centric software for performing whole slide scans and PhenoCycler experiments. The PhenoImager Fusion software runs on the workstation connected to the PhenoImager Fusion instrument.

Phenochart

Phenochart™ is a whole-slide viewer for fluorescent and bright images acquired on Akoya Biosciences' instruments. Annotations made in this software are readable by inForm® Tissue Finder image analysis software. Phenochart is freely distributed.

inForm Tissue Finder

inForm Tissue Finder is software used for the analysis of PhenoImager Fusion images. This software supports features such as spectral unmixing, tissue classification, spatial phenotyping, protein expression measurements, and data export. It can be run on the PhenoImager Fusion computer or other Microsoft® Windows® 10 computers. Additional inForm software seats separate from those that come with each PhenoImager Fusion system are available for purchase.

Example Applications

Examples of PhenoImager Fusion applications include:

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

KEY CONCEPTS OF MULTISPECTRAL IMAGING

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

- Autofluorescence
- Photobleaching
- Multispectral Imaging
- Multispectral Analysis

Autofluorescence

Many biological materials are naturally fluorescent or autofluorescent (AF). Many vitamins, some hormones, and a variety of biological enzymes and structural proteins exhibit autofluorescence. (See Figure 1.1.) These materials often fluoresce strongly enough to interfere with specific fluorescence labeling studies.

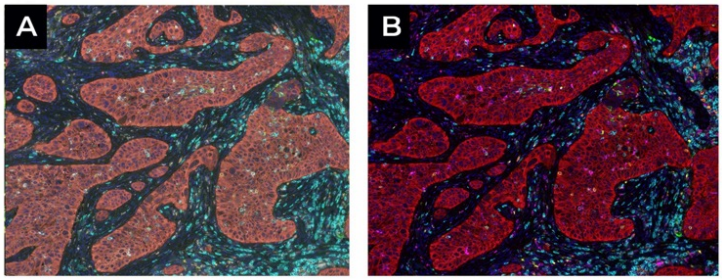


Figure 1.1. Human lung cancer tissue with autofluorescence (A) and the same tissue with reduced autofluorescence (B).

Photobleaching

Dyes can be damaged by intense light, reducing the emission signal through a process called photobleaching. (See Figure 1.2.) Therefore, it is important to limit the time the dyes are exposed to excitation light or to bright light during routine handling.

Usually, blue or UV light is the most damaging. The PhenoImager Fusion uses an electronically gated excitation source synchronized with its camera to limit photobleaching. The sample is only exposed to light while the camera is taking an image. This setup enables repeated measurements with minimal effect on the sample.

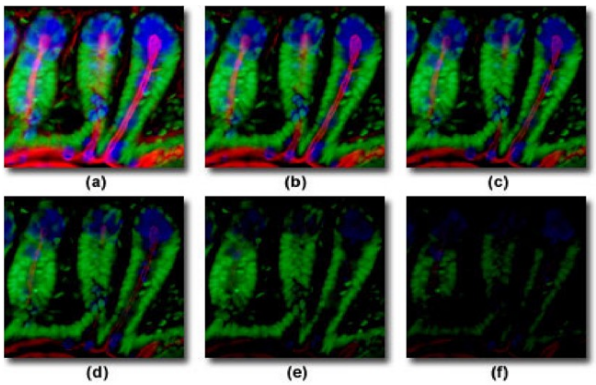


Figure 1.2. Differential photobleaching in multiplex tissue. Emission signals from 3 different dyes in 2-minute time intervals from a-f show the effects of photobleaching. Image from Molecular Expressions™ Optical Microscopy Primer: Fluorescence - Photobleaching - Interactive Tutorial, https://micro.magnet.fsu.edu/primer/ java/fluorescence/photobleaching/

Multispectral Imaging

Filters used for fluorescence imaging are often designed so they only transmit a very narrow range of wavelengths of light where the dye is inherently most responsive. This helps distinguish the desired dye from other dyes or autofluorescence signals in the sample. Using only narrowband filtering, one can typically distinguish up to ~5 dyes.

To distinguish >5 dyes on the same sample, PhenoImager Fusion uses unique multispectral filter cubes and unmixing algorithms. This approach allows separation of up to 7 colors on the same sample (6-plex + DAPI) by identifying which dye/s are present and at what amounts in each pixel. Unmixing also enables the analysis software to identify and remove contributions from autofluorescence. (See Figure 1.3.)

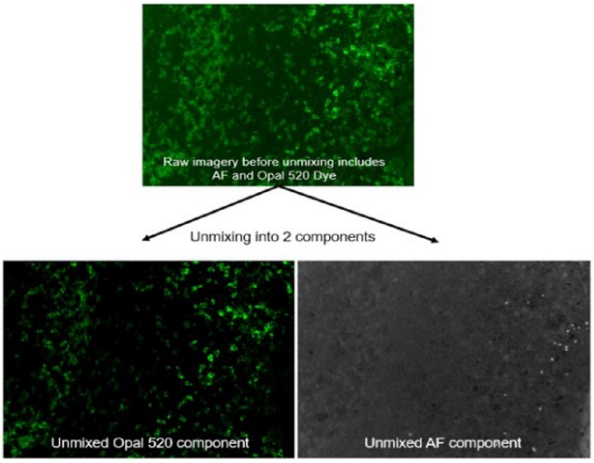


Figure 1.3. Removing autofluorescence with multispectral imaging.

Multispectral Analysis

The PhenoImager Fusion imaging system offers a unique solution to the problem of whole-slide imaging of multiplexed samples.

Multispectral analysis requires all fluorescent materials to produce a unique spectral emission. When stimulating a material and examining the emitted fluorescence over a range of wavelengths, the resulting emission intensities can generate an emission spectrum.

In general, multispectral analysis generates the spectral curves for the various fluorescent dyes or materials in a specimen. It also generates a spectral curve for the autofluorescence that is almost always present to some degree.

Using spectral analysis algorithms, the contribution of the individual fluorescence spectra is separated. The result is a set of images representing each spectrum that contributes to the final image. (See Figure 1.4.)

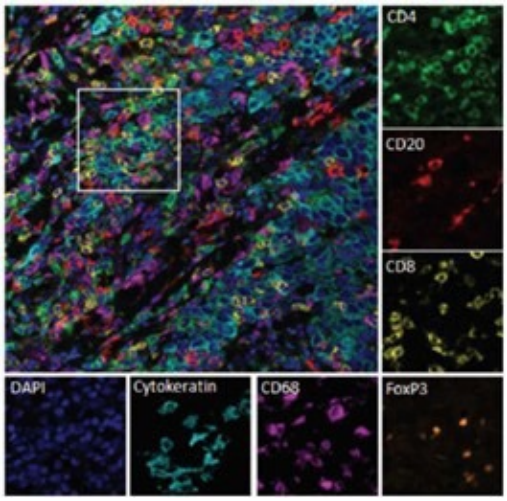


Figure 1.4. Unmixed multispectral image of human breast cancer tissue stained against CD4 (green), CD20 (red), CD8 (yellow), FoxP3 (orange), CD68 (purple), Cytokeratin (cyan), and DAPI (dark blue) using Opal reagents.

Chapter 2: Instrument Overview

SYSTEM OVERVIEW

This chapter describes the PhenoImager Fusion instrument hardware. The PhenoImager Fusion is a standalone automated microscope that allows for PhenoImager scanning of up to 4 slides at a time. When paired with PhenoCycler fluidics instrument, it can be used for PhenoCycler experiments.

This section introduces the hardware components of the PhenoImager Fusion instrument. The main components include:

- Automated XYZ Stage.
- Slide Carriers (PhenoImager Slide Carrier or PhenoCycler Flow Cell Slide Carrier).
- Imaging Module.
- Status LED.
- Filter Turret.
- Objective Turret.
- Power Switch.
- Ventilation Outlets.
- Input/Output Connections.
- Carrying Handle.

LEFT-SIDE VIEW

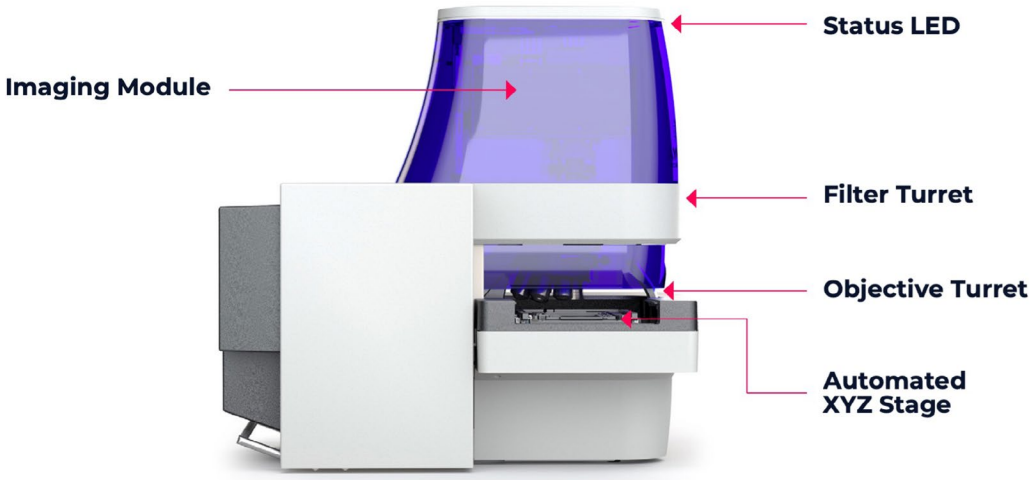


Figure 2.1. The left-side view of the PhenoImager Fusion Instrument. Parts are described in Table 2.1.

Table 2.1. Left-side view of hardware components

Part	Description
Imaging Module	Contains a scientific CMOS camera.
Status LED	LED strip that indicates status and health of the instrument.
Filter Turret	A motorized turret containing epi-fluorescence filters. **IMPORTANT:** Do not move the epi-fluorescence filters from their designated and marked positions unless directed to do so by an Akoya technician.
Objective Turret	A motorized turret containing microscope objectives. **IMPORTANT:** Do not move the objectives from their designated and marked positions unless directed to do so by an Akoya technician.
Automated XYZ Stage	The stage holds the Slide Carrier and moves it in X, Y, and Z directions via software control.

RIGHT-SIDE VIEW

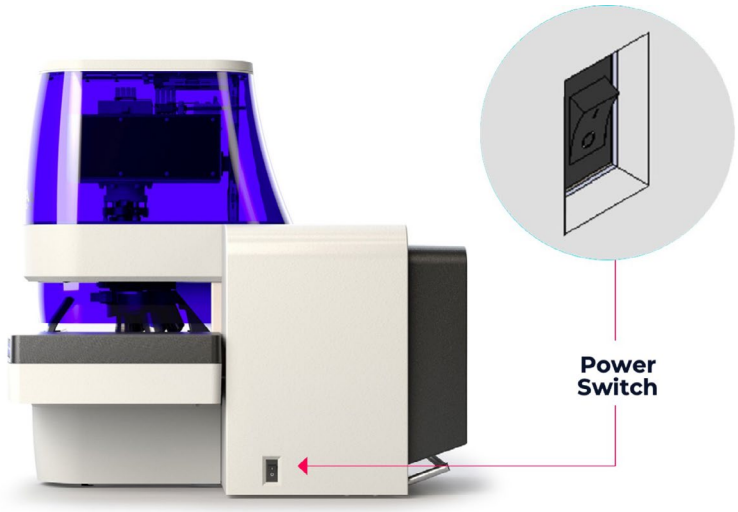


Figure 2.2. The right-side view of the PhenoImager Fusion. Parts are described in Table 2.2.

Table 2.2. Right-side view of hardware components

Part	Description
Power Switch	Turns the PhenoImager Fusion Instrument ON or OFF.

REAR VIEW

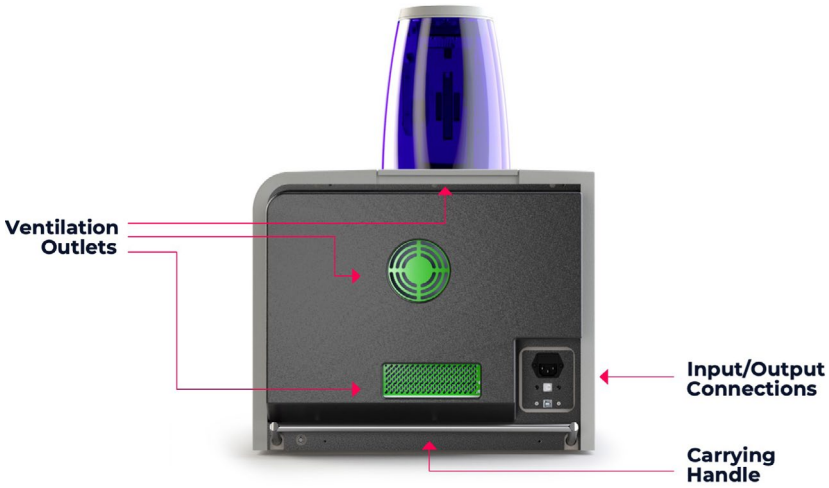


Figure 2.3. PhenoImager Fusion rear view.

Table 2.3. Rear view of hardware components

Part	Description
Ventilation Outlets	See Ventilation Requirements for details on ventilation.
Input/Output Connections	See Input/Output Connections for details on connections.
Carrying Handle	Used by Akoya Biosciences personnel to lift the instrument. **IMPORTANT:** Do not lift or move the PhenoImager Fusion instrument. Installing, servicing, and moving the PhenoImager Fusion instrument should be performed only by qualified Akoya Biosciences service personnel. Contact Akoya Biosciences Technical Support support@akoyabio.com.

VENTILATION REQUIREMENTS

There are 3 ventilation outlets on the enclosures of the PhenoImager Fusion. (See Figure 2.3.)

For proper cooling and ventilation, provide a minimum of 6 inches (15.25 cm) of clearance between the back of the instrument and the wall and above the top of the instrument.

INPUT/OUTPUT CONNECTIONS

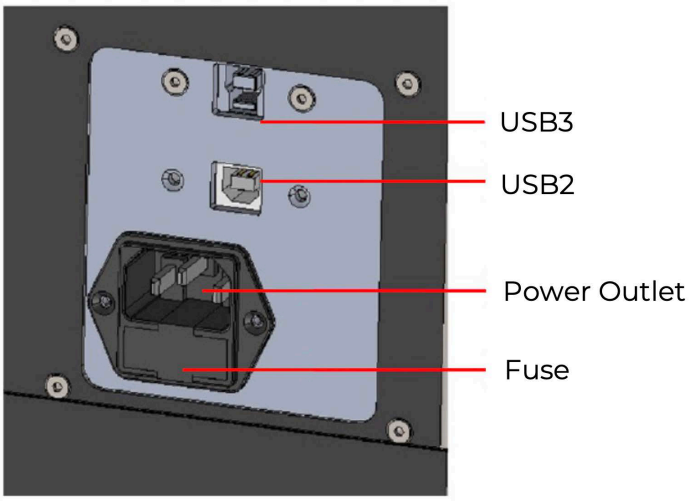


Figure 2.4. Details of input/output connections on the rear panel.

Table 2.4. Input/output connections on rear panel

Input/Output Connections	Description
USB2 Port	Connects instrument to the computer.
USB3 Port	Connects camera to the computer.
Power Outlet	Provides a port for the power cord.
Fuse Box	Contains the fuses for the system.

SYSTEM COMPUTER AND MONITOR

The PhenoImager Fusion system includes a widescreen monitor and a computer pre-installed with Fusion Acquisition Software, Phenochart, and the inForm software. A wireless keyboard and mouse are also included. Computer connections are described in Table 2.5.

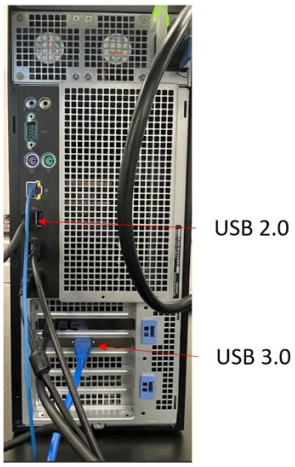


Figure 2.5. PhenoImager Fusion computer connections.

Table 2.5. System computer connections

Input/Output Connection on Rear Panel	Description
USB2 Port	Connects a USB 2.0 cable to the USB 2.0 connector on the rear of the instrument.
USB3 Port	Connects a USB 3.0 HUB to the USB 3.0 HUB connector on the rear of the instrument.

SLIDE CARRIERS

Slide Carriers are integral components of the PhenoImager Fusion.

- The PhenoImager Slide Carrier is used for PhenoImager imaging of stained and coverslipped microscope slides. This carrier is not intended for other types of slides besides stained and coverslipped slides.
- The PhenoImager is compatible with #1 and #1.5 coverslips.
- The PhenoCycler Flow Cell Slide Carrier is used to perform PhenoCycler experiments on slides that are assembled into Flow Cells. This carrier is not meant for other types of slides besides Flow Cells. For details see PhenoCycler Flow Cell Slide Carriers.
- Either of the Slide Carriers can be used in the Automated XYZ Stage.
- The slide dimensions that are compatible with both carriers are listed in Table 2.6.
- The area of the slide that is accessible for scanning is shown for the PhenoImager Slide Carrier in Figure 2.6 (in gray) and for the PhenoCycler Flow Cell Slide Carrier in Figure 2.7 (in gray).

Table 2.6. Slide formats compatible with the PhenoImager Slide Carrier and the PhenoCycler Flow Cell Slide Carrier

Carrier Type	Width	Height	Thickness
PhenoImager Slide Carrier	25.0 ± 1.0 mm	75.0 ± 1.0 mm	1.00 ± 0.1 mm
PhenoCycler Flow Cell Slide Carrier	25.4 mm	76.2 mm	1.00 ± 0.1 mm

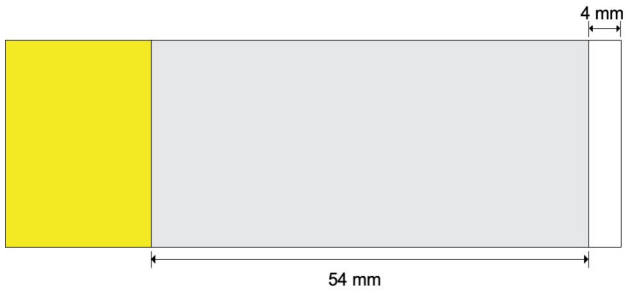


Figure 2.6. Approximate accessible imaging area (in gray) for PhenoImager Slide Carrier. The small square on the left (in yellow) indicates the slide label.

NOTE ***The PhenoImager is compatible with #1 and #1.5 coverslips.***

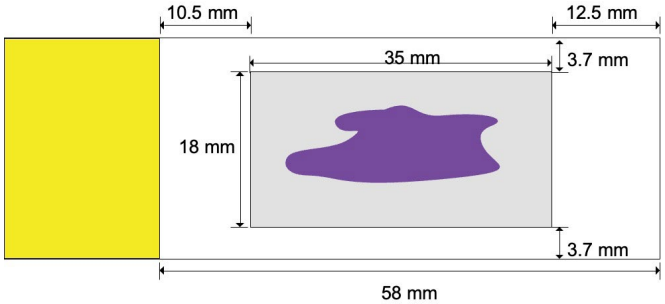


Figure 2.7. Approximate accessible imaging area (in gray) for PhenoCycler Flow Cell Carrier. The sample is illustrated in the center of the imaging area (in purple). The small square on the left (in yellow) indicates the slide label.

PHENOIMAGER SLIDE CARRIER

The PhenoImager Slide Carrier holds up to 4 microscope slides and helps protect slides from damage. (See Figure 2.8.) The slide positions are labeled from 1 to 4 and correspond to slide identification in the PhenoImager Fusion software. Parts are described in Table 2.7.

Figure 2.8. PhenoImager Slide Carrier for the PhenoImager workflow.

Table 2.7. PhenoImager Slide Carrier hardware components

Parts	Description
Slide Position	Each slide position can hold one slide. Slide position 1 is on the far left and slide position 4 is on the far right.
Slide	Each microscope slide is manually loaded into the slide position. Slide formats are listed in Table 2.6.
Insert Indicator	An arrow icon that indicates the side of the Slide Carrier to be inserted into the Automated XYZ Stage.
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 carrier handle is the side of the Slide Carrier to hold when inserting it and removing it from the Automated XYZ Stage. A unique number is printed at the top of the handle to identify the Slide Carrier.

PHENOCYCLER FLOW CELL SLIDE CARRIER

A Flow Cell is installed on the PhenoCycler Flow Cell Slide Carrier and is used for PhenoCycler experiments.

The PhenoCycler Flow Cell Slide Carrier interfaces with the PhenoCycler system via 2 fluidics connectors.

The top view of the PhenoCycler Flow Cell Slide Carrier is shown in Figure 2.9. Components are described in Table 2.8.

TOP VIEW

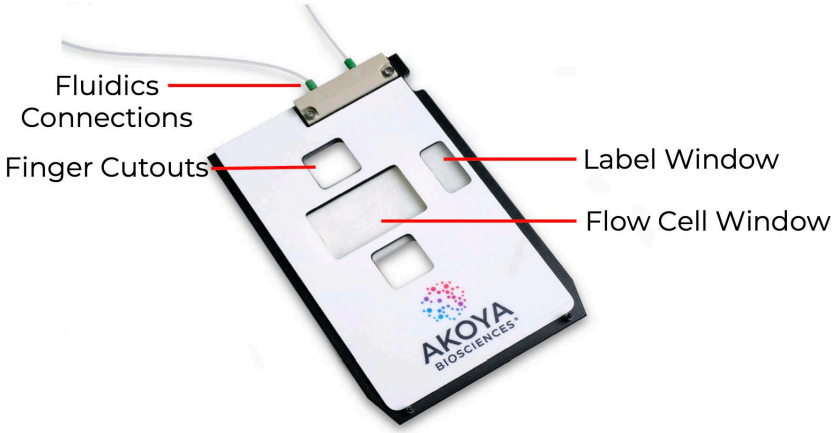


Figure 2.9. Top view of PhenoCycler Flow Cell Slide Carrier.

Table 2.8. PhenoCycler Flow Cell Slide Carrier, top view of hardware components

Carrier Component	Description
Flow Cell Window	Allow imaging of the sample in the Flow Cell through a clear cutout.
Label Window	Allow imaging of the slide label.
Finger Cutouts	Provide access to remove the Magnetic Slide Retainer.
Fluidics Connections	Connect the PhenoCycler Flow Cell Slide Carrier to the PhenoCycler unit by 2 Quick Connect fittings (not shown).

BOTTOM VIEW

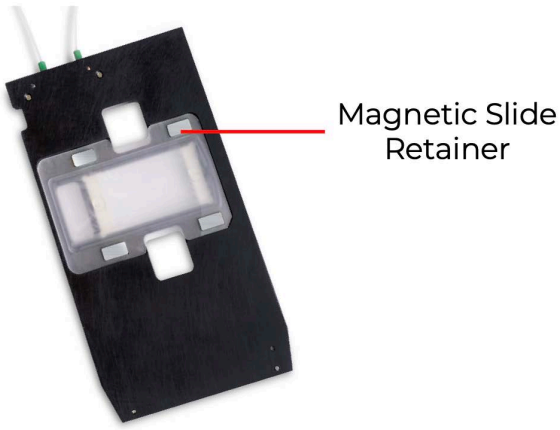


Figure 2.10. Bottom view of PhenoCycler Flow Cell Slide Carrier.

Table 2.9. PhenoCycler Flow Cell Slide Carrier, bottom view of hardware components

Carrier Component	Description
Magnetic Slide Retainer	Securely holds the Flow Cell in the Slide Carrier by way of transparent retaining tray.

Figure 2.11. Assembled Flow Cell. For details on loading the Flow Cell into the Carrier, see Setting Up PhenoCycler Flow Cell Slide Carriers.

Chapter 3: Getting Started

INTRODUCTION

The chapter describes how to operate the PhenoImager Fusion hardware. It is important to read and understand all safety considerations. Before using the system, see Chapter 1: Introducing the PhenoImager Fusion.

If not familiar with the PhenoImager Fusion hardware, review the description of each hardware component in the PhenoImager Fusion system in Chapter 2: Instrument Overview.

SYSTEM STARTUP

Start the PhenoImager Fusion system in 2 steps:

1. Turn on the PhenoImager Fusion Instrument.
2. Launch the PhenoImager Fusion Software.

Turning on the PhenoImager Fusion Instrument

To start the PhenoImager Fusion instrument:

1. If necessary, plug the PhenoImager Fusion power cord into an appropriate power outlet.
2. Turn on the computer and allow Windows 10 to start.
3. Switch the Power Switch to the ON (I) position.

	***Warning: Use only the power supply cord set provided with the PhenoImager Fusion 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.*
	***Warning: Use only a properly grounded power outlet when connecting the system to power.***
	***Warning: Appliance inlet is where the cord is connected and disconnected from the device.*** *Place device or equipment in a manner so the disconnecting device is accessible at all times.*

LAUNCHING THE PHENOIMAGER FUSION SOFTWARE

To launch the PhenoImager Fusion software:

1. Double-click the PhenoImager Fusion icon on the Windows 10 desktop.
2. The PhenoImager Fusion home page opens. See Chaper 4: PhenoImager Fusion Software Overview.

SETTING UP PHENOIMAGER SLIDE CARRIERS

Inspecting Slides and Slide Carriers

Before inserting slides into Slide Carriers, both items should be inspected for potential defects. This section describes how to properly inspect the slides and Slide Carriers.

Inspecting Slides

When inspecting the slides:

- Verify the slides meet the required formats and dimensions. (See Table 2.6)
- Do not use broken or damaged slides.
- Only use slides that are free of debris such as fingerprints, dust, and hair.

Inspecting PhenoImager Slide Carriers

To inspect the PhenoImager Slide Carrier:

- 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 surface, clean the Slide Carrier before use. (See Chapter 8: Cleaning and Maintaining the PhenoImager Fusion.)

Loading Slides into the PhenoImager Slide Carrier

After the slides and Slide Carriers have been successfully inspected, slides can be loaded into the PhenoImager Slide Carriers. (See Figure 3.1.) For information on PhenoImager Slide Carriers, see Chapter 2: Instrument Overview.

To load a slide into a PhenoImager Slide Carrier:

1. Place the Slide Carrier on 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 desired Slide Position until the slide is up against the Spring-Loaded Tab. (See Figure 3.3.)
4. Gently lay the label end of the slide completely into the slide position. The tab should push the slide against the opposite wall of the slide position.
5. Load up to 4 slides into each Slide Carrier. Slides do not need to be contiguous.
6. To load additional slides into another Slide Carrier, repeat steps 2 to 4 above. Slide Carriers may be stacked on top of one another for easy handling and storage. (See Figure 3.2.)

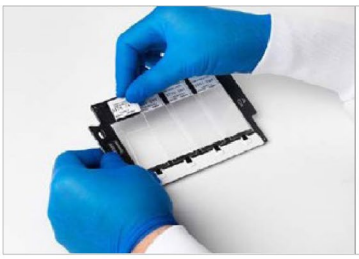


Figure 3.1. Insert slide into Slide Carrier.

Figure 3.2. Slide Carrier Stack.

Figure 3.3. Carrier Retaining Gate.

Loading the PhenoImager Slide Carrier into the Automated XYZ Stage

1. Launch the PhenoImager Fusion software and wait for the system to complete initialization.
2. Slide in the carrier until the whole carrier clears the gate. (See Figure 3.4.)

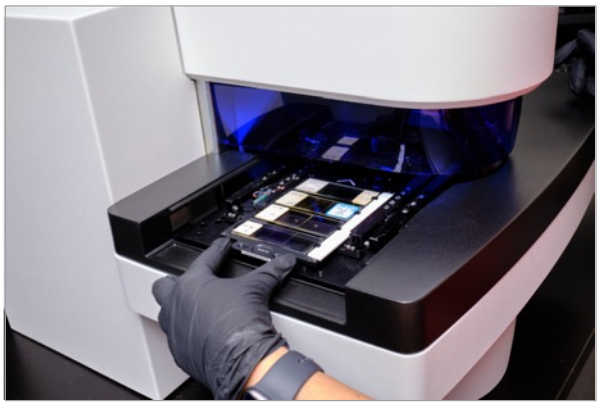


Figure 3.4. Loading the Slide Carrier into the Fusion Automated XYZ Stage.

Removing the PhenoImager Slide Carrier from The Automated XYZ Stage

To remove any Carrier from the Automated XYZ Stage:

1. Ensure that the stage is in the load position with the retaining gate open. If it is not, return to the software home page.
2. Slide the carrier out of the stage until the whole carrier clears the carrier retaining gate.

Removing Slides from the PhenoImager 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 your thumb and index finger.
4. Remove the remaining end of the slide from the Slide Carrier.

SETTING UP PHENOCYCLER FLOW CELL SLIDE CARRIERS

1. Handling the Next Gen Flow Cell Assembly Device

The device can be moved by holding the lower shoulders. (See Figure 3.5.)

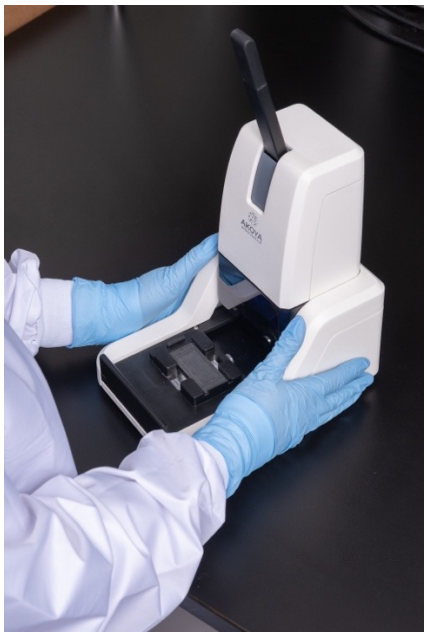


Figure 3.5. Correct handling of the assembly device using the lower shoulders.

CRITICAL ***DO NOT lift the device by the raised central cover. (See Figure 3.6.)***

Figure 3.6. Incorrect handling of the assembly device using the central cover.

2. Assembling the Flow Cell Using the Next-Gen Flow Cell Assembly Device

Table 3.1. List of materials

Item	Vendor	P/N
Stained sample on a charged microscope slide*	Leica Slide White Apex™ Superior Adhesive or Fisherbrand™ Superfront™ Plus	3800080 or 12-550-15
10X Buffer for PhenoCycler Buffer Additive for PhenoCycler-Fusion	Akoya Biosciences	7000019
1X PBS	Thermo Fisher Scientific	14190144
Coplin jar	Customer Choice	MLS**
Kimwipes™ or lint-free cloth	Customer Choice	MLS
Flow Cells	Akoya Biosciences	240205 (10 pk) 240204 (2 pk)
Next-Gen Flow Cell Assembly Device	Akoya Biosciences	SF-000085

**Major Laboratory Supplier (MLS)

CRITICAL ***If the sample slide has a sticker label like the one seen below, remove the label before Flow Cell assembly. Presence of any sticker label can cause the Flow Cell or the slide to break during assembly.***

Remove the sticker label before Flow Cell assembly to prevent:

a. Flow Cell/Sample slide breakage.

b. Compromised seal resulting in a leak.

This procedure uses the Next-Gen Flow Cell Assembly Device to adhere the Flow Cell on top of the antibody-stained sample slide. (Figure 3.7.)

NOTE ***Do not assemble the Flow Cell until ready to run the experiment. Store the antibody-stained slide/s in a Coplin jar with Storage Buffer at 4°C for up to 5 days prior to running the PhenoCycler experiment.***

Prepare 1X Phenocycler-Fusion Buffer

To prepare 1 L of 1X PhenoCycler-Fusion Buffer using components from 10X Buffer kit for PhenoCycler-Fusion (P/N 7000019):

1. In a clean glass beaker (or similar container) add 800 mL of ddH_2O.
2. Pipet 100 mL of 10X Buffer for PhenoCycler.
3. Pipet 100 mL of Buffer Additive for PhenoCycler-Fusion.
4. Use the same pipet for the buffer additive to pipet up and down at least 5X to ensure complete transfer of solution.
5. Mix by pipetting up and down with pipet aid or by using magnetic stir bar.

NOTE ***The 1X Buffer for PhenoCycler-Fusion including additive is referred to as 1X PhenoCycler-Fusion Buffer.***

Scale this recipe to the amount of 1X PhenoCycler-Fusion Buffer needed for the experiment run.

NOTE ***The 1X PhenoCycler-Fusion Buffer should be stored at room temperature (RT) and is stable for 2 weeks.***

Figure 3.7. Next-Gen Flow Cell Assembly Device.

Assemble the Flow Cell

1. Retrieve Coplin jar of Storage Buffer containing the stained sample slides from 4°C storage.
2. Fill a second Coplin jar with 50 mL of 1X PBS and label the jar.
3. Fill a third Coplin jar with 50 mL of 1X PhenoCycler-Fusion Buffer and label the jar.
4. Move stained sample slide from Storage Buffer to the Coplin jar filled with 1X PBS.
5. Incubate for 10 minutes. Once 10 minutes has elapsed with the sample slide in the Coplin jar of 1X PBS, proceed to the next step. If there are air bubbles present refer to Appendix A: Inspecting Air Pockets in the Assembled Flow Cell.

CRITICAL ***A 10-minute incubation is critical to ensure the slide is at RT during Flow Cell assembly. A cold slide will negatively affect the adhesion process.***

6. Open the case containing the Flow Cell. (See Figure 3.8.)

Figure 3.8. Case containing 2 Flow Cells.

NOTE ***Inspect Flow Cells for dust and cracks and ensure they are within the expiration date.***

7. Remove and dispose of the plastic cover slide. (See Figures 3.9 and 3.10.)

Figure 3.9. Flow Cell with plastic cover intact.

Figure 3.10. Remove the plastic cover slide (left) to reveal a Flow Cell without protective plastic cover (right).

8. Remove the peel-off that covers the adhesive on the Flow Cell by pulling on the tab using fingers or forceps. (Figure 3.11.)

NOTE ***The tab can sometimes break off. If this occurs, use forceps to pry away the cover.***

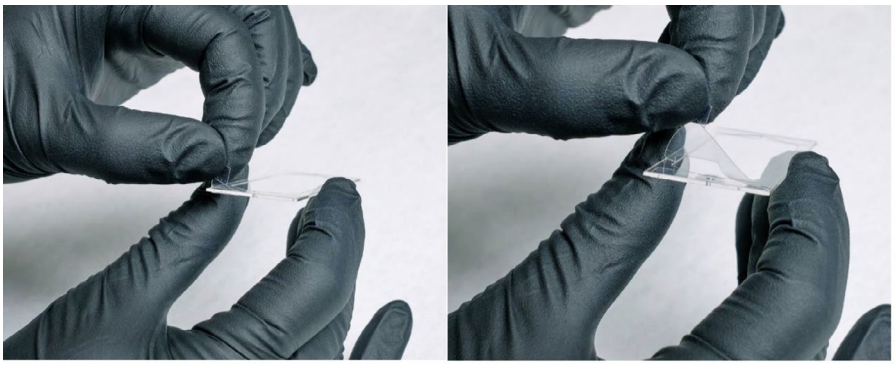


Figure 3.11. Pull the tab portion of the peel-off to reveal the adhesive.

NOTE ***Be sure to note which side of the Flow Cell is adhesive. Once the adhesive protective plastic is peeled off, hold the Flow Cell only by the edges. Avoid touching the sticky adhesive, which could cause poor adhesion to the slide.***

9. Raise the handle of the Flow Cell Assembly Device and then gently pull out the drawer. (See Figure 3.12, left.)
10. Place the Flow Cell in the pocket (Figure 3.12, right) within the drawer of the Flow Cell Assembly Device (See Figure 3.13). Ensure that the **adhesive side is facing up.**

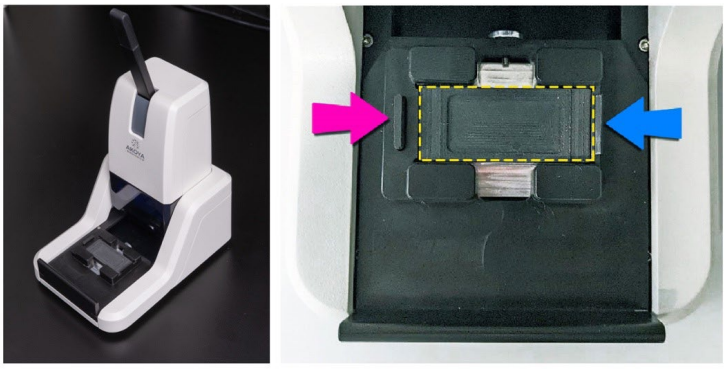


Figure 3.12. Flow Cell in Assembly Device with handle raised and drawer pulled out (left). Close-up of the Device Drawer, top view (right). The pink arrow on the left points to the spacer bar; the blue arrow on the right points to the stop bar. The dashed yellow box outlines the pocket for Flow Cell placement.

Figure 3.13. Flow Cell placed in the pocket of the Assembly Device with adhesive side facing up. The pink arrow on the left points to the spacer bar; the blue arrow on the right points to the stop bar.

11. Remove the sample slide from the Coplin jar containing 1X PBS.
12. Dry the back of the slide by wiping with a Kimwipe (or equivalent). (See Figure 3.14, left.)
13. Lift the label side of the slide to allow excess PBS to run off the opposite edge and onto a Kimwipe.
14. Carefully use a Kimwipe to dry excess PBS around the tissue. Do not leave the tissue exposed to air for extended periods. (See Figure 3.14, right.)

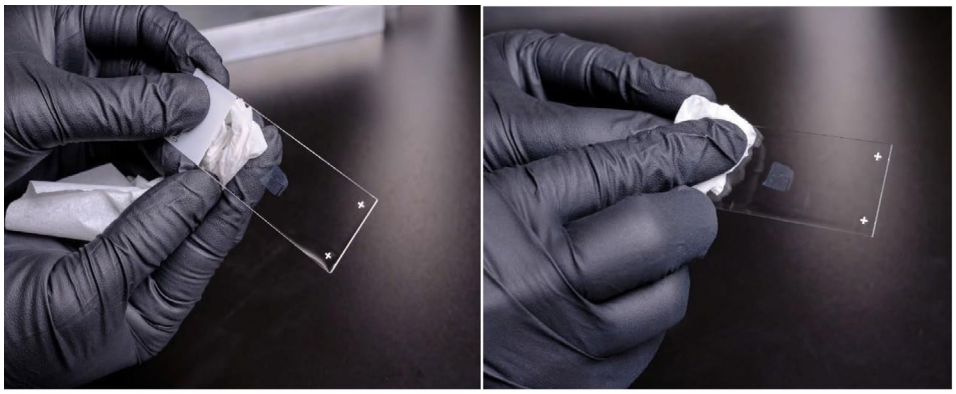


Figure 3.14. Sample slide dried with Kimwipe (left). Drying the sample slide while avoiding the sample (right).

15. Place the sample slide with the **sample facing down** on top of the Flow Cell.

NOTE ***The label side will be held up by the spacer bar. Ensure the non-label side is against the stop bar indicated by the blue arrow. (See Figure 3.15, left.)***

CRITICAL ***It is critical to align the slide and the Flow Cell as illustrated. (See Figure 3.15, right.)***

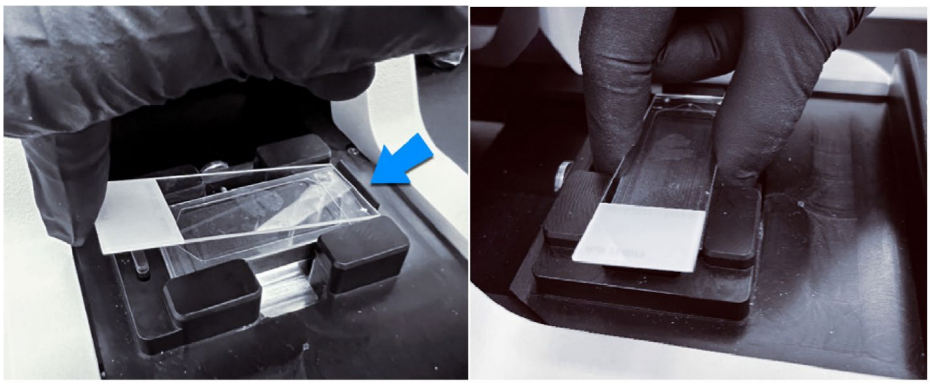


Figure 3.15. Placing the slide tissue-side down on top of Flow Cell with the non-label slide against the stop bar, as indicated by the blue arrow (left). Aligning the slide and the Flow Cell (right).

16. Gently slide the drawer all the way into the close/assembly position. (See Figure 3.16, right.)

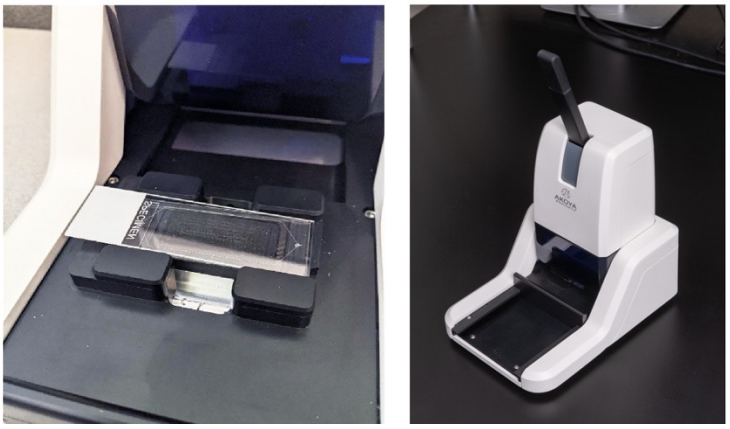


Figure 3.16. Flow Cell and Slide ready for assembly (left). The Flow Cell in the Assembly Device with the drawer in the close/assembly position (right).

17. Lower the handle all the way down to assemble the Flow Cell. The handle should seat
18. easily and stay down on its own. (See Figure 3.17.)

Figure 3.17. Assembly Device when the handle is completely pressed down.

CRITICAL ***The handle must be down for at least 30 seconds to ensure a proper seal and prevent leaks.***

18. Raise the handle and then pull out the drawer to remove the assembled Flow Cell with the slide adhered to it.
19. Ensure there is no crack on the Flow Cell or the sample slide.
20. Submerge the slide vertically in the Coplin jar with 50 ml of 1X PhenoCycler-Fusion Buffer from Step 3. Ensure the label is visible at the top and the top port is NOT submerged in buffer. (See Figure 3.18.) The bottom port of the Flow Cell (away from the label) must be fully submerged to allow the Buffer to flow inside.

NOTE ***This step allows buffer to rehydrate the tissue while the adhesive cures to the microscope slide.***

Figure 3.18. Adhered Flow Cell placed vertically in Coplin jar with 50 mL of 1X PhenoCycler-Fusion Buffer.

22. Leave the assembled Flow Cell in 1X PhenoCycler-Fusion Buffer for **at least 10 minutes** to allow the Flow Cell to completely adhere to the slide.
23. When ready to assemble the Slide Carrier, carry the assembled Flow Cell to the PhenoCycler-Fusion system in the Coplin jar containing 1X PhenoCycler-Fusion Buffer.

NOTE ***It is essential to wipe the Flow Cell (staying away from the ports), with ddH2O and 70% ethanol prior to loading it into the Flow Cell Slide Carrier.***

For instructions on how to set up the PhenoCycler-Fusion system for the run, see Chapter 6: PhenoCycler-Fusion Workflow.

Inspecting Assembled Flow Cell Slides

When inspecting slides:

- Verify the slides meet the required formats and dimensions.
- Do not use broken or damaged slides and/or Flow Cells.
- Use only slides that are free of debris, fingerprints, and dust.
- Ensure assembled Flow Cell is lined up with the slide.

Inspecting PhenoCycler Flow Cell Slide Carriers

- Do not use Slide Carriers that are warped, bent, or cracked.
- If any liquid or sticky residue is on the carrier, clean the Slide Carrier before use. (See Cleaning the Slide Carrier).

CRITICAL ***The Flow Cell Slide Carrier is fragile. Please handle with care.***

Loading Assembled Flow Cell Slides into the PhenoCycler Flow Cell Carrier

NOTE ***Perform when prompted by the software, after the PhenoCycler is primed and PhenoCycler-Fusion is ready for the sample slides.***

1. Turn the carrier over so the Magnetic Slide Retainer is facing up.
2. Remove the Magnetic Slide Retainer using the finger cutouts.
3. Remove the blank Flow Cell and set aside.
4. Remove sample slide Flow Cell from Coplin jar with 1X Buffer for PhenoCycler.
5. Wipe a Kimwipe slowly across the surface in one direction to clean the excess buffer on the outside of the sample Flow Cell.

NOTE ***Use ddH2O and 70% ethanol to clean.***

6. Place the Flow Cell slide into the carrier so the slide label is visible through the window. (See Figure 3.19.)

NOTE ***Ensure the sample Flow Cell is in the correct slot based on where the experiment template was loaded.***

7. Replace the Magnetic Slide Retainer and ensure that its surface is flush with the bottom of the carrier.

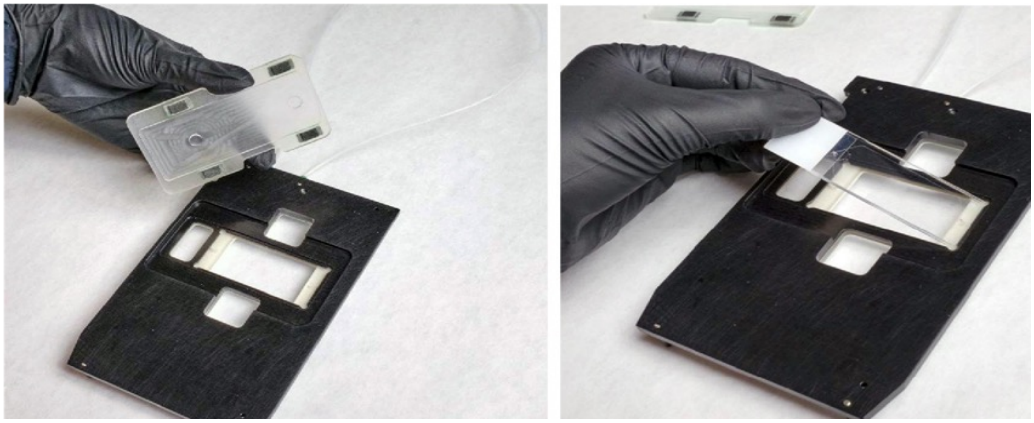


Figure 3.19. Carrier as the Magnetic Slide Retainer is being removed (left). Carrier being loaded with a Flow Cell Slide (right).

LOADING THE SLIDE CARRIER INTO THE AUTOMATED XYZ STAGE

If the Flow Cell Slide Carrier is not already connected to the PhenoCycler, attach the 2 connectors to the PhenoCycler fluidics before loading the carrier onto the automated stage. (See Figure 3.20.)

CRITICAL ***Before placing the Slide Carrier into the Fusion system, the software will prompt a check for leaks. Inspect the top of the Slide Carrier for buffer accumulating on top of the Flow Cell. Then, turn the Slide Carrier over to ensure buffer has not accumulated inside the Magnetic Slide Retainer.***

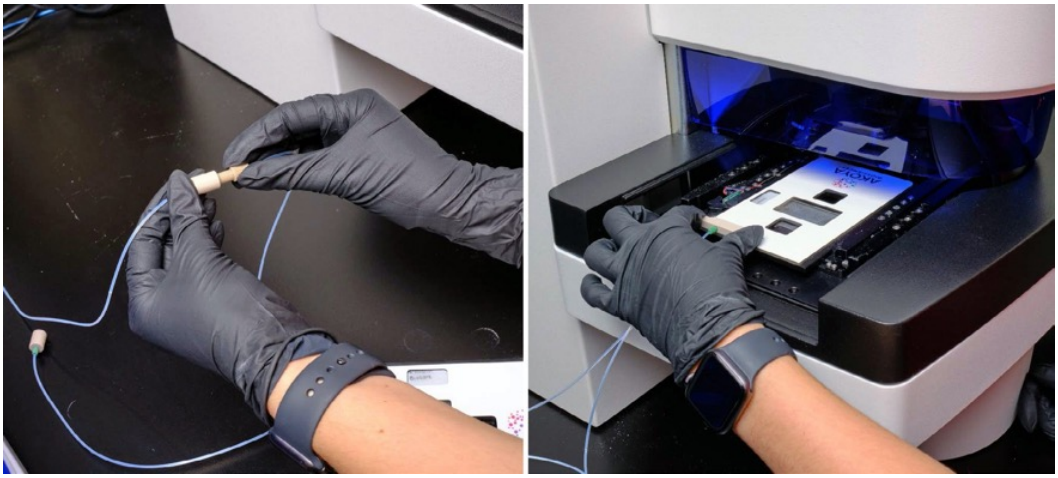


Figure 3.20. Fluidics connectors from the Flow Cell Slide Carrier (left) and the corresponding connectors on the PhenoCycler instrument (right).

NOTE ***To ensure a proper seal, turn the fittings until they click.***

Once prompted by the software, slide the carrier in until the whole carrier clears the gate. If the Flow Cell Slide Carrier does not slide in easily, it is not assembled correctly.

CRITICAL ***Do not move the XYZ stage while inserting the carrier.***

Removing the Slide Carrier from the Automated XYZ Stage

1. Using the tan manifold, pull the Slide Carrier out of the stage until the whole carrier clears the carrier retaining gate.

CRITICAL ***Do not pull the Slide Carrier by the tubing.***

Removing a Flow Cell from a Flow Cell Slide Carrier

1. Turn the carrier over so the Magnetic Slide Retainers are facing up.
2. Remove the Magnetic Slide Retainer using the finger cutouts. (See Figure 3.19.)

Cup your hand over the entire slide and gently turn the carrier over to allow the Flow Cell slide to drop into your hand.

CRITICAL ***The Flow Cell Slide Carrier is fragile. Please handle with care.***

Chapter 4: PhenoImager Fusion Software Overview

PHENOIMAGER FUSION SOFTWARE HOME PAGE

The Home Page links to the pages needed to maintain and run the PhenoImager Fusion. (See Figure 4.1.)

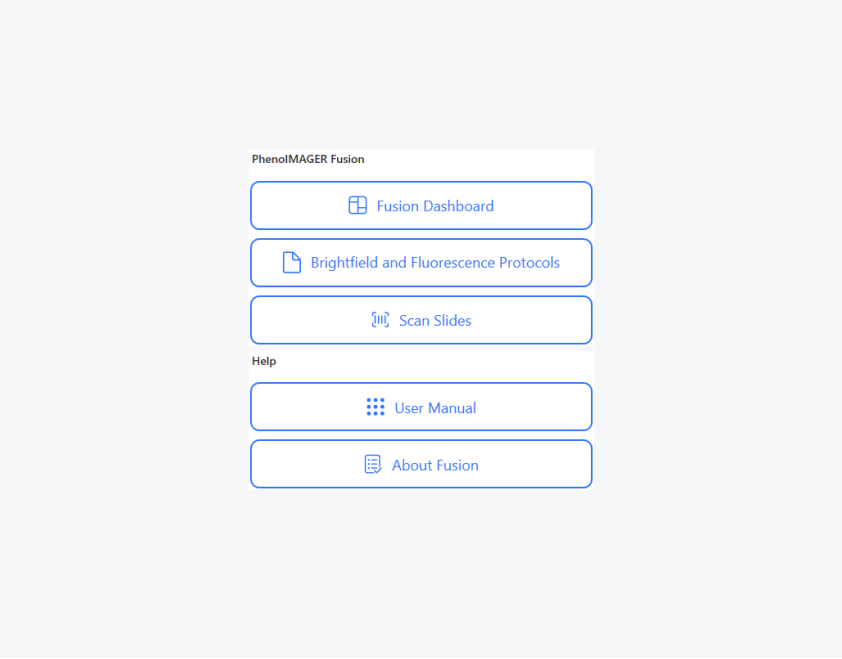


Figure 4.1. PhenoImager Fusion Home Page view when configured **without** the PhenoCycler instrument.

The Home Page will display additional buttons if the system includes a PhenoCycler instrument. (See Figure 4.2.)

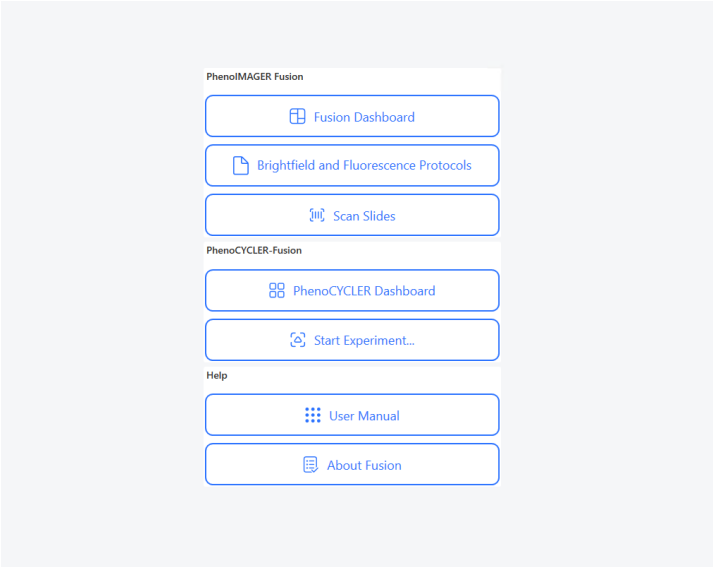


Figure 4.2. PhenoImager Fusion Home Page view when configured **with** the PhenoCycler Instrument.

The Home Page buttons link to the following pages:

- **Fusion Dashboard** – Maintenance tasks related to the PhenoImager Fusion microscope.
- **Brightfield and Fluorescence Protocols** – Create or edit scanning protocols for conventionally coverslipped brightfield (BF) and fluorescent (FL) slides. (See Chapter 5: PhenoImager Workflow with Brightfield and Fluorescence.)
- **Scan Slides** – Select the scanning rules and scan the slides. (See Chapter 5: PhenoImager Workflow with Brightfield and Fluorescence.)
- **PhenoCycler Dashboard** – Maintenance tasks related to PhenoCycler fluidics. (See Chapter 7: Cleaning and Maintaining PhenoCycler-Fusion.)
- **Start Experiment** – Run the PhenoCycler experiment, including selecting the rules. (See Chapter 6: PhenoCycler-Fusion Workflow.)
- **User Manual** – Displays installed user guide.
- **About Fusion** – Fusion software plus patent information.

FUSION DASHBOARD

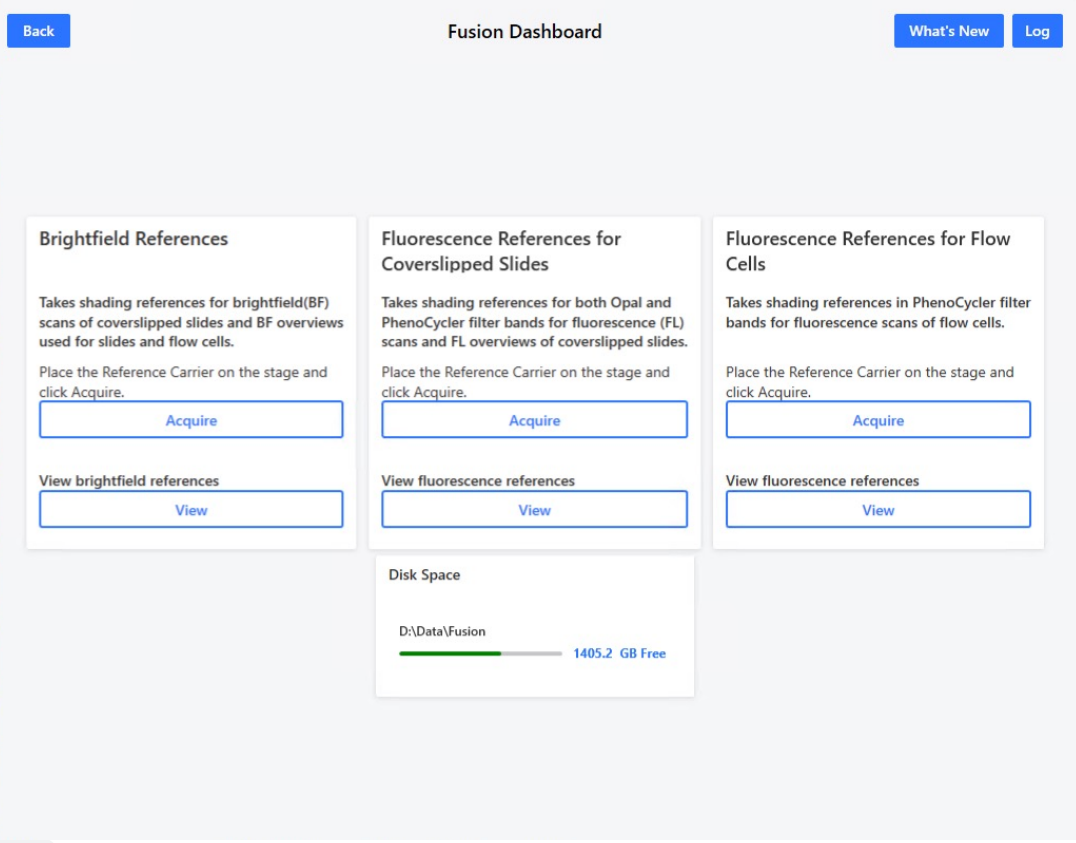


Figure 4.3. PhenoImager Fusion Dashboard page.

Brightfield References

Acquiring Brightfield References

Click Acquire to take new brightfield references using the Reference Carrier. The system will automatically move to the appropriate slide locations to acquire reference imagery for each objective. This process will take approximately 5 minutes, during which time the system can be left unattended. The software will display an estimated completion time..

Viewing Brightfield References

Click View to see brightfield reference imagery and information for the 4x overview, 20x and 40x resolution references. Export For Diagnostics will save the set of reference images to aid in technical support.

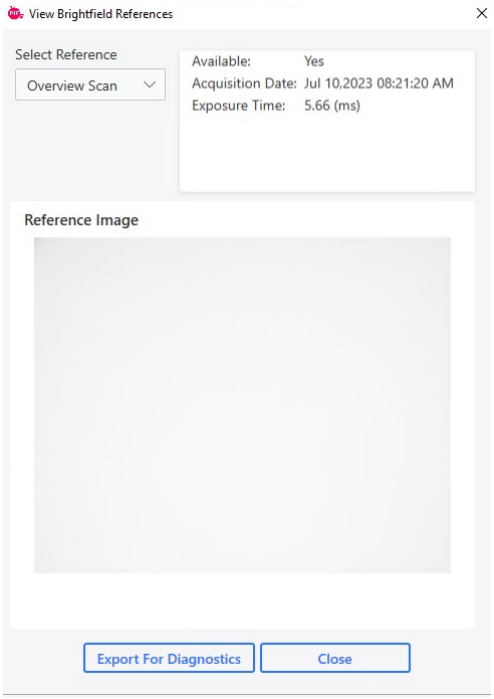


Figure 4.4. View Brightfield References window.

Fluorescence References for Coverslipped Slides

Acquiring Fluorescence Referencesfor Coverslipped Slides

Click Acquire to take new fluorescent references using the Reference Carrier. The system will automatically move to the appropriate slide locations to acquire reference imagery for each combination of objective and filter. This process will take approximately 20 minutes, during which time the system can be left unattended. The software will display an estimated completion time.

Viewing Fluorescence References for Coverslipped Slides

Click View to see reference imagery and information for the 4x overview, 20x and 40x resolution references for each filter. Export For Diagnostics will save the set of reference images to aid in technical support.

Click Export For Diagnostics to save the set of reference images to aid in technical support.

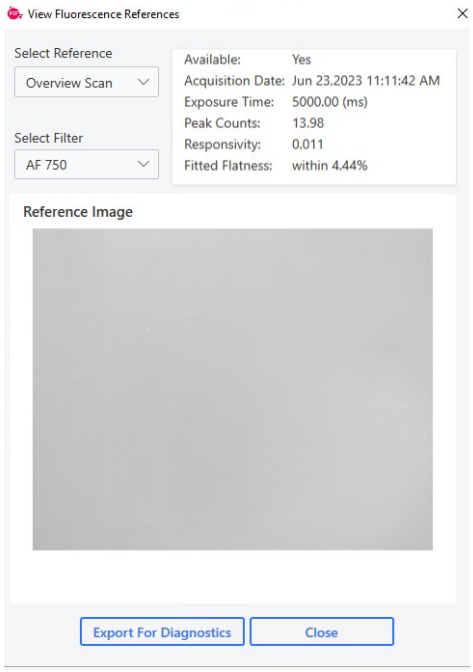


Figure 4.5. View Fluorescence References window.

Fluorescence References for Flow Cells

Acquiring Fluorescence References for Flow Cells

Click Acquire to take new Fluorescence References for Flow Cells using the Reference Carrier. The system will automatically move to the appropriate slide locations to acquire reference imagery using each PhenoCycler workflow-specific filter. This process will take approximately 10 minutes, during which time the system can be left unattended. The software will display an estimated completion time.

Viewing Fluorescence References for Flow Cells

Click View to see reference imagery and information for the 20XLWD objective references for each PhenoCycler workflow-specific filter. Export For Diagnostics will save the set of reference images to aid in technical support.

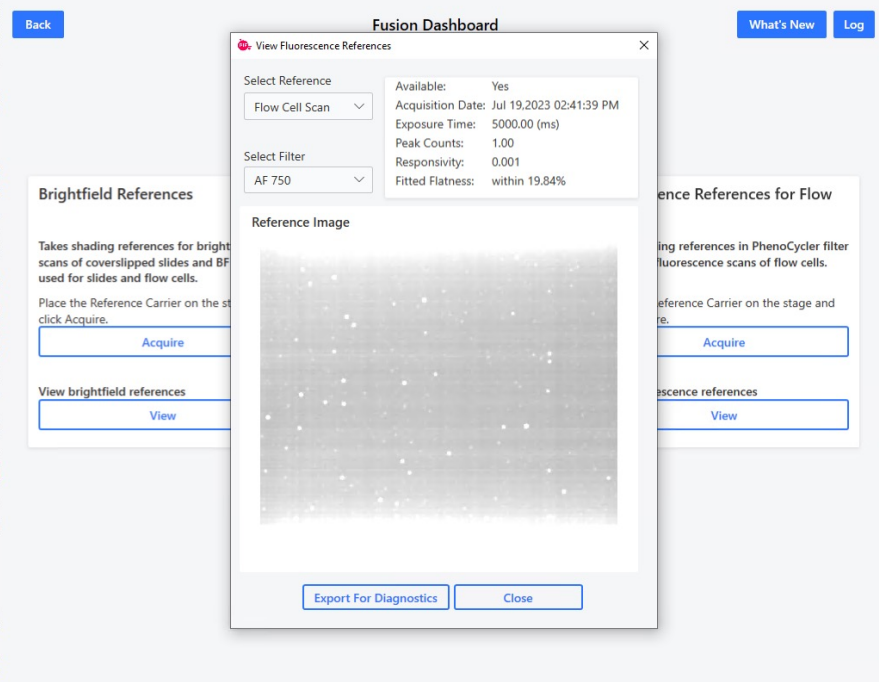


Figure 4.6. View Fluorescence References for Flow Cells window.

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 simple color images (e.g., a whole slide scan), the transmission is mapped from 0-255.

Fluorescence

Acquired images are normalized by a shading pattern derived from the reference images. The shading pattern, which is the reference image divided by the mean intensity in the center, is applied on a pixel-by-pixel basis, with one pattern per epi-filter. Here, center means the central 1/16th of the image area. While the exact shape of the shading pattern varies per instrument, the overall effect is to increase the signal near the image edges with minimal effect to the signal from the center of the image.

Disk Space

The disk space widget shows the used space on the Fusion Acquisition PC storage. The colored bars—green, yellow, or red—indicate the status in relation to the average required storage to successfully complete the runs or scans.

We recommend that the disk space is cleared up and maintained at green.

A red status bar indicates the user cannot complete a full run or scan.

Chapter 5: PhenoImager Workflow with Brightfield and Fluorescence

PhenoImager Fusion allows scanning of slides in Brightfield and Fluorescence. This section describes the steps involved in the PhenoImager workflow, including:

- Creating and Editing Protocols
- Scanning Slides

CREATING PROTOCOLS

Studies

- A study is a group of ≥2 slides that belong together. PhenoImager Fusion stores scan data by the study. This could be an experimental study (e.g., Ki67 markers in breast cancer tissue) with all slides from one source, or together with other groupings. Each slide may be scanned more than once, if needed.
- The default location for a study is D:\Data\Fusion\ [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 Scanning Slides section for more details about imagery.
- Protocol defines the set of rules to be used during the PhenoImager workflow, including imaging mode, pixel resolution, filter cubes, exposure times, and other parameters. Protocols have the file extension “.fpr” and are saved in D:\Data\Fusion\ [Study].

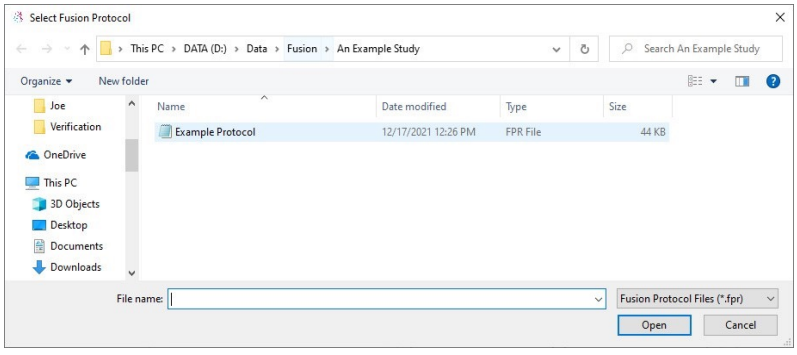


Figure 5.1. Opening a protocol.

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 Opals and other fluorescent dyes.

Create New Protocol

1. Select Brightfield and Fluorescence Protocols from the PhenoImager Fusion Home Page.
2. Click the New button. The Create Protocol window opens. (See Figure 5.2.)
3. Enter a Protocol name.
4. Select Brightfield or Fluorescence under the imaging mode.
5. If you selected Fluorescence, three Filter Set options will be shown. Select the appropriate option.
6. Select a previously created Study or create a new Study.
7. To select a previously created study, click on the study in the Available Studies list to highlight it.
8. To create a new study, enter the Study Name in the text box and click the + button. The new study will be added to the Available Studies list where it can then be selected.
9. Click the Create Protocol button to create the protocol in the selected study. To load an existing protocol, click Load and select the protocol from the study folder.

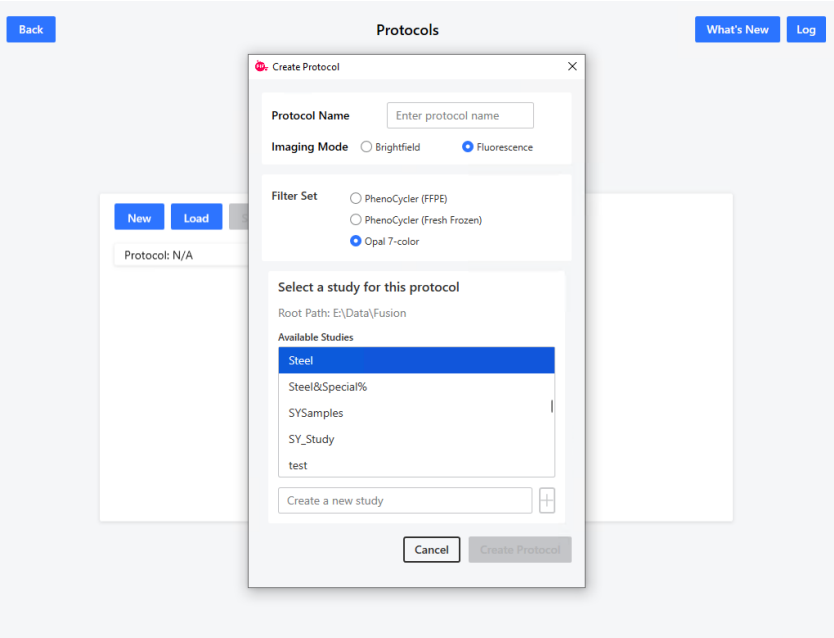


Figure 5.2. Create a protocol.

EDITING PROTOCOLS

After creating a BF or FL protocol and assigning it to a study, use the Edit Protocol screen to add specific details to the protocol. The next sections are organized by these 2 types of protocols:

- Brightfield Protocols
- Fluorescence Protocols

Brightfield Protocols

After creating a BF protocol and assigning it to a study, the Edit Protocol window for brightfield protocols opens. (See Figure 5.3.)

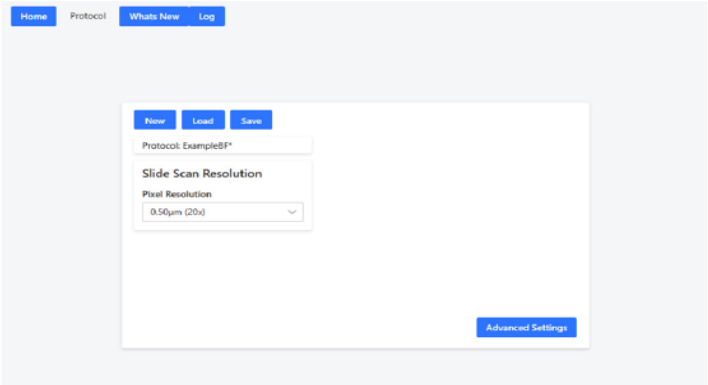


Figure 5.3. Edit protocol window.

Under Slide Scan Resolution, choose the Pixel Resolution while imaging. The options include:

- 0.50 µm per pixel (20x)
- 0.25 µm per pixel (40x)

Advanced Settings

Click the Advanced Settings button to access additional options. (Figure 5.4.)

Sample Finding

PhenoImager Fusion has a specialized algorithm for finding TMA samples. The algorithm expects TMA cores that are 0.6 - 2.5 mm in diameter, arranged in grids of at least 2x2. It will search for multiple grids on the same slide. If samples fit this description, select Sample is a TMA.

Compression

By default, BF whole slide scans 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 in the drop-down box.

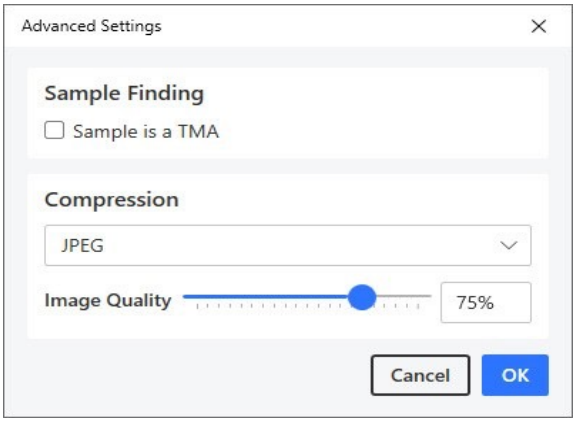


Figure 5.4. Advanced settings for Brightfield protocol.

Fluorescence Protocols

After creating the fluorescence protocol and assigned it to a study, the Edit Protocol window for fluorescence protocols opens. (Figure 5.5.)

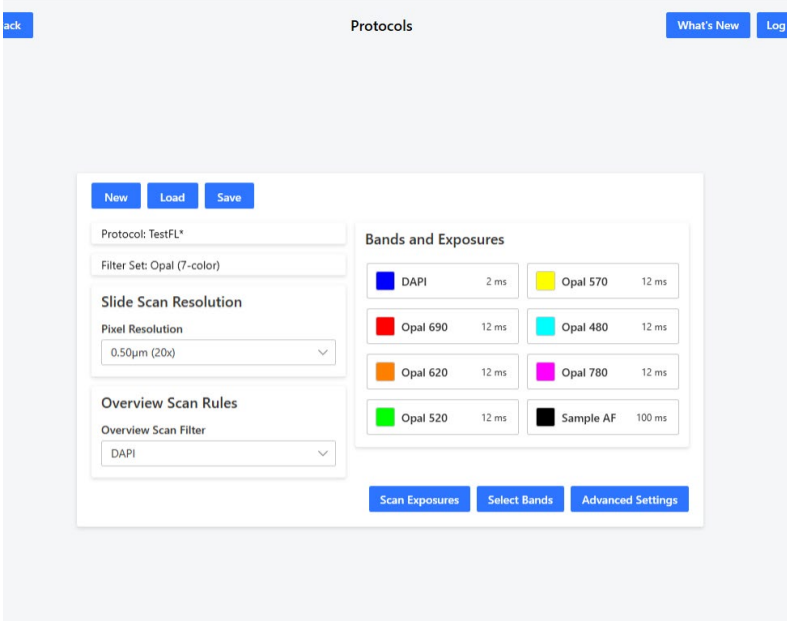


Figure 5.5. Edit Protocol window.

Slide Scan Resolution

Under Slide Scan Resolution, select the Pixel Resolution to be used while imaging the slide:

- 0.50 µm per pixel (20x)
- 0.50 µm per pixel (20x) [binned from 40x]
- 0.25 µm per pixel (40x)

Overview Scan Rules

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 no counterstain, then choose a filter that aligns with the expression of the most common stain or autofluorescence.

Filter Bands

The Bands and Exposure section shows the current color and exposure time selected for each filter. Edit the filters by selecting the Select Bands button.

- After clicking the Select Bands button, the select filters window opens, which allows one to choose the filters used to take the slide scan. (See Figure 5.6.) Imagery for each band will be taken in the order it is shown. To remove bands, uncheck the band. The colors associated with each band can also be changed; these are the colors that Phenochart will use to display the scan.

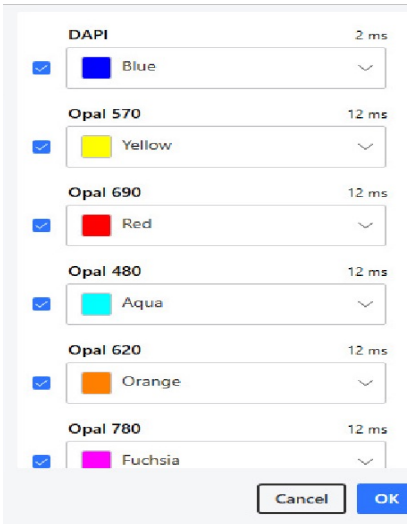


Figure 5.6. Select Filters window.

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

Scan Exposures

Setting Exposures

Edit exposure times by clicking the Scan Exposures button to open the Scan Exposures window (See Figure 5.7). This allows editing of the exposure times in milliseconds for each filter by manually or by clicking the Autoexpose button.

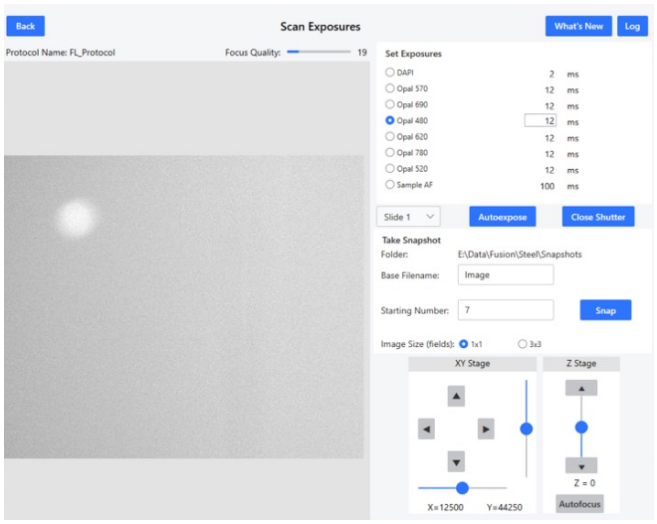


Figure 5.7. Scan Exposures window.

To use the autoexpose function, first navigate to a representative slide using the Slide drop-down. 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 Set Exposures panel identifies which filter band is currently shown.

Refine the sample position by using the XY Stage arrow buttons (for small movements) or sliders (for larger movements). Click Autofocus or use the Z Stage slider to bring the live view into focus.

Click Autoexpose to have the system find the best exposure for that filter band.

- After autoexposing, auto-focus and auto-expose again to refine the focus and exposure estimates.
- Override the autoexposed value by typing a value in the highlighted text box. Values must be between 0.1 and 2000 ms.

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

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

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.

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. Pick a Base Filename, select an Image Size, then click the Snap button. Navigate to new areas and take as many snapshots as desired. They will be numbered incrementally.

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

Advanced Settings

Click the Advanced Settings button to access additional options. (See Figure 5.8.)

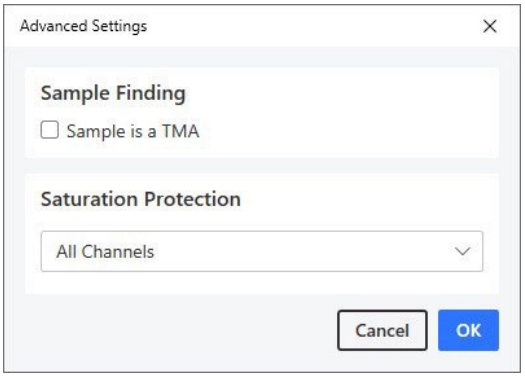


Figure 5.8. Advanced Settings window.

Sample Finding

PhenoImager Fusion has a specialized algorithm for finding TMA samples. The algorithm expects TMA cores that are 0.6-2.5 mm in diameter, arranged in grids of at least 2x2. It will search for multiple grids on the same slide. If samples fit this description, select Sample is a TMA.

Saturation Protection

Saturation Protection helps prevent overexposure when acquiring fluorescence imagery. If this option is selected and saturated pixels are detected in the imagery, PhenoImager Fusion will lower the exposure time for the entire scan to reduce the number of saturated pixels.

Saturation Protection can reduce exposure times by up to 4-fold when scanning using the 20x or 40x resolution options and up to 16-fold when using the 20x (binned from 40x) resolution options. Saturation protection may be applied to all channels or to only the DAPI channel.

The recommended option for PhenoImager experiments is the All Channels setting.

NOTE ***Using Saturation Protection does not increase scan times noticeably because Fusion can record each image using 2 camera settings within 1 readout.***

CRITICAL ***Critical If the sample has bright debris that will saturate at much lower exposure times than the true sample, disable Saturation Protection. Note that this could cause exposure times to be reduced too low for proper visualization of the sample.***

SCANNING SLIDES

The section describes the process of scanning slides.

The PhenoImager Slide Carrier

Insert slides into the 4-Slide Carrier as illustrated in Figure 3.1 and load the carrier into the XYZ Stage.

Editing Slides Within the Carrier

Click Scan BF and Opal slides from the main menu. A window will appear. (See Figure 5.9.)

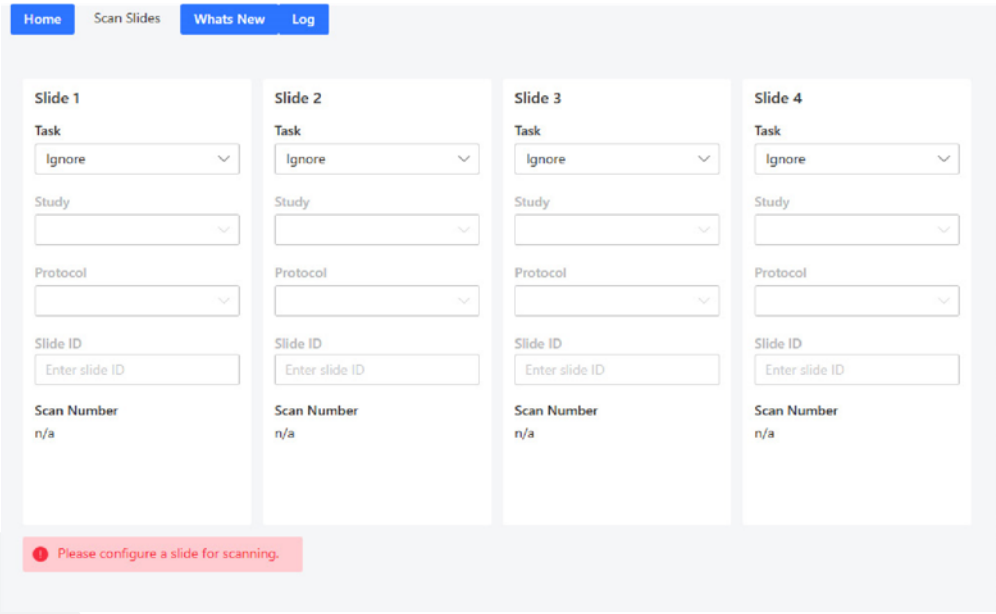


Figure 5.9. Edit Slides window.

For each slide, the following must be entered:

- **Task** - Choose Scan Slide or Ignore. Select Ignore if there is no slide in the slot.
- **Study** - Select a study.
- **Protocol** - Select the appropriate protocol saved within the study.
- **Slide ID** - Add slide ID.

Scanning

The Scan Slides button is enabled when at least one slide is ready to be scanned. Click Scan Slides to start scanning. The PhenoImager Fusion will report the scanning progress for each slide on the screen during the scan.

When PhenoImager Fusion has completed scanning, a pop-up window summarizes the result of each slide. (See Figure 5.10.)

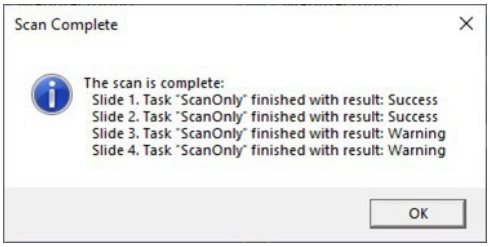


Figure 5.10. Results of Scan Complete pop-up window.

Possible results include:

- **Success** – The slide was scanned successfully.
- **Warning** – The slide was scanned, but with a warning.
- **Failed** – The slide did not finish scanning due to an error.

Chapter 6: PhenoCycler-Fusion Workflow

OVERVIEW

In a PhenoCycler-Fusion experiment, mixtures of Reporters are sequentially dispensed onto a tissue section stained with PhenoCycler antibodies. After each incubation of PhenoCycler Reporters, the Fusion microscope scans in 4 fluorescence channels followed by a gentle wash to dehybridize and remove the Reporters.

The tissue staining protocols and the 96-well Reporter plate preparation are outlined in Chapter 3 and Chapter 4 of the PhenoCycler-Fusion User Guide. Assembling the sample slide Flow Cells is described in Chapter 3.

The following formula estimates the run time of PhenoCycler-Fusion experiments:

Run Time = (fluidics time [45 minutes] + scanning time) x (number of cycles)

The total fluidics time is approximately 45 minutes per cycle. The scanning time depends on the size of the tissue being imaged and is approximately 25 minutes for a 1.5 cm x 1.5 cm scan area.

CRITICAL ***It is recommended to perform the Post Experiment Clean after every run.***

PRE-EXPERIMENT PREPARATION

Material Provided

Table 6.1. Material available for purchase from Akoya

Item	Storage	P/N
Flow Cell (10 pk)	Room Temperature	240205
10X Buffer Kit for PhenoCycler-Fusion		7000019
PhenoCycler Flow Cell Slide Carrier		Included with PhenoCycler instrument.

Materials Not Provided

Table 6.2. Material not available for purchase from Akoya

Type	Item	Vendor	P/N
Solvents	DMSO - ACS reagent, ≥99.9%	Sigma-Aldrich	472301-4L
	ddH_2O or Milli-Q® H_2O	Customer Choice	MLS
Plastic Consumables/ Tools	Squeeze bottle for water	Customer Choice	MLS
	Compressed air duster	Customer Choice	MLS
	Kimwipes	Customer Choice	MLS
	Additional buffer reservoirs	Beckman Coulter	BK372795
	Sterile disposable filter unit	Nalgene™ Rapid-Flow™ (Recommended)	156-4020

Set up the PhenoCycler Instrument for a PhenoCycler-Fusion Run
The Fusion software includes a Preflight Routine to guide the user through setting up buffers, priming the instrument and checking for leaks. The software also performs fluidics and microscope setting pre-checks. Once the pre-checks are passed, the user can start the run.

EQUILIBRATE REAGENTS TO RT

- Retrieve the sealed, pre-loaded 96-well Reporter plate containing the Reporter Master Mix solutions from 4°C. For Reporter plate preparation, see PhenoCycler-Fusion User Guide, Chapter 5.
- **Allow equilibration at RT for 15 minutes** before setting up the instrument. Failure to do so may impact data quality.
- Ensure reservoirs are clean and dry if used from a previous run. To minimize debris, the reservoirs should be air dried as opposed to wiped dry.

NOTE ***Prepare the appropriate 96-well Reporter plate prior to setting up the PhenoCycler-Fusion system for a run.***

PREPARE 1X PHENOCYCLER-FUSION BUFFER

To prepare 1 L of 1X PhenoCycler-Fusion Buffer using components from 10X Buffer kit for PhenoCycler-Fusion (P/N 7000019):

1. In a clean glass beaker (or similar container) add 800 mL of ddH_2O.
2. Pipet 100 mL of 10X Buffer for PhenoCycler.
3. Pipet 100 mL of Buffer Additive for PhenoCycler-Fusion.
4. Use the same pipet for the buffer additive to pipet up and down at least 5X to ensure complete transfer of solution.
5. Mix by pipetting up and down with pipet aid or by using magnetic stir bar.

NOTE ***The 1X Buffer for Phenocycler-Fusion including additive is referred to as "1X PhenoCycler-Fusion Buffer."***

Scale this recipe to the amount of 1X PhenoCycler-Fusion Buffer needed for the experimental procedure.

NOTE ***Avoid shaking to prevent bubble formation. If using a bottle to mix, do not invert a lid-screwed bottle to mix. This prevents leaking. If spills occur, clean with 70% ethanol.***

Storage

The 1X PhenoCycler-Fusion Buffer should be stored at RT and is stable for 2 weeks.

Use

This 1X PhenoCycler-Fusion Buffer is used:

- To fill the buffer bottle in the PhenoCycler sidecar. (See Figure 6.1.)
- For the reporter stock solution in the 96-well plate. (See Chapter 5 of the PhenoCycler-Fusion User Guide.)
- For the incubation step after mounting the Flow Cell.

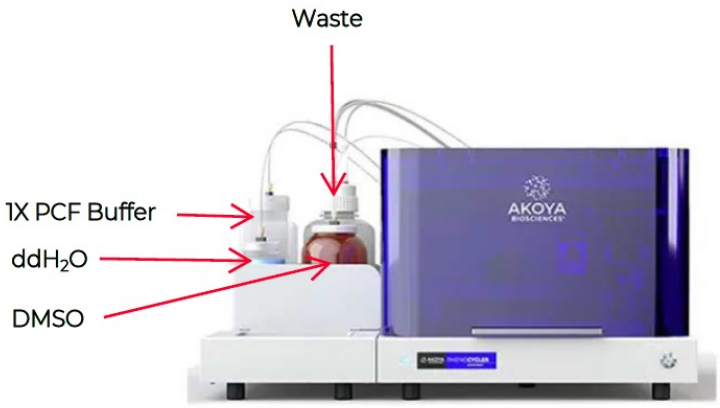


Figure 6.1. Sidecar containing buffers.

PHENOCYCLER SCAN PAGE

To set up a PhenoCycler scan, click on the Start Experiment button from the Home Page to display the Load Experiments screen. (See Figure 6.2.)

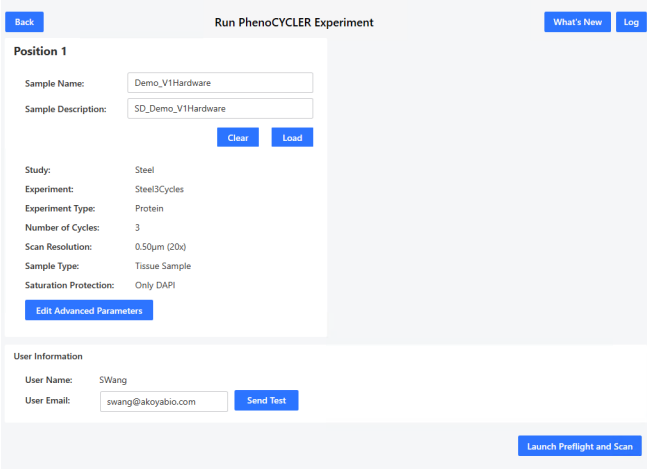


Figure 6.2. Load Experiment screen.

1. Enter the Sample Name and Sample Description.
2. Load an .xpd file made from the Experiment Designer by clicking the Load button in the Position 1 pane. The Load Experiment Design window will appear. (See Figure 6.3.)

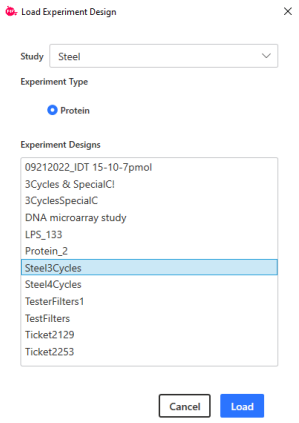


Figure 6.3. Load Experiment Design window.

3. Select a study from the Study drop-down. Then, select a file made from the experimental design application under the Experimental Designs pane and click the Load button. The details of the experiment will be displayed.
4. Use the Clear button to clear the loaded .xpd file, Sample Name, and/or Sample Description entries, as needed.

ADVANCED SETTINGS

For .xpd files created using Experiment Designer earlier than Experiment Designer 2.0.0, click the Edit Advanced Parameters button to access additional options (see Figure 6.4) which include:

- Scan Resolution.
- Saturation Protection.
- Sample Type.
- Sample Finding.

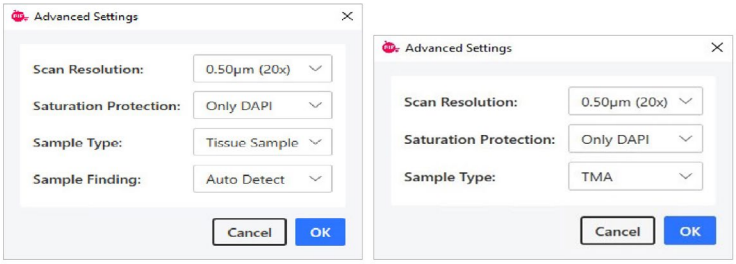


Figure 6.4. Advanced Settings windows. Screenshots compare a Sample Type of Tissue Sample (left) with a Sample Type of TMA (right).

Scan Resolution

Select the appropriate µm resolution for the scan.

Saturation Protection

Saturation Protection helps prevent overexposure when acquiring fluorescence imagery. If this option is selected and saturated pixels are detected in the imagery, Fusion will lower the exposure time for the entire scan to reduce the number of saturated pixels.

The recommended option for PhenoCycler experiments is Only DAPI.

NOTE ***Using Saturation Protection does not increase scan times noticeably because PhenoCycler-Fusion can record each image using 2 camera settings within 1 readout.***

Consider disabling Saturation Protection if the sample has bright debris that will saturate at much lower exposure times than the true sample. This could cause the exposure times to be reduced too low for proper visualization of the sample.

Sample Type

PhenoCycler-Fusion has a specialized algorithm for finding whole tissue samples vs finding TMA samples. The TMA algorithm expects TMA cores that are 0.6-2.5 mm in diameter, arranged in grids of at least 2x2. It will search for multiple grids on the same slide. If samples fit this description, select a Sample Type of TMA. This setting can be adjusted in the Preflight Routine when assessing the Sample Mask.

Sample Finding

The Sample Finding setting does not need to be adjusted because Sample Finding sensitivity will be set during the Preflight Routine. (See Figure 6.5.)

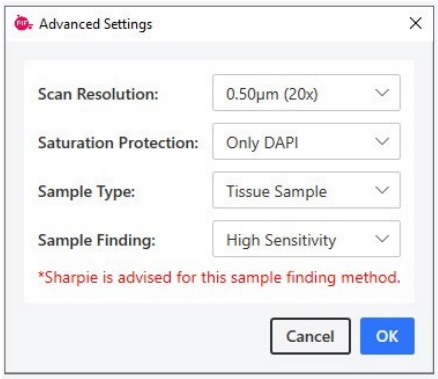


Figure 6.5. Sample Finding: High Sensitivity.

Email

Enter one email address in the text box under user information to receive email notifications related to scanning progress. If the information is entered, an email will be received under the following conditions:

1. When clicking the Send Test Email button.
2. At the start of every new cycle.
3. At the end of the last blank cycle.
4. When post processing has been completed (marking the end of the experiment).
5. If an error occurs at any point during the experiment.

After entering all of the experiment information, click Launch Preflight and Scan to start the Preflight Routine.

PREFLIGHT ROUTINE

The Preflight Routine is performed immediately before starting a scan to ensure a safe and successful PhenoCycler-Fusion experiment. The preflight routine may be canceled at any time. A series of dialog windows shown in the figures and steps in this section will guide the user through the process.

The Preflight Routine has 5 dialog windows:

- Get Ready.
- Blank Flow Cell.
- Sample Flow Cell.
- Tubing and Carrier Tray Check.
- Sample Validation.

Many of the instrument status indicators on the dialog windows are continuously refreshed by the software. All indicators must be green and all check boxes checked before the Next button will be enabled.

Get Ready

Get Ready is the first dialog box in the Preflight Routine. (See Figure 6.6.)

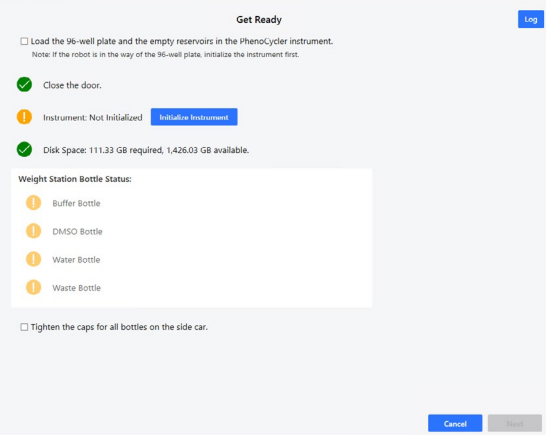


Figure 6.6. Dialog window, Get Ready.

1. Ensure that the 1X PhenoCycler-Fusion Buffer solution was made within 2 weeks.
2. Ensure the reporter plate corresponding to the selected experiment design is loaded into the PhenoCycler instrument.
3. Ensure the empty reservoirs are loaded into the PhenoCycler instrument.

NOTE ***Do not cover the reservoirs with foil or any other material.***

4. Close the PhenoCycler instrument door. The Door checkbox will turn green when the software determines the door has been closed.
5. If the instrument is not initialized, click the Initialize Instrument button.
6. If the Disk Space checkbox is not green, then clear disk space until the Disk Space checkbox turns green.

7. Ensure there is sufficient volume of 1X PhenoCycler-Fusion Buffer, DMSO, and water in the appropriate bottles.
8. Empty the waste bottle.
9. The software will weigh the installed bottles and indicate if the correct weight has been determined.
10. Confirm that the caps on the bottles within the sidecar have been tightened by selecting the checkbox.

NOTE ***Bottle caps should be tightened by rotating the bottle rather than the cap or tubing. Tightening the bottles completely is critical for preventing bubble formation.***

NOTE ***All 4 bottles must remain on the sidecar with tightened caps to avoid introducing bubbles.***

NOTE ***The software will latch the PhenoCycler door when Next button is clicked.***

The Get Ready dialog box is complete. (See Figure 6.7.)

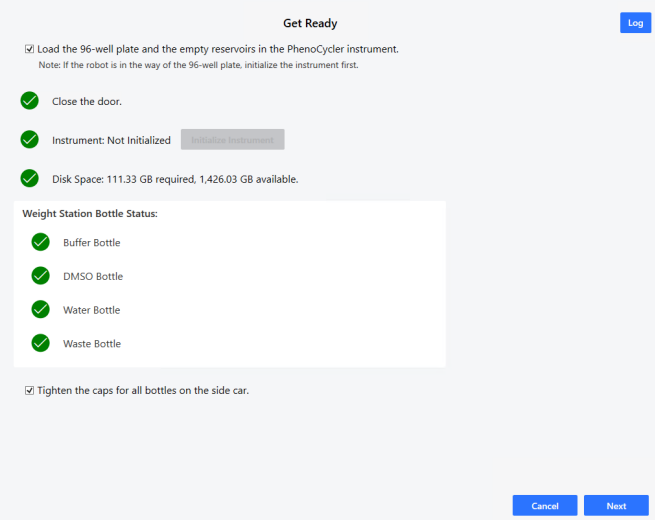


Figure 6.7. Dialog window, Get Ready complete.

CAUTION ***A correctly loaded 96-well plate will have A1 closest to the front of the instrument and will lie flat.***

Blank Flow Cell

A blank Flow Cell is used to prime the instrument lines. Blank Flow Cell is the second dialog box in the Preflight Routine. (See Figure 6.8.)

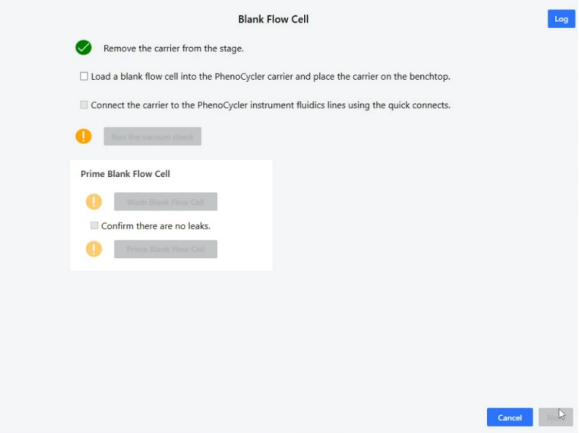


Figure 6.8. Dialog window, Blank Flow Cell.

1. Remove the carrier from the stage.
2. Insert a blank Flow Cell into the PhenoCycler carrier to check vacuum pressure.
3. Connect the fluidic lines of the PhenoCycler Flow Cell Slide Carrier to the PhenoCycler instrument.
4. Click the Run the vacuum check button. If the vacuum check does not turn green, check the vacuum waste bottle cap. If the cap is on tightly and the vacuum check does not turn green, contact Akoya Biosciences Technical Support at support@akoyabio.com.
5. Click the Wash Blank Flow Cell button and watch the blank Flow Cell as fluid is pumped in by the PhenoCycler and check for leaks. If a leak is detected, confirm that the Flow Cell is not broken or cracked and is seated correctly.
6. Inspect the carrier for leaks. If none are observed, check heck the Confirm there are no leaks checkbox. If leaks are observed, reseat the blank flow cell then click Wash Blank Flow Cell again to reperform the leak check.
7. Click the Prime Blank Flow Cell button as many times as needed.
8. The Blank Flow Cell dialog box is complete. (See Figure 6.9.)
9. Click the Next button to move to the Sample Flow Cell.

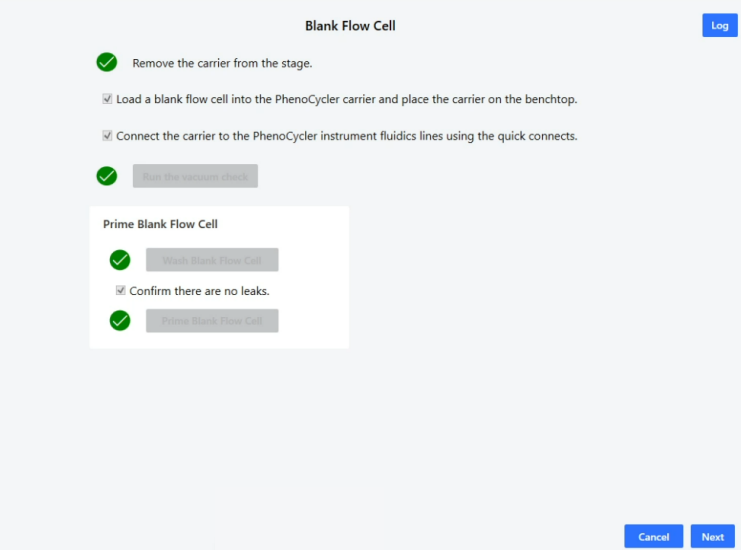


Figure 6.9. Dialog window, Blank Flow Cell complete.

Sample Flow Cell

Sample Flow Cell is the third dialog box in the Preflight Routine. (See Figure 6.10.)

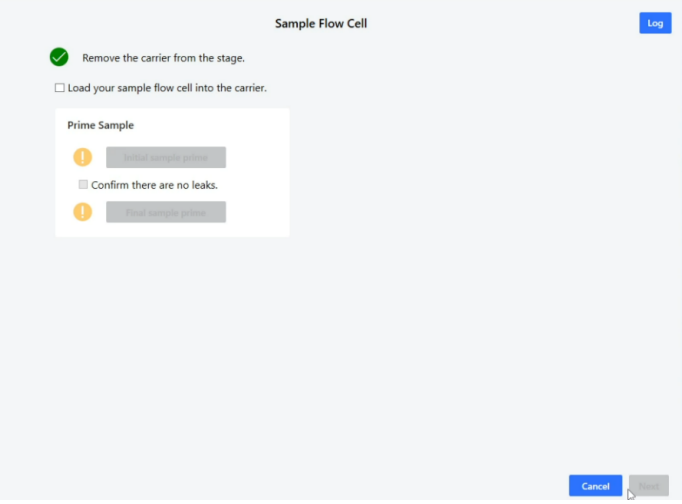


Figure 6.10. Dialog window, Sample Flow Cell.

1. Collect sample Flow Cell. Soak a Kimwipe with DI water. Wipe the Kimwipe across the top of the Flow Cell in a single direction slowly. Perform this wipe again with a second Kimwipe soaked with 70% ethanol. It is critical that both the top and bottom of the assembled Flow Cell is clean and the magnetic tray are clean and clear of debris to prevent focus issues.
2. Remove the carrier from the stage.
3. Remove the blank Flow Cell and insert the sample Flow Cell into the same Fusion carrier. Click the checkbox to indicate the sample Flow Cell is in the carrier.

NOTE ***The blank Flow Cell can be reused up to 6 months.***

4. Click the Initial Sample Prime button to start priming. Watch the sample Flow Cell as fluid is pumped in by the PhenoCycler and check for leaks.
5. Click the checkbox to indicate there are no leaks. If a leak is detected, confirm that the Flow Cell is not broken or cracked and is seated correctly.
6. Click the Final Sample Prime button to prime. Prime up to 5 times.
7. The Sample Flow Cell dialog box is complete. (See Figure 6.11.)
8. Click the Next button to move to the Tubing Check.

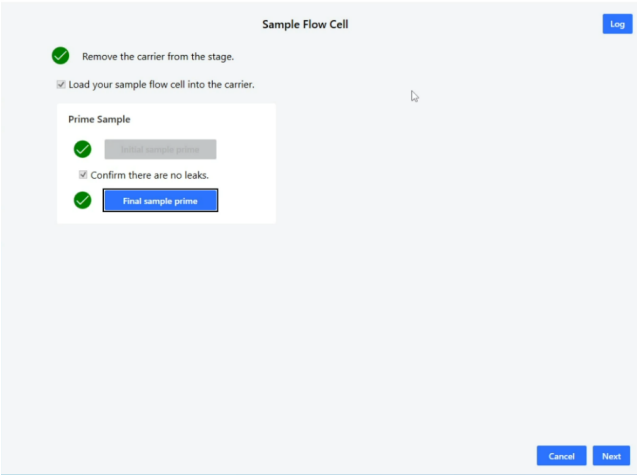


Figure 6.11. Dialog window, Sample Flow Cell complete.

Tubing and Carrier Tray Check

Tubing and Carrier Tray Check is the fourth dialog box in the Preflight Routine. (See Figure 6.12.)

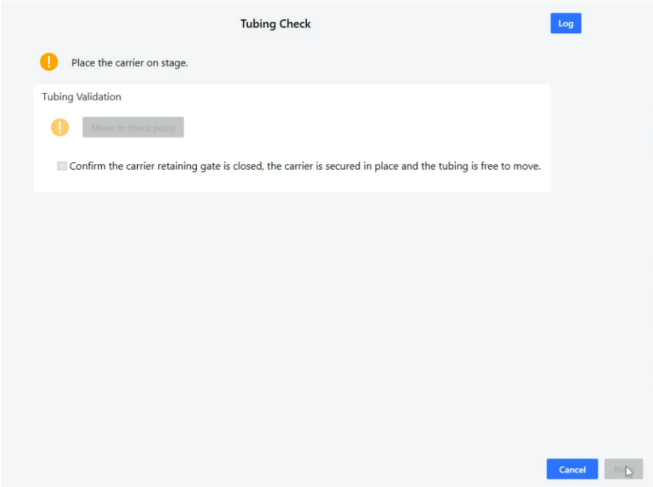


Figure 6.12. Dialog window, Tubing and Carrier Tray Check.

1. Load the Flow Cell carrier onto the stage.
2. Click the Move to check point button to move the carrier to the imaging position.
3. Inspect and make sure the carrier retaining gate is closed when the carrier is in the imaging position. If the gate is not closed, then push the carrier in to the close the gate.
4. Adjust the carrier tubing so it has enough slack in the line to move freely.
5. Click the checkbox to confirm the carrier retaining gate is closed and the tubing is free to move.
6. The Tubing Check dialog box is complete. (See Figure 6.13.)
7. Click the Next button to move to Sample Validation.

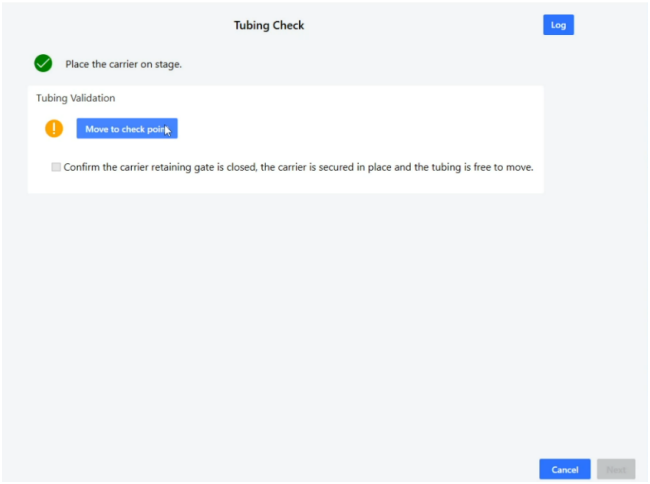


Figure 6.13. Dialog window, Tubing and Carrier Tray Check intermediate step.

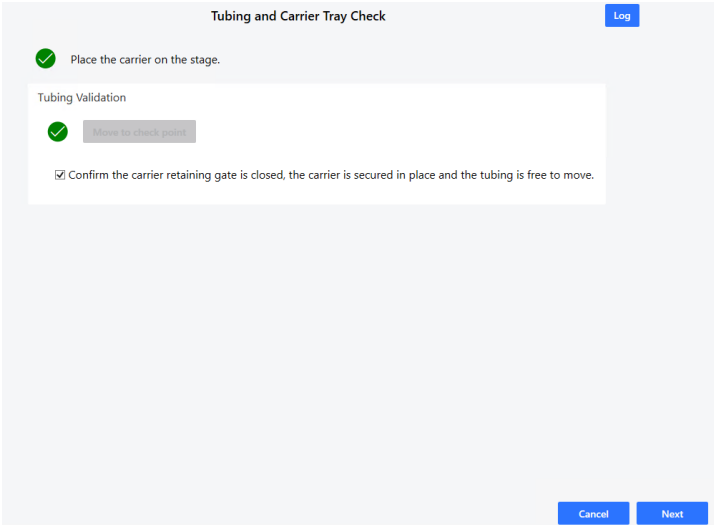


Figure 6.14. Dialog window, Tubing and Carrier Tray Check complete.

Sample Validation

Sample Validation is the fifth and final dialog box in the Preflight Routine. (See Figure 6.15.)

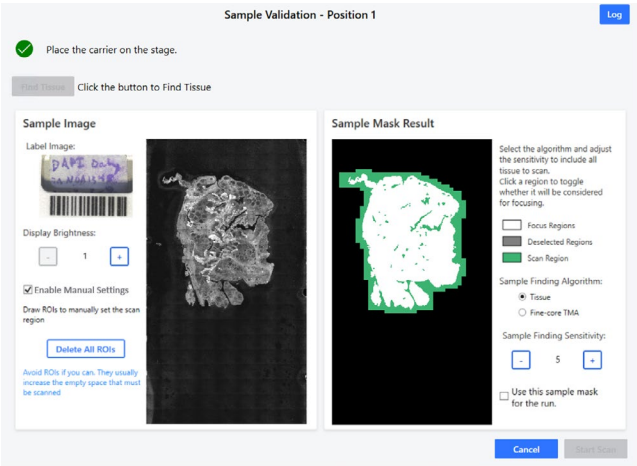


Figure 6.15. Dialog window, Sample Validation.

1. Load the Flow Cell carrier onto the stage.
2. Click Find Tissue to take an image of the sample. Use the Brightness controls to decrease and increase the display brightness so each tissue piece can be easily distinguished from the background. NOTE: This adjustment affects the display only and does not impact sample finding results.
3. Adjust the Sample Finding Sensitivity and/or Sample Finding Algorithm (see Table 6.4) to ensure all tissue pieces are masked as Focus Regions. NOTE: If the algorithm masks some non-tissue areas as Focus Regions, click on those regions in the Sample Mask to de-select them.
4. If settings cannot be found that detect all tissue pieces, better results may be obtained by drawing ROIs around tissue pieces. To do so, click Enable Manual Settings.
5. Once the sample image looks good, adjust the sample mask settings if needed. (See Table 6.4.)

Table 6.4. Sample Mask Results, settings descriptions

Item	Description
Scan Region	Area that will be scanned. Click and drag the mouse to create ROIs* indicated by yellow,
Focus Region	Area that will be used to focus. Click-and-drag the mouse to create focus points indicated by white,
Deselected Region	Area that will not be scanned
Sample Finding Algorithm	Tissue - select for all other samples. Fine-core TMA - PhenoImager Fusion has a specialized algorithm for finding TMA samples. The algorithm expects TMA cores that are 0.6-2.5 mm in diameter, arranged in grids of at least 2 x 2. It will search for multiple grids on the same slide. If the samples fit this description, select Sample is a TMA.
Sample Finding Sensitivity	Adjust the sensitivity using the controls.

*Region of interest (ROI).

6. Once the sample mask is complete, check the Use this sample mask checkbox.
7. The Start Scan button will be enabled. (See Figure 6.16.)

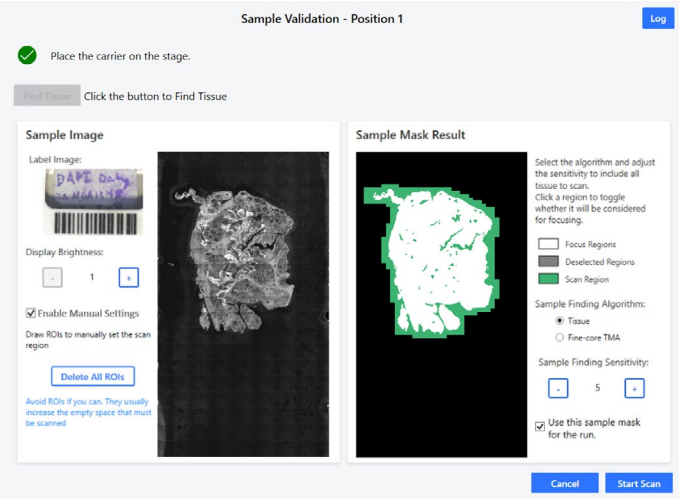


Figure 6.16. Dialog window, Sample Validation complete.

The Preflight Routine is now complete. Click the Start Scan button to begin the PhenoCycler experiment.

OPTIONAL CANCEL RUN

If the user needs to cancel the run, click the Cancel Run button to stop operations will stop as soon as possible. (See Figure 6.17.) If the robot or fluidics is in the middle of an operation, it will first complete the action. Once completed, the robot will return to the home position and the PhenoCycler door will unlock.

Once the run is canceled, the user will have the option to perform a Post-Experiment Clean or start another run. (See Figure 6.18.)

NOTE ***If a run is canceled, a final qptiff will NOT be generated.***

EXAMPLE 1: CANCELING DURING A PHENOCYCLER RUN

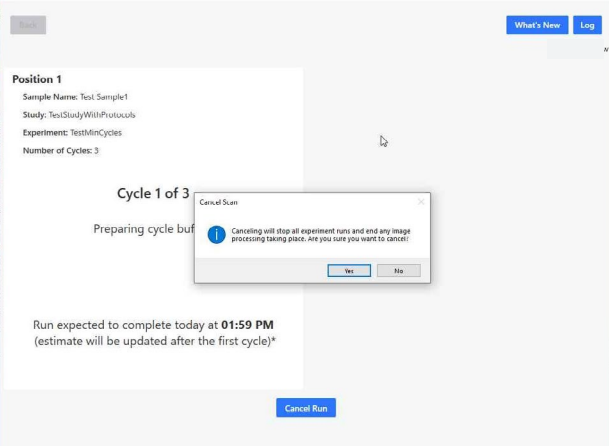


Figure 6.17. Dialog window, Preparing Cycle Buffers, Cancel Run.

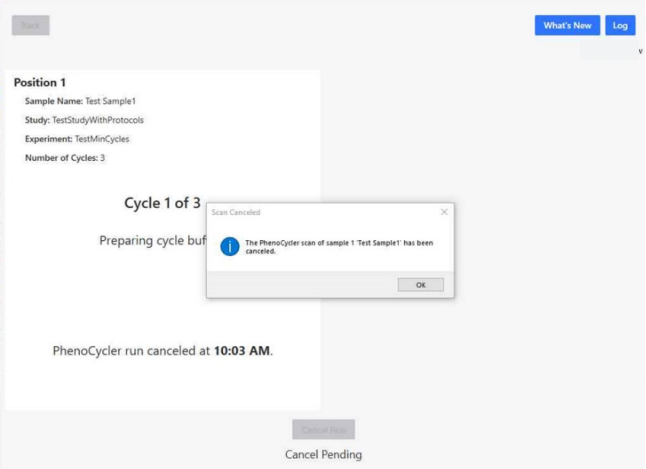


Figure 6.18. Dialog window, PhenoCycler run canceled.

EXAMPLE 2: CANCELING DURING POST-EXPERIMENT CLEAN

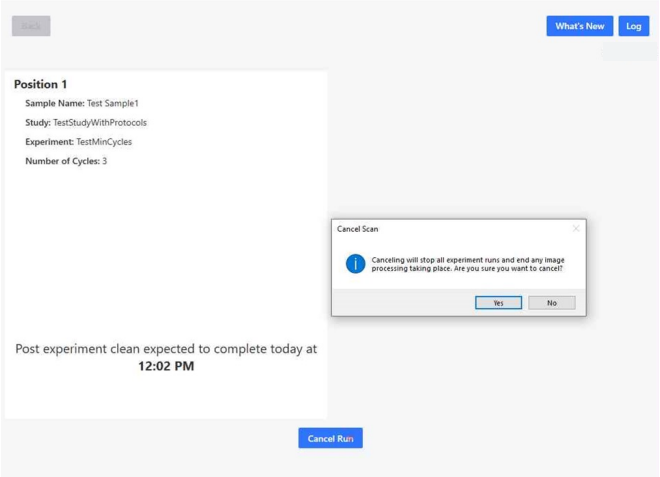


Figure 6.19. Dialog window, Performing post-experiment clean, Cancel Run.

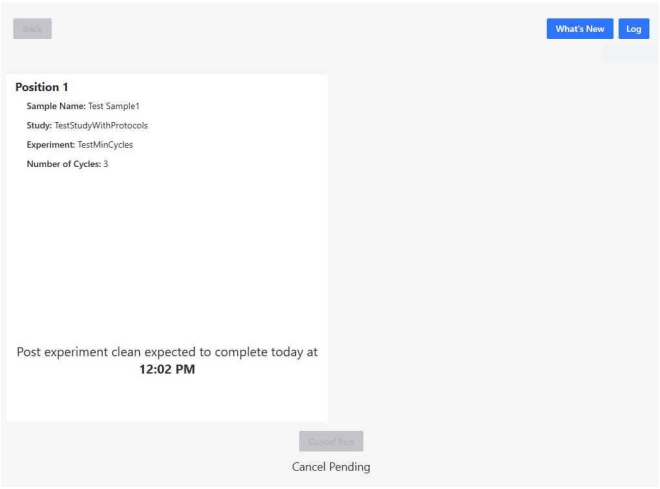


Figure 6.20. Dialog window, Performing post-experiment clean, Cancel Pending.

STATUS AND ESTIMATED TIME OF COMPLETION INDICATORS DURING A PHENOCYCLER RUN

Throughout the run, various status panels indicate the ongoing steps within the PhenoCycler run and display the estimated run completion time.

EXAMPLE STATUS PANELS ARE SHOWN BELOW:

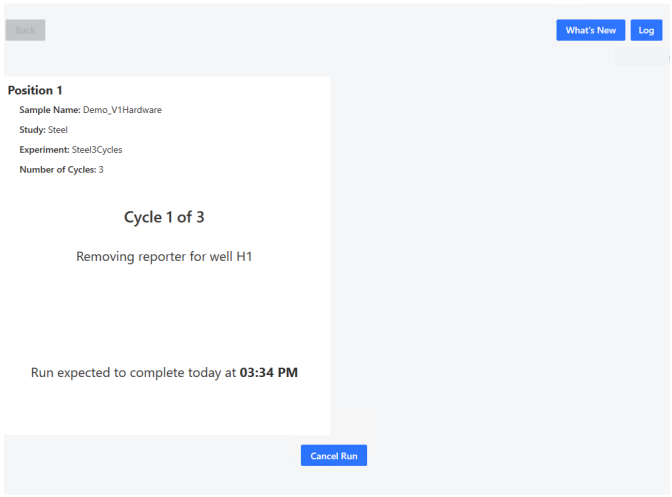


Figure 6.21. Dialog window, Example status panel, Removing reporter for wells.

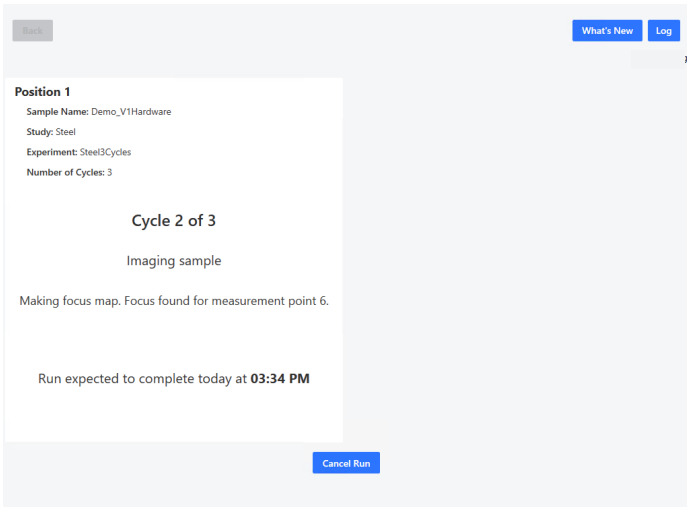


Figure 6.22. Dialog window, Example status panel, Imaging sample, Making focus map.

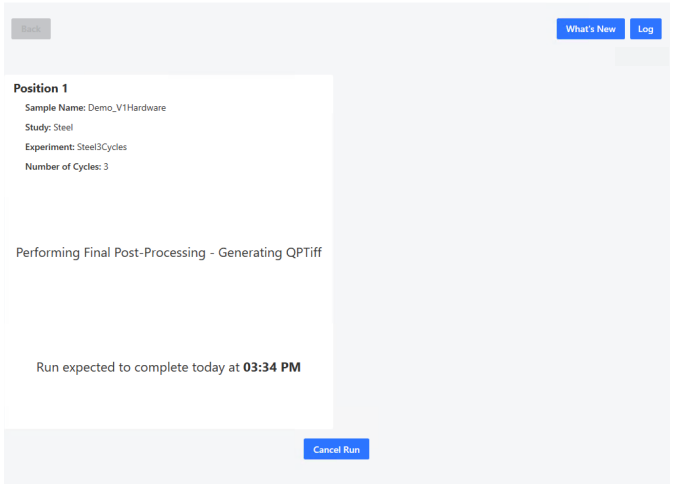


Figure 6.23. Dialog window, Example status panel, Performing Final Post-Processing – Generating QPTiff.

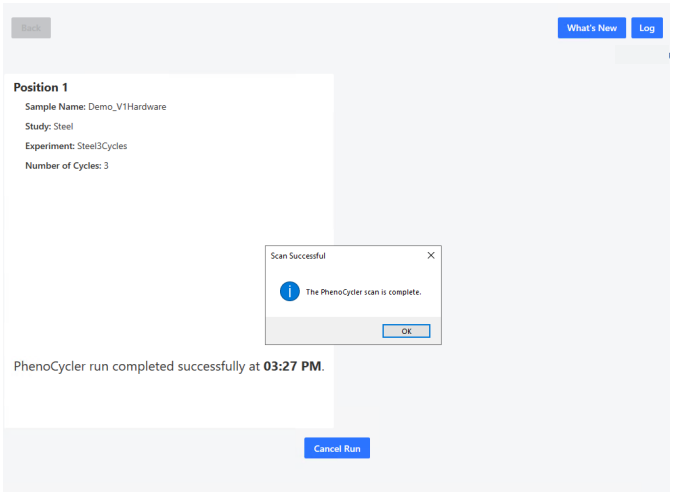


Figure 6.24. Dialog window, Example status panel, PhenoCycler run completed successfully. Scan Complete.

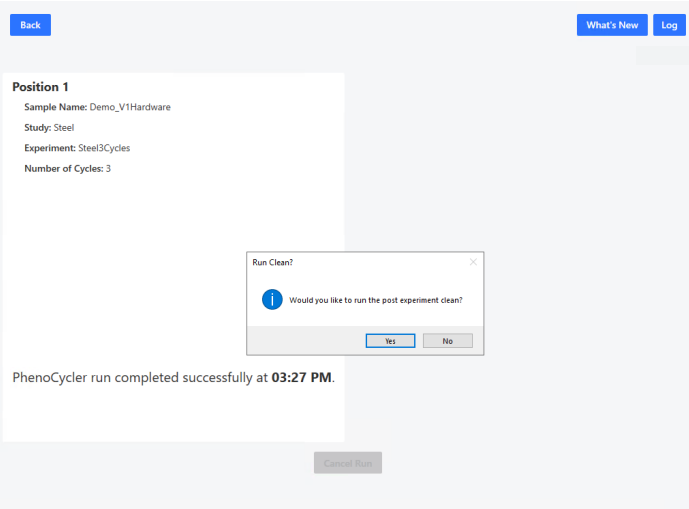


Figure 6.25. Dialog window, Example status panel, PhenoCycler run completed successfully. Run Clean?

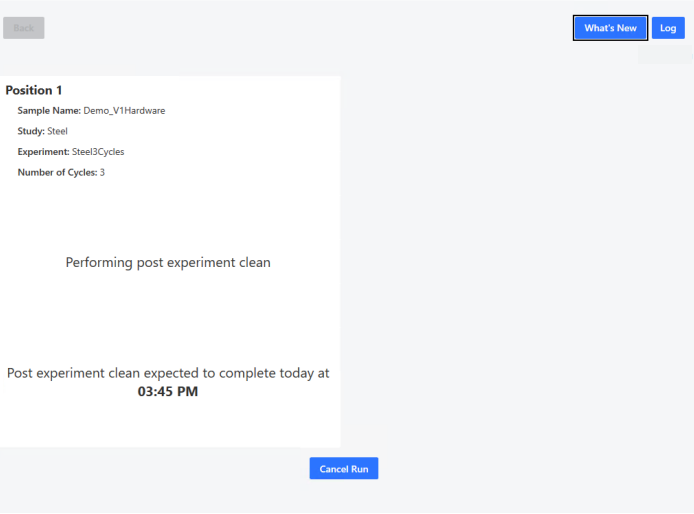


Figure 6.26. Dialog window, Example status panel, Performing post experiment clean.

Chapter 7: Cleaning and Maintaining PhenoCycler

Please see the PhenoCycler-Fusion User Guide for complete instructions on PhenoCycler cleaning and maintenance. Some steps require use of the Fusion software via the PhenoCycler Dashboard. Those steps are described below.

PHENOCYCLER DASHBOARD SOFTWARE

The PhenoCycler Dashboard can be accessed from the main page. This page contains Post Experiment Clean, Maintenance Wash, and Clear Tissue. (See Figure 7.1.)

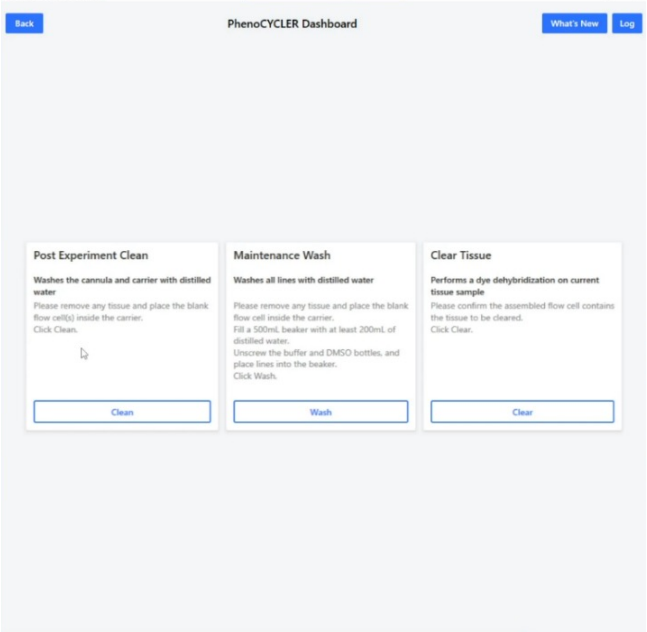


Figure 7.1. PhenoCycler Maintenance Dashboard.

Post Experiment Clean

The post-experiment clean must be performed immediately after every run to maintain the instrument.

Be sure to remove the tissue sample and insert a blank Flow Cell before running. If this step is performed with the tissue sample loaded, that tissue will not be reusable for further studies. To perform this step, select the Clean button after loading the blank Flow Cell.

Maintenance Wash

The maintenance wash clears and washes the lines of residual buffers and DMSO to maintain optimal instrument performance. To perform the Maintenance Wash, follow these steps:

- Load a blank Flow Cell into the PhenoCycler Flow Cell Slide Carrier.
- Fill a beaker with at least 200 mL of water and place near the PhenoCycler.
- Remove the tops of Bottle 1 (PhenoCycler Buffer) and Bottle 2 (DMSO) and place the fluidic lines into the beaker of water.

  NOTE ***When attaching or removing bottles from the PhenoCycler instrument, keep the caps still while turning the bottles. Turning the caps will twist the lines.***

- Click the Wash button.

Clear Tissue

If the experiment run was interrupted for any reason and it is possible that reporters are still on the sample, they must be cleared from the sample while still connected to the PhenoCycler instrument to reuse the sample.

If the tissue is not needed for future runs, this step is not necessary.

NOTE ***Remove the tissue section from the stage as soon as possible if it needs to be preserved for future studies. Place the tissue in Storage Buffer at 4°C without letting it dry during the transfer.***

Chapter 8: Cleaning and Maintaining the PhenoImager Fusion

CLEANING THE EXTERIOR

Clean the PhenoImager Fusion instrument exterior as needed. Wipe down the non-electrical exterior parts of the PhenoImager Fusion 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 Fusion instrument. *Sprays and liquids that come into contact with the PhenoImager Fusion 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.*

CLEANING THE MONITOR

Clean the monitor as needed. Wipe the monitor 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.*

CLEANING THE POWER & COMMS PORTS

Remove dust from the power and communication ports by spraying 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: Electric Shock Hazard. Do not attempt to replace the fuses. Only qualified Akoya Biosciences personnel can replace the fuses in this instrument.

CLEANING THE SLIDE CARRIER

It is important to keep carriers free from sticky debris caused by slide labels, tape, or mounting media. Clean the Slide Carriers using soap and water with a soft cloth or using an ultrasonic bath.

REPLACING PARTS ON THE PHENOIMAGER FUSION SYSTEM

Replacing the Fuses: Contact Akoya Biosciences Technical Support for blown fuses or to order replacement fuses.

	*Warning: Turn off the electrical power to the PhenoImager Fusion system (as described in "System Shutdown") before cleaning any part of the instrument where electrical or fiber optic connections are made.*

Appendix A: Inspecting Air Pockets in the Assembled Flow Cell (Optional)

If large air pockets are still present after the 10-minute incubation during Flow Cell assembly, a manual syringe can be used to remove from individual Flow Cell samples one at a time. (See Figure A.1.) Most air pockets typically clear during software pre-flight checks.

1. Retrieve the Flow Cell Slide Carrier from PhenoCycler.
   a. Retrieve the carrier that will be used for optional bubble removal.
   b. Connect the 2 lines attached to the PhenoCycler.
   c. Connect the 2 lines attached to the Flow Cell Slide Carrier.

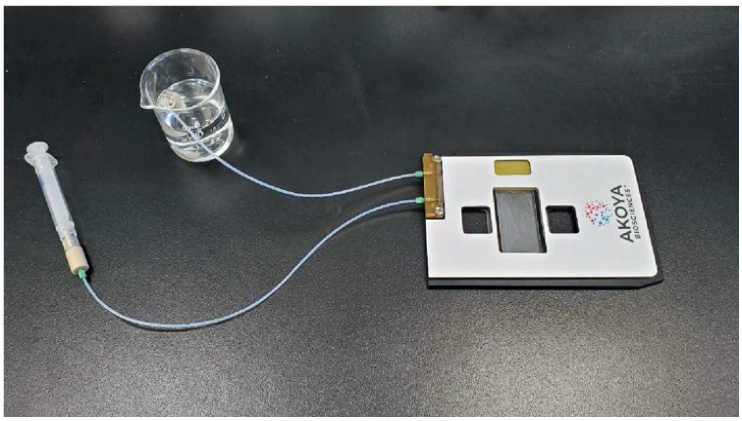


Figure A.1. Flow Cell Carrier connected to a manual syringe.

2. Once back at bench area, retrieve a 50 mL beaker with 1X PhenoCycler-Fusion buffer.
3. Unscrew quick connections.
4. Place inlet tubing of Flow Cell Slide Carrier into the beaker containing the buffer.
5. Attach a manual syringe to the outlet tubing of the Flow Cell Slide Carrier.
6. Slowly open the syringe, pulling buffer from beaker and into the Flow Cell. This should remove all air pockets present in the Flow Cell.

Appendix B: Bottle Cleaning

This section provides instruction for cleaning bottles for DMSO, and 1X PhenoCycler-Fusion buffer.

1. Rinse the empty bottles with ddH2O and dispose of the rinsed water in hazardous waste.

   NOTE ***Clean all bottles thoroughly with soap and water every 3 months.***

2. Allow bottles to air dry after rinsing. Do not use paper towels to dry bottles.

   NOTE ***Store bottles dry with lids tightened to prevent dust accumulating inside the bottles.***

3. After bottles are dry, fill bottle with minimum required volumes for experiment run.
4. Replace bottle lid and tighten.

NOTE ***All reagents should be completely disposed according to your institution's hazardous waste guidelines after 2 weeks.***

CRITICAL ***Do not re-use reagents past 2 weeks.***

If unable to allow bottles to air dry, perform the following for each individual reagent bottle:

1. Add 25 mL of the appropriate freshly made reagents. For example, in the DMSO bottle, add 25 mL of DMSO solution.
2. Replace bottle lid and tighten.
3. Swirl the 25 mL of solution throughout the bottle.
4. Dispose of the 25 mL in hazardous waste.
5. Fill bottle with minimum required volumes for experiment run.
6. Replace bottle lid and tighten.

Appendix C: Specifications

SYSTEM SPECIFICATIONS

Table C.1.

SYSTEM SPECIFICATIONS	
Size (L x W x H)	25” (64 cm) L x 20” (51 cm) W x 26” (66 cm) H
Weight (Instrument)	~ 120 lbs. (54.4 kg)
Weight (Total System)	~ 162 lbs. (74 kg)
Spectral Range	440 nm to 780 nm
Pixel Resolution	0.25 µm, 0.5 µm
File Format	Akoya Biosciences QPTIFF format for whole slide scans
Operating System	Microsoft® Windows 10, 64-bit
RAM	32 GB

ENVIRONMENTAL CONDITIONS

Table C.2.

ENVIRONMENTAL CONDITIONS	
Operating Temperature	+20 to +26˚C (68˚F to 80˚F)
Operating Humidity	30% - 60% non-condensing
Storage Temperature	+10˚C to +30˚C (59˚F to 86˚F)
Storage Humidity	20% - 60% non-condensing
Shipping Temperature (up to 72 hours max)	-29˚C to +38˚C (14˚F to 113˚F)
Shipping Humidity (up to 72 hours max)	10%-85% non-condensing

ELECTRICAL SPECIFICATIONS

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

Table C.3.

ELECTRICAL SPECIFICATIONS	
Input Voltage and Frequency	100-120V, 50/60Hz (±10%) 200-240V, 50/60Hz (±10%) System does not have transient over voltage protection.
Computer Interface	USB 2.0, USB 3.0
Fuse	4A Littelfuse® 250V, 5 mm x 20 mm

Appendix D: Akoya TIFF Specifications

BACKGROUND

This appendix 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 the 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 Big TIFF images, depending on image size, with multiple images per file. For images larger than about 2K x 2K pixels, tiled format is used, and the image is provided in several resolutions (pyramidal tiled images). Tile size is 512 x 512 pixels. Images smaller than 2K x 2K use stripped format.

The highest resolution (baseline) image(s) appear first in the file. For each resolution there are N baseline images where N depends on the contents. For BF images, N=1 and each image is an RGB image. For FL images or unmixed component images, N = number of bands, which is usually > 1, and each image is a grayscale image.

A thumbnail RGB image is provided. This is a good image to use as an icon in graphical image lists. After the baseline images, it is the second image in BF (RGB) images and the (N+1) 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 described in Table D.1.

Table D.1. TIFF and BigTIFF images data format

Description	RGB/mom	Title/Strip	Resolution	Note
Baseline Image	Varies	Varies	Full	Tiled if > 2K x 2K RGB for BF, else mono.
More full-resolution images	Mono	Varies	Full	If N > 1.
Thumbnail	RGB	Stripped	~500 x 500	
Half-resolution images	Varies	Varies	Half	Only if baseline is tiled.
Macro (overview) image of whole slide	RGB	Stripped	~2000 x 4000	Required for whole-slide scans. Optional for simple RGB images and extracted components.
Label image	RGB	Stripped	~500 x 500	Optional, whole-slide scans.

DETECTION

Readers can recognize Akoya tissue images via the contents of the "Software" TIFF tag (see Table D.2). 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

The metadata is contained in two locations. Within standard TIFF tags as listed in the table below is one location. Within the ImageDescription string, using a set of XML tags described in Table D.3 is the second. These are provided for each image (IFD) in the file and 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 D.2. TIFF tags

TIFF Tag	Optional	Description of Content
Software	No	Starts with "PerkinElmer-QPI".
ImageDescription	No	Further metadata in XML format (see next section).
ImageWidth	No	Width of the image in pixels.
ImageLength	No	Height of the image in pixels.
ResolutionUnit	No	Unit used for resolution and position (see below).
XResolution	No	Pixel X resolution (see below).
YResolution	No	Pixel Y resolution (see below).
XPosition	Yes	Sample X location in ResolutionUnits. This is ULHC location except for Macro image which reports its image center.
YPosition	Yes	Sample Y location in ResolutionUnits. This is ULHC location except for Macro image which reports its image center.
SampleFormat	No	Integer (1) for BF, FL; or float (3) for extracted components.

TIFF Tag	Optional	Description of Content
SMinSampleValue	No	Minimum signal value in the image.
SMaxSampleValue	No	Maximum signal value in the image.
BitsPerSample	No	8 (FL); 8, 8, 8 (RGB); or 32 (unmixed component).
SamplesPerPixel	No	1 (FL or unmixed component) or 3 (RGB).
NewSubfileType	No	0 for full-resolution images, 1 for reduced res images.
TileWidth	Yes	Tile width (512) if tiled format is used.
TileLength	Yes	Tile height (512) if tiled format is used
TileOffsets	Yes	List of tile offsets, if tiled format is used.
TileByteCounts	Yes	Size of each (compressed) tile, if tiled format is used.
StripOffsets	Yes	List of strip offsets, if tiled format is not used.
RowsPerStrip	Yes	Number of rows per strip, if tiled format is not used.
StripByteCounts	Yes	Size of each (compressed) strip, if tiled format is not used.
PlanarConfiguration	No	1 (chunky) for RGB images, 2 (planar) otherwise.
Photometric Interpretation	No	2 (RGB) for RGB images, 1 (BlackIsZero) otherwise.
DateTime	No	The DateTime is the acquisition time.
Compression	No	Compression may be shown as None, CCITT Group 3, PackBits, LZW, or JPEG.
JPEG fields	Yes	JPEG fields are defined when JPEG compression is used.

ResolutionUnit, XResolution, and YResolution are required fields in a valid TIFF file. When the true resolution of the image is known, ResolutionUnit will be 3 (cm). XResolution and YResolution will be expressed in pixels/cm. When the true resolution is not known, ResolutionUnit will be 2 (inch). XResolution and YResolution will be expressed in 96 (pixels/inch). Pixels from Akoya Biosciences instruments are always square, so XResolution and YResolution will always have the same value.

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

IMAGE DESCRIPTION CONTENTS

The ImageDescription tag contains a string in XML format. The string contains a top-level < PerkinElmerQPI-ImageDescription> element. Nested within this element are child elements with the tag names and values as listed in Table D.3. Elements appear in the order listed.

Table D.3. IMAGE DESCRIPTION TAGS

TIFF Tag	Optional	Description of Content
ImageType	No	A string identifying the type of image within the file (Table D.1), with the following values: • FullResolution • ReducedResolution • Thumbnail • Overview • Label
Is Unmixed Component	No	"True" for unmixed multispectral images, otherwise "False."
Exposure Time	No	Exposure time equals the exposure expressed in an integer number in 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).
Signal Units	No	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 For example, a value of 68 (hex 44) encodes OD units with peak-signal weighting.
Name	Yes	Band name for FL whole slide scans, component name for extracted components. Not present for RGB images.
Color	Yes	Color to use when rendering this band, as decimal r, g, b byte triplet. Present for FL whole slide scans or extracted components. Not present for RGB images.
Responsivity	Yes	Instrument responsivity, if available, for FL whole-slide and unmixed images. See below for details.
Objective	Yes	Objective name, if known, otherwise not present.
ScanProfile	Yes	Element containing scan and/or unmix parameters. It is valid XML whose contents are opaque to most readers. It is only provided on the first (baseline) image and is omitted from all other IFDs.
ProtocolName	Yes	Name of the acquisition protocol.
ValidationCode	Yes	Used for internal data integrity checks – readers can ignore this.
ImagingCycle	Yes	Zero-based acquisition cycle for Fusion imagery.
Biomarker	Yes	String indicating target such as CD8, Ki67, etc.
ScaleFactor	Yes	After allowing for bit-depth conversion, pixel values in this dataset have been scaled by this factor. For example, if a 12-bit image is stored in an 8-bit image, and counts were scaled from 4095 to 255, this would be 1; if counts were scaled from 3102 to 255, ScaleFactor would be 1.3201. The default is 1.
PartNo	No	String with the manufacturer part number.
Bands	No	Collection of bands each as described by sub- elements below.
Band	No	Description of a single band with sub-elements noted below.
Name	No	String with the band name.
Cut-on	No	Cut-on wavelength of this band in nm.

TIFF Tag	Optional	Description of Content
Cut-off	No	Cut-off wavelength of this band in nm.
CameraSettings	Yes	Description of the camera settings used for this image with subelements listed below. If present, all its sub-elements must be provided.
Gain	No	Represents the Gain factor, such as 1, 3, etc.
Binning	No	Binning extent, such as 1 for full-resolution, 2 for 2x2 binning.
OffsetCounts	No	Offset count setting for the camera.
ExcitationFilter	Yes	Description of excitation filter with sub-elements listed below. If present, all its sub-elements must be provided.
Name	No	String with the filter public name.
Manufacturer	No	String with the manufacturer or OEM supplier.
PartNo	No	String with the manufacturer part number.
Bands	No	Collection of bands each as described by sub- elements below.
BitDepth	No	Bit resolution used to read out the camera. This may differ from the resolution used to store this image. For example, it may be acquired with 12-bit resolution but saved in a QPTIFF file with 8-bit resolution.
Orientation	No	String containing one of the following values: 'Normal' (default) 'Rotate180' 'MirrorV' 'MirrorH'
ROI	Yes	Description of the sensor ROI contributing to the image. Coordinates are sensor pixels before binning. Even if the Camera Settings are provided, this field is optional.
X	No	X origin of ROI. Left-most pixel in sensor array is 0.
Y	No	Y origin of ROI. Top-most pixel in sensor array is 0.
Width	No	Width of ROI.
Height	No	Height of ROI.

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

INSTRUMENT RESPONSIVITY

The <Responsivity> tag is a container for a list of normalized instrument response values. This tag is present for whole-slide FL images from PhenoImager Fusion.

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

For unmixed component images, the <Responsivity> tag will contain one <Band> tag for each band in the original image file. The <Band> tags are repeated for each unmixed component image. The overview, thumbnail and label images do not have <Responsivity> tags. Each <Filter> or <Band> tag describes the instrument responsivity for acquisitions using that filter or band. The contents of the <Filter> and <Band> tags are described Table D.4.

Table D.4. CONTENT OF FILTER AND BAND TAGS

Tag	Description of Contents
Name	The name of the filter (whole-slide image) or band (component image).
Response	Response compares the instrument response to the reference artifact, normalized for exposure. The formula is: raw counts/ (2bit depth × exposure time × gain × binning arbit depth is the bit deptagery, 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 IMAGE DESCRIPTION

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

<?xml version=”1.0” encoding=”utf-8”?>
<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</</Filter>
</Responsivity>
<Objective>4x</Objective>
<ScanProfile><! -- this will be a serialized scan protocol. It is valid XML but otherwise </ScanProfile>
<ValidationCode>4281ff86778db65892c05151d5de738d</ValidationCode>

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