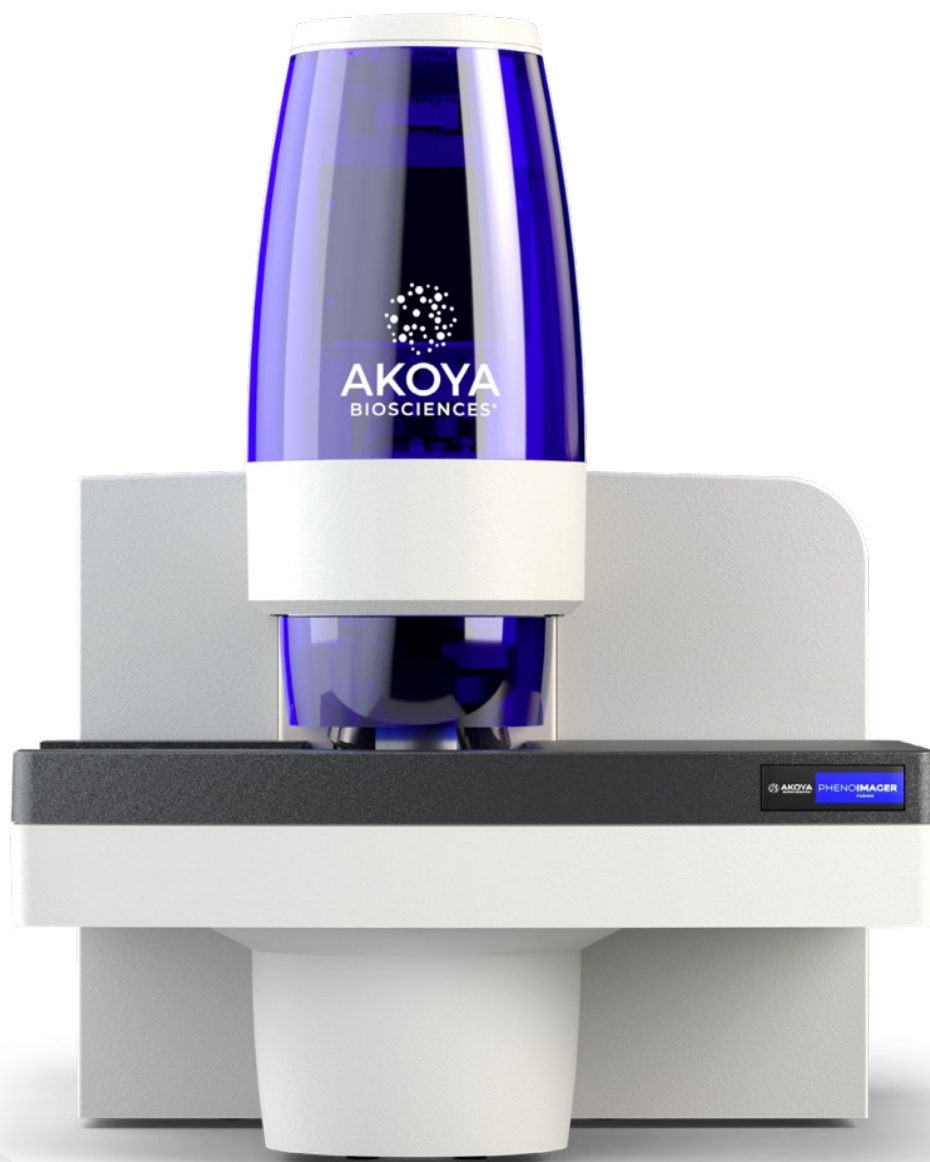


Phenolmager Fusion 2.0

USER GUIDE

For use with PhenoCycler-Fusion 2-Flow Cell Configuration and Fusion 4-Slide Configuration



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Contents

Copyright and Disclaimers.....	2
Preface	6
Intended Audience for this Document.....	6
Associated Documents.....	6
Guide Conventions.....	6
Product Service and Customer Support Plans.....	7
CE.....	7
Korean Certifications (KC).....	7
Definition of Symbols (Labeling of Equipment and Location).....	8
Safety Considerations	10
Required Training	10
Power Cord Selection	11
Fuses.....	11
Cables and Adapters.....	11
Chapter 1: Introducing the PhenolMager Fusion	12
Intended Use.....	12
Principles of Operation	12
Akoya Software	13
Key Concepts of Multispectral Imaging	13
Chapter 2: Instrument Overview	16
System Overview.....	16
Left-Side View.....	16
Right-Side View.....	18
Rear View.....	19
Ventilation Requirements.....	19
Input/Output Connections.....	20
System Computer and Monitor.....	21
PhenolMager Slide Carriers.....	24
PhenoCycler Flow Cell Slide Carriers.....	24
Bottom View.....	25
Chapter 3: Getting Started	26
Introduction.....	26
System Startup.....	26
Setting Up PhenolMager Slide Carriers.....	27
Setting Up PhenoCycler Flow Cell Slide Carriers	28
Loading The Flow Cell Slide Carrier into The Automated XYZ Stage	39

Chapter 4: PhenolMager Fusion Software Overview.....	42
PhenolMager Fusion Software Home Page	42
Fusion Dashboard	44
Chapter 5: PhenolMager Workflow with Brightfield and Fluorescence.....	48
Creating Protocols.....	48
Editing Protocols	50
Acquiring Snapshots for Spectral Libraries.....	54
Scanning Slides	56
Chapter 6: PhenoCycler-Fusion Workflow (High Plex Protein)	58
Overview.....	58
Buffer Preparation.....	59
Using the Run Buffer Calculator for PhenoCycler-Fusion 2-Slide Protein Runs	60
PhenoCycler Scan Page	66
Preflight Routine	69
Status and Estimated Time of Completion Indicators.....	81
Chapter 7: Cleaning and Maintaining PhenoCycler.....	83
PhenoCycler Dashboard Software	83
Chapter 8: Cleaning and Maintaining the PhenolMager Fusion	85
Cleaning the Exterior.....	85
Cleaning the Monitor	85
Cleaning the Power & Comms Ports	85
Cleaning the Slide Carrier	86
Cleaning After a Leak	86
Replacing Parts on the PhenolMager Fusion System	86
Appendix A: Troubleshooting	87
Hardware	87
Software	91
Appendix B: Bottle Cleaning	93
Appendix C: Inspecting Air Pockets in the Assembled Flow Cell (Optional)	94
Appendix D: Installing and Uninstalling the PhenolMager Fusion Software	95
Appendix E: Specifications	97
System Specifications.....	97
Environmental Specifications.....	97

Electrical Specifications	98
Appendix F: Akoya TIFF Specifications	99
Background	99
Data Format.....	99
Detection	100
Metadata	100
Image Description Contents.....	101
Instrument Responsivity.....	104
Sample Image Description	105
Appendix G: PhenolMager Fusion EULA	106

Preface

Welcome to the Akoya Biosciences Phenolmager® 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 Phenolmager 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:

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

PRODUCT SERVICE AND CUSTOMER SUPPORT PLANS

Akoya offers a full range of services to ensure success. From the 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.

Programs can include such useful services including:

- 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

	<i>This device complies with all CE rules and requirements.</i>
---	--

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 Phenolmager 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)



Phenolmager Nameplate



Serial Number		  
PFYYWWNxxxx 		
Part No. / Rev.		R-R-AK0-Fusion
AB-000002/x		Amp/Type/Volt
4 T 250		
V~		Hz
100-240		50/60


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 Marlborough, MA USA
 01752
 (855) 896-8401

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www.akoyabio.com



Serial Label

SAFETY CONSIDERATIONS

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

	<i>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.</i>
---	--

DANGER, WARNINGS, AND CAUTION SIGNS

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

GENERAL HAZARDS

None.

ELECTRICAL SAFETY

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

	<i>Warning: Do not operate the system in an environment with explosive or flammable gases. 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.</i>
	<i>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 at support@akoyabio.com.</i>
	<i>Warning: Turn off the electrical power to the Phenolmager Fusion system by shutting down the system before cleaning any part of the instrument where electrical or fiber optic cables connect.</i>
	<i>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.</i>

POWER CORD SELECTION

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

	<i>Warning: Use only the power supply cord set provided with the PhenolMager 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.</i>
	<i>Warning: Use only a properly grounded power outlet when connecting the system to power.</i>
	<i>Warning: The appliance inlet is a disconnecting device. Place the device or equipment where disconnecting the device is always accessible.</i>

FUSES

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

	<i>Warning: The fuses in this instrument are only replaceable by trained Akoya Biosciences personnel.</i>
---	---

CABLES AND ADAPTERS

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

	<i>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.</i>
---	---

MECHANICAL SAFETY

	<i>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.</i>
---	---

WEIGHT WARNING

	<i>Warning: LIFTING HAZARD. The PhenolMager Fusion instrument weighs ~120 lbs. (~54 kg). Do not move the PhenolMager Fusion instrument. Installing, servicing, and moving the PhenolMager 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.</i>
---	---

BRIGHT LIGHT

	<i>Warning: BRIGHT LIGHT HAZARD. The interior of the PhenolMager 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.</i>
---	--

Chapter 1: Introducing the Phenolmager Fusion

Section	Page
Intended Use	12
Principles of Operation	12
Akoya Software	13
Key Concepts of Multispectral Imaging	13

INTENDED USE

- The Phenolmager® 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 Phenolmager Fusion should only be used by appropriately trained operators. Akoya highly recommends reading this guide prior to system operation.
- Failure to comply with the instructions in this guide may pose a danger to the operator.

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

PRINCIPLES OF OPERATION

- Akoya's Phenolmager 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 Phenolmager Fusion is configured to visualize up to 6-plex Opal staining on a Slide Carrier that can carry up to 4 slides.
- The Phenolmager Fusion paired with PhenoCycler® also enables imaging of 50+ biomarkers per sample using the PhenoCycler®-Fusion workflow.
- The Phenolmager 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 Phenolmager Fusion system includes the following Akoya software:

Phenolmager Fusion

Phenolmager Fusion is an operator-centric software for performing whole slide scans and PhenoCycler Protein experiments. The Phenolmager Fusion software runs on the workstation connected to the Phenolmager 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 used for the analysis of Phenolmager 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 Phenolmager Fusion computer or other Microsoft® Windows® 10 computers. Additional inForm software seats separate from those that come with each Phenolmager Fusion system are available for purchase.

Example Applications

Examples of Phenolmager 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 Phenolmager 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.

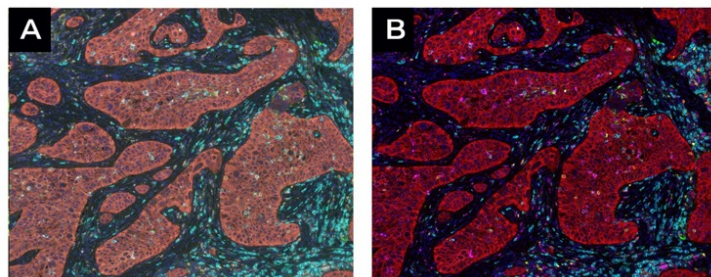


Figure 1.1. Human lung cancer tissue with autofluorescence (A) and the same tissue with reduced autofluorescence (B).

Photobleaching

Dyes can be damaged by intense light that reduces 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.

Blue light or UV light are generally the most damaging. The PhenolMager 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 the image. This setup enables repeated measurements with minimal effect on the sample.

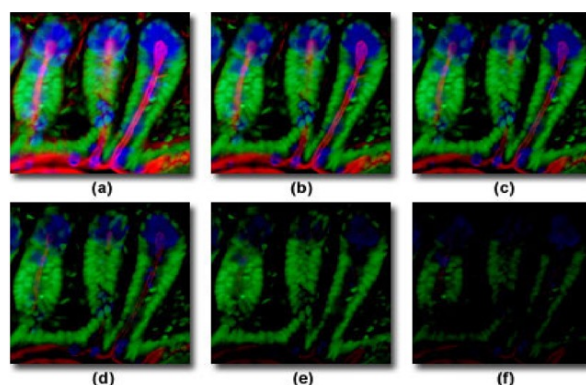


Figure 1.2. Differential photobleaching in multiplex tissue. Emission signals from 3 different dyes in 2-minute time intervals from a-f show the effects of photobleaching. Image from Molecular Expressions™ Optical Microscopy Primer: Fluorescence - Photobleaching - Interactive Tutorial, <https://micro.magnet.fsu.edu/primer/java/fluorescence/photobleaching/>

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, PhenolMager 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.)

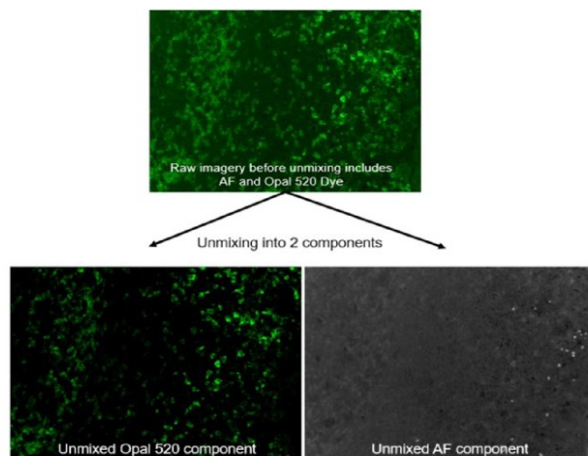


Figure 1.3. Removing autofluorescence with multispectral imaging.

Multispectral Analysis

The PhenolMager 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.)

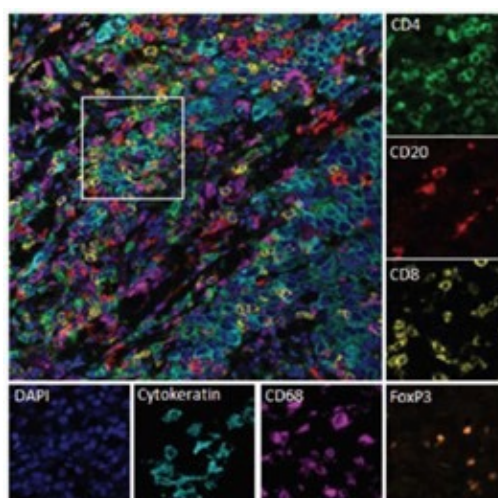


Figure 1.4. Unmixed multispectral image of human breast cancer tissue stained against CD4 (green), CD20 (red), CD8 (yellow), FoxP3 (orange), CD68 (purple), Cytokeratin (cyan), and DAPI (dark blue) using Opal reagents.

Chapter 2: Instrument Overview

Section	Page
System Overview	16
Left Side View	16
Right Side View	18
Rear View	19
Ventilation Requirements	19
Input/Output Connection	20
System Computer and Monitor	21
Phenolmager Slide Carriers	24
PhenoCycler Flow Cell Slide Carriers	24
Bottom View	25

SYSTEM OVERVIEW

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

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

- Automated XYZ Stage.
- Slide Carriers (Phenolmager 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

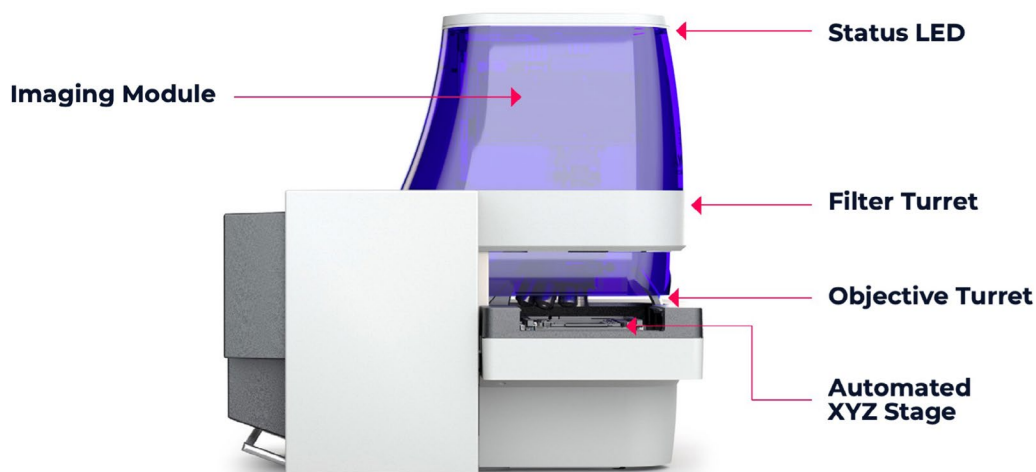


Figure 2.1. The left-side view of the PhenolMager Fusion Instrument. Parts are described in Table 2.1.

Table 2.1. Left-side view of hardware components

Part	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

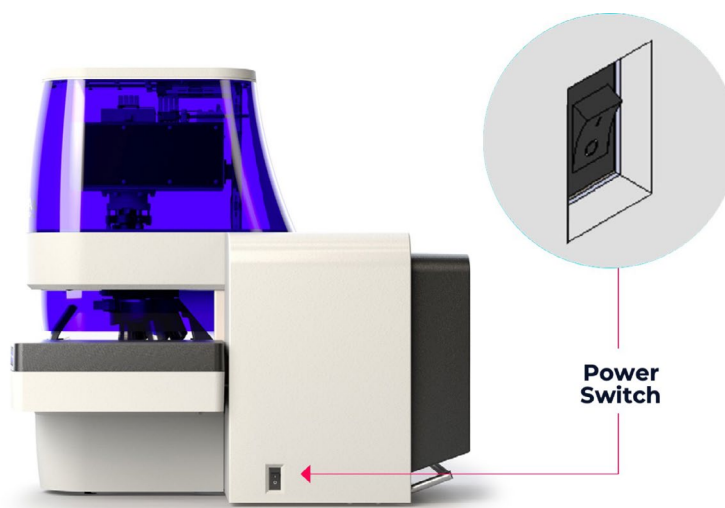


Figure 2.2. The right-side view of the PhenolMager Fusion. Parts are described in Table 2.2.

Table 2.2. Right-side view of hardware components

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

REAR VIEW

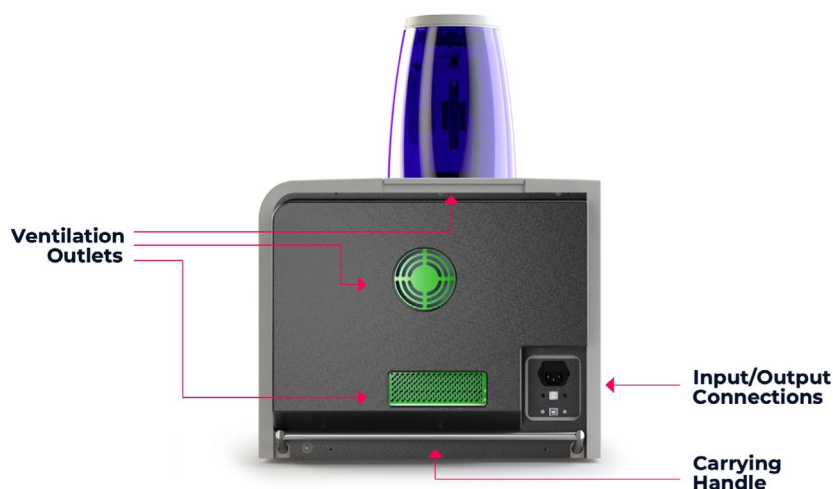


Figure 2.3. Phenolmager Fusion rear view.

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 Phenolmager Fusion instrument. Installing, servicing, and moving the Phenolmager 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.

VENTILATION REQUIREMENTS

There are 3 ventilation outlets on the enclosures of the Phenolmager 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

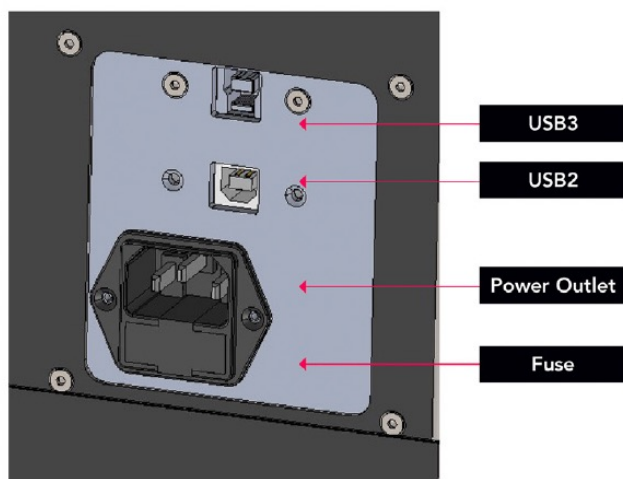


Figure 2.4. Details of input/output connections on the rear panel.

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 Phenolmager 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.

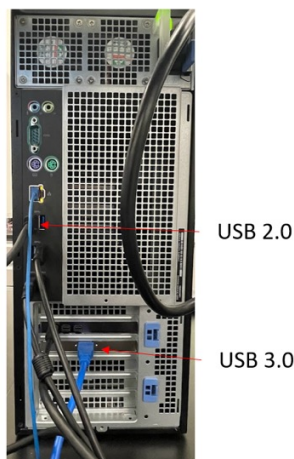


Figure 2.5. Phenolmager Fusion computer connections.

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.

PHENOIMAGER SLIDE CARRIERS

- The Phenolmager Slide Carrier is used for Phenolmager imaging of stained and coverslipped microscope slides. This carrier is not intended for other types of slides besides stained and coverslipped slides.
- The Phenolmager 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 assembled 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 Phenolmager 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 Phenolmager Slide Carrier and the PhenoCycler Flow Cell Slide Carrier

SLIDE Carrier	Width	Height	Thickness
Phenolmager 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

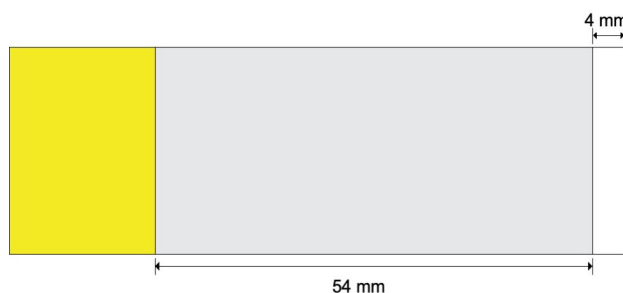


Figure 2.6. Approximate accessible imaging area (in gray) for Phenolmager Slide Carrier. The smaller square on the left (in yellow) indicates the slide label.

NOTE The Phenolmager is compatible with #1 and #1.5 coverslips.

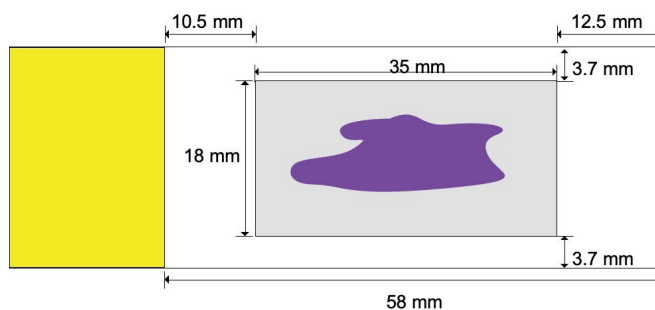


Figure 2.7. Approximate accessible imaging area (in gray) for PhenoCycler Flow Cell Carrier. The sample is illustrated in the center of the imaging area (in purple). The smaller square on the left (in yellow) indicates the slide label.

The PhenolMager 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 PhenolMager Fusion software. Parts are described in Table 2.7.



Figure 2.8. PhenolMager Slide Carrier for the PhenolMager workflow.

Table 2.7. PhenolMager Slide Carrier hardware components

Components	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 CARRIERS

Assembled Sample Flow Cells are used for PhenoCycler experiments and can be placed in either Position 1 or Position 2, or both, depending on the number of samples (1 or 2) applied in a run.

The PhenoCycler Flow Cell Slide Carrier interfaces with the PhenoCycler system via 4 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.

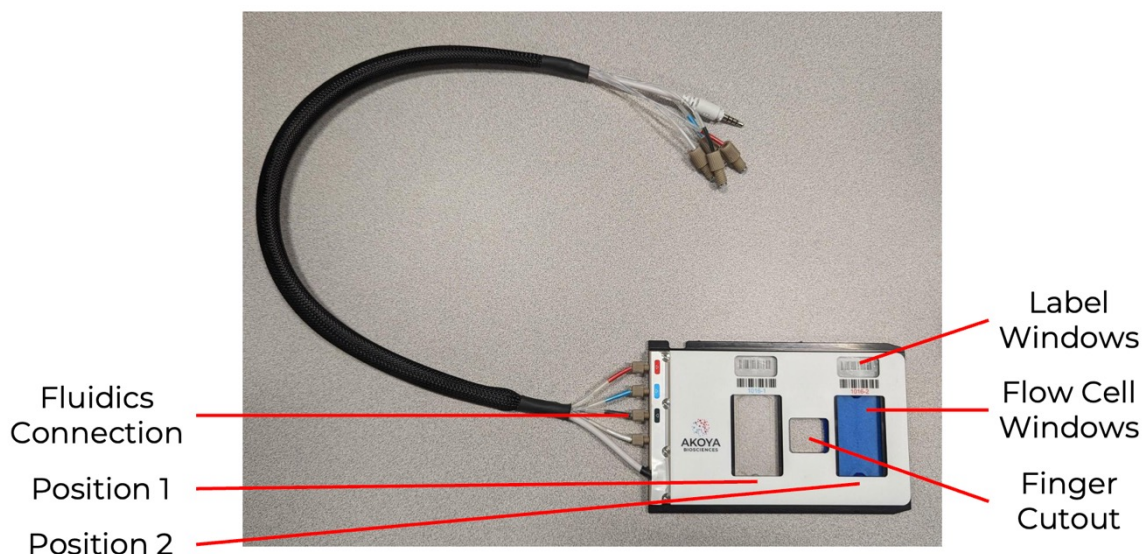


Figure 2.9. Top view of PhenoCycler Flow Cell Slide Carrier.

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

Carrier Component	Description
Flow Cell Windows	Allow imaging of the sample in the Flow Cell through a clear cutout.
Label Windows	Allow imaging of the slide label.
Finger Cutout	Provides access to remove the Magnetic Slide Retainer.
Fluidics Connections	Connect the PhenoCycler Flow Cell Slide Carrier to the PhenoCycler unit by 4 Quick Connect fittings (not shown).

BOTTOM VIEW

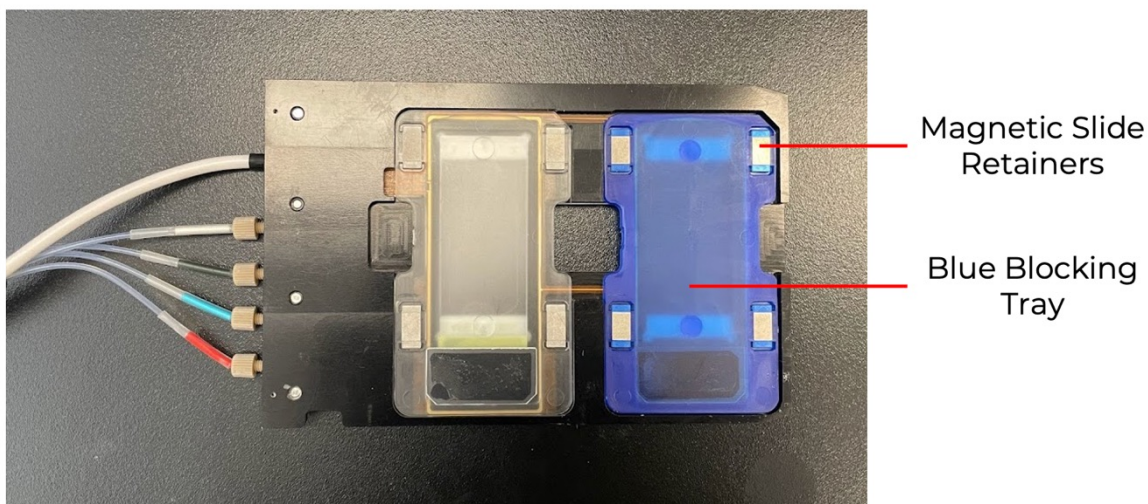


Figure 2.10. Bottom view of PhenoCycler Flow Cell Slide Carrier with Blue Blocking Tray (right).

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

Carrier Component	Description
Magnetic Slide Retainers	Securely hold the Flow Cell in the Slide Carrier by way of transparent retaining trays.
Blue Blocking Tray	Blocks Flow Cell carrier inlet and outlet ports in unused Flow Cell slot.



Figure 2.11. Assembled Sample Flow Cell. For details on loading the Sample Flow Cell into the carrier, see [Setting Up PhenoCycler Flow Cell Slide Carriers](#).

Chapter 3: Getting Started

Section	PAGE
Introduction	26
System Startup	26
Setting Up Phenolmager Slide Carriers	27
Setting Up PhenoCycler Flow Cell Slide Carriers	28
Loading the Flow Cell Slide Carrier into the automated XYZ Stage	39

INTRODUCTION

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

If not familiar with the Phenolmager Fusion hardware, review the description of each hardware component in the Phenolmager Fusion system in [Chapter 2: Instrument Overview](#).

SYSTEM STARTUP

Start the Phenolmager Fusion system in 2 steps:

1. Turn on the Phenolmager Fusion instrument.
2. Launch the Phenolmager Fusion software.

Turning on the Phenolmager Fusion Instrument

To start the Phenolmager Fusion instrument:

1. If necessary, plug the Phenolmager 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 Phenolmager 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 Phenolmager Fusion Software

To launch the Phenolmager Fusion software:

1. Double-click the Phenolmager Fusion icon on the Windows 10 desktop.
2. The Phenolmager Fusion home page opens. See [Chapter 4: Phenolmager 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 2.5)
- Do not use broken or damaged slides.
- Only use slides that are free of debris such as fingerprints, dust, and hair.

Inspecting Phenolmager Slide Carriers

To inspect the Phenolmager Slide Carrier:

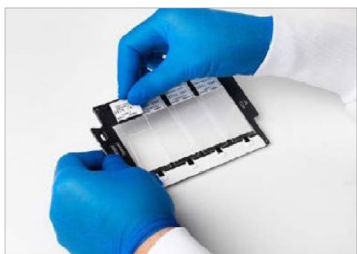
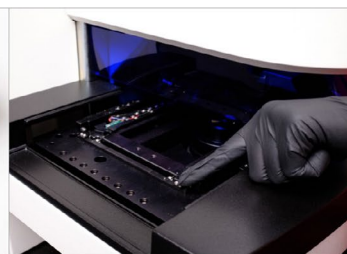
- Verify the Slide Carrier Tab Cover is secure.
- Do not use Slide Carriers that are warped or bent.
- If any sticky residue is on the carrier handle or outer surface, clean the Slide Carrier before use. (See [Chapter 8: Cleaning and Maintaining the Phenolmager Fusion](#).)

Loading Slides into the Phenolmager Slide Carriers

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

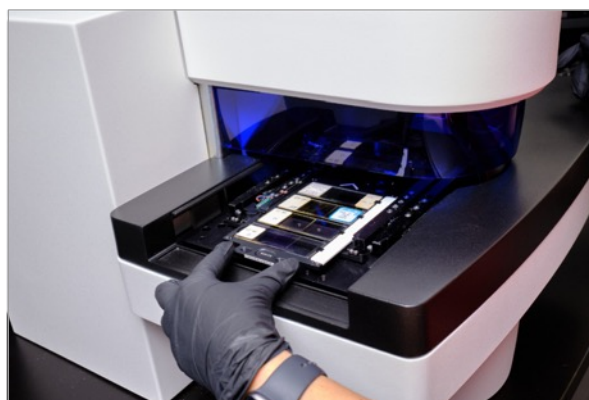
To load a slide into a Phenolmager Slide Carrier:

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 3.1.)
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 Phenolmager Slide Carrier into the Automated XYZ Stage

1. Launch the Phenolmager Fusion software and wait for the system to complete initialization.
2. Slide in the carrier until the whole carrier clears the gate. (See Figures 3.3 and 3.4.)

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

Removing the Phenolmager 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 Phenolmager Slide Carriers

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

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.

Assembling the Sample 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™	3800080 or 12-550-15
10X Buffer for PhenoCycler	Akoya Biosciences	7000019
Buffer Additive for PhenoCycler-Fusion		
1X PBS	Thermo Fisher Scientific	14190144
Coplin jar	Customer Choice	N/A
Kimwipes® or lint-free cloth	Customer Choice	N/A
Flow Cells	Akoya Biosciences	240205 (10 pk) 240204 (2 pk)
Next-Gen Flow Cell Assembly Device	Akoya Biosciences	SF-000085
Compressed Air	Customer Choice	N/A
Reservoirs with Magnets, Left-Hand Side	Akoya Biosciences	SF-000113
Reservoirs with Magnets, Right-Hand Side (Note: Only needed when running DNA workflows)	Akoya Biosciences	SF-000114

CRITICAL **If the sample slide has a sticker label like the one seen below, remove the label before sample 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:

- Flow Cell/Sample slide breakage.
- 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 sample 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.*

For direction on buffer preparation see Chapter 6, Buffer Preparation.



Figure 3.7. Next-Gen Flow Cell Assembly Device.

Assemble the Sample 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 air bubbles are present, refer to [Appendix C: Inspecting Air Pockets in the Assembled Flow Cell](#).

CRITICAL A 10-minute incubation is critical to ensure the slide is RT during sample 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. (See 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**.

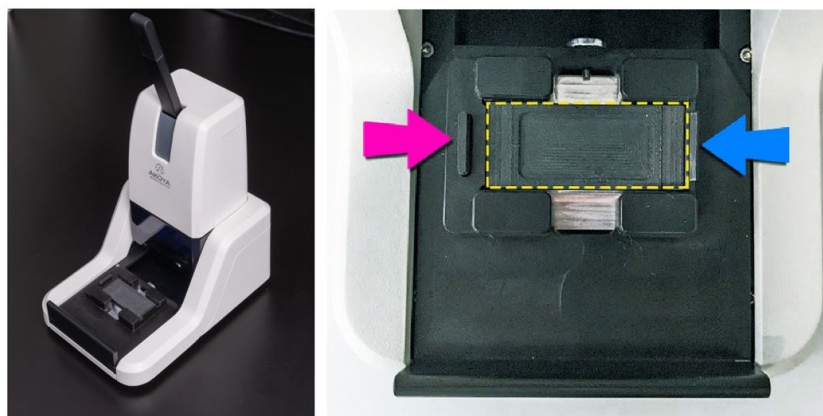


Figure 3.12. Flow Cell in the Assembly Device with handle raised and drawer pulled out (left). Close-up of the Device Drawer, top view (right). The pink arrow on the left points to the spacer bar; the blue arrow on the right points to the stop bar. The dashed yellow box in the center outlines the pocket 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. Check that the adhesive is free of lint or debris.
12. Remove the sample slide from the Coplin jar containing 1X PBS.
13. Dry the back of the slide by wiping with a Kimwipe (or equivalent). (See Figure 3.14, left.)
14. Lift the label side of the slide to allow excess PBS to run off the opposite edge and onto a Kimwipe.
15. 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.)

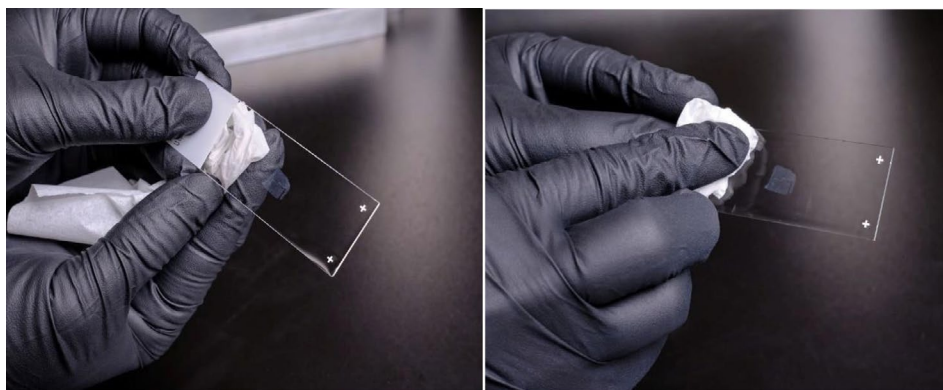


Figure 3.14. Sample slide dried with Kimwipe (left). Drying the sample slide while avoiding the sample (right).

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

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

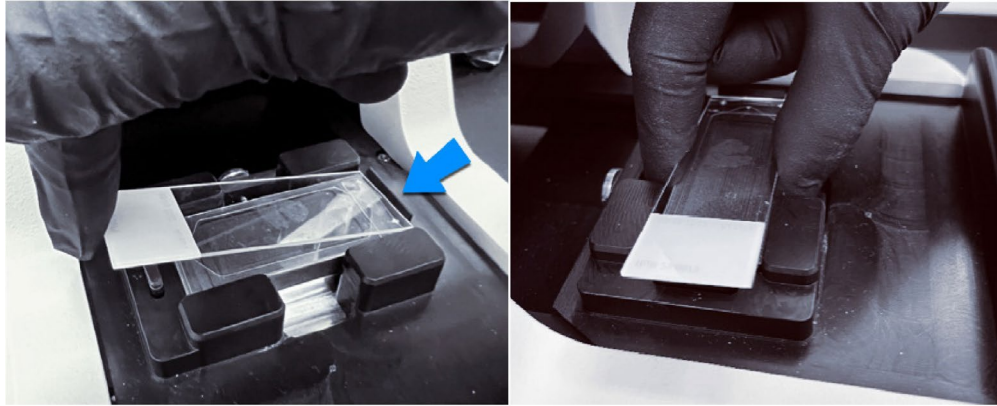


Figure 3.15. Placing the slide tissue-side down on top of the Flow Cell with the non-label side against the stop bar, as indicated by the blue arrow (left). Aligning the slide and the Flow Cell (right).

- 17.** Gently slide the drawer all the way into the close/assembly position as illustrated. (See Figure 3.16, right.)

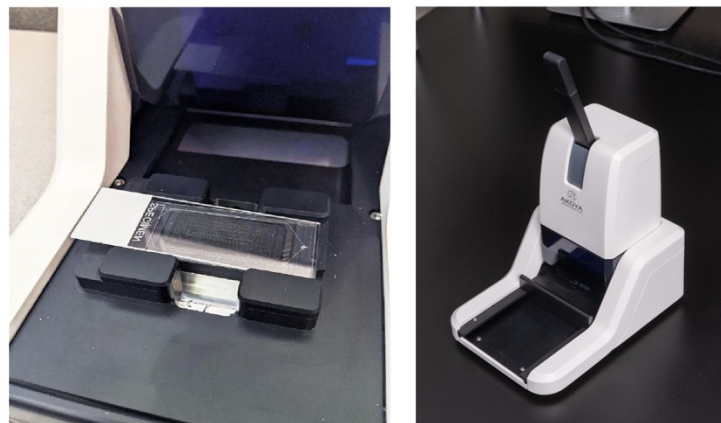


Figure 3.16. Flow Cell and Slide ready for assembly (left). The Flow Cell in the Assembly Device with the drawer in the close/assembly position (right).

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



Figure 3.17. Assembly Device with the handle completely pressed down.

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

19. Raise the handle and then pull out the drawer to remove the assembled Flow Cell with the slide adhered to it (sample Flow Cell).
20. Ensure there is no crack on the Flow Cell or the sample slide.
21. Ensure the flow cell adhesive is transparent and free of lint or debris.

CRITICAL *If the flow cell adhesive appears cloudy or there is visible lint or debris inside the adhesive, the seal is compromised and the sample flow cell should not be used.*

22. 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 sample 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.

23. Leave the assembled sample Flow Cell in 1X PhenoCycler-Fusion Buffer for **at least 10 minutes** to allow the Flow Cell to completely adhere to the slide.
24. When ready to assemble the Slide Carrier, carry the assembled sample 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 Sample Flow Cells

When inspecting sample Flow Cell components:

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

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 Sample Flow Cells 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 Flow Cells.*

1. Turn the carrier over so the Magnetic Slide Retainers are facing up.
2. Remove the Magnetic Slide Retainer using the finger cutout.
3. Remove the blank Flow Cell and set aside.
4. Remove the sample Flow Cell from the Coplin jar containing 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. Carefully wipe both the top of the flow cell and the bottom slide.

NOTE *If salt precipitates are observed, use ddH₂O followed by 70% ethanol to clean.*

6. Place the sample 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.*

NOTE *Ensure the leak detection circuit is clean and dry. See instructions in [Appendix A: Troubleshooting](#).*

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

NOTE *If running a single protein assay, insert sample Flow Cell into Position 1 and use the Blue Blocker Tray in Position 2.*

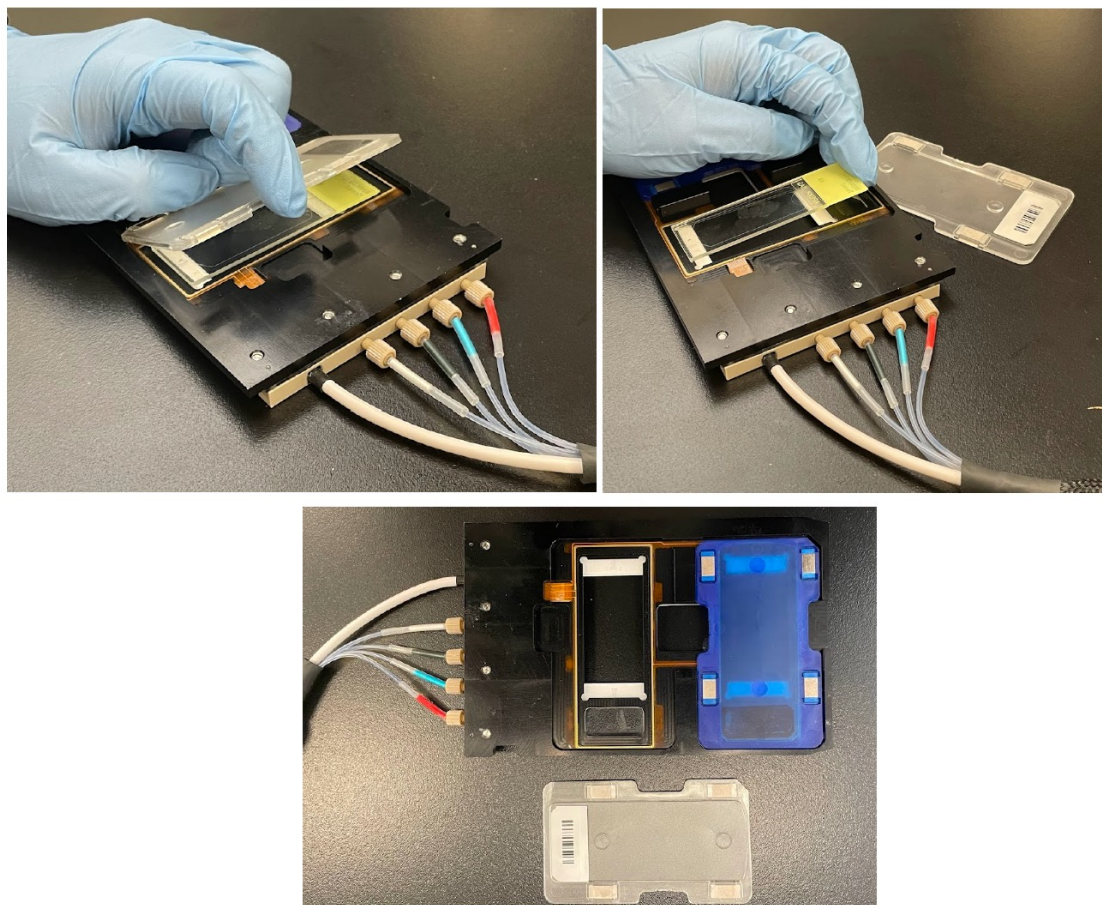


Figure 3.19. View of carrier as the Magnetic Slide Retainer is being removed (top left). Carrier being loaded with a Flow Cell into Position 1 (top right). Carrier with the sample Flow Cell successfully loaded (bottom).

LOADING THE FLOW CELL SLIDE CARRIER INTO THE AUTOMATED XYZ STAGE

If the Flow Cell Slide Carrier is not already connected to the PhenoCycler, attach the 4 tubing connectors and leak detection cable to the PhenoCycler fluidics by matching the color indicators. (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.

NOTE After leak check, ensure the Flow Cell sample does not have air pockets. If air pockets are visible, remove the Flow Cell sample and use manual syringe to remove air pockets as instructed in [Appendix C: Inspecting Air Pockets in the Sample Flow Cell](#).

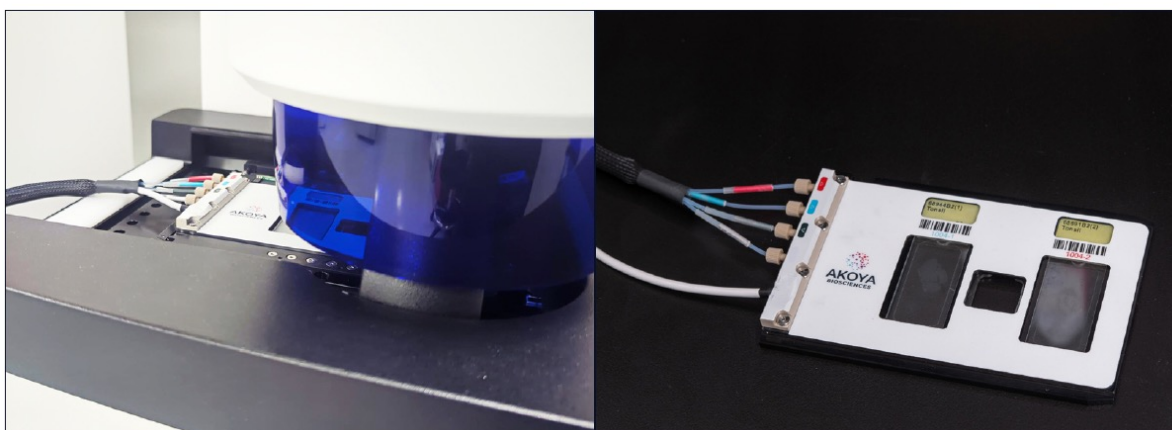


Figure 3.20. Fluidics connectors from the Flow Cell Slide Carrier (left) and the corresponding connectors on the PhenoCycler instrument (right).

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

CRITICAL All tubing connections must be connected to the Flow Cell Slide Carrier and instrument regardless of running 1 or 2 samples.

Once prompted by the software, slide the Flow Cell Slide Carrier into position. If the carrier doesn't slide in easily, re-check the fit of the Magnetic Slide Retainer and/or the fit of the Flow Cell in the carrier. Proper whole-system setup is illustrated in Figure 3.21.

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

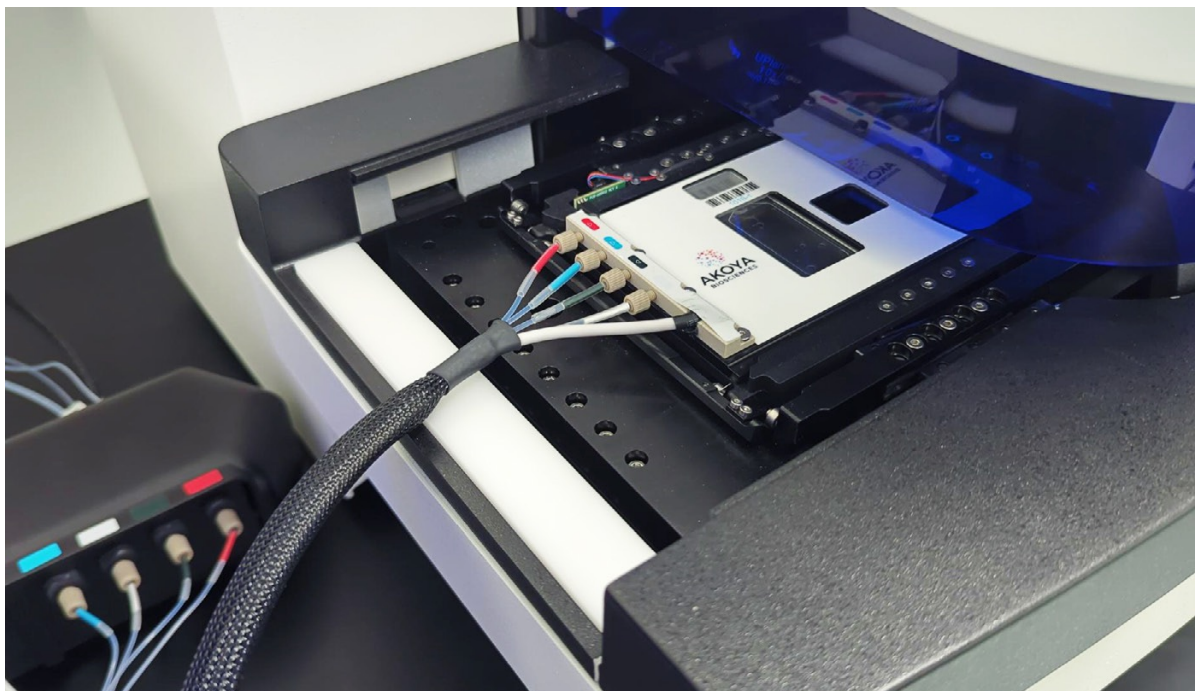


Figure 3.21. Whole-system setup.

Removing the Slide Carrier from the Automated XYZ Stage

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

CRITICAL Do not pull the Slide Carrier by the sheath, tubing, or leak detector cable.

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.)
3. Carefully remove each Flow Cell from the Slide Carrier by the sample slide label. (See Figure 3.19).

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

Using the Blue Blocker Tray

If running a single protein sample, place Flow Cell sample in Position 1 and use a Blue Blocker Tray in Position 2, as shown in Figure 3.22.

NOTE Before inserting the Blue Blocker Tray, ensure the Leak Detection Circuit is clean and dry. See instructions in [Appendix A: Troubleshooting](#).



Figure 3.22. Blue Blocker Tray in Position 2.

Chapter 4: Phenolmager Fusion Software Overview

Section	Page
Phenolmager Fusion Software Home Page	42
Fusion Dashboard	44

PHENOIMAGER FUSION SOFTWARE HOME PAGE

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

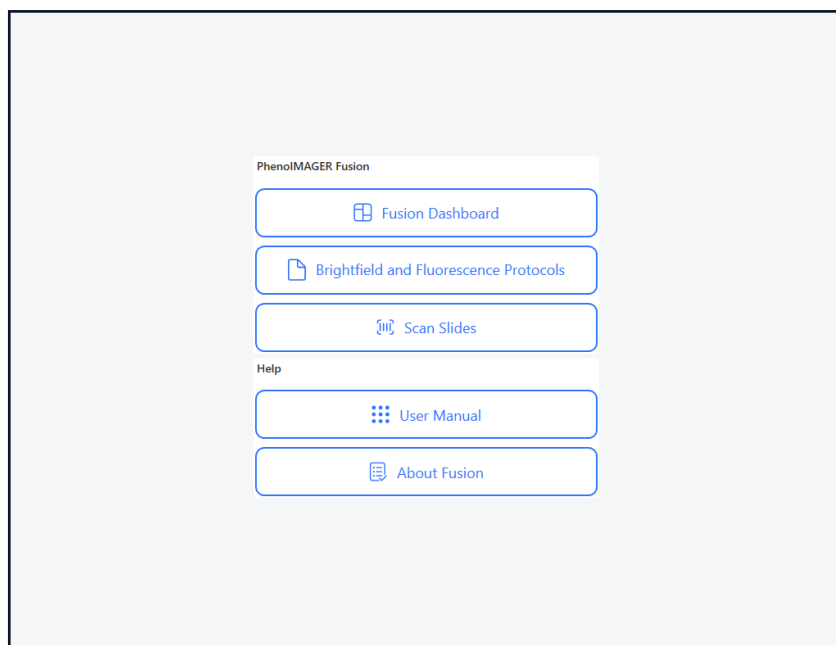


Figure 4.1. Phenolmager Fusion Home Page view when configured **without** the PhenoCycler instrument.

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

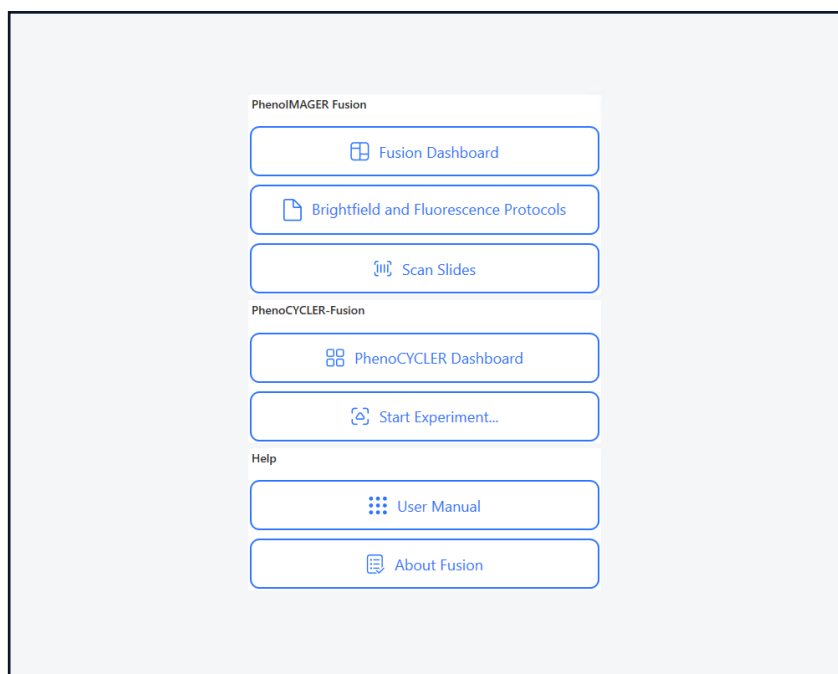


Figure 4.2. Phenolmager Fusion Home Page view when configured **with** the PhenoCycler instrument.

The Home Page buttons link to the following pages:

- **Fusion Dashboard** — Maintenance tasks related to the Phenolmager Fusion microscope.
- **Brightfield and Fluorescence Protocols** — Create or edit scanning protocols for conventionally coverslipped brightfield (BF) and fluorescent (FL) slides. (See [Chapter 5: Phenolmager Workflow with Brightfield and Fluorescence](#).)
- **Scan Slides** — Select the scanning rules and scan the slides. (See [Chapter 5: Phenolmager 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 the installed user guide.
- **About Fusion** — Fusion software plus patent information.

FUSION DASHBOARD

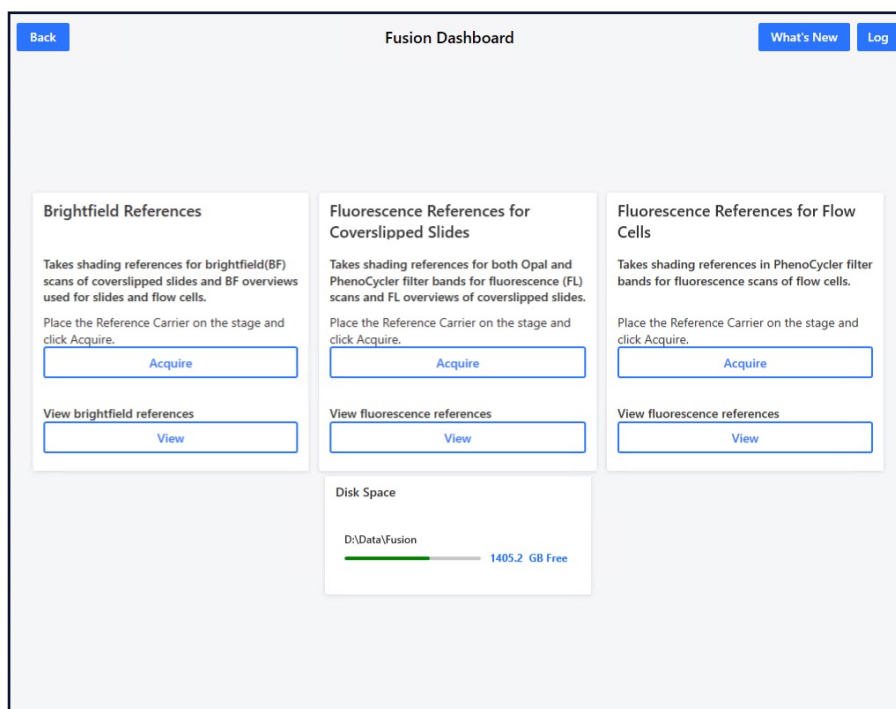


Figure 4.3. PhenolMager Fusion Dashboard page.

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.

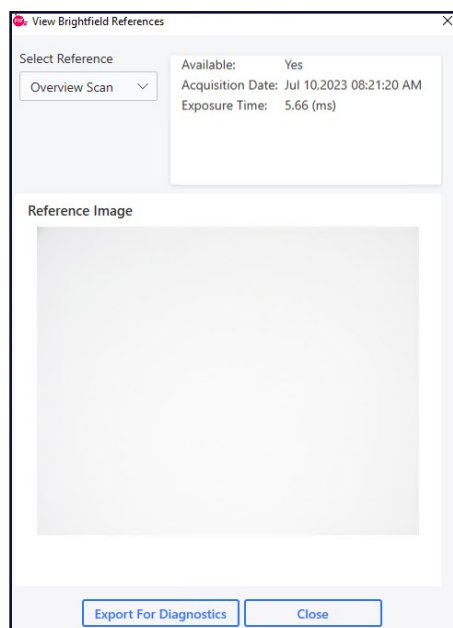


Figure 4.4. View Brightfield References window.

Fluorescence References for Coverslipped Slides

Acquiring Fluorescence References for 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.

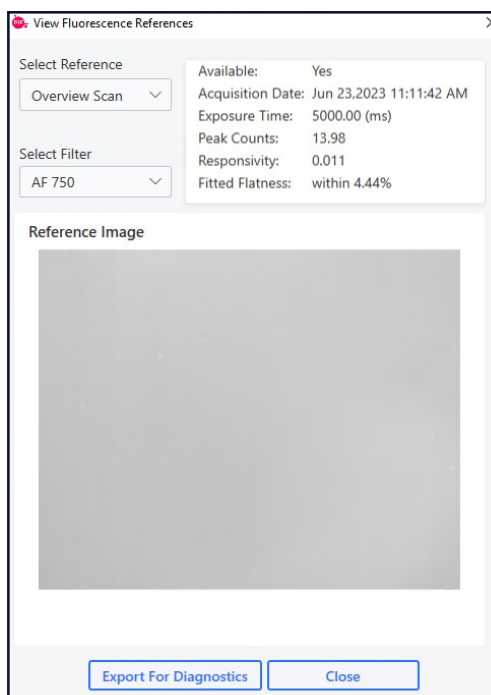


Figure 4.5. View Fluorescence References window.

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.

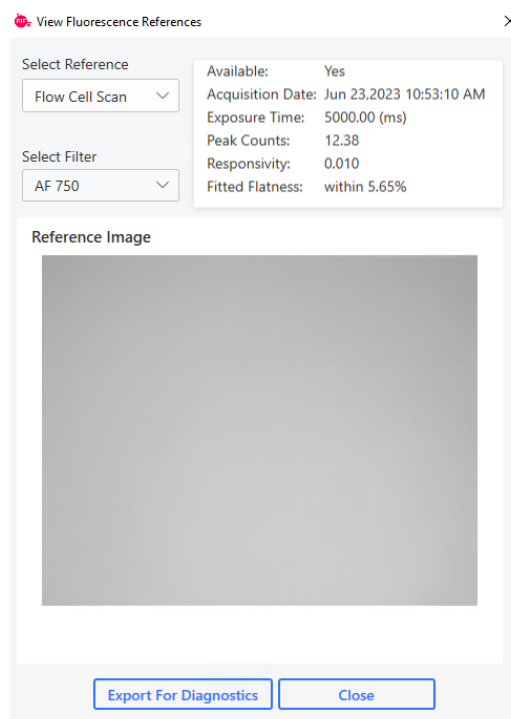


Figure 4.6. View Fluorescence References for Flow Cells window.

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 $1/16^{\text{th}}$ 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 be cleared up and maintained at green.

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

Chapter 5: Phenolmager Workflow with Brightfield and Fluorescence

Section	Page
Creating Protocols	48
Editing Protocols	50
Acquiring Snapshots for Spectral Libraries	54
Scanning Slides	56

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

- Creating and Editing Protocols
- Acquiring Snapshots for Spectral Libraries
- Scanning Slides

CREATING PROTOCOLS

Studies

- Phenolmager Fusion stores scanned data by the study. A study is a group of ≥ 2 slides that belong together. 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]. 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 Phenolmager 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].

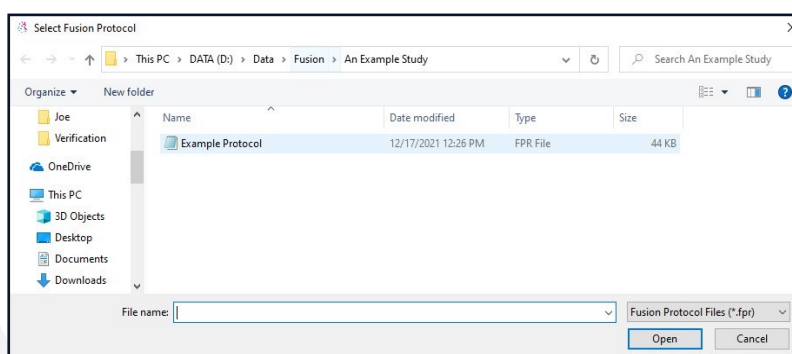


Figure 5.1. Opening a protocol.

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 PhenolMager 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.

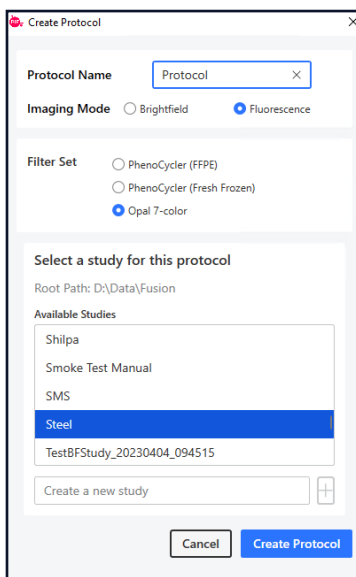


Figure 5.2. Create a protocol.

EDITING PROTOCOLS

After creating the BF or FL protocol and assigned it to a study, use the Edit Protocol screen to add specific details to the protocol. While editing fluorescence protocols, snapshot images can also be acquired. The next sections are organized by these 2 types of protocols:

- Brightfield Protocols
- Fluorescence Protocols

Brightfield Protocols

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

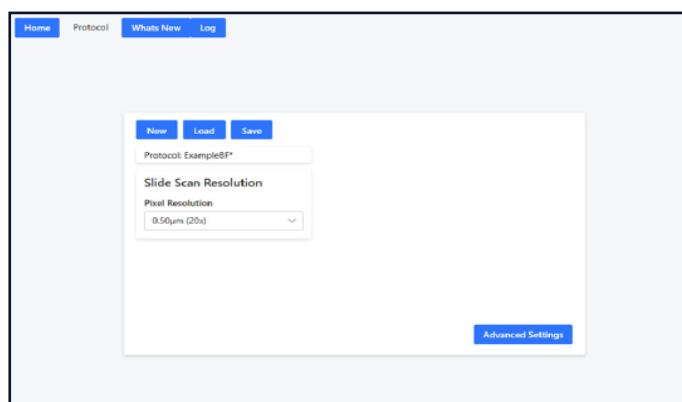


Figure 5.3. Edit protocol window.

Under Slide Scan Resolution, choose the Pixel Resolution to be used 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. (See Figure 5.4.)

Sample Finding

PhenolMager 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 the 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 you prefer lossless compression instead, select LZW rather than JPEG in the drop-down box.

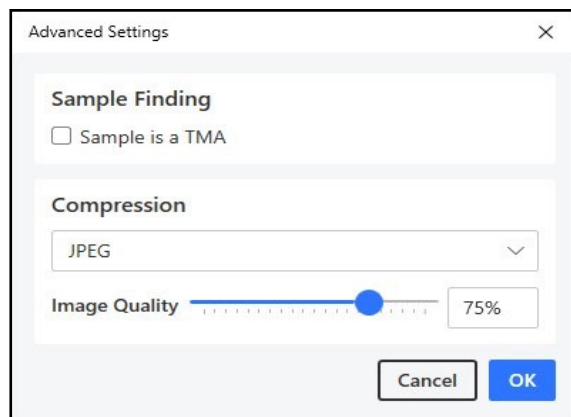


Figure 5.4. Advanced settings for brightfield protocol.

Fluorescence Protocols

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

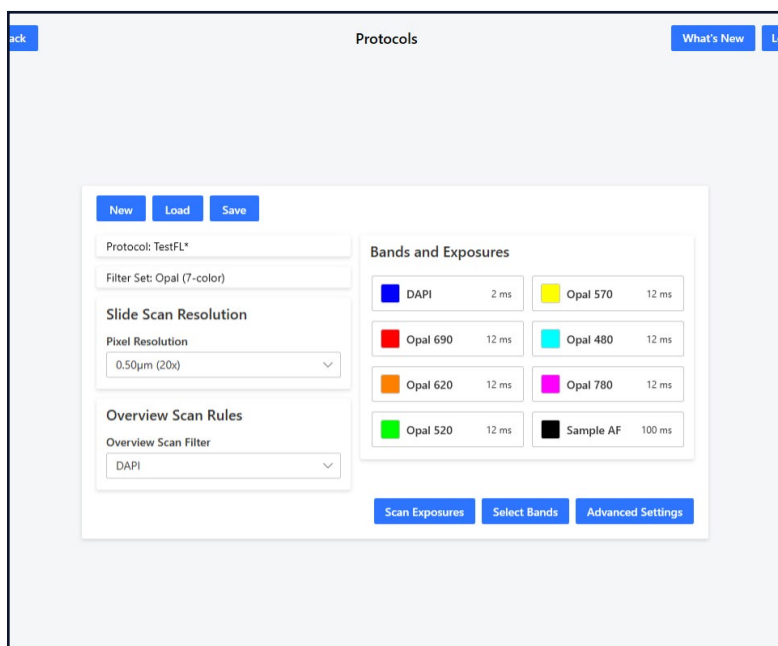


Figure 5.5. Edit Protocol window.

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

Filter Bands

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

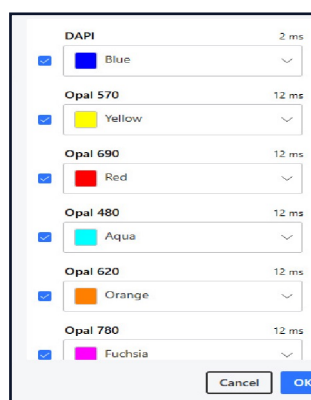


Figure 5.6. Set Filters window.

NOTE If the Overview or Focus band are removed, PhenolMager Fusion will select new bands. Navigate 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. This allows editing of the exposure times in milliseconds for each filter by manually or by clicking the Autoexpose button.

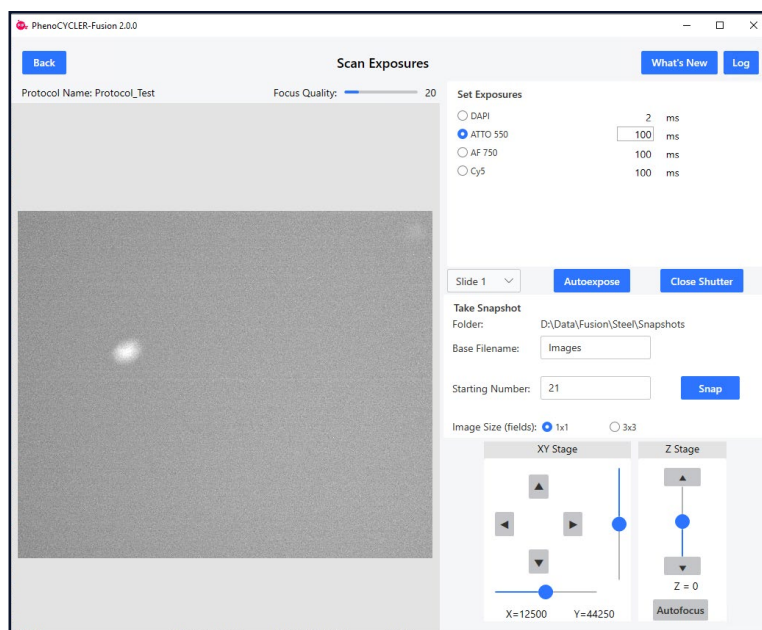


Figure 5.7. Scan Exposures window.

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 filter 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.)

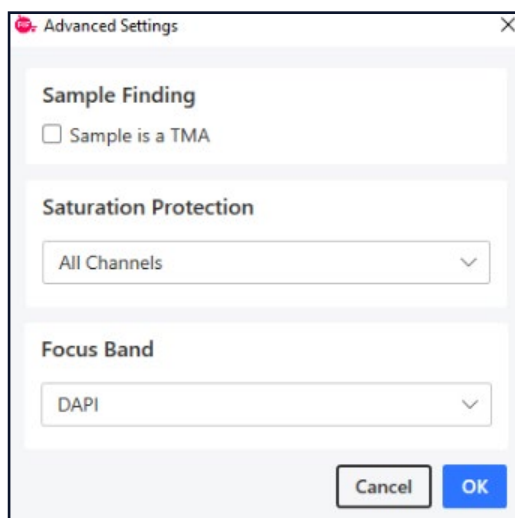


Figure 5.8. Advanced Settings window.

Sample Finding

Phenolmager 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 the samples fit this description, select Sample is a TMA.

Final Data Format

The Final Data Format setting allows one to choose whether to save the resulting QTIFF in a Standard 8-bit file format or in an Extended Range 16-bit file format. The 16-bit option provides higher dynamic range while resulting in files that are 2-3X larger than the Standard 8-bit.

Focus Band

The Focus Band setting allows one to change the filter that will be used to generate the focus map for scanning. Typically, using the default DAPI band will provide the best results. However, if the sample does not have DAPI counterstain, then choose a filter that aligns with the expression of the most common stain or autofluorescence.

ACQUIRING SNAPSHOTS FOR SPECTRAL LIBRARIES

Akoya Biosciences recommends that library spectra are updated for each instrument every 6 months or after changing the light source or the liquid light guide (this happens during preventative maintenance) to account for changes in light source intensity over time. Library slides are single-stained serial sections of tissue that are used to capture the library spectrum of each fluor to provide the most accurate unmixing.

Guidance on staining Opal spectral library slides can be found in the Opal Multiplex IHC Assay Development Guide and Image Acquisition Information.

Acquiring Library Scans in Fusion Software

1. Create a new fluorescent protocol named for each library slide.
2. Set the Overview Scan Filter to the filter that aligns with the single stained slide. Set exposure times according to the chart below. (The same exposure times apply to all library slides.)

Step	Procedure
1	Slide preparation
2	DAPI counterstain and mount

Acquiring Library Snapshots in Fusion Software

Follow the instructions below to acquire snapshot images of library slides for spectral extraction.

1. Create a new Opal 7-color protocol.
2. Navigate to an area on the first library slide that shows abundant positive staining. Auto-focusing may be necessary.
3. Set exposure times according to Table 5.2.

NOTE Column headings in Table 5.1 indicate bands in the protocol. Row headings indicate the fluor present on the slide.

Table 5.1. Guidance for setting exposures when acquiring snapshots of spectral library slides

	DAPI	Opal 480	Opal 520	Opal 570	Opal 620	Opal 690	Opal 780	Sample AF
DAPI	AE*	AE	AE	Dark**	Dark	Dark	Dark	AE
Opal 480	AE	AE	AE	Dark	Dark	Dark	Dark	AE
Opal 520	AE	AE	AE	Dark	Dark	Dark	Dark	AE
Opal 570	AE	AE	AE	AE	Dark	Dark	Dark	AE
Opal 620	AE	AE	AE	Dark	AE	Dark	Dark	AE
Opal 690	AE	AE	AE	Dark	Dark	AE	Dark	AE
Opal 780	AE	AE	AE	Dark	Dark	Dark	AE	AE
AF	AE	AE	AE	Dark	Dark	Dark	Dark	AE

*Autoexposed (AE): Autofluorescence bands that should be autoexposed.

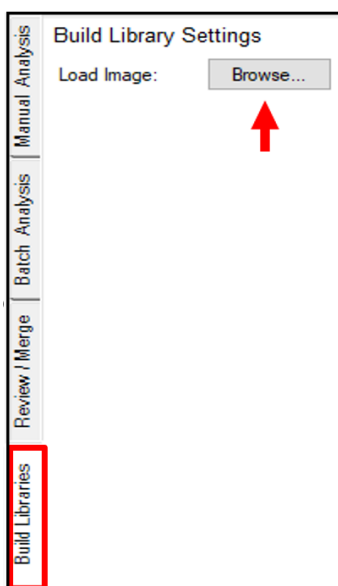
**Dark: Should be manually set to the appropriate exposure listed in Table 5.2.

Shaded cells: (running diagonally down the center of the table) Represent signal bands that should be autoexposed.

Table 5.2. Exposure times for dark bands per resolution option

0.50 μm per pixel (20x)	0.50 μm per pixel (20x) [binned from 40x]	0.25 μm per pixel (40x)
50 ms	3 ms	12 ms

4. Acquire a Snapshot of this library slide.
5. Repeat Steps 2 - 4 for each library slide. Make sure to adjust the exposure times according to Table 5.2 for each slide.
6. Open the .qptiff slide scan in Phenochart and create a stamp annotation for inForm project.
7. Follow the guidance in the inForm User Guide to extract library spectra from the Snapshots. (See Figure 5.9.)

**Figure 5.9.** Build Libraries – Browse.

SCANNING SLIDES

This section describes the process of scanning slides.

The Phenolmager 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 5.10.)

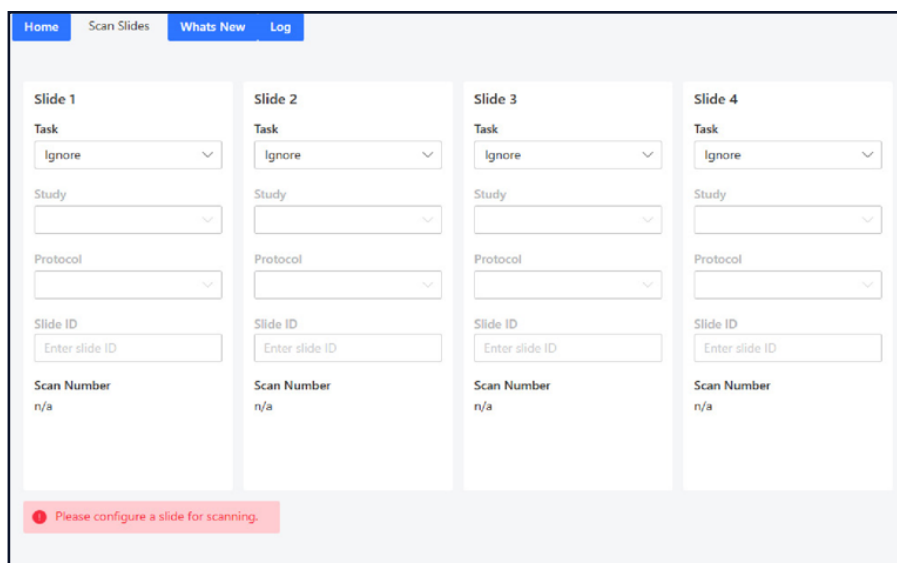


Figure 5.10. Edit Slides window.

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 the 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 Phenolmager Fusion will report the scanning progress for each slide on the screen during the scan.

When Phenolmager Fusion has completed scanning, a pop-up window summarizes the result of each slide. (See Figure 5.11.)

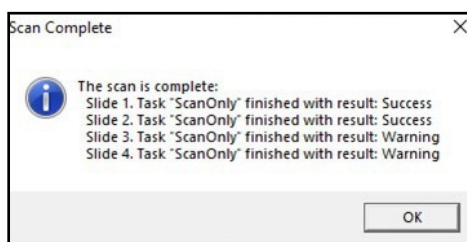


Figure 5.11. Results of Scan Complete pop-up window.

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 (High Plex Protein)

Section	Page
Overview	58
Buffer Preparation	59
Using the Run Buffer Calculator for PhenoCycler-Fusion 2-Slide Protein Runs	60
PhenoCycler Scan Page	66
Preflight Routine	69
Status and Estimated Time of Completion Indicators	81

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 are outlined in PhenoCycler-Fusion User Guide, Chapter 3 and the 96-well reporter plate preparation is outlined in PhenoCycler-Fusion User Guide, Chapter 4. Assembling sample Flow Cells is described in this [PhenolMager Fusion User Guide, Chapter 3](#).

One-sample PhenoCycler-Fusion Experiment

The following formula estimates the run time of a 1-sample PhenoCycler-Fusion experiment:

$$\text{Run Time(1-sample)} = [\text{fluidics time} + \text{scanning time}] \times (\text{number of cycles})$$

The total fluidics time is approximately 22 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.

Two-sample PhenoCycler-Fusion Experiment

When running a 2-sample PhenoCycler-Fusion experiment, the total experiment time will vary based on the size of both samples and the number of cycles for each sample. During a 2-sample experiment, fluidics operations for one sample happen while the other sample is being scanned. Since scanning times are typically longer than fluidics time, each cycle time is typically determined by the total scanning time of both samples.

The following formula estimates the run time of a 2-sample PhenoCycler-Fusion experiment

where both experiments include the same number of cycles:

- **Run Time(2-sample) = initial fluidics time + (sample 1 scanning time + sample 2 scanning time) x (number of cycles)**

The total fluidics time is approximately 22 minutes per cycle. The scanning time depends on the size of each of the tissues being imaged and is approximately 25 minutes for a 1.5 cm x 1.5 cm scan area.

If one sample includes n fewer cycles than the other sample, then the scanning time for that sample should be replaced by the fluidics time for n cycles in the formula above.

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

BUFFER PREPARATION

Materials 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.
Reservoirs with Magnets, Left-Hand Side		Qty 1 included with kit/instrument. Additional units can be purchased using P/N: SF-000113.
Reservoirs with Magnets, Right-Hand Side (Note: Only needed when running RNA workflows)		Qty 1 included with kit/instrument. Additional units can be purchased using P/N: SF-000114.

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 ₂ O or Milli-Q® H ₂ O	Customer Choice	N/A
Plastic Consumables/Tools	Compressed Air Duster	Customer Choice	N/A
	Kimwipes or lint-free cloth	Customer Choice	N/A
	Magnetic stir plate and bar	Customer Choice	N/A

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 run can be started.

Equilibrate Reagents to Room Temperature (RT)

- Retrieve the sealed, pre-loaded 96-well reporter plate containing the Reporter Master Mix solutions from 4°C. For reporter plate preparation, see the 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 for 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.

Below are two methods of calculating buffer preparation. The PhenoCycler-Fusion Run Buffer Calculator available in the PhenoCycler-Fusion Experiment Designer Software will ensure there is enough buffer prepared for the intended experiments. Alternatively, bulk buffer can be prepared using the amounts given or scaled accordingly.

USING THE RUN BUFFER CALCULATOR FOR PHENOCYCLER-FUSION 2-SLIDE PROTEIN RUNS

The PhenoCycler-Fusion 2.0 Run buffer calculator is found on the Home screen of the PhenoCycler Experiment Designer software (see Figure 6.1) and is designed to assist in estimating the volumes of 1X PhenoCycler-Fusion buffer, High DMSO, and Low DMSO buffers needed for a multi-cycle protein experiment on the PhenoCycler-Fusion 2.0 system. The calculator provides the volumes for each component needed in the corresponding sidecar bottle during the experimental run.

For a single slide (position 1) experiment, the maximum number of total cycles the PhenoCycler Fusion 2.0 system can run is 64 cycles (including blank cycles). For a 2-slide run using both positions 1 and 2, you may run 63 cycles (including blank cycles) distributed across the two positions.

1. Design reporter plate using the PhenoCycler Experiment Designer app.
2. Return to the home screen. (See Figure 6.1.)
3. Calculate to determine the required volumes of 1X PCF buffer, High DMSO, and Low DMSO buffers for an experimental run.

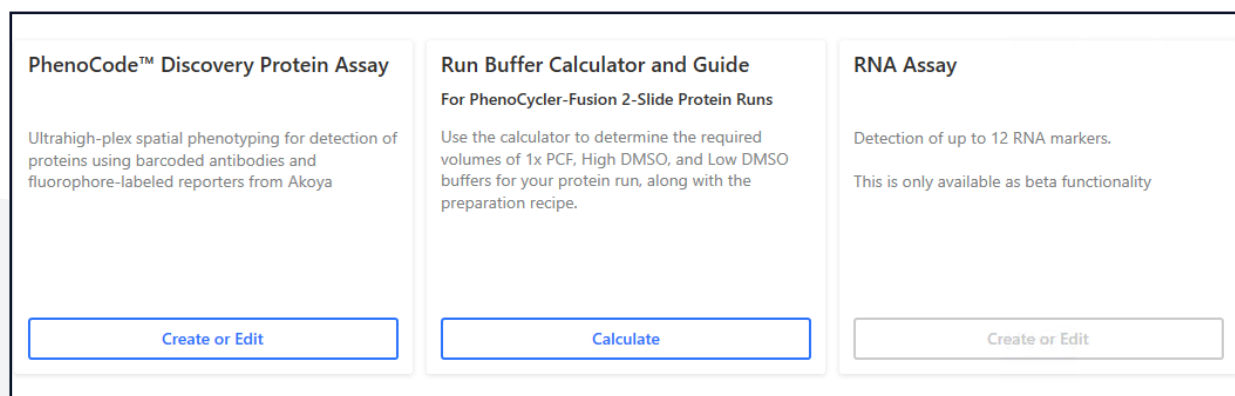


Figure 6.1. Home screen of PhenoCycler Experiment Designer.

- Click on the Load button for each individual position to load a previously designed experiment. (See Figure 6.2.)

The screenshot shows the 'PhenoCycler Experiment Designer' window. At the top left is a 'Home' button. Below it, under 'Experiments to Run:', there are two boxes for 'Position 1' and 'Position 2'. Each box contains a 'Load' button and the following text: 'Study: N/A', 'Experiment: N/A', and 'Total Number of Cycles: N/A'. Below these is the 'Run Buffers Required:' section, which contains three rows of buffer requirements. Each row has a table with two columns: 'Total volume for bottle:' and 'Recipe'. The first row is for '1x PCF Buffer', the second for 'High DMSO Buffer', and the third for 'Low DMSO Buffer'. All values in the tables are currently 'N/A mL'.

Figure 6.2. Starting Run Buffer Calculator and Guide.

NOTE If running a dual slide experiment, load an experiment into position 1 first, then load position 2.

NOTE Click on the Clear button to remove the loaded experiment into either position if needed.

- The volumes will automatically update with the required buffer volumes for the loaded experiment/s. (See Figure 6.3.)

The screenshot shows the 'PhenoCycler Experiment Designer' window with experiments loaded. The 'Experiments to Run:' section now shows 'Position 1' and 'Position 2' with 'Clear' and 'Load' buttons. The text for Position 1 is 'Study: Steel_WPA_Test', 'Experiment: Mincycles1', and 'Total Number of Cycles: 3'. The text for Position 2 is 'Study: Steel_WPA_Test', 'Experiment: Mincycles2', and 'Total Number of Cycles: 3'. The 'Run Buffers Required:' section now shows populated values for the three buffer types. The first row is for '1x PCF Buffer' with a total volume of 350 mL and a recipe of 35 mL 10x Buffer, 35 mL Buffer Additive, and 280 mL DI water. The second row is for 'High DMSO Buffer' with a total volume of 100 mL and a recipe of 90 mL DMSO and 10 mL 1x PCF Buffer. The third row is for 'Low DMSO Buffer' with a total volume of 150 mL and a recipe of 30 mL DMSO and 120 mL 1x PCF Buffer.

Figure 6.3. Example buffer Calculator with experiments loaded in both positions.

6. Transfer the volumes displayed to a secondary container using a serological pipet, graduated cylinder, or other volume measuring device.
7. Gently mix the solutions thoroughly using a serological pipet or magnetic stir bar.
8. Allow solutions to cool for at least 15 minutes before adding to the appropriate sidecar bottle for an experimental 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 ddH₂O.
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.

Storage

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

Use

This 1X PhenoCycler-Fusion Buffer solution is used:

- To fill the buffer bottle in the PhenoCycler sidecar. (See Figure 6.4.)
- For the reporter stock solution in the 96-well plate.
(See PhenoCycler-Fusion User Guide, Chapter 5.)
- For the incubation step after mounting the [Flow Cell](#).
- To dilute the DMSO for High and Low DMSO buffers.

Prepare the Low DMSO and High DMSO Buffer Solutions

Preparation and storage of low and high DMSO solutions should occur in separate, clean, amber, glass bottles at ambient RT. See Total Final Volume in Table 6.3 to determine the volume size of the amber bottle required.

CRITICAL *Use a graduated cylinder or pipet aid to measure and transfer DMSO and 1X PhenoCycler-Fusion Buffer. Inaccurate dilution can result in poor signal or carryover between cycles.*

1. Identify and label 2 appropriate amber glass containers for the preparation and storage of the low DMSO and high DMSO solutions. Place a PTFE- or Teflon-coated mixing bar into the bottom of each container if using a mixing bar to stir. Include the date of preparation on the label to track solution usability.
2. Following the Table 6.3, transfer the appropriate volumes of DMSO into their designated low DMSO and the high DMSO containers.
3. Following the Table 6.3, transfer the appropriate volumes of 1X PhenoCycler-Fusion Buffer into the corresponding low DMSO container and high DMSO container.
4. Mix the low DMSO and high DMSO solutions with the mixing bar and a magnetic stir plate set on medium for 5 minutes with the container lid off. The stirring motion should be gentle with no vigorous agitation; adjust the magnetic stir plate setting accordingly. Alternatively, using a pipet aid, pipet up and down at least 15 times to thoroughly mix.
5. Allow both solutions to cool for at least 15 minutes.
6. Remove the low DMSO and high DMSO bottles from the sidecar of the PhenoCycler instrument for filling. Do not fill the bottles while they are still on the sidecar to avoid spills.
7. Pour the low and high DMSO solutions into corresponding bottles from the sidecar of the PhenoCycler instrument. Store any excess solution in a cool, dry, dark place.
8. Return the filled low and high DMSO bottles to the appropriate locations on the sidecar of the PhenoCycler instrument. (See Figure 6.4.)

CRITICAL *Carefully inspect the sidecar bottle labels when pouring DMSO solutions. Swapping DMSO solutions in the sidecar bottles will result in poor signal, high background, or carryover between cycles.*

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.*

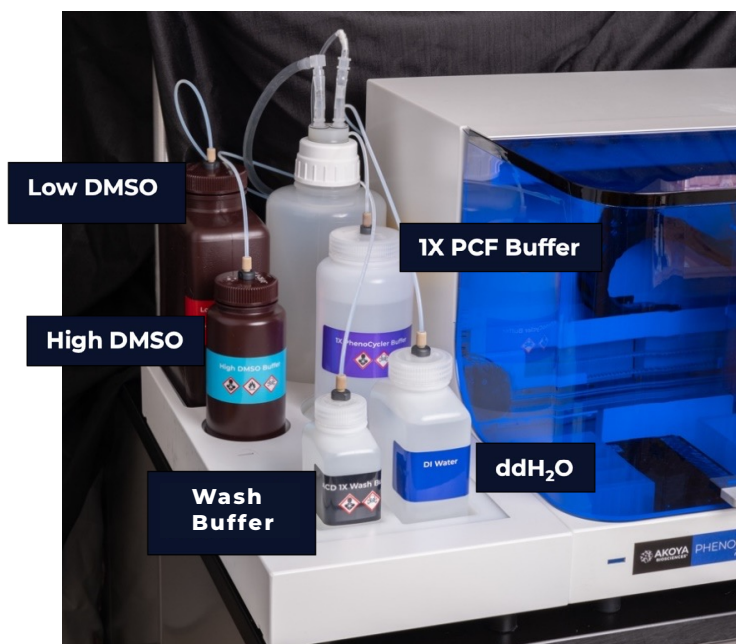


Figure 6.4. PhenoCycler-Fusion 2.0 sidecar containing buffers.

NOTE The low DMSO and high DMSO solutions can be made in advance and stored at RT for up to 2 weeks. Using solutions older than 2 weeks will jeopardize staining quality. Expired solutions should be discarded according to your institution's waste protocols. For guidance with bottle cleaning procedures, see [Appendix B: Bottle Cleaning](#).

CRITICAL DMSO solutions must remain at RT. If DMSO solutions are accidentally stored in the fridge, discard them. Solutions that have cooled to fridge temperatures can yield poor results even after re-equilibrating to RT. For guidance with bottle cleaning procedures, see [Appendix B: Bottle Cleaning](#).

TABLE 6.3. Volumes of Low DMSO, High DMSO, and 1X PhenoCycler-Fusion Buffer with Additive required for 1 slide position based on the number of marker cycles. If running 2 positions, sum up the volumes.

# of Marker Cycles	Low DMSO			High DMSO			1X PCF Buffer with Additive (mL)
	Volume of Pure DMSO (mL)	Volume of 1X PCF Buffer with Additive (mL)	Total (mL)	Volume of Pure DMSO (mL)	Volume of 1X PCF Buffer with Additive (mL)	Total (mL)	
5	40	160	200	90	10	100	150
10	50	200	250	135	15	150	150
15	60	240	300	135	15	150	200
20	80	320	400	135	15	150	200
25	90	360	450	180	20	200	250
30	100	400	500	180	20	200	250
35	120	480	600	180	20	200	300
40	130	520	650	180	20	200	350
45	150	600	750	225	25	250	350
50	160	640	800	225	25	250	400
55	170	680	850	225	25	250	400
60	190	760	950	270	30	300	450

The 2 blank cycles in each scenario are already taken into consideration, e.g., the first row shows the volumes needed for 5 marker cycles (+2 blank cycles).

This table provides the total necessary volumes of Low DMSO, High DMSO, and 1X PhenoCycler-Fusion Buffer with Additive for a single slide position, considering the number of marker cycles. In the case of running 2 slide positions, simply sum the volumes needed. The table also shows the volumes of pure DMSO and 1X PhenoCycler-Fusion Buffer with Additive required to create low DMSO and high DMSO buffer solutions.

Example: If you are running two 20-marker cycle experiments within the same run, you will need $400 + 400 = 800$ ml of low DMSO buffer and $150 + 150 = 300$ ml of high DMSO buffer. When running a single slide of 40 marker cycles, you will need 650 ml of low DMSO buffer and 200 ml of high DMSO buffer.

NOTE The volumes in the table account for the different minimum requirements of each bottle. PhenoCycler-Fusion 2.0 can run up to a total of 64 cycles (60 marker cycles). If running both slide positions, make sure the combined number of cycles does not exceed the maximum limit of 60 marker cycles.

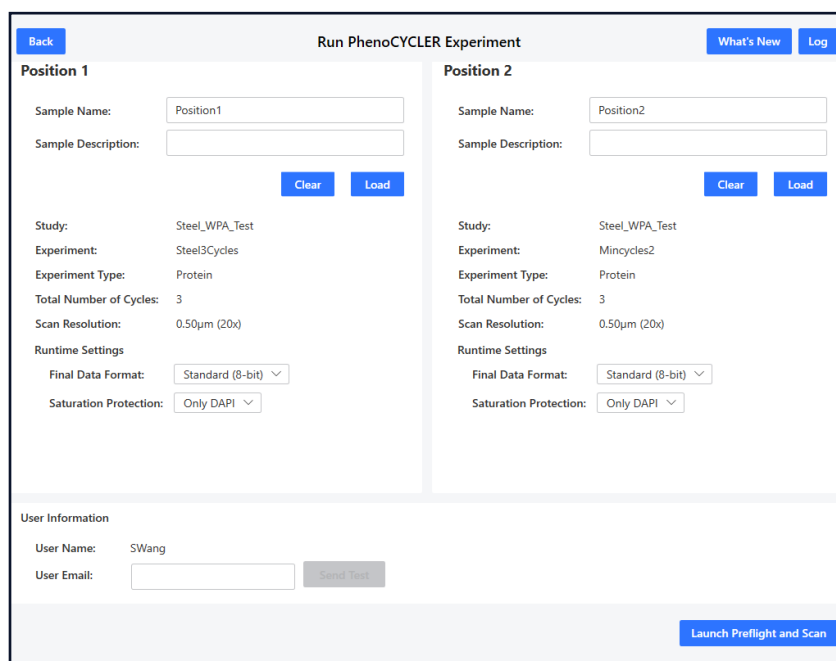
CRITICAL Avoid shaking to prevent bubble formation. If using a bottle to mix, do not invert a lid-screwed bottle to mix. This prevents leaking. If spills occur, clean with 70% ethanol.

Table 6.4. Volumes required to make low DMSO and high DMSO buffer solutions. Recommended when making buffer solutions for runs planned for the whole week.

	Volume of Pure DMSO (mL)	Volume 1X PCF Buffer with Additive (mL)	Total Final Volume (mL)
Low DMSO	100	400	500
	200	800	1000
	400	1600	2000
High DMSO	225	25	250
	450	50	500
	900	100	1000

PHENOCYCLER SCAN PAGE

To set up a PhenoCycler scan, using the PhenolMager Fusion software, click on the Start Experiment button from the Home Page to display the Load Experiments screen. (See Figure 6.5.)



Run PhenoCYCLER Experiment

Position 1

Sample Name:

Sample Description:

Study: Steel_WPA_Test

Experiment: Steel3Cycles

Experiment Type: Protein

Total Number of Cycles: 3

Scan Resolution: 0.50µm (20x)

Runtime Settings

Final Data Format:

Saturation Protection:

Position 2

Sample Name:

Sample Description:

Study: Steel_WPA_Test

Experiment: Mincycles2

Experiment Type: Protein

Total Number of Cycles: 3

Scan Resolution: 0.50µm (20x)

Runtime Settings

Final Data Format:

Saturation Protection:

User Information

User Name: SWang

User Email:

Figure 6.5. Load Experiments 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 either the Position 1 or Position 2 pane. The Load Experiment Design window will appear. (See Figure 6.6.)

NOTE If only running a single protein experiment, then Position 1 must be used.



Figure 6.6. 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.

NOTE *If 2 protein experiments are loaded, the software will perform the following validation:*

- Sample names must be unique if part of the same study.
 - If running 2 experiments (using position 1 and position 2) then they must both be protein.
 - Unique wells are selected (no overlap) in the loaded protein experiments in positions 1 and 2.
 - Waste capacity is checked (1.7 liters).
4. Use the Clear button to clear the loaded .xpd file, Sample Name, and/or Sample Description entries, as needed.
 5. Under Runtime Settings select Final Data Format and Saturation Protection from the respective pulldown menus.

The Final Data Format setting allows one to choose whether to save the final combined QTIFF in a Standard 8-bit file format or in an Extended Range 16-bit file format. The 16-bit option provides higher dynamic range while resulting in a file that is 4-5X larger than the Standard 8-bit.

CRITICAL *The Extended Range (16-bit) final QTIFF files are 4-5 X larger in size compared to Standard (8-bit) files. (Figure 6.7.)*

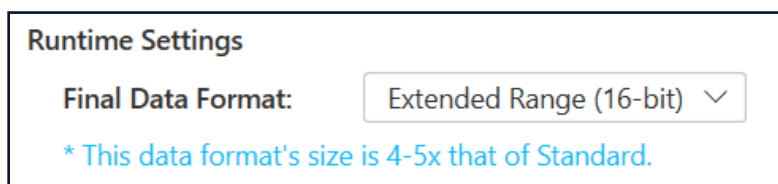


Figure 6.7. Extended range QPTIFF.

Saturation Protection option is available with the Standard (8-bit) format. It helps prevent overexposure when acquiring fluorescence imagery.

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 signal to be reduced too low for proper visualization of the sample.

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:

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

After entering 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 6.8.)

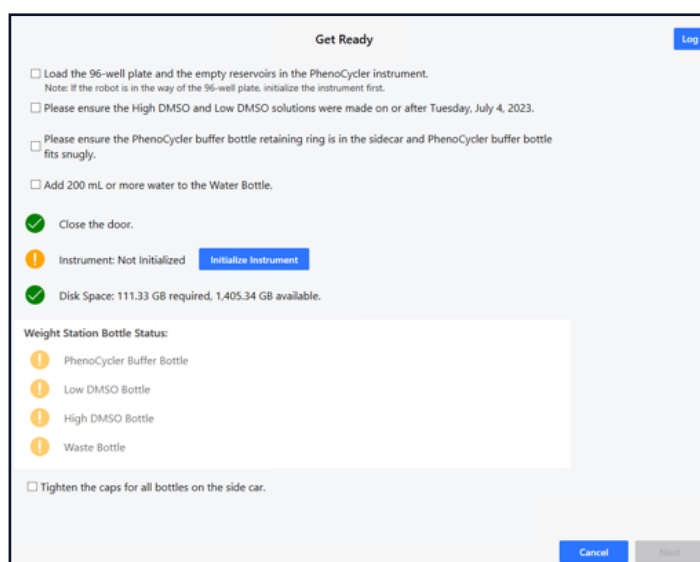


Figure 6.8. Dialog window, Get Ready.

1. Ensure that the High DMSO, Low DMSO, and 1X PhenoCycler-Fusion Buffer solutions were made within 2 weeks. For guidance on bottle cleaning, see [Appendix B: Bottle Cleaning](#).
2. Ensure the PhenoCycler buffer bottle retaining ring is in place and the bottle fits snugly.
3. Ensure the correct reporter plate corresponding to the selected experiment designs are loaded into the PhenoCycler instrument.

CAUTION A correctly loaded 96-well plate will have A1 closest to the front of the instrument and will lie flat. If the silver under the plate is visible, the 96-well plate has been loaded incorrectly. (See Figure 6.9.)

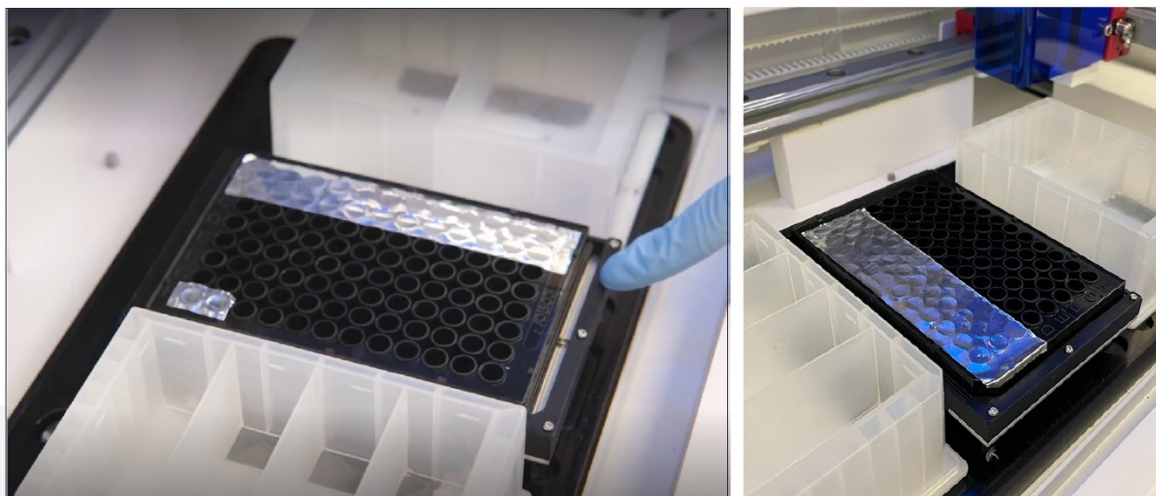


Figure 6.9. Incorrectly loaded 96-well plate (left) and correctly loaded plate (right).

4. Ensure the empty reservoirs are loaded into the PhenoCycler instrument.

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

5. Close the PhenoCycler Instrument door. The Door checkbox will turn green when the software determines the door has been closed.
6. If the instrument is not initialized, click the Initialize Instrument button.
7. If the Disk Space checkbox is not green, then clear disk space until the Disk Space checkbox turns green.
8. Ensure there is sufficient volume of 1X PhenoCycler-Fusion Buffer, High and Low DMSO, and ddH₂O in the appropriate bottles.

NOTE *The bottle for 1X PhenoCycler-Fusion Buffer should fit snugly in the sidecar without risk of tipping. If the bottle is not snug, the bottle retaining ring may have been removed.*

9. Empty the waste bottle.
10. The software will weigh the installed bottles and indicate if the correct weight has been determined.
11. 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 6 bottles must remain on the sidecar with tightened caps to avoid introducing bubbles. Do not remove unused bottles.*

12. The Get Ready dialog box is complete. (See Figure 6.10.)

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

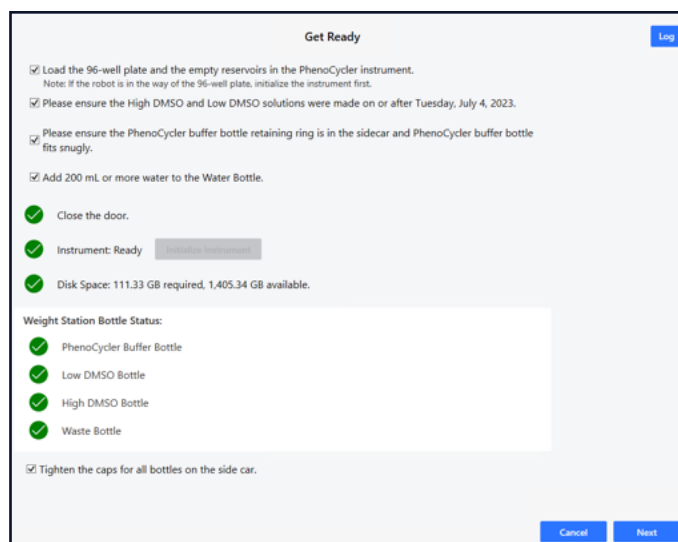


Figure 6.10. Dialog window, Get Ready complete.

Blank Flow Cell

Blank Flow Cell is the second dialog box in the Preflight Routine. (See Figure 6.11.) A blank Flow Cell is used to prime the instrument lines.

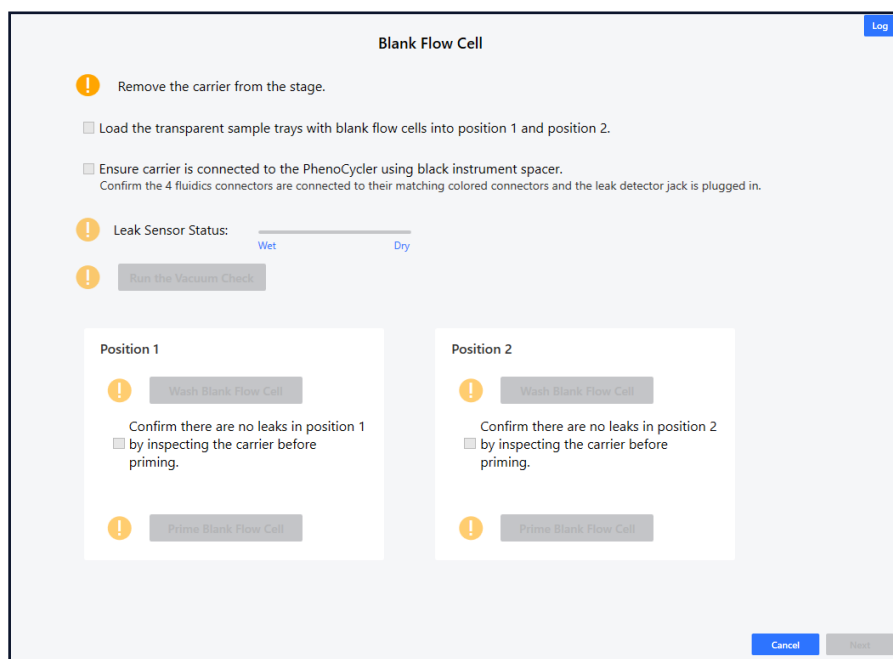


Figure 6.11. Dialog window, Blank Flow Cell.

1. Remove the carrier from the stage.
2. Insert a blank Flow Cell into the PhenoCycler Flow Cell Slide Carrier at each position that will be used for the experiment in preparation for the vacuum and leak checks.

NOTE Before loading the magnetic tray, ensure the leak detection circuit is clean and dry. See instructions in [Appendix A: Troubleshooting](#).

3. Connect the fluidic lines of the PhenoCycler Flow Cell Slide Carrier to the PhenoCycler instrument. Refer to [Loading The Flow Cell Slide Carrier into the Automated XYZ Stage](#).
4. Confirm that the Leak Sensor Status is Dry. If the Leak Sensor Status is Wet, dry it by following the procedure in [Appendix A: Troubleshooting](#).
5. 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 Bioscience Technical Support at support@akoyabio.com.
6. Click the Wash Blank Flow Cell button and watch for leaks as fluid is pumped into the blank Flow Cell by the PhenoCycler. If a leak is detected, confirm that the Flow Cell is not broken or cracked and is seated correctly following the procedure for [Appendix A: Troubleshooting](#).
7. Inspect the carrier for leaks. If none are observed, check the “Confirm there are no leaks” checkbox. If leaks are observed, then refer to [Appendix A](#) for instructions on how to dry the sensor, reseal the blank flow cell, then click Wash Blank Flow Cell again to reperform the leak check.
8. Click the Prime Blank Flow Cell button.
9. The Blank Flow Cell dialog box is complete. (See Figure 6.12.)
10. Click the Next button to move to the Sample Flow Cell.

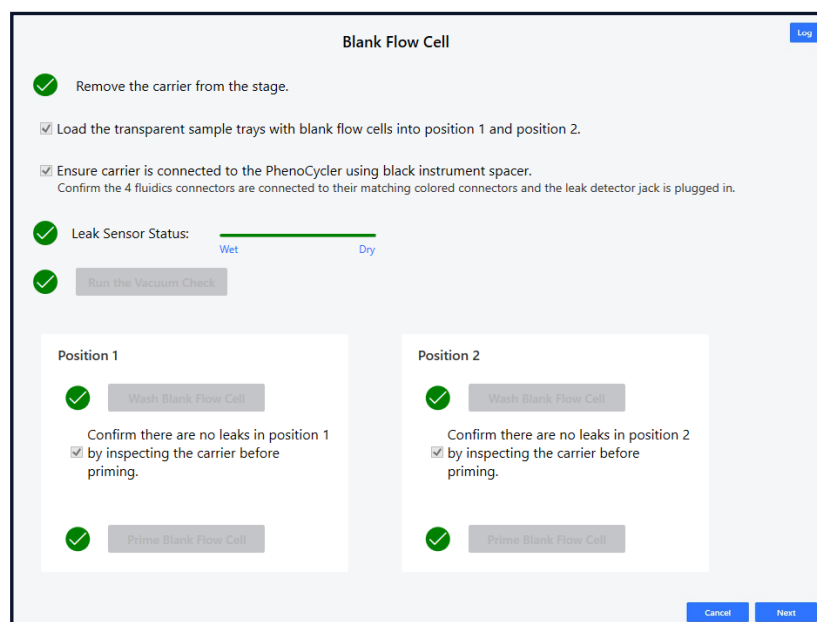


Figure 6.12. Dialog window, Blank Flow Cell complete.

Sample Flow Cell

Sample Flow Cell is the third dialog box in the Preflight Routine. (See Figure 6.13.)

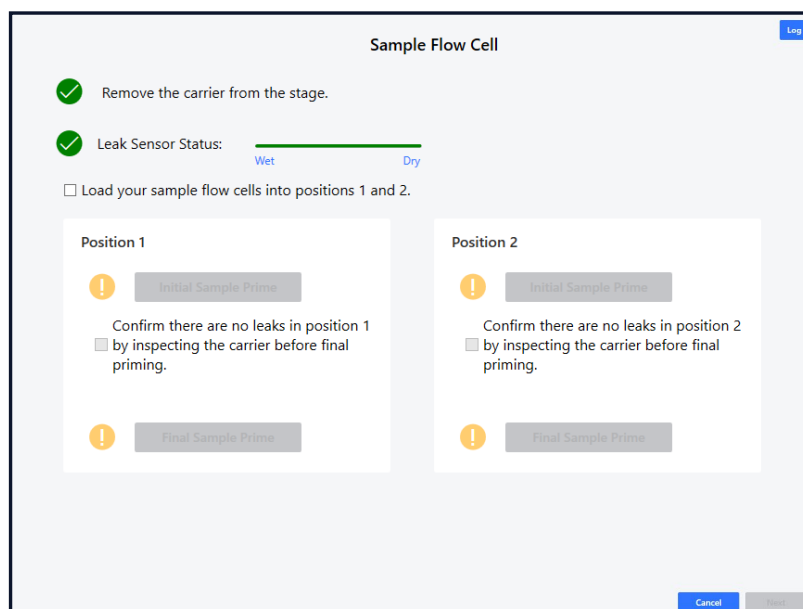


Figure 6.13. Dialog window, Sample Flow Cell.

Prepare sample Flow Cells.

1. Retrieve a sample Flow Cell from the Coplin jar containing 1X PhenoCycler-Fusion Buffer. Dampen a Kimwipe with ddH₂O. Wipe the Kimwipe across the top of the Flow Cell in a single direction slowly. Perform this wipe again with a second Kimwipe dampened with 70% ethanol. It is critical that both the top and bottom of the assembled Flow Cell and magnetic tray are clean and clear of debris.
2. Ensure the Flow Cell Carrier is removed from the Automated XYZ Stage.
3. Remove the blank Flow Cell and insert the sample Flow Cell into the same flow cell carrier. Click the checkbox to indicate the sample Flow Cell is in the carrier.

NOTE *The blank Flow Cell can be reused for multiple experiments.*

NOTE *Before loading the Magnetic Slide Retainer, ensure the leak detection circuit is clean and dry. See instructions in [Appendix A: Troubleshooting](#).*

4. Click the Initial Sample Prime button to start priming. Watch the sample Flow Cell for leaks as fluid is pumped in by the PhenoCycler.
5. Inspect the carrier for leaks. If none are observed, click the checkbox to indicate there are no leaks. If a leak is detected, refer to [Appendix A](#) for instructions on how to dry the sensor then confirm that the Flow Cell is not broken or cracked and is seated correctly before reperforming the leak check.
6. Click the Final Sample Prime button to prime. Prime up to 5 times (to remove all air bubbles if present).
7. The sample Flow Cell dialog box is complete. (See Figure 6.14.)
8. Click the Next button to move to the Tubing Check.

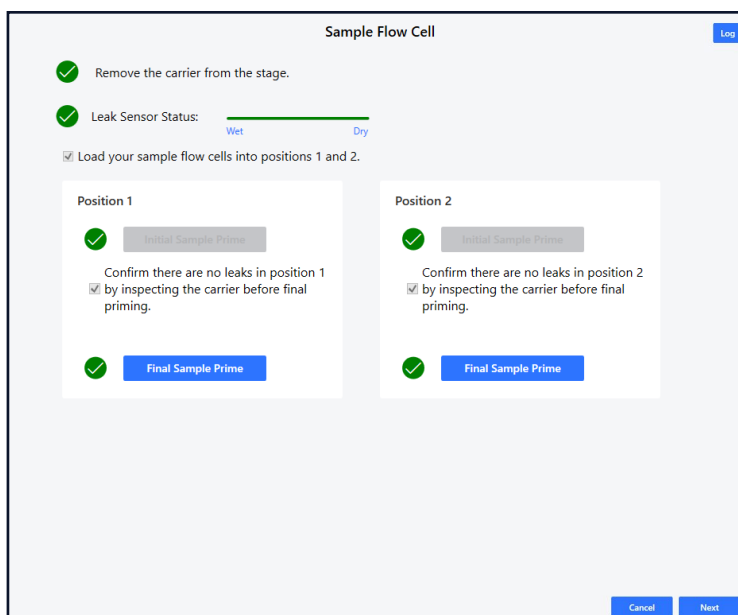


Figure 6.14. Dialog window, Sample Flow Cell complete.

Tubing and Carrier Tray Check

Tubing Check is the fourth dialog box in the Preflight Routine. (See Figure 6.15.)

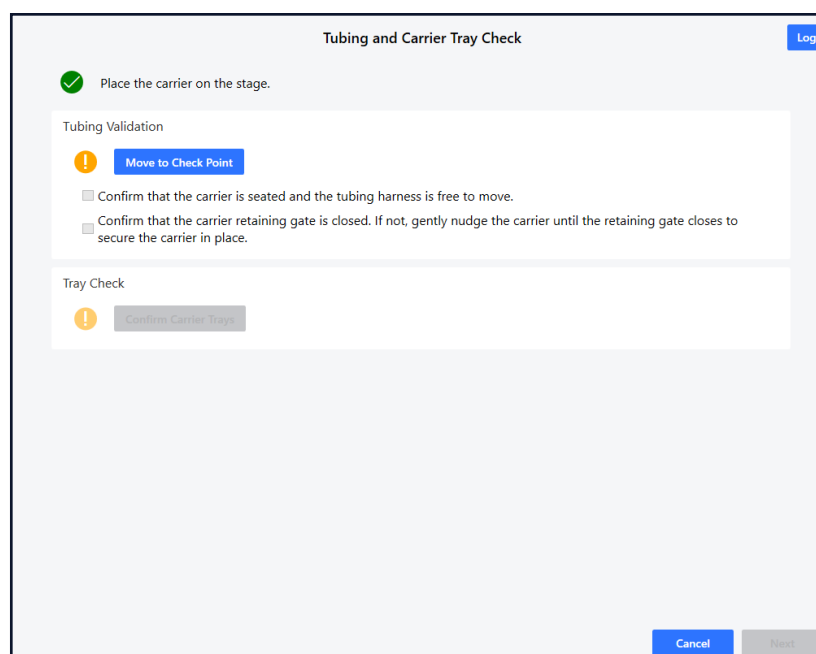


Figure 6.15. Dialog window, Tubing and Carrier Tray Check.

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 to ensure 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 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. Click the Confirm Carrier Trays button to check for the presence of the Blue Blocker Tray.
7. The Tubing and Carrier Tray Check dialog box is complete. (See Figure 6.16.)
8. Click the Next button to move to Sample Validation.

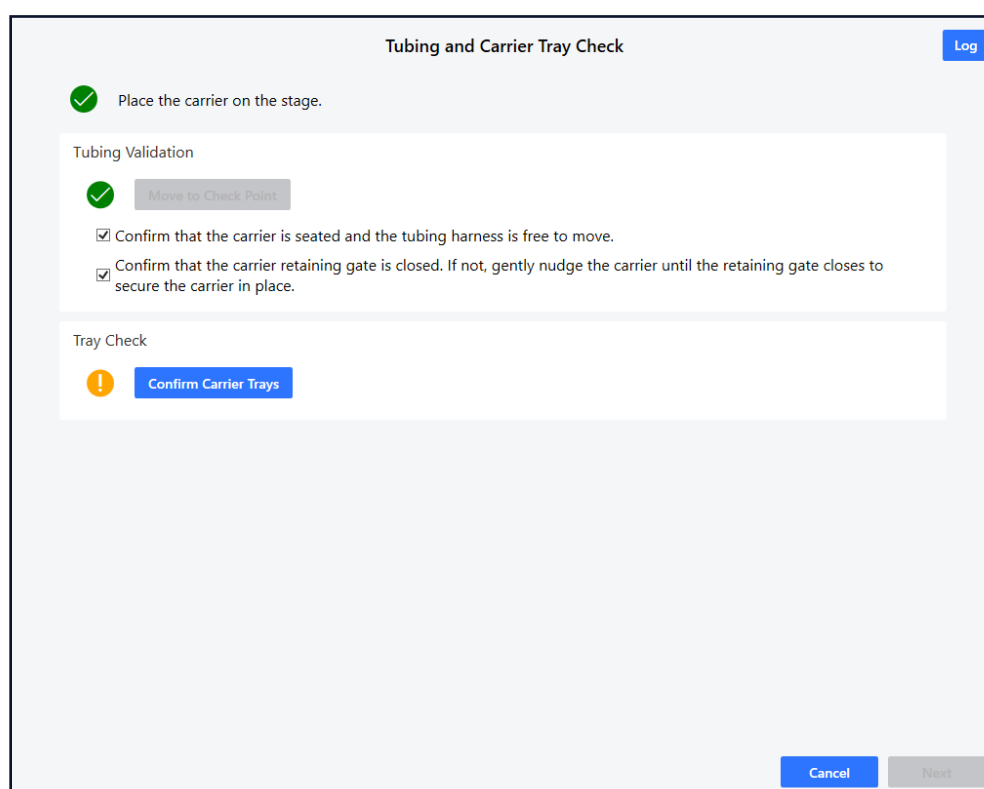


Figure 6.16. Dialog window, Tubing and Carrier Tray Check complete.

Sample Validation

Sample Validation is the fifth and final dialog box in the Preflight Routine. (See Figure 6.17.) Perform a visual check to confirm the label is visible in the Slide Label window.

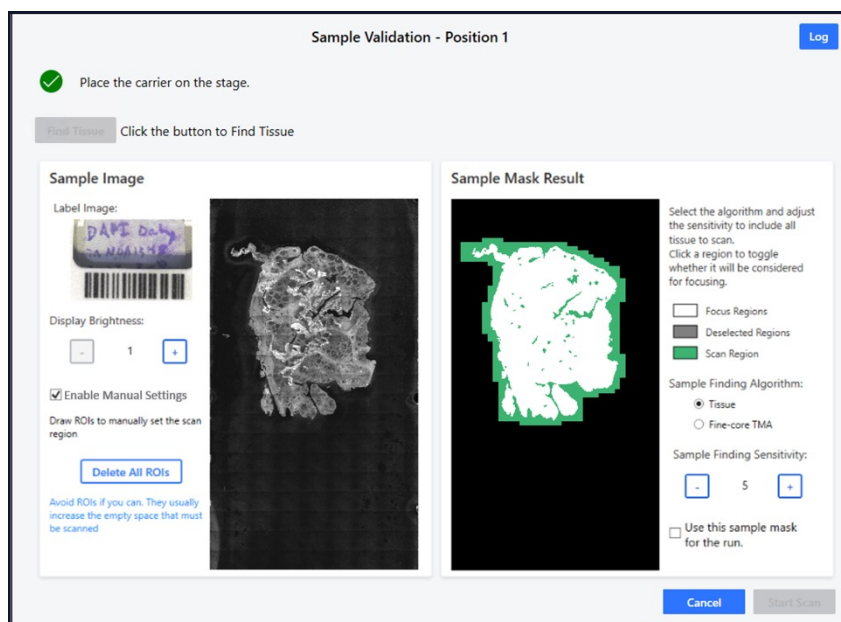


Figure 6.17. Dialog window, Sample Validation.

NOTE *Ensure that Windows display settings are at 100%.*

1. Click Find Tissue to take an image of the sample. Use the brightness controls to decrease and increase the 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.*

2. Adjust the Sample Finding Sensitivity and/or Sample Finding Algorithm (see Table 6.5) 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.*

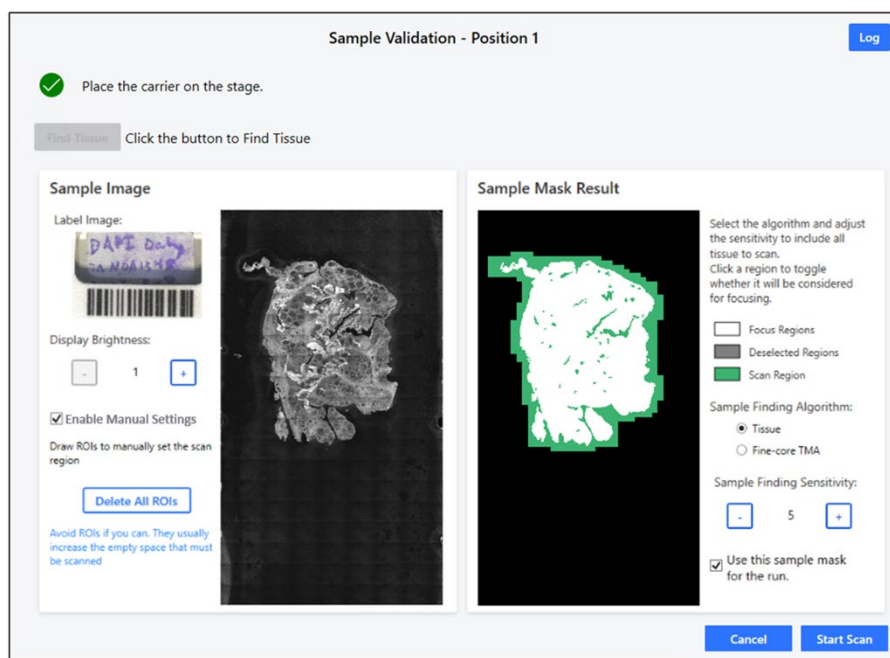
3. If settings cannot be found that detect all tissue pieces, better results may be obtained by drawing ROIs around tissue pieces. To do so, click Enable Manual Settings.
4. Once the sample mask looks good adjust the sample mask settings, if needed. (See Table 6.5.)

Table 6.5. Sample Mask Results, settings descriptions

Item	Description
Scan Region	Area that will be scanned. Click-and-drag the mouse to create ROIs* indicated in yellow.
Focus Region	Area that will be used to focus. Click the mouse to create focus points indicated in white.
Deselected Region	Area that will not be scanned.
Sample Finding Algorithm	Fine core TMA: Select to use a specialized algorithm that detects TMA cores that are 0.6-2.5 mm in diameter, arranged in grids of at least 2x2. The algorithm will search for multiple grids on the same slide. Tissue: Select for all other samples.
Sample Finding Sensitivity	Adjust the sensitivity using the controls.

*ROI: region of interest.

- Once the sample mask is complete, check the Use this Sample Mask checkbox.
- If scanning samples in 2 positions, then the Next button will be enabled. Click Next to repeat the process for position 2.
- When creating the sample masks is complete, the Start Scan button will be enabled. (See Figure 6.18.)

**Figure 6.18.** 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 run must be cancelled, click the Cancel Run button to stop operations will stop as soon as possible. (See Figure 6.19.) 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.

NOTE If 2 experiments are running, canceling will apply to both.

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

Example 1: Canceling During A PhenoCycler Run

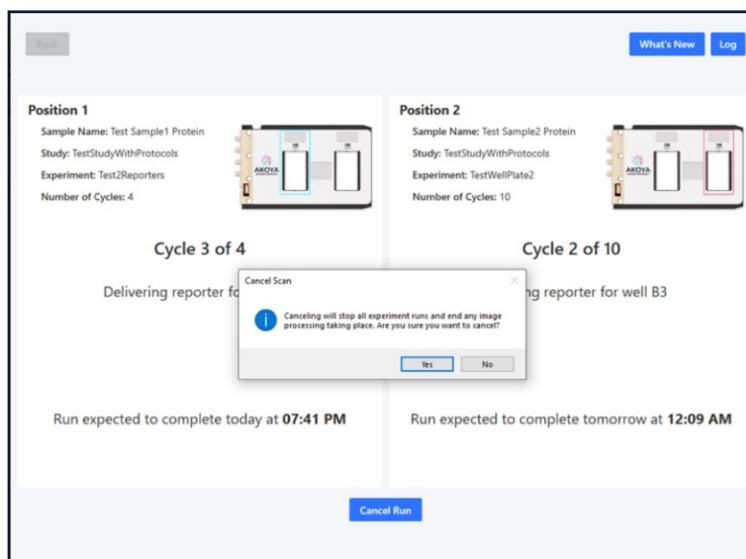


Figure 6.19. Dialog window, Delivering Reporters, Cancel Run.

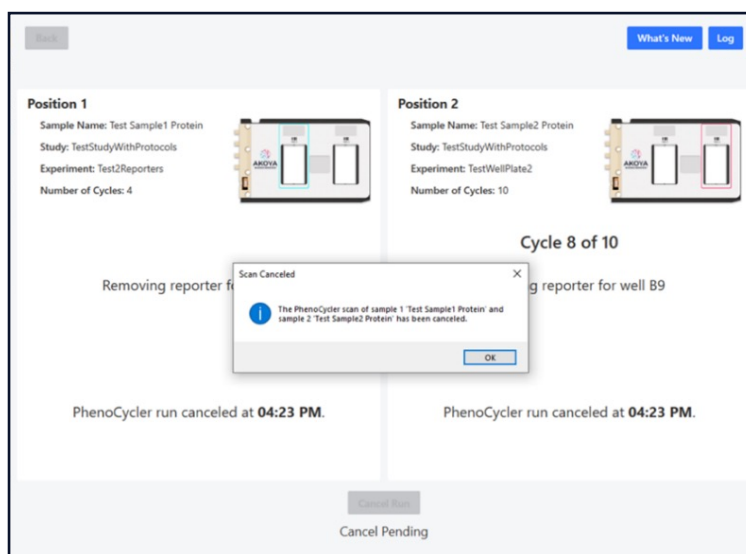


Figure 6.20. Dialog window, PhenoCycler run canceled.

Example 2: Canceling During Post-Experiment Clean

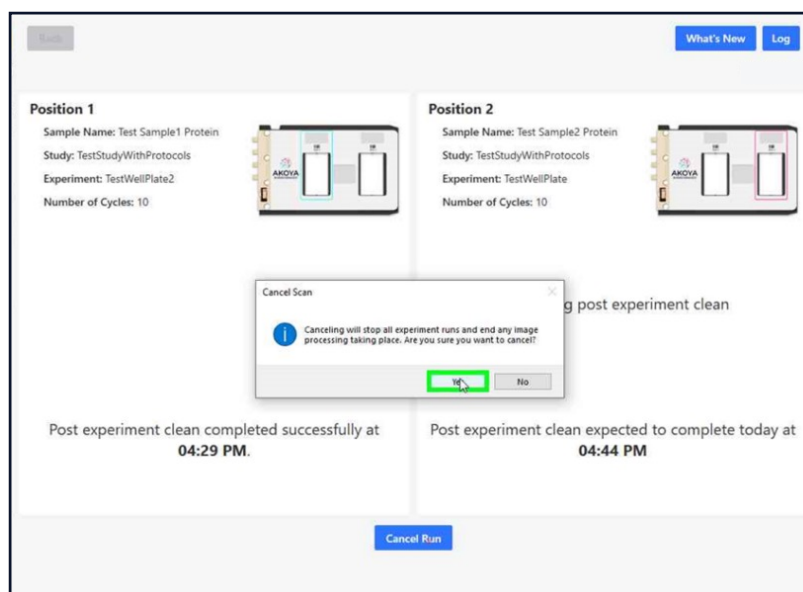


Figure 6.21. Dialog window, Performing post-experiment clean, Cancel Run.

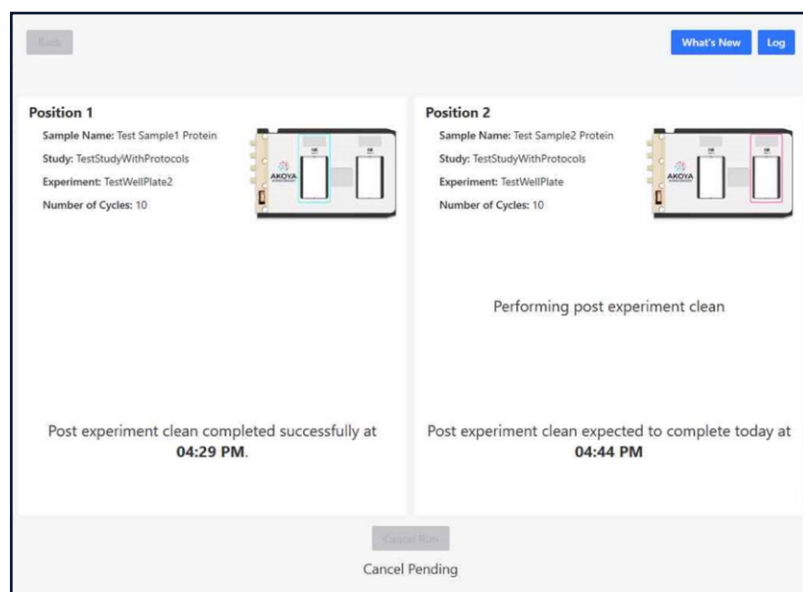


Figure 6.22. Dialog window, Performing post-experiment clean, Cancel Pending.

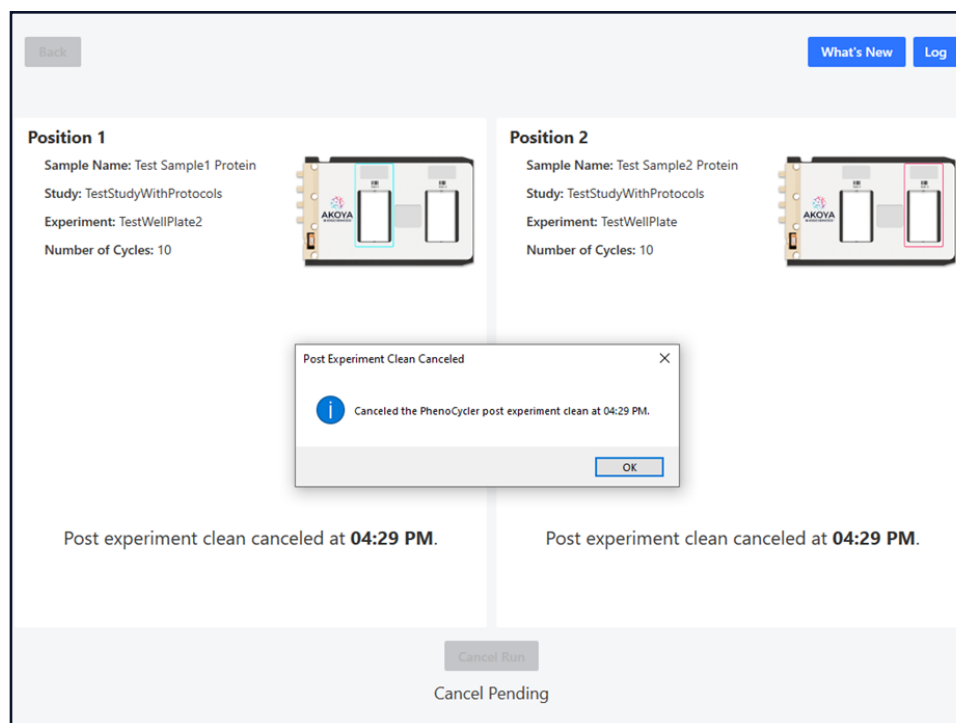


Figure 6.23. Dialog window, Post-experiment clean canceled.

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:

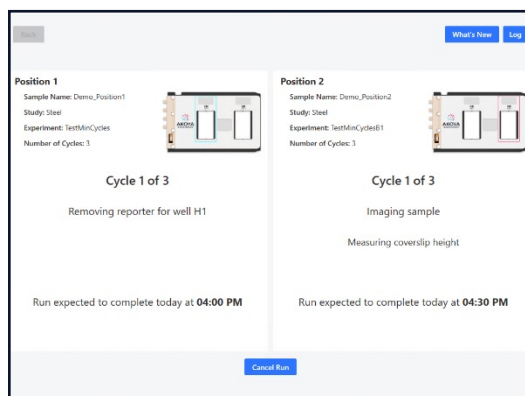


Figure 6.24. Dialog window, Example status panel, Removing reporter for wells.

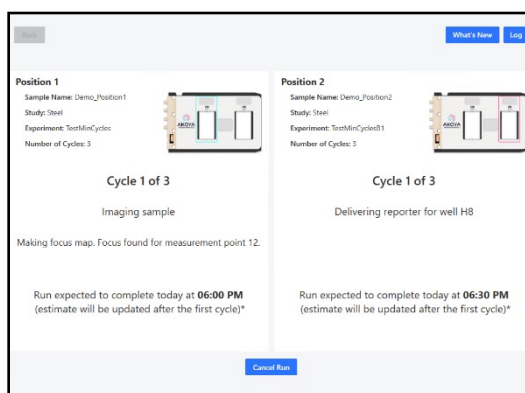


Figure 6.25. Dialog window, Example status panel, Imaging sample, Making focus map.

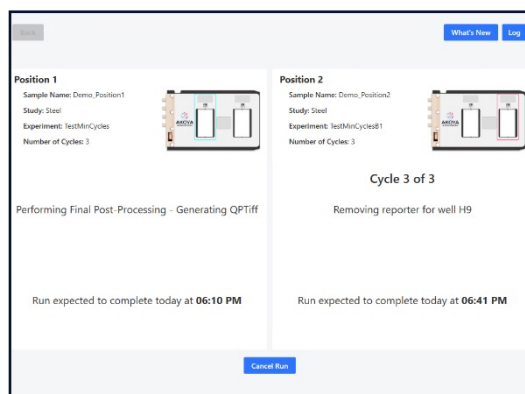


Figure 6.26. Dialog window, Example status panel, Performing Final Post-Processing — Generating QPTiff.

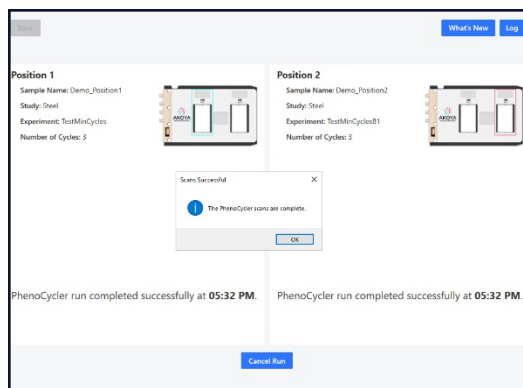


Figure 6.27. Dialog window, Example status panel, PhenoCycler run completed successfully. Scan Complete.

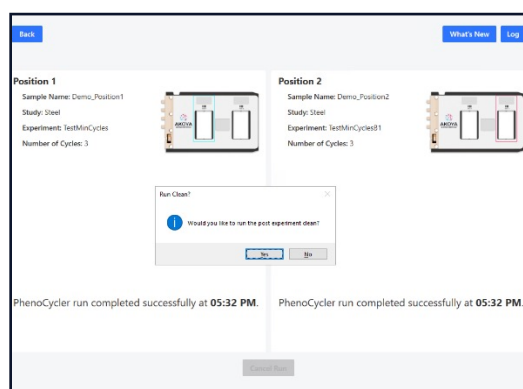


Figure 6.28. Dialog window, Example status panel, PhenoCycler run completed successfully. Run Clean?

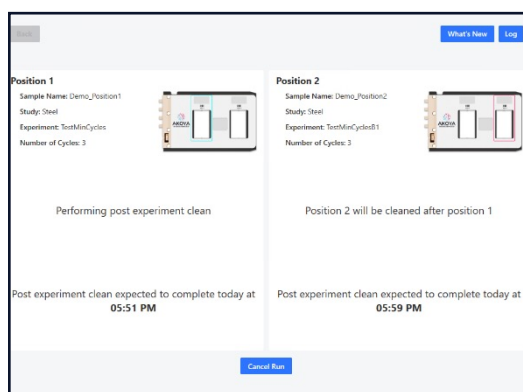


Figure 6.29. Dialog window, Example status panel, Performing post experiment clean.

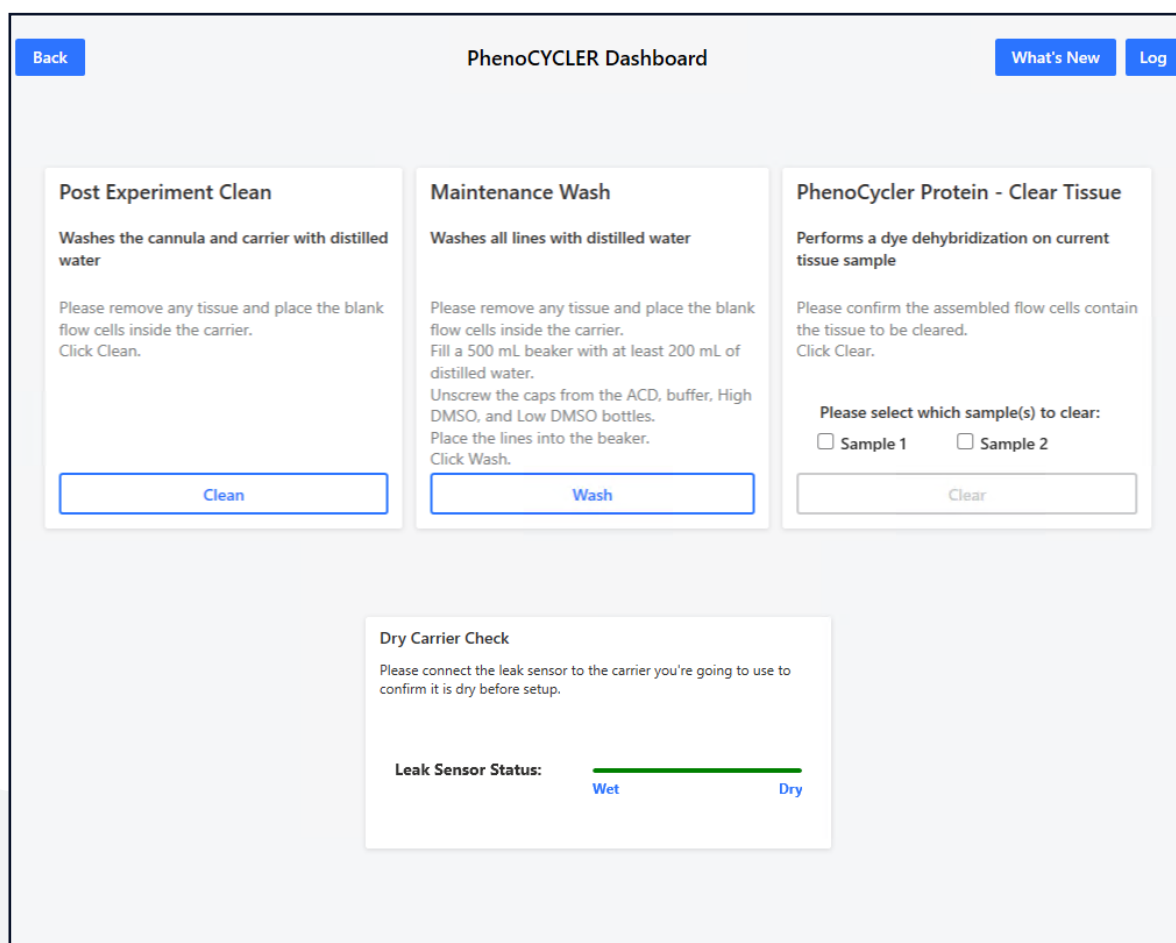
Chapter 7: Cleaning and Maintaining PhenoCycler

Section	Page
PhenoCycler Dashboard Software	83

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.)



The screenshot shows the PhenoCYCLER Dashboard interface. At the top, there are buttons for 'Back', 'What's New', and 'Log'. The dashboard is divided into three main sections: 'Post Experiment Clean', 'Maintenance Wash', and 'PhenoCycler Protein - Clear Tissue'. Each section contains instructions and a button to perform the action. Below these sections is a 'Dry Carrier Check' section with a 'Leak Sensor Status' indicator showing a green bar between 'Wet' and 'Dry'.

PhenoCYCLER Dashboard

Post Experiment Clean
Washes the cannula and carrier with distilled water
Please remove any tissue and place the blank flow cells inside the carrier.
Click Clean.
Clean

Maintenance Wash
Washes all lines with distilled water
Please remove any tissue and place the blank flow cells inside the carrier.
Fill a 500 mL beaker with at least 200 mL of distilled water.
Unscrew the caps from the ACD, buffer, High DMSO, and Low DMSO bottles.
Place the lines into the beaker.
Click Wash.
Wash

PhenoCycler Protein - Clear Tissue
Performs a dye dehybridization on current tissue sample
Please confirm the assembled flow cells contain the tissue to be cleared.
Click Clear.
Please select which sample(s) to clear:
☐ Sample 1 ☐ Sample 2
Clear

Dry Carrier Check
Please connect the leak sensor to the carrier you're going to use to confirm it is dry before setup.
Leak Sensor Status: ————— **Wet** **Dry**

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 blank Flow Cells before running. If this step is performed with the tissue sample loaded, that tissue will not be reusable for further studies. Note that both positions will be cleaned. To perform this step, select the Clean button after loading the blank Flow Cells.

Maintenance Wash

The maintenance wash clears and washes the lines of residual buffers and DMSO to maintain optimal instrument performance. Note that both positions will be washed in the process. To perform the Maintenance Wash, follow these steps:

Load blank Flow Cells into the PhenoCycler Flow Cell Slide Carrier, ensuring that the leak detection circuit is clean and dry. See instructions in [Appendix A: Troubleshooting](#).

- Fill a beaker with at least 200 mL of ddH₂O and place near the PhenoCycler.
- Remove the tops of the Low DMSO Buffer bottle and the High DMSO Buffer bottle and place the fluidic lines into the beaker of water.

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

- Click the Wash button.

Clear Tissue

If a protein 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. The option is given to clear position 1, position 2, or both positions.

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.*

NOTE See [Chapter 8: Cleaning and Maintaining the Phenolmager Fusion](#) for instruction on cleaning the Slide Carrier.

Dry Carrier Check

The dry carrier check widget shows the status of the leak detection sensor. The colored bars—green, yellow, or red—indicate the status in relation to the wetness.

A red status bar indicates the sensor is detecting a leak and cannot complete a dashboard function. See [Appendix A: Troubleshooting](#).

Chapter 8: Cleaning and Maintaining the Phenolmager Fusion

Section	Page
Cleaning the Exterior	85
Cleaning the Monitor	85
Cleaning the Power & Comms Ports	85
Cleaning the Slide Carrier	86
Cleaning After a Leak	86
Replacing Parts on the Phenolmager Fusion System	86

CLEANING THE EXTERIOR

Clean the Phenolmager Fusion instrument exterior as needed. Wipe down the non-electrical exterior parts of the Phenolmager 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 Phenolmager Fusion instrument. Sprays and liquids that come into contact with the Phenolmager 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 ddH₂O with a soft cloth or using an ultrasonic bath.

CLEANING AFTER A LEAK

For details, see [Appendix A: Troubleshooting](#).

REPLACING PARTS ON THE PHENOIMAGER FUSION SYSTEM

To replace the fuses (blown fuses or to order replacement fuses), contact Akoya Biosciences Technical Support at support@akoyabio.com.



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

Appendix A: Troubleshooting

HARDWARE

Recovering from a leak

This section describes the recommended process to follow after the Leak Detection Circuit detects a leak, including identifying the leak source, steps for cleaning to begin a new run, and troubleshooting tips. The leak detection sensor in the PhenoCycler-Fusion 2.0 is located on the carrier and connects to the PhenoCycler instrument via a 1/8-inch port (similar to a headphone jack); the plug can stay connected to complete the following steps. Leaks are detected by creating a conductive path between the traces that circle each Flow Cell location in the carrier, which is typically caused by liquid that might otherwise spill onto the system.

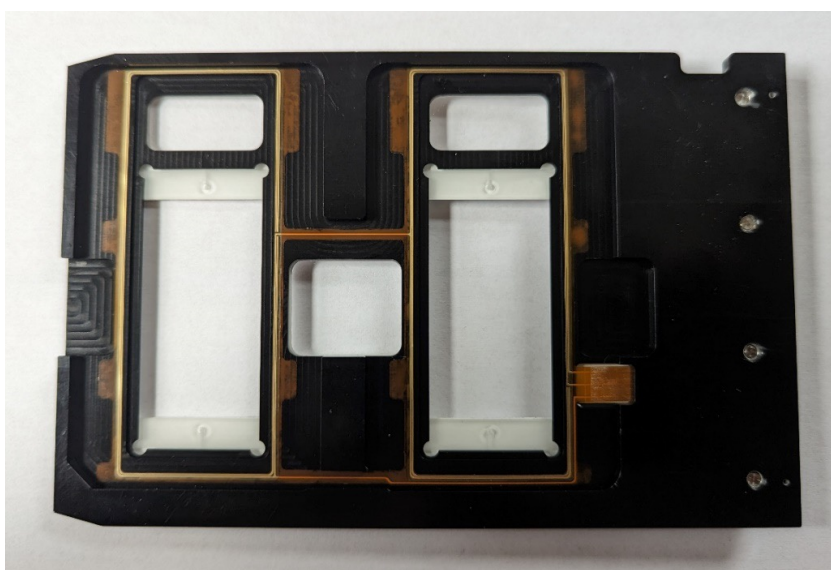


Figure A.1. Bottom of PhenoCycler Flow Cell Slide Carrier without Magnetic Slide Retainer to show the Leak Detection Circuit.

A leak in a sample Flow Cell is characterized by liquid pooling in the Magnetic Slide Retainer under the Flow Cell. Leaks can occur due to either Flow Cell adhesive failure or improper Flow Cell assembly. If a leak occurs, note the liquid location before disturbing the sample Flow Cell in the Slide Carrier. Note the lot number and expiration date of the Flow Cells and contact Akoya Biosciences Technical Support at support@akoyabio.com.

When liquid pools on top of the sample Flow Cell in the Slide Carrier, this indicates either the Flow Cell was not seated properly in the Slide Carrier or the gasket has a leak path. In this case, turn the Slide Carrier over and check the Magnetic Slide Retainer. The Magnetic Slide Retainer should be flush with the back of the Slide Carrier and not protruding past the aluminum base. If the Magnetic Slide Retainer is bulging outwards, check that the Flow Cell is seated correctly by carefully removing the Magnetic Slide Retainer. The sample Flow Cell should be seated in the region defined in Figure A.2. If the Flow Cell appears correctly seated, remove it and check for contamination on the seals. Hair and other debris can create a leak path and must be physically removed.

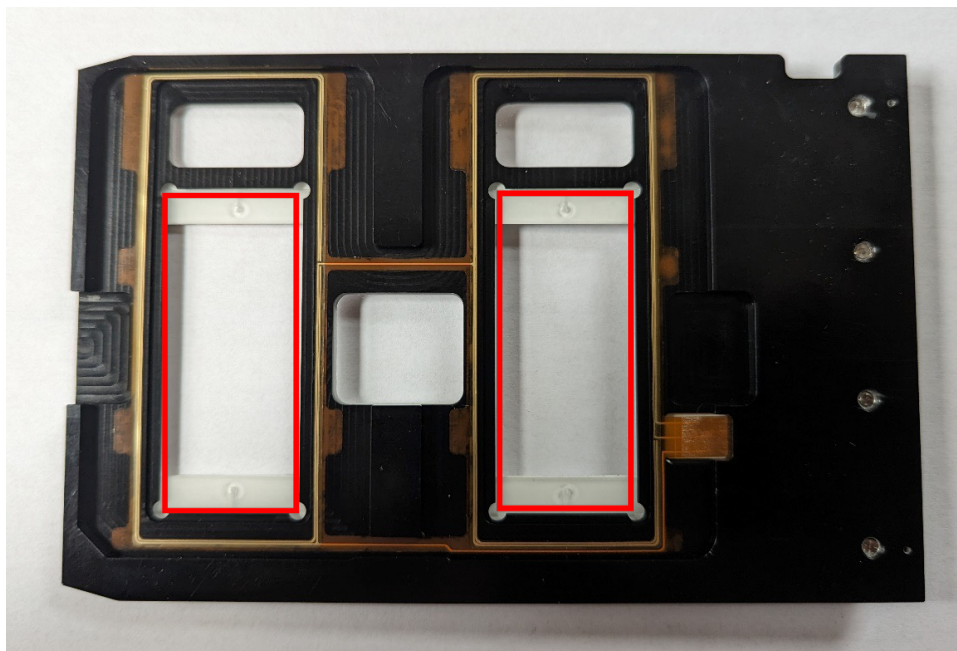


Figure A.2. Sample Flow Cell attached to Slide Carrier seating region (highlighted in the red outlines).

A leak may be detected from liquid leaking in either Flow Cell location. If only a single sample is being imaged, the Blue Blocker Tray will contain no liquid. The purpose of this tray is to prevent potential drips from affecting leak detection. If the Blue Blocker Tray has liquid in it, contact Akoya Biosciences Technical Support at support@akoyabio.com.

If a leak occurs while the carrier is on the stage, follow these steps to clean the instrument:

1. Quit the PhenolMager Fusion software.
2. Remove the carrier from the stage then inspect the instrument as soon as possible to see if any liquid has escaped the carrier onto the stage or condenser. To inspect the stage, power off the Fusion microscope and manually move the stage around to look for visible liquid.
3. Clean off any liquid on the stage with a dry Kimwipe. Subsequently, clean the stage surface with a Kimwipe soaked in 70% ethanol.

NOTE Please avoid contact with the stage rails when cleaning with alcohol.

NOTE Do not spray ethanol onto the stage; soak the wipe separately.

NOTE Take care to not contaminate the objective lenses when wiping up leaks.

4. Clean off any drips on the condenser lens surface using optical wipes.
5. Contact support@akoyabio.com if:
 - a. The leak was more than ~ 5 mL.
 - b. Errors are encountered on subsequent runs.

For all detected leaks, follow the steps below to clean the carrier and samples:

The steps below describe how to clean and dry the carrier and samples after a detected leak.

If the leak was detected during the Preflight Routine, begin the procedure below and stop after the Preflight Routine dialog shows a Dry reading. After the Preflight Routine records a Dry reading, it is important not to add any more liquids to the carrier, so continue the rest of the procedure but skip steps 3, 4, and 8 if you have not done them already.

If the leak was detected during an experiment, perform all the steps below and confirm that the leak circuit is dry using the Dry Carrier Check in the PhenoCycler Dashboard. The Dry Carrier Check will continuously report the detector status:

1. Remove the leaking Magnetic Slide Retainer/s and sample Flow Cell/s.
2. Examine the Leak Detection Circuit. (see Figure A.1) for liquid or salt residue to confirm the leak location. Wipe away any remaining liquid with a dry Kimwipe or lint-free cloth.
3. Wipe the Leak Detection Circuit to remove any salt residue using a Kimwipe or lint-free cloth soaked in ddH₂O. Pay special attention to Figure A.3.
4. Wipe the Leak Detection Circuit using a lint-free cloth or Kimwipe soaked in 70% ethanol. Pay special attention to Figure A.3.
5. Wipe the Leak Detection Circuit using a dry lint-free cloth or Kimwipe. Visually inspect the entire area of the Leak Detection Circuit to be sure all fluid has been wiped away.
6. Wipe the Magnetic Slide Retainer/s using a Kimwipe or lint-free cloth soaked in ddH₂O. Pay special attention to where it contacts the Leak Detection Circuit.
7. Wipe the Magnetic Slide Retainer/s using a Kimwipe or lint-free cloth soaked in 70% ethanol—especially where it contacts the Leak Detection Circuit.
8. Repeat steps 3 through 5 to ensure all salt residue is fully removed the Leak Detection Circuit.
9. Let the slide carrier sit with the Leak Detection Circuit facing up for at least 2 minutes. Make sure to dry any liquid around the ports of the slide carrier before letting the carrier sit, and continuously dry any liquid that may come out of the ports.
10. Clean the Sample Flow Cell(s) with 70% ethanol to remove any buffer residue that may interfere with image quality. Dry the sample Flow Cell using a Kimwipe or lint-free cloth. Leftover liquid might trip the Leak Detection Circuit.
11. Reassemble the sample Flow Cell/s into the Slide Carrier with the Magnetic Slide Retainer/s.

NOTE *If the plug for leak detection is disconnected, ensure the connector is dry before reconnecting to the system.*

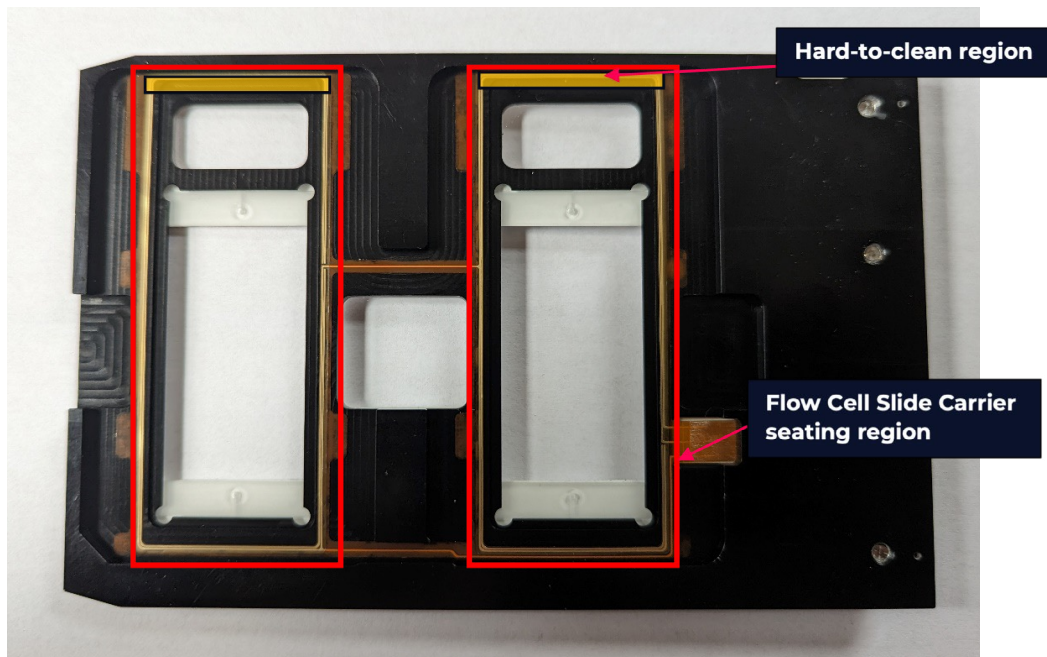
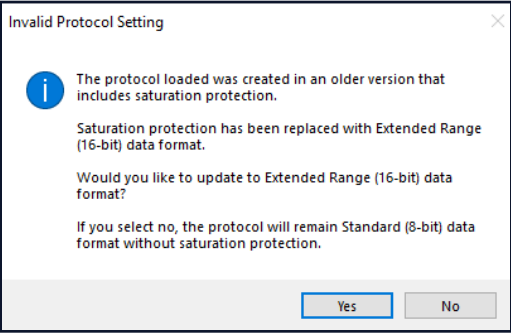


Figure A.3. Leak Detection Circuit with hard-to-clean regions (highlighted in yellow boxes) within the Flow Cell Slide Carrier seating region (highlighted in the red outlines).

The Pre-flight Routine can be used to check the status of the leak detection circuit. In some cases, multiple rounds of cleaning may be necessary to reset the leak status. If leak detection is preventing the start of the experiment after cleaning multiple times, contact Akoya Biosciences Technical Support at support@akoyabio.com.

SOFTWARE

Table A.1. Troubleshooting software

Message	Possible Cause	Solution
Pop-up window: Run Failed to Complete	An error occurred during the sample scan	Reboot the software.
	An unsupported file was loaded	Load supported file.
Software not running after installation	Corrupted config files	Run a configuration file health check: - Navigate to the following folder location: C:\ProgramData\Akoya\Fusion. - Open the Fusion.config file and compare this file with the created backup. - Navigate to the following folder location: C:\ProgramData\Hardware\PhenoCycler. - Open the Fluidics.config file and compare this file with the created backup.

Appendix B: Bottle Cleaning

These instructions are for cleaning bottles for High DMSO, Low 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 High DMSO bottle, add 25 mL of High 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: 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 C.1.) Most air pockets typically clear away during software pre-flight checks.

1. Retrieve the Flow Cell Carrier from the PhenoCycler.

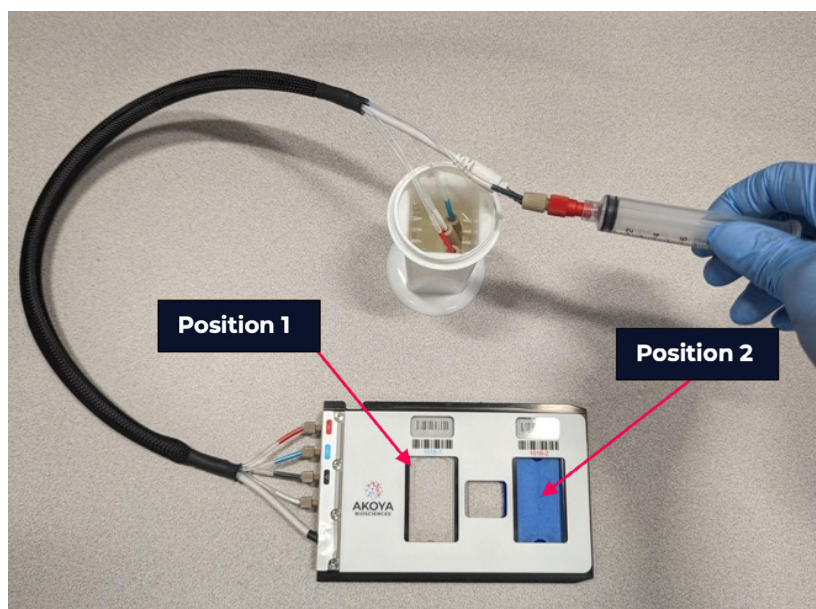


Figure C.1. Flow Cell Carrier connected to a syringe.

2. Retrieve a small beaker with approximately 50 ml of 1X PhenoCycler-Fusion Buffer.
3. Attach a syringe to the outlet tubing of the Flow Cell Slide Carrier that correlates to the Flow Cell position to be flushed.
 - a. Black is the outlet for position 1 in the Flow Cell and is the configuration shown in Figure C.1.
 - b. White is the outlet for position 2 in the Flow Cell.
4. Place the remaining 3 tubes into the beaker containing the buffer.
5. Slowly open the syringe, pulling buffer from the beaker and into the Flow Cell. This action should remove all air pockets present in the Flow Cell.

NOTE Do not push buffer into the flow cell. Always pull the buffer solution through. Pushing can cause damage to the flow cell.

Appendix D: Installing and Uninstalling the Phenolmager Fusion Software

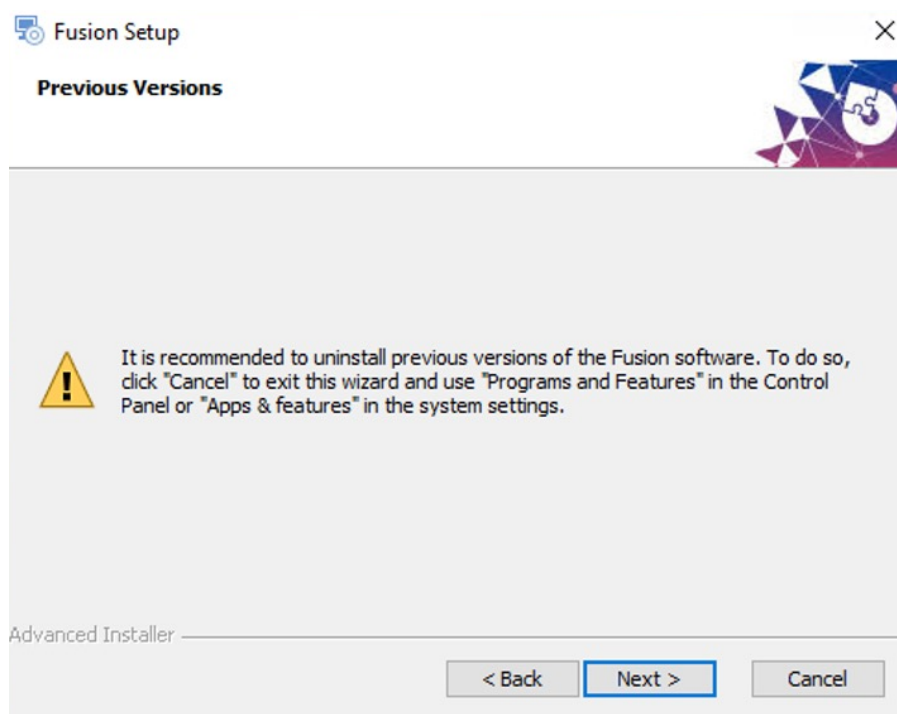


Figure D.1. Phenolmager software installation wizard.

1. Create a backup.

Before starting the Fusion software installer, make a backup of the following folder which contains the Fusion configurations. It is critical to have a backup of the configurations before uninstalling or installing any software.

- C:\ProgramData\Akoya

Once the backup is created, uninstall the software from the “programs and features” in the control panel or “apps & features” in the system settings. It is recommended to restart the computer after uninstallation.

2. Install the latest version of Fusion by the provided installer.

3. Configuration File Check (Fusion.config)

- a. Navigate to the following folder location: C:\ProgramData\Akoya\Fusion.
- b. Confirm the Fusion.config exists.
- c. Navigate to the following folder location: C:\ProgramData\Hardware\PhenoCycler.
- d. Confirm the Fluidics.config file exists.

NOTE For those users who don't have file extensions enabled, the configuration files will appear without a suffix (e.g., Fusion, Fluidics).

4. Run Fusion 2.x.x software.
 - a. If the configuration file checks are successful, launch the Fusion 2.x.x software.
 - b. If the software silently fails to initialize, please move to the “configuration file health check” step in [Appendix A: Troubleshooting](#).
 - c. Acquire new brightfield and fluorescence references by clicking every “Acquire” button in the Fusion Dashboard.
 - d. Optionally, run a small PhenoCycler-Fusion experiment to confirm the software functionality.

NOTE If you encounter issues operating the software post-installation, follow the steps outlined in [Appendix A: Troubleshooting](#) to perform a Configuration File Health Check.

Appendix E: Specifications

SYSTEM SPECIFICATIONS

Table E.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 μ m, 0.5 μ m
File Format	Akoya Biosciences QTIFF format for whole slide scans
Operating System	Microsoft® Windows 10, 64-bit
RAM	32GB

ENVIRONMENTAL CONDITIONS

Table E.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 E.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 F: 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 F.1.

Table F.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 tags (See Table F.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 F.2 is the second location. 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 F.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 F.3. Elements appear in the order listed.

Table F.3. IMAGE DESCRIPTION TAGS

TIFF Tag	Optional	Description of Content
ImageType	No	A string identifying the type of image within the file (Table F.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 wwwt 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 wwwt 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 CameraSettings are provided, this field is optional.
X	No	X origin of ROI. Left-most pixel in sensor array is 0.
Y	No	Y origin of ROI. Top-most pixel in sensor array is 0.
Width	No	Width of ROI.
Height	No	Height of ROI.

**For whole slide images (BF and FL), SignalUnits will be 64 (hex 40) (raw counts, peak signal).
For unmixed images, 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 PhenolMager 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 F.4.

Table F.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>
```

Appendix G:

Phenolmager Fusion EULA

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

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