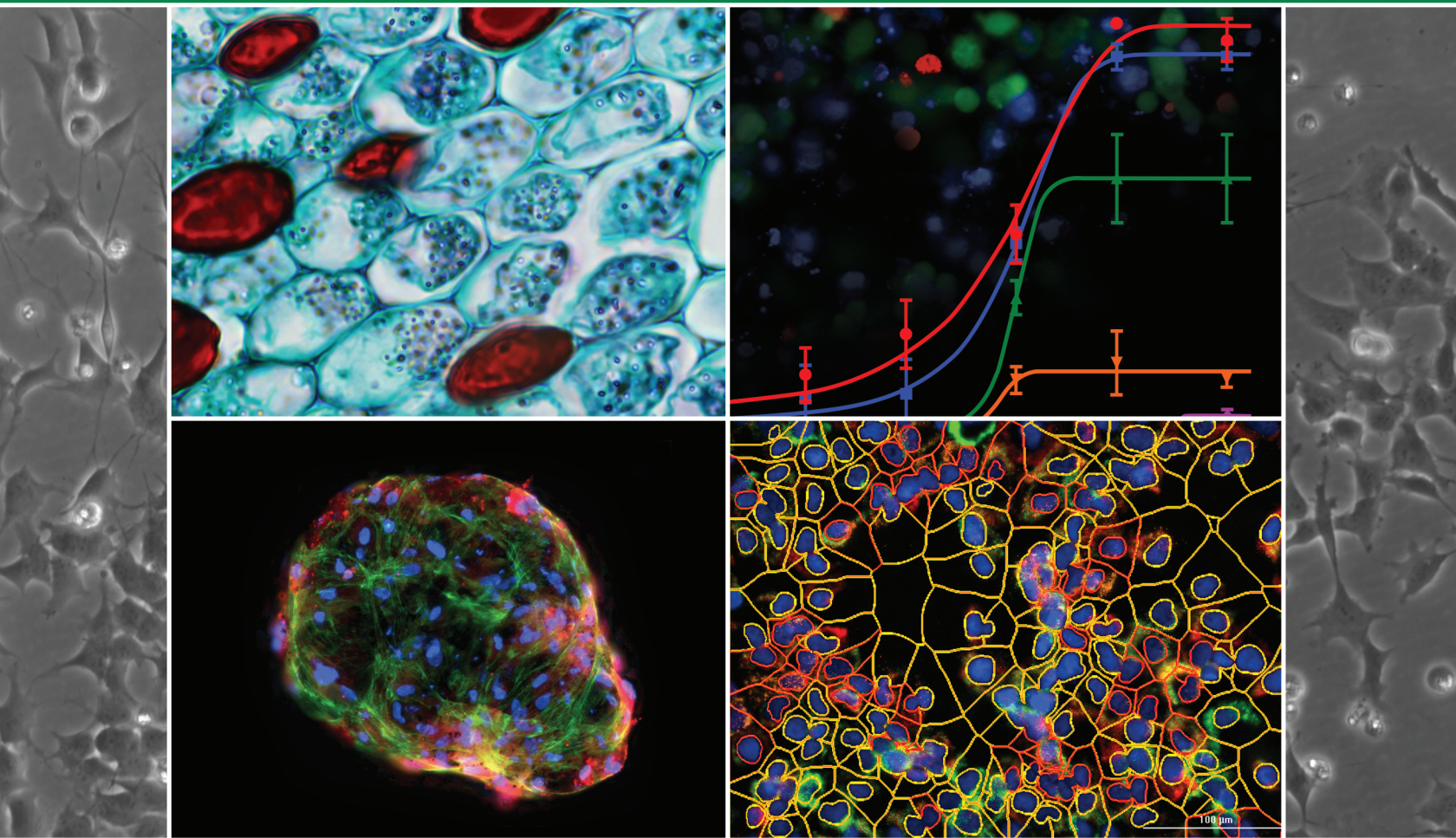


# Imaging Training Manual



ERRATA NOTICE: This document contains references to BioTek. Please note that BioTek is now Agilent. For more information, go to [www.agilent.com/lifesciences/biotek](http://www.agilent.com/lifesciences/biotek).



# Imaging Training Manual

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February 2022



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## Introduction

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This introductory chapter will guide you through the features and workflow of using Gen5 for microscopy. These overview topics include references to more specific information provided in subsequent chapters. Whether you are a new user or need a refresher on how to use Gen5, this is the place to start.

**Note:** Gen5 version 3.12 or higher is required to perform all features herein described.

This guide assumes user familiarity with basic digital microscopy concepts such as objectives, fluorescence, filters, etc.

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This manual is intended for use with the following instruments and software:

### Instruments

|              |   |
|--------------|---|
| Lionheart FX | LFX, LFXW   |
| Lionheart LX | LLX, LLXW   |
| Cytation C10 | C10PHC2, C10MPHC2, C10PWC, C10MPWC, C10PW, C10MPW   |
| Cytation 7   | CYT7U, CYT7UW, CYT7UMW, CYT7UM  |
| Cytation 5   | CYT5FV, CYT5FW, CYT5FAV, CYTFAW, CYT5MV, CYT5MW, CYT5MPV, CYT5MPW, CYT5MFV, CYT5MFW, CYT5MFAV, CYT5MFAW, CYT5V, CYT5W, CYT5PV, CYT5PW |
| Cytation 1   | CYT1FAV, CYT1V  |

### Gen5 Software

- GEN5
- GEN5SECURE
- GEN5IPLUS
- GEN5IPRIME

## Introduction

### 1.1 - The Gen5 Microscopy Workflow

This introductory chapter ensures you will be prepared for success. Before you begin using your imager, read and understand this important information.

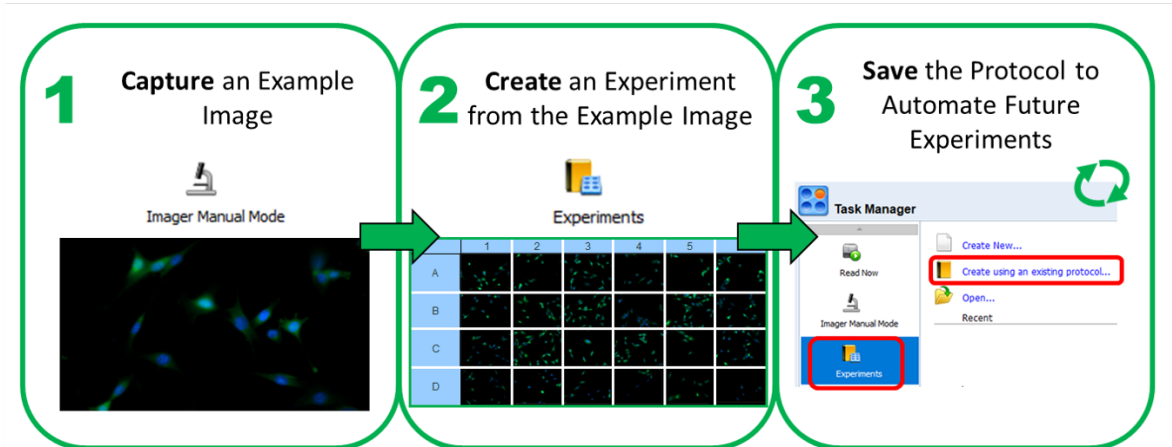
The first thing you may notice about your Cytation or Lionheart is the absence of eyepieces! Instead, all imaging on Agilent microscopes is performed digitally using Agilent **Gen5** software. Gen5 software also contains image processing and analysis tools. The equivalent of eyepieces in Gen5 is **Imager Manual Mode**. This mode (described in 2 - Quick Guide to Imaging on page 36) lets you view your samples and operate the microscope just like a manual microscope.

However, Gen5 is designed to assist in automating your microscopy workflows (termed "Protocols" in Gen5). Gen5 allows you to easily repeat previously saved protocols. To properly follow along with these instructions you need a sample plate ready to image.

As you will soon see, the best way to define a Gen5 Protocol is to use one of your own biological samples. This eliminates guesswork on the best settings. The remaining sections in this chapter will walk you through the process of creating a new protocol from scratch. If this is your first time working with Gen5 (or if you want a refresher), start with 1.2 - Important Gen5 Imaging Concepts on the facing page, which defines key terms and concepts for successfully using Gen5.

The process to create a new protocol follows three steps.

1. **Capture** an Example Image. Start by capturing a new image. Acquiring a good image requires properly adjusting focus and exposure settings. This image is used to test image processing and analysis steps. Covered in 1.3 - Capture an Example Image on page 18.
2. **Create** an Experiment from the Example Image. The captured image is used to create an experiment. A Gen5 Experiment is the automated acquisition, processing, and analysis of images across a microplate or other vessel. This is covered in 1.4 - Create an Experiment from the Example Image on page 21.
3. **Save** the Protocol to Automate Future Experiments. After creating a perfect protocol, you will want to save your hard work to use it again. 1.5 - Saving Gen5 Files and Defining the Image Library on page 26 covers saving a protocol and using it in future experiments.



Finally, 1.6 - Example Exercises as shown on page 30 contains exercises to test your understanding.

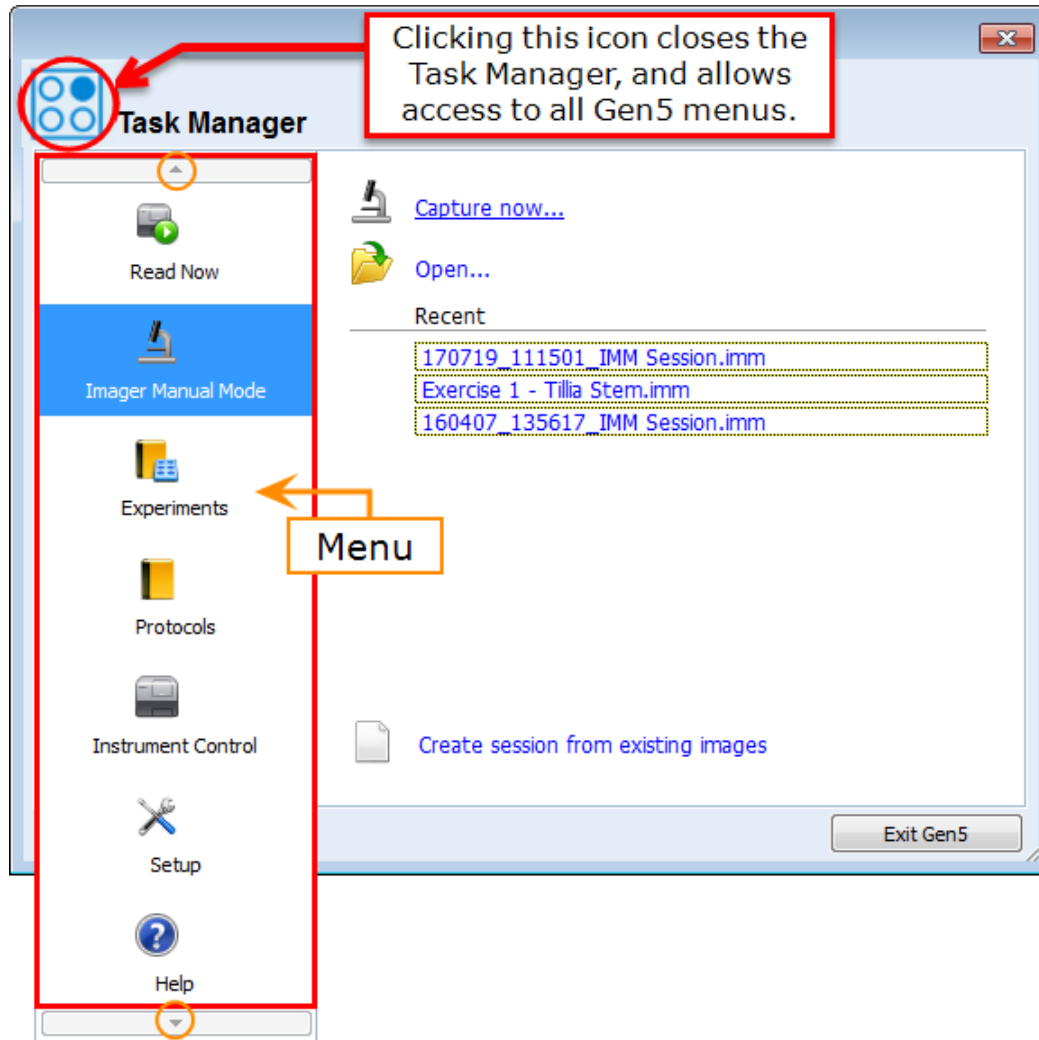
## Introduction

### 1.2 - Important Gen5 Imaging Concepts

Start by launching Gen5.



The Task Manager will be displayed. Select a Task Manager menu item to display related controls for that task.



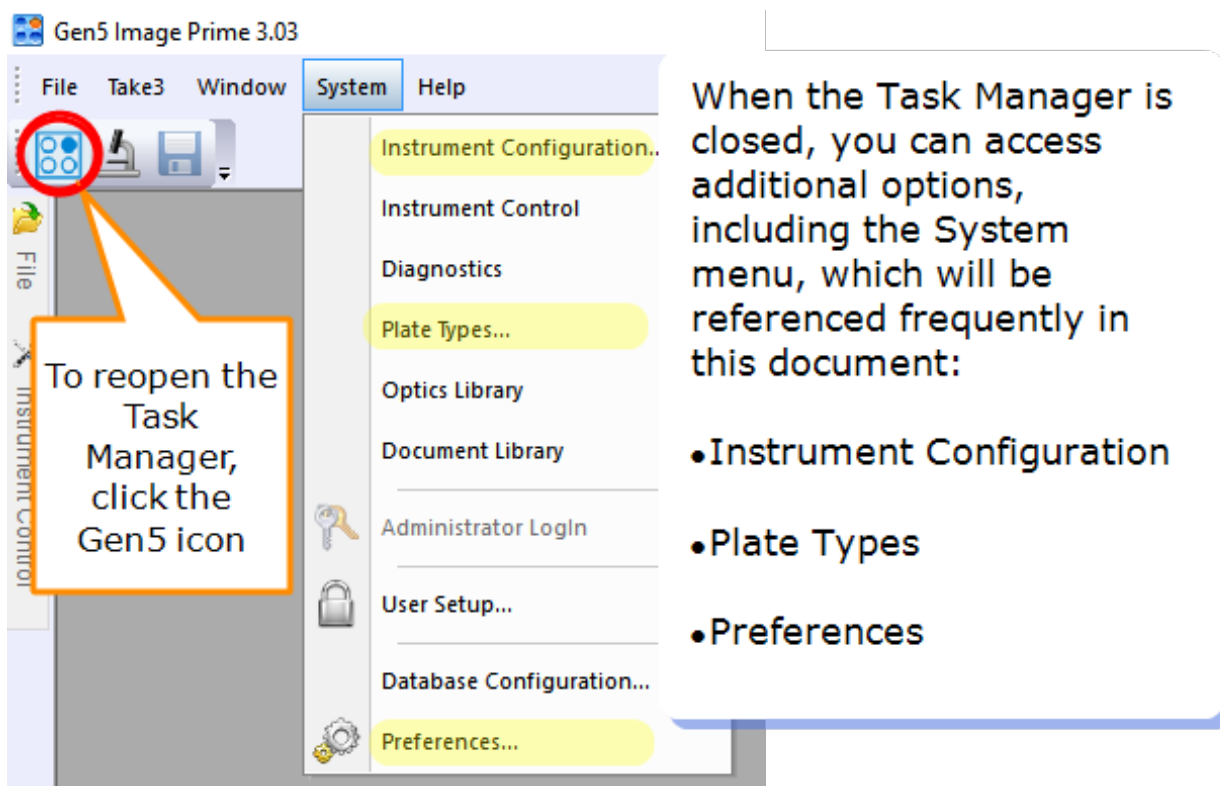
- **Read Now:** Immediately run an experiment.
- **Imager Manual Mode:** Use the imager as a manual microscope to view your sample live and capture one image at a time.
- **Experiments:** For automated acquisition and analysis: run an existing experiment or create new experiments.
- **Protocols:** Experimental design without data.



## Introduction

- **Instrument Control:** To control the imager independently, including incubation, opening/closing the carrier, priming and dispensing, gas recharge, etc.
- **Setup:** Configure instrument; define preferences, security, and System Menu.
- **Help:** Access the Gen5 Help topics, FAQs, Sample Files, and the contact information for Technical Support.

The above tasks will be referenced in the following sections. You will use the **Imager Manual Mode** and **Experiments** tasks most often. Also note that the Task Manager can be closed to access the menus at the top of the Gen5 window. The Task Manager may be reopened at any time by clicking the Gen5 icon.



The following key concepts are essential for understanding the microscopy workflow in Gen5. Several of these concepts are covered in more detail at the end of this section.


- **Manual Mode vs. Experiments:** Gen5 offers two ways of imaging. Manual mode (1.3 - Capture an Example Image on page 18 and 2.1 - Acquiring an Image on page 37) is used to manually customize all aspects of the image acquisition. This mode is most commonly used to take a few images or take a representative image for the creation of an experiment. Experiment mode is for automated acquisition of images across a microplate or vessel. Images are automatically acquired based on settings defined for a representative image captured in manual mode. The settings for an experiment are saved in a Protocol file.
- **Plate Types:** For automated microscopy, the dimensions of the microplate must be known by Gen5 to properly image the wells. The plate definition also contains important information: the bottom thickness of the plate and the default focus location (referred to in Gen5 as the Bottom Elevation). Gen5 has pre-made definitions for many common microplates. Selecting the correct plate type is critically important for successful imaging. If you need assistance selecting the

## Introduction

correct plate type or creating a new plate type, contact Technical Support or your Field Applications Scientist.

**Important:** *Never* use the plate types named “WELL PLATE” (e.g., 6 WELL PLATE, 96 WELL PLATE, etc.) for imaging experiments. These plates are in Gen5 for compatibility for non-imaging workflows. For more information about plate types: See Everything you ever wanted to know about Plate Types on the next page.

- **Bottom Elevation:** The autofocus default starting location for the Plate Type. It is critical to optimize this before using the plate in Experiment mode; otherwise, poor autofocus performance is expected. Gen5 offers several ways to set the Bottom Elevation, including the method described here: **Setting the Bottom Elevation**.
- **Correction Collars:** Correction collars make fine adjustments in the high-power objectives (20x, 40x, and 60x air) to compensate for changes in the vessel thickness to still achieve crisp focus. You must adjust the correction collar on objectives to match the bottom thickness of the sample vessel. If the collar is improperly adjusted images will be blurry and autofocus may have difficulty. See **Adjusting the Correction Collar on page 15** for more details.
- **File Types:** Gen5 uses three different file types and an image storage directory for storing imaging data:
  - **Image Manual Mode Sessions (.IMM):** These files store Manual Mode sessions. They can be re-opened to view images, take additional images in the session, or create new experiments. The default name is DATE\_TIME\_IMM Session.imm where DATE & TIME are the date and time that the session was started. The DATE is in year, month, day (YYMMDD) format. The TIME is in hour (24-hour), minute, second format. The raw images are not stored in the .IMM file. Instead an image storage folder containing the raw image files is created, called **Gen5 Image Library**.

 Image files are large and storing them can quickly exceed your computer’s memory. An external hard drive or similar device is recommended for storing the Gen5 Image Library. Using a cloud drive for storing image files is **not** recommended.

**Important:** Both the image storage folder (Gen5 Image Library) and the IMM file are necessary for opening the Gen5 Manual Mode session. Both must be copied to a new computer to open the file.

- **Experiment Files (.XPT):** The experiment file contains settings and data collected during an experiment from Experiment Mode. The raw image files are not stored in the experiment file. Instead an image storage folder containing the raw image files is created, called **Gen5 Image Library**.

**Important:** Both the image storage directory and the XPT file are necessary for opening the Gen5 experiment. Both must be copied to a new computer to open the file.

- **Protocol Files (.PRT):** The Protocol file contains only the settings to create an experiment. It is different from an Experiment file in that it does not contain data. Its purpose is to create

## Introduction

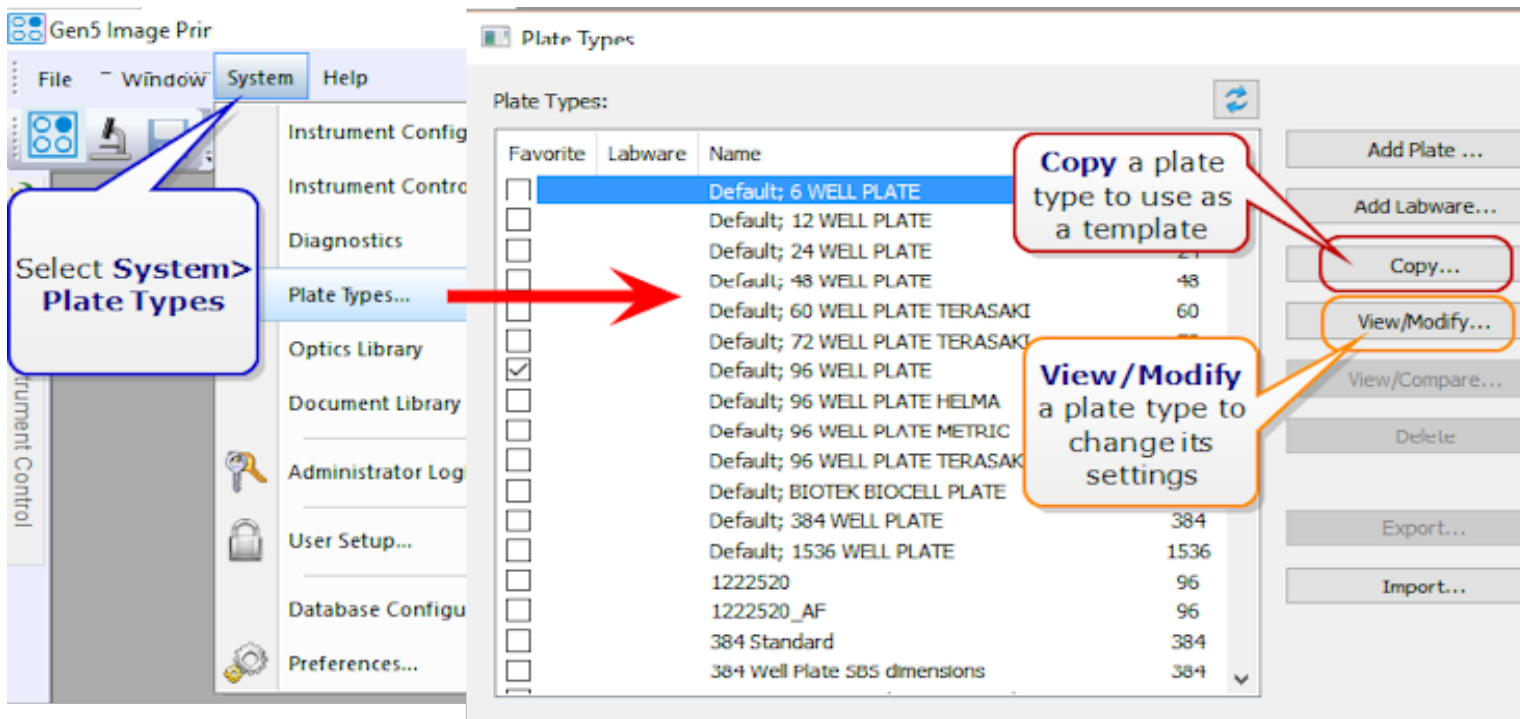
new experiment files with the same settings to repeat experiments, run experiments using the BioSpa 8, or run experiments with a 3rd party automation platform.

- **Image Storage Directory:** The location of the Gen5 Image Library is defined when Gen5 is installed (1.5 - Saving Gen5 Files and Defining the Image Library on page 26). Gen5 creates a separate folder in the image library to store the acquired images of each imaging session. The name of the folder contains a DATE and TIME prefix (see the DATE & TIME formats in the Manual Mode section above for the format) followed by the name of the Manual Mode session or Experiment file. These folders are essential for opening Gen5 files. To open an imaging session on another computer, both the corresponding image folder and .IMM or .XPT file must be copied or moved to the same location on that computer. This can be done manually or using a zip file created in Gen5 (*as described on page 200*). In addition, Gen5 can export images into a variety of formats including color or grayscale TIFF, PNG, JPEG, etc.

## Everything you ever wanted to know about Plate Types

The plate definition is more than just a map of your wells. It provides **critical information** to enable the imager to find focus and protect your objectives and samples. To access the Plate Type Library, close the Task Manager and:

- In Gen5, select **System>Plate Types** and **Copy** the plate type that is most similar to your vessel.
- **You need:** The plate/vessel with properly prepared samples.



**Important:** At a minimum, you must set the Bottom Elevation and Bottom Thickness to achieve the best results!

# Introduction

**Plate Description**

Name: Greiner 96 Black Flat Bottom Fluotrac  
Manufacturer: Greiner  
Catalogue #: 655073-655079

Number of Rows: 8  
Number of Columns: 12

Plate Width: 85480  $\mu\text{m}$   
Plate Length: 127760  $\mu\text{m}$

Plate Height: 14400  $\mu\text{m}$   
Stacked Height:  $\mu\text{m}$

Plate Lid adds: 3500  $\mu\text{m}$   
 Include Lid Parameters

Wells

Top Left Y: 11240  $\mu\text{m}$   
Top Left X: 14380  $\mu\text{m}$

Bottom Right Y: 74240  $\mu\text{m}$   
Bottom Right X: 113380  $\mu\text{m}$

Well Diameter: 6960  $\mu\text{m}$

Slide Holder

**Plate Description - Imaging Parameters**

Bottom Elevation (1): 3500  $\mu\text{m}$  (Distance from top of carrier to bottom elevation)

Define Accessible Bottom Focus Region

X/Y Positions (3)

Generate default values from existing well definitions

Top Left Y: 1500  $\mu\text{m}$   
Top Left X: 1500  $\mu\text{m}$

Bottom Right Y: 83980  $\mu\text{m}$   
Bottom Right X: 126260  $\mu\text{m}$

Bottom thickness (2): 100  $\mu\text{m}$  (Distance from bottom elevation to lowest position in bottom region)

Max distance below carrier: 0  $\mu\text{m}$  (Max distance below the top of the carrier outside the bottom region)

Show 2.5 mm grid

Update Display

then, change these parameters

Click here first

Imaging Parameters...

Auto Map...

OK Cancel Help


## Introduction

### Setting the Bottom Elevation

The bottom elevation may be defined either in Manual Mode or in the Plate Types window. However, be aware that the bottom elevation is a global change to the currently selected plate type and your changes will affect all other users who share this plate type. A safer practice is to create your own "Copy" of the plate type and make changes to that plate type record. This is particularly true if multiple users use different types of samples on the same plate type. The directions below show how to update the bottom elevation from the plate types window.

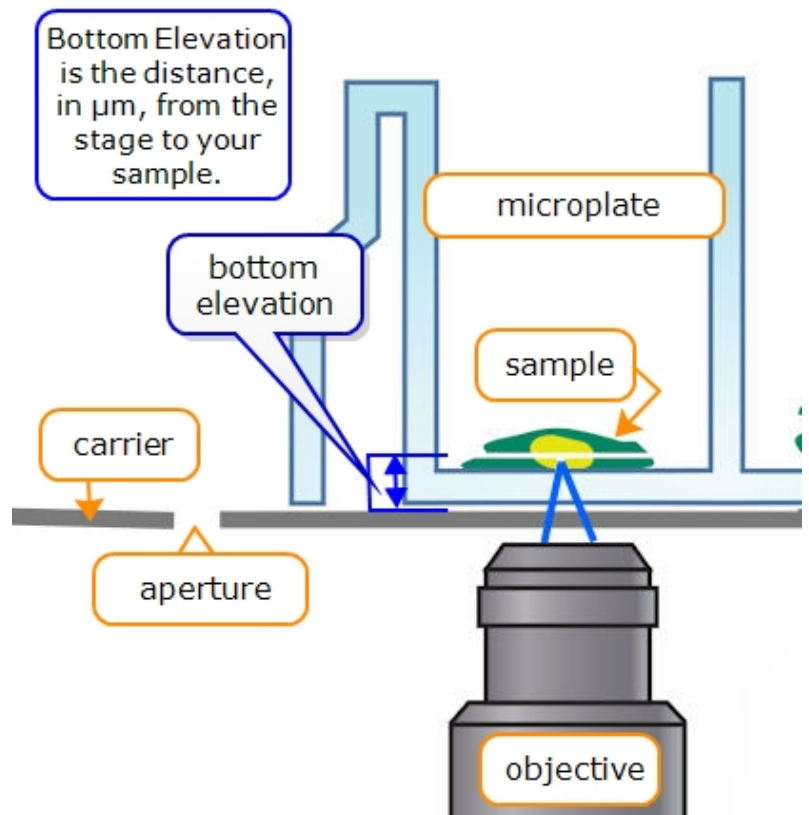
#### Bottom Elevation:

To define Bottom Elevation:

1. Put the sample plate on the carrier.
2. Click 
3. Focus on the sample; check the plate center and corners. When the image is satisfactory, click

**Save settings**

**Note:** Do not use the **Finder Scope** of the upright imager to determine bottom elevation.



#### Setting the Bottom Thickness:

This value comes **from the plate manufacturer** and can be found on the specification sheet or by calling the manufacturer.

**See also 11.4 - Labware on page 233.**

## Introduction

### Setting the Accessible Bottom Focus Region

For objectives 20x and higher, the accessible bottom focus region must also be defined.

Define Accessible Bottom Focus Region **1** Fill checkbox

X/Y Positions (3)

**2** Generate default values from existing well definitions

|                 |                                    |    |                 |                                     |    |
|-----------------|------------------------------------|----|-----------------|-------------------------------------|----|
| Top Left Y:     | <input type="text" value="7940"/>  | µm | Top Left X:     | <input type="text" value="1500"/>   | µm |
| Bottom Right Y: | <input type="text" value="81430"/> | µm | Bottom Right X: | <input type="text" value="126250"/> | µm |

Bottom thickness (2):  µm (Distance from bottom elevation to lowest position in bottom region)

Max distance below carrier:  µm (Max distance below the top of the carrier outside the bottom region)

1. Fill the checkbox to "Define Accessible Bottom Focus Region."
2. Click the button to "Generate default values from existing well definitions."
3. Enter a **Bottom thickness** - review specifications from vessel manufacturer or begin with a guesstimate, e.g., 500 µm for a standard microplate, 200 µm for a slide holder.  
"Max distance below carrier" is typically 0. This parameter supports hanging-drop plates and special vessels like T25 flasks in an adapter.
4. Save settings.

## Introduction

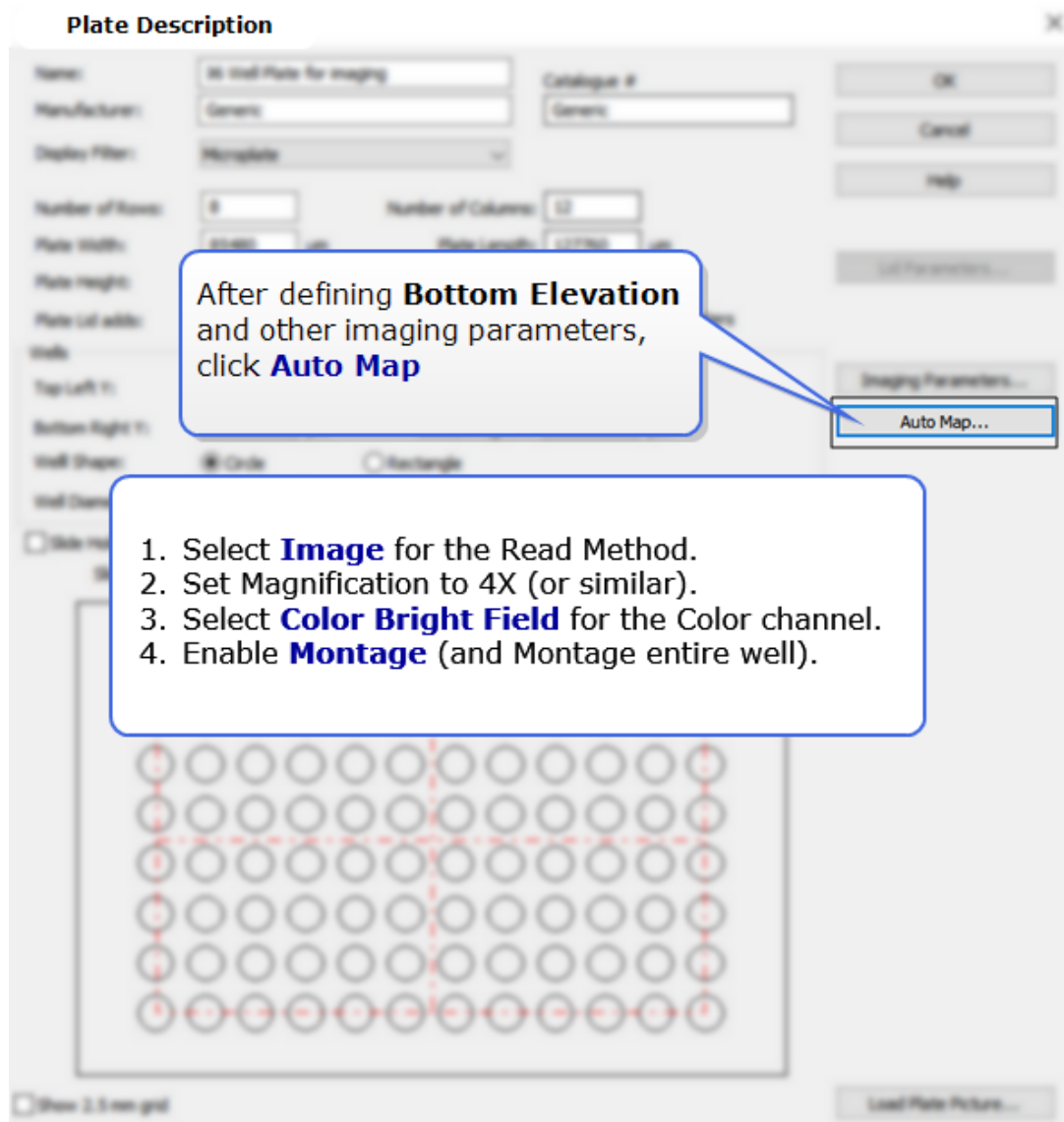
### Use AutoMapping to Precisely Define Well Position

Auto Map...

Gen5's Auto Map feature makes it easy to precisely define the position of wells in your plate type. This is an important first step to performing ELISPOT assays, and other whole-well assays. Run Auto Map to tell Gen5 exactly how to center wells in images.

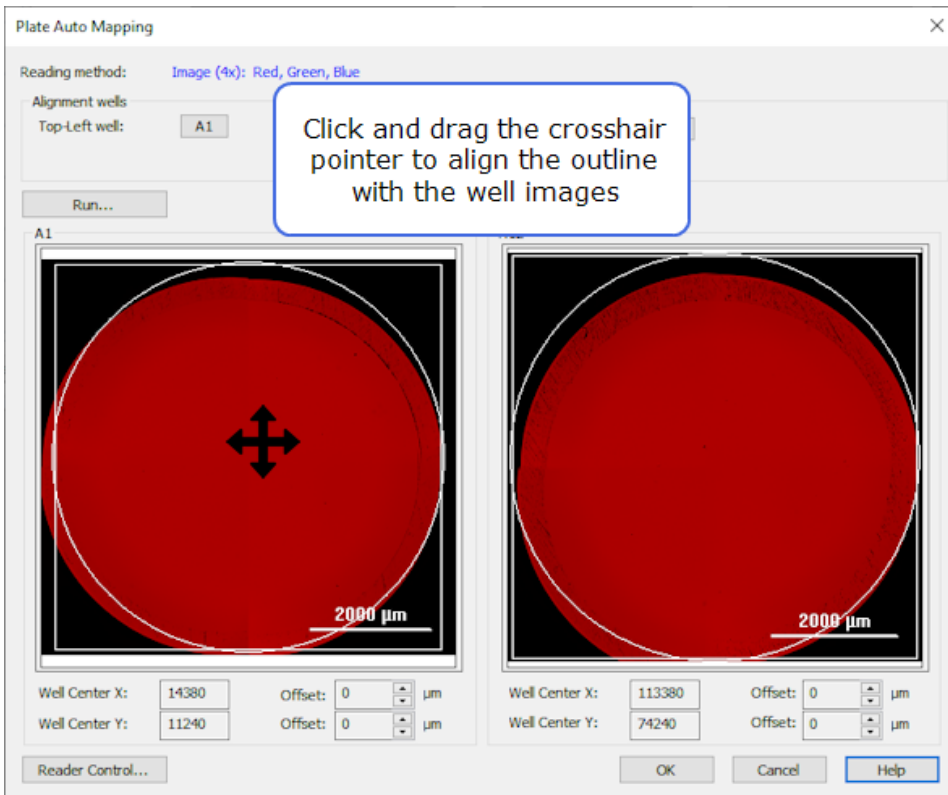
**Note:** This feature does not support slide holders, flasks, or similar labware. It is designed for standard microplates.

**Upright imager:** Transmitted light source is a good choice for Auto Map with an empty plate.



## Introduction

Using an empty plate , click **Run** and follow the prompts.



When both wells appear to be positioned correctly, click **OK** to save the offsets.

Gen5 will update the plate type record.



## Introduction

### Adjusting the Correction Collar

Correction collars make fine adjustments in the objective to compensate for changes in the vessel to still achieve crisp focus. You must adjust the correction collar on high-power objectives, 20X, 40X, and 60X air, to **match the bottom thickness of the sample vessel**. If the collar is improperly adjusted images will be blurry and autofocus may have difficulty. Redo this step for new or different vessels, as needed.



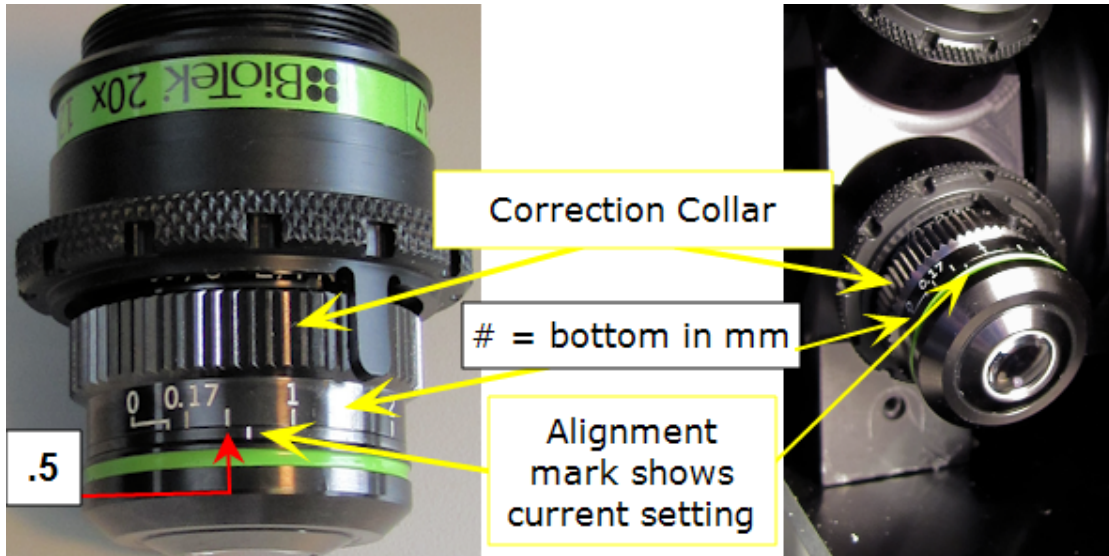
Use Manual Mode to access the objective:

The screenshot displays the microscope's software interface. On the left, the 'EXPOSURE' and 'FOCUS' panels are visible. The 'FOCUS' panel has a yellow box around it with a red '1' next to it, and the 'FM Scan...' button is highlighted with a yellow box. A red box around the 'Access Objective' button is labeled with a red '3'. On the right, the 'Focus Metric Scan' window is open, showing a graph of 'Focus Metric Ratio' vs 'Focus Position (µm)'. The graph has a peak at approximately 2059.8 µm. A red box around the 'Stop Scan' button is labeled with a red '2'. A text box in the center contains the following instructions:

1. Expand the **Focus** panel and click **FM Scan**
2. In the Focus Metric Scan window, click **Stop Scan**
3. Click **Access Objective**

When you click **Access Objective**, the turret will lower and rotate to position the objective you are currently using for removal. Open the access door and identify the objective. Objectives can be identified by the colored band around the objective.

## Introduction



| Magnification | Color  | Magnification                        | Color       |
|---------------|--------|--------------------------------------|-------------|
| 1.25X         | Black  | 20X*                                 | Green       |
| 2.5X          | Brown  | 40X*                                 | Light Blue  |
| 4X            | Red    | 60X*                                 | Cobalt Blue |
| 10X           | Yellow | * objectives with correction collars |             |

After identifying the objective, locate the correction collar, scale, and alignment mark. (It is **not** necessary to remove the objective from the turret.) Turn the collar until the alignment mark indicates the bottom thickness of your vessel in mm. **This should be the same value you input for the Bottom Thickness of your plate definition, adjusted to mm.**

For example, a Bottom Thickness of 500  $\mu\text{m}$  will have a correction collar set to 0.5.



Adjust focus ring and click OK to return objective to run-time position.

OK

After adjusting the correction collar, close the access door and click **OK**. The instrument will return to the same x-, y- and z-coordinates as prior to the correction. You will need to re-focus the field.

For reference here are some common Bottom Thickness values:

## Introduction

| Plate Type                                       | Typical Bottom Thickness*    |
|--|------------------------------|
| Glass-bottom microplate                          | 0.17 mm                      |
| Film-bottom imaging plastic microplate           | 0.19 or 0.5 mm               |
| Generic plastic microplates**                    | 1.3 mm (can be up to 2.0 mm) |
| Other plastic labware (Petri dish, T-25 flask)** | 1.3 mm (can be up to 2.0 mm) |
| Slide with glass cover slip                      | 0.17 mm                      |

\*These are typical bottom thicknesses. Check your plate manufacturer's specifications for the actual bottom thickness.

\*\*Although correction collars can adjust to these larger thicknesses, the best image quality at higher magnifications is always achieved with film or glass bottom microplates (or other vessels) with a bottom thickness of 0.5 mm or less.



Objectives can be accessed using the **Reader Setup** button on the toolbar or via the Gen5 menu: **System>Instrument Configuration>View/Modify>Setup**. Click **Access** next to the objective of interest.

Learn more about the imaging objectives and LED filter cubes: See 11.1 - Instruments and Accessories on page 215.

## Introduction

### 1.3 - Capture an Example Image

#### Preparing to Capture an Image



Before you begin, prepare your sample for imaging and identify which **Plate Type** corresponds to the vessel containing your sample (See Everything you ever wanted to know about Plate Types on page 9.)

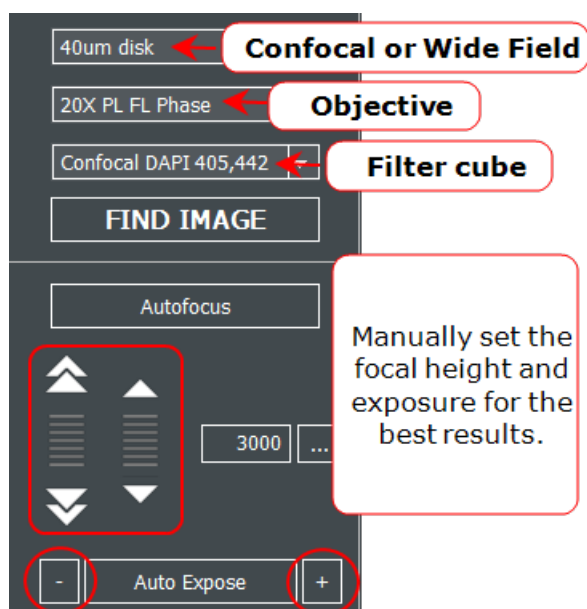
Click the **Manual Mode** button to begin. Select the Plate Type and load the plate into the instrument.

#### Guidelines for Capturing an Image in Manual Mode

See also 2.1 - Acquiring an Image on page 37 and 2.5 - Additional Acquisition Options on page 51.

Chapter 2 of this training guide covers Manual Mode in detail. The purpose of this section is to outline how to use imager manual mode to accomplish your scientific goals. You may wish to reference section 2.1 before starting this section if you have never used manual mode before (or need a refresher) to see how to accomplish each of these steps and for more fine details on the operation of manual mode.

The first step is to bring an image into focus. Select a **well** that contains sample; a **color channel** that is present in the sample; and the desired **magnification**. (If you are unsure how to do this or any of the following steps, stop now and read section 2.1.)

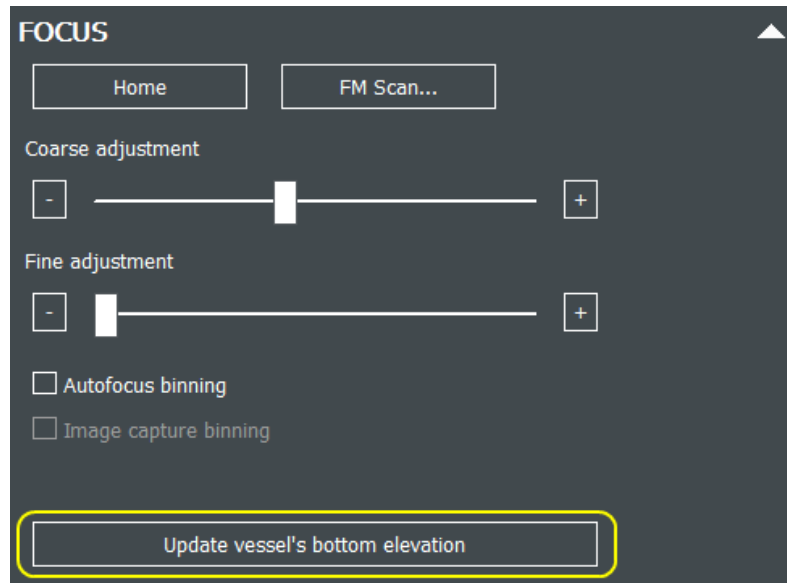


Next, click **Find Image** to automatically adjust the focus and exposure. If this is the first time using the plate definition, the bottom elevation may be incorrect and autofocus will fail. If this happens, manually adjust the focus to bring the sample into focus.

**Update the Bottom Elevation:** If this is the first time you have used this plate definition and/or a large change in focus was required from the default position (> 100 um), it is critically important to update the bottom elevation of the plate. The bottom elevation is the default focus location. Updating it will save you time in the future when using this plate (manual mode will start at the bottom elevation setting) and most importantly the bottom elevation is the starting point for the autofocus routine in experiment mode. Most

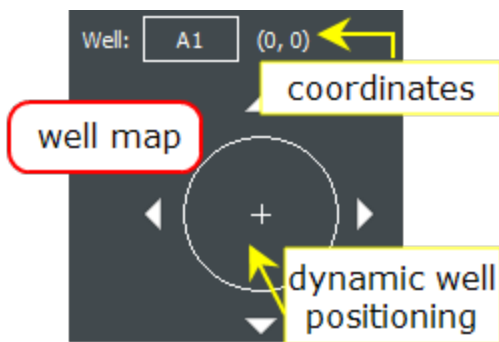
## Introduction

issues with the autofocus in experiment mode stem from having an incorrect bottom elevation setting. To update the bottom elevation: expand the **Focus** panel, and select **Update vessel's bottom elevation**.



For more information see: **Setting the Bottom Elevation**.

Once the bottom elevation is set, the next step is to capture a reference image. This paragraph describes how to capture a single reference image. If your experiment requires a montage or z-stack, read through the directions here, as the same principles apply, and then see the "Multidimensional" paragraph below.



The best practice is to set the exposure and focus settings on the brightest well in your sample (often the positive control). Navigate to that well. (Click the **Well** button.)

The most important choice is the first color to capture. The first color channel is used for autofocus in Experiment mode. This color channel must be present in every well and should be bright with good contrast. Common choices for the focus channel include nuclear stains (e.g. DAPI, Hoechst, DRAQ5), brightfield, or phase contrast.

Choose the first channel and adjust the exposure and focus.

When the first channel is captured, switch to the next color channel, adjust the exposure and focus, and capture it. Repeat until all of the colors required for your experiment have been captured for a single image. When all channels are captured, click **Process / Analyze**, described below.

**Note:** If using a 20x, 40x, or 60x air objective and the image cannot be focused manually, the correction collar may need to be adjusted. See Adjusting the Correction Collar on page 15.

Multidimensional acquisition is defined on the **Imaging Mode** panel. Options include montage (3.3 - Image Montage on page 64), z-stack (3.4 - Z-Stacking on page 72), and kinetic (3.5 - Kinetic Image Capture as shown on page 79). The montage and z-stack acquisitions are useful for creating an image

## Introduction

set to create a protocol. The kinetic settings are used for imaging a specific cell location. All of these options can be added later; and, in most cases kinetics should be added to an experiment later.

### Guidelines for Processing Images in Manual Mode

See 2.3 - Image Processing and Analysis on page 45; or See 6 - Image Processing on page 138.

Image Processing methods are discussed in detail in Chapter 6. Here are more general guidelines on when to apply them:

- **Image Preprocessing** is the most commonly used processing method. Image Preprocessing uses a rolling ball algorithm to smooth uneven backgrounds. Typically recommended for fluorescent images with their dark backgrounds, applying Image Preprocessing prevents future samples from having background issues. Only apply it to brightfield images that need to be quantified. The size of the rolling ball may be adjusted for each channel and should be set to 2-3 times the size of the object of interest in most circumstances.
- **Image Deconvolution** applies a 2D deconvolution to the image. Deconvolution most improves images of 20x and higher magnification of small objects (< 500 nm in size). If deconvolution is used, it should be applied to the raw image.
- **Image Stitching** is only applied to image sets with a montage. It stitches the image together to provide a single, seamless image for analysis and export. Only images with overlap need to be stitched.
- **Z-Projection** reduces the z-stack from a series of z-images to a single image.
- **Kinetic Frame Alignment** aligns the images from a kinetic series. This tool is qualitative only for movie export and viewing. It does not affect any analysis results.
- **Digital Phase Contrast** converts a standard brightfield image into a phase-like image (background is changed from light to dark). This is useful for full well images in 96- and 384-well plates that have a large meniscus effect in phase contrast.

### Guidelines for Analyzing Images in Manual Mode

- 2.3 - Image Processing and Analysis on page 45.
- 7 - Basic Image Analysis on page 148.
- 8 - Advanced Analysis as shown on page 168.

All of the analysis in Gen5 is accomplished using **Cellular Analysis** or **Image Statistics**.

**Image Statistics** is primarily used to measure the physical size (length, width, area) of the image for use in other calculations (e.g., confluence). The primary tool is **Cellular Analysis**: described in Chapter 7 (Image+ & Image Prime) and Chapter 8 (Image Prime). Specific details for defining cellular analysis depend on the sample and the scientific goals of the analysis.

However, some general principles include: All cellular analysis begins with a count of a specific color channel. What is counted depends on the analysis goals. For example, counting cells would select a marker (e.g., DAPI) that identifies each nucleus/cell or, if confluence wants to be measured, a whole cell stain would be selected (e.g., Calcein). From there "subpopulations" can be identified to perform additional counts based upon unique criteria. For example, in a live/dead experiment where two nuclear stains are used, the initial count would count the color that stains all cells live or dead (e.g., Hoechst). Then a subpopulation counts the number of cells that are also stained with the dead cell color (e.g., propidium iodide). Details on how to perform each of these steps are given in Chapter 7 & section 2.3.

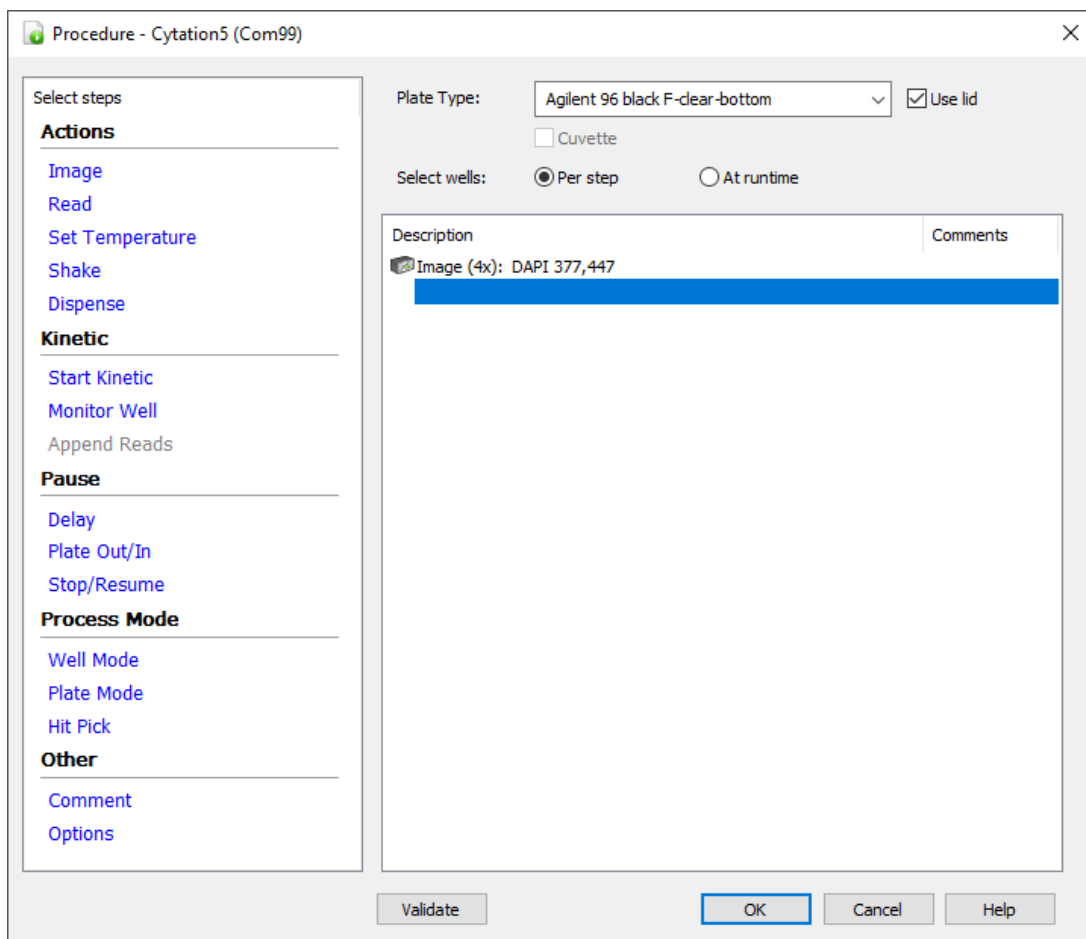
## Introduction

### 1.4 - Create an Experiment from the Example Image



After the example image is captured, processed, and analyzed, the entire image session can be converted to an Experiment to batch process the entire plate and save a protocol to use for future experiments.

The Experiment is created by first selecting the image and then either clicking on the **Create Experiment** button or right-clicking on the thumbnail and clicking "Create Experiment from this image set." Both options open a new Gen5 Experiment window with the Procedure settings displayed. All the acquisition settings from the example image session have been copied to the **Procedure** window. Any processing or analysis steps have been copied to the **Data Reduction** window (which will be addressed later in this section). Several common settings that you may want to add to your protocol are addressed in the next sections. If you do not need to add any of these, you may skip ahead to the Data Reduction section.



### Selecting Wells To Image

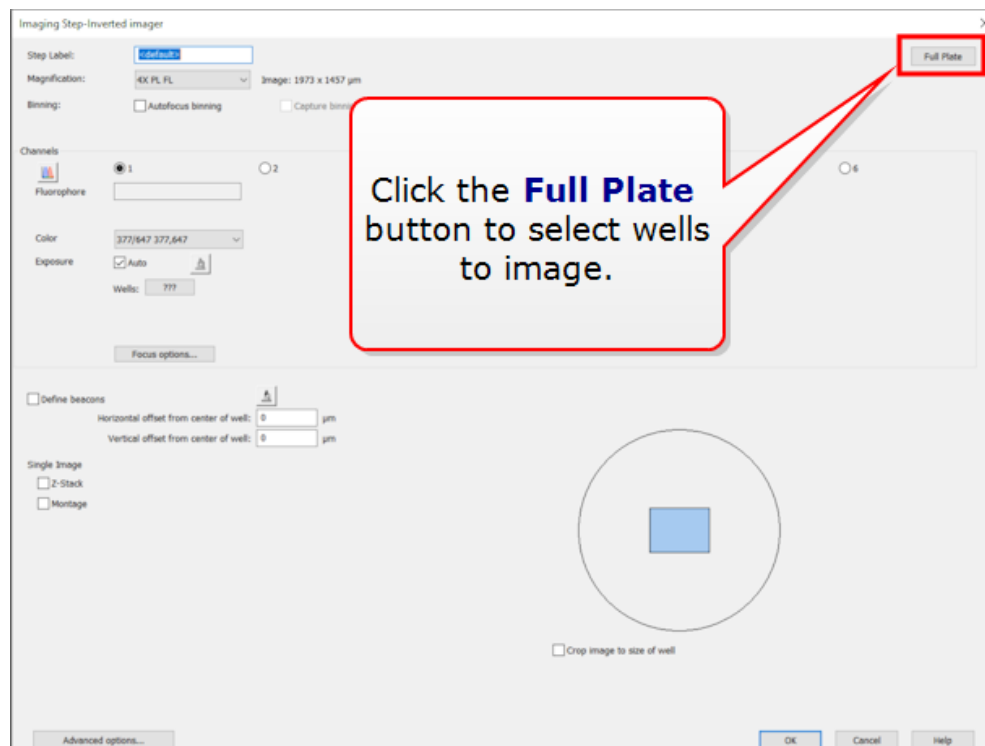
By default, every well on the plate will be imaged. Two options let you select specifically which wells to image on the plate.

The options are chosen with the **Select wells** buttons immediately below the **Plate Type** field in the Procedure.

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Plate Type:   Use lid  
 Cuvette  
Select wells:  Per step  At runtime

1. **Select wells: Per step.** This is the default option. This option will run the wells configured on each imaging step. To select the wells, open the imaging step by double clicking on it and select the box in the upper right corner. By default this button says **Full Plate**, but if wells were previously selected it will have the range or number of wells listed.



This option is useful if the same wells will be read every time the protocol is run. It is also required when using the Biostack, BioSpa, or another robotic integration using Gen5 OLE.

Plate Type:   Use lid  
 Cuvette  
Select wells:  Per step  At runtime

2. **Select wells: At runtime.** The option will ask you each time the protocol is run which wells to image. The same wells will be imaged on each step of the procedure (if there are multiple imaging steps). This option is not compatible with the BioSpa or another robotic integration using Gen5 OLE.

## Autofocus Settings

See 5 - Understanding AutoFocus on page 124.

Successful imaging requires understanding and choosing the best autofocus method for your sample. By default, Gen5 uses an image-based autofocus that is the same as clicking "Auto Focus" in imager manual

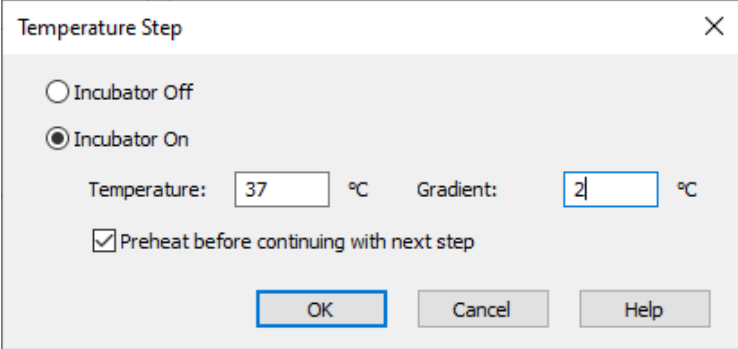


## Introduction

mode. The most common adjustment is to use the Laser AutoFocus (LAF) accessory. See [5.2 - Laser AutoFocus](#) on page 132.

## Temperature



For applications requiring a specific temperature setting (e.g., live cell imaging), the **Temperature** step should be the first step in the protocol. This will bring the microscope up to temperature before asking you to load your plate. To add a temperature step, click **Set Temperature** under Actions in the left panel.



The dialog box titled "Temperature Step" contains the following elements:

- Two radio buttons: "Incubator Off" (unselected) and "Incubator On" (selected).
- Two input fields: "Temperature: 37 °C" and "Gradient: 2 °C".
- A checked checkbox labeled "Preheat before continuing with next step".
- Three buttons at the bottom: "OK", "Cancel", and "Help".

Set the desired temperature. For brightfield or phase contrast imaging, it is recommended to add a 2°C gradient to keep condensation from forming on the lid. If the temperature step is not listed first in the protocol, click and drag it to the first spot.

| Description   | Comments |
|---|----------|
|  Temperature: Setpoint 37 °C |          |
|  Image (4x): DAPI 377,447    |          |

## Kinetic Acquisition

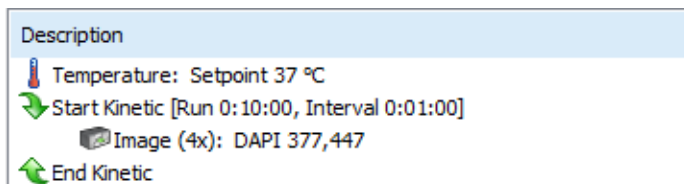
See [3.5 - Kinetic Image Capture](#) on page 79.

There are two options for kinetics in Gen5. Setting up the kinetics options differ and the choice depends on the experimental needs. The two options can also be combined in an Experiment.

1. **Kinetics.** The standard kinetics option is used when you want Gen5 to time the experiment. The plate will remain in the instrument during the entire experiment. This is typically done for rapid kinetics.
2. **Discontinuous Kinetics.** Discontinuous kinetics allows for the removal of the plate between each time point. The timing of the plate reads is controlled by the user. This is typically used for long term assays where the instrument needs to be used between each read. This is also commonly used for BioSpa and Gen5 OLE kinetic protocols.
3. **Combining Kinetic and Discontinuous Kinetics.** When both kinetics options are applied, Gen5 appends the data for each standard kinetic image set for analyzing all the results collected during the time span. Set both kinetics options if this is useful for the experiment.

To configure standard kinetics, click **Start Kinetic** under Kinetic on the left panel of the Procedure window. The kinetic run is defined by setting the total time (duration) and the interval (image capture frequency). The Image step needs to be inside the kinetic loop as shown below.

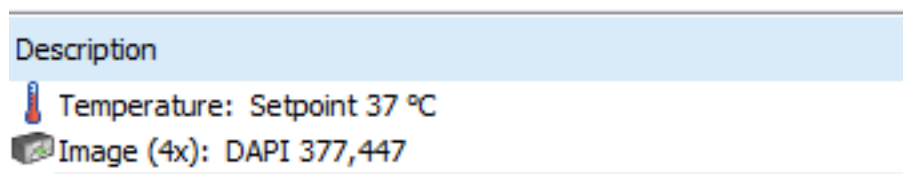
## Introduction



If the steps are not properly positioned, drag and drop them in the Procedure window.

To turn on **Discontinuous Kinetics**, click **Options** under Other on the left panel of the Procedure window.

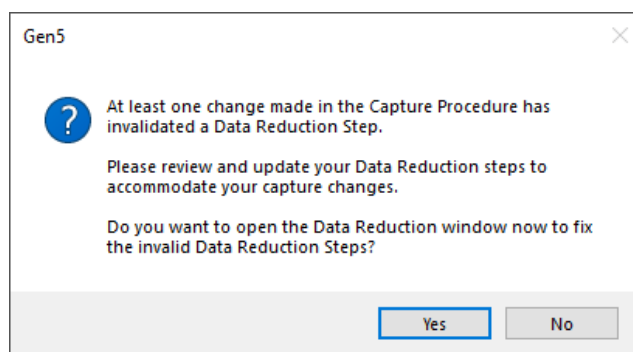
The Discontinuous Kinetic run is defined by setting the total time and interval. These are only estimates. The final time stamps are recorded when the plates are read. If you are unsure about the total number of time points, it is best to overestimate. There is no kinetic loop or other indicator shown for a Discontinuous Kinetic, so the Procedure window will look the same before and after it is added. See [Discontinuous Kinetics on page 80](#).



## Data Reduction


- 6 - Image Processing on page 138
- 7 - Basic Image Analysis on page 148
- 8 - Advanced Analysis on page 168

Many of the above adjustments to the Procedure will invalidate a Process or Analysis step that was defined in manual mode. In experiment mode, all of the Process and Analysis steps are listed under **Data Reduction**. If that is the case, the message below will appear.



Every step that is covered with a "red no" symbol needs to be addressed. In most cases, double clicking the step and then clicking OK to close it will clear the issue. However, in some cases you will need to select a new data set input. In these cases, a message will appear when clicking "OK".

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| Description   | Data Out          |
|---|-------------------|
|  Image Preprocessing | Tsf[DAPI 377,447] |
|  Cellular Analysis   | Cell Count        |

More Data Reduction steps can be added to do transformations that cannot be performed in imager manual mode. These are covered in the Gen5 online help in **Help > Help Topics > Define Data Reduction Requirements**.

## Introduction

### 1.5 - Saving Gen5 Files and Defining the Image Library

Image file sizes are significantly larger than other data files.

Widefield (standard field-of-view) image files are at least 2 MB each.

A 4x4 montage per well of a 96-well plate will require **3327 MB of storage space**.

Confocal and Wide Field of View (WFOV) camera imaging create files around 7.57 MB.

A 4x4 montage per well of a 96-well plate will require **11.6 GB of storage space**.

Using an external hard drive or another form of additional storage for images is strongly recommended. However, storing images on a cloud drive is **not** recommended.

During installation of your imager and Gen5 software, it is likely that default settings were retained. To check or change these settings, close the Task Manager and select **System > Preferences** from the Gen5 menu. **Changes are global** and will change storage location of future experiment, protocol, and image files.

The image shows a screenshot of the Gen5 software interface. On the left, the 'System' menu is open, with 'Preferences...' highlighted at the bottom. A red arrow points from the 'System' menu to the 'System Preferences' dialog box on the right. In the 'System Preferences' dialog, the 'File Storage' section is highlighted with a blue box. This section contains two radio buttons: 'Gen5 Database' (selected) and 'Windows File System'. A blue callout box points to these options with the text: 'Gen5 Experiment and Protocol files can be stored in the Gen5 Database or Windows File System'. Below this, an orange callout box states: 'Targeted Apps, like the Scratch Assay App, must use the Windows File System'. At the bottom of the 'System Preferences' dialog, 'Image Save Options' is highlighted with a red box, and a red callout box points to it with the text: 'Create an Image Library'. A red arrow also points from the 'Image Save Options' box back to the 'System Preferences' dialog.

Select: **System>Preferences**

**File Storage**

Storage option for Experiment and Protocol files:

- Gen5 Database
- Windows File System

Gen5 Experiment and Protocol files can be stored in the Gen5 Database or Windows File System

Targeted Apps, like the Scratch Assay App, must use the Windows File System

Create an Image Library

Image Save Options

## Introduction

### Image Save Options

Raw image files are large compared to other Gen5 data files, so they are handled differently. Images are stored in the Windows File System in a user-specified location. Click **Browse** to redirect your image files.

**Image Save Options**

Gen5 Image Library

Pathname:  Browse...

Experiment Folder

Name:

Example:

Plate Folder

Name:

Example:

Image File Naming Convention

Name:

Example:

Image files are stored in the Gen5 Image Library in a user-specified location in the Windows File System

See also 9.1.2 - Strategies for Data Management on page 201.

### Save the Protocol to Automate Future Experiments

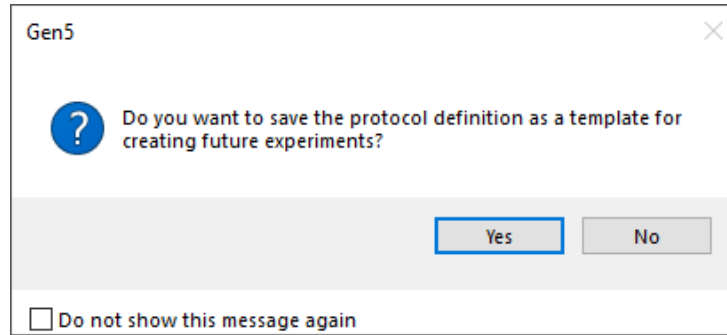
The Protocol file (file extension .PRT) stores all of the experiment settings for repeating the same experiment. In other words, the steps described above in sections 1.3 & 1.4 only need to be performed once per experimental design.



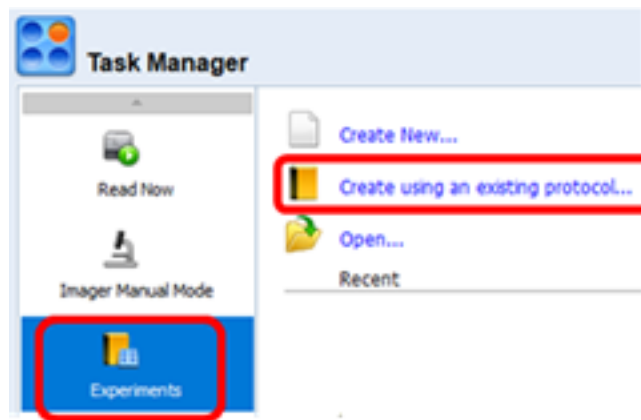
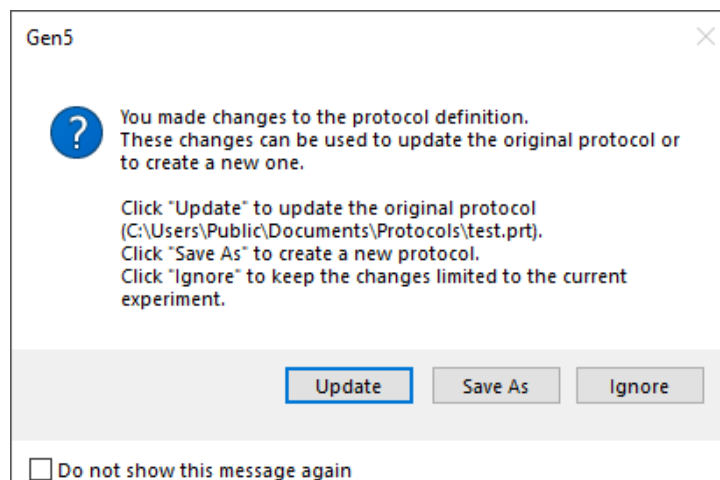
When you are ready to execute the experiment click the "Read New" button that looks like a play button in green. Gen5 will ask you to save the experiment (.XPT file) and then execute the protocol as designed.

The protocol is not saved when the experiment is saved. (Remember, experiments and protocols are two separate files.) The Protocol file can be saved at any time from an experiment by selecting **File > Save Protocol As**. And, when closing an experiment, you will be prompted to save the protocol file if it hasn't been saved before.

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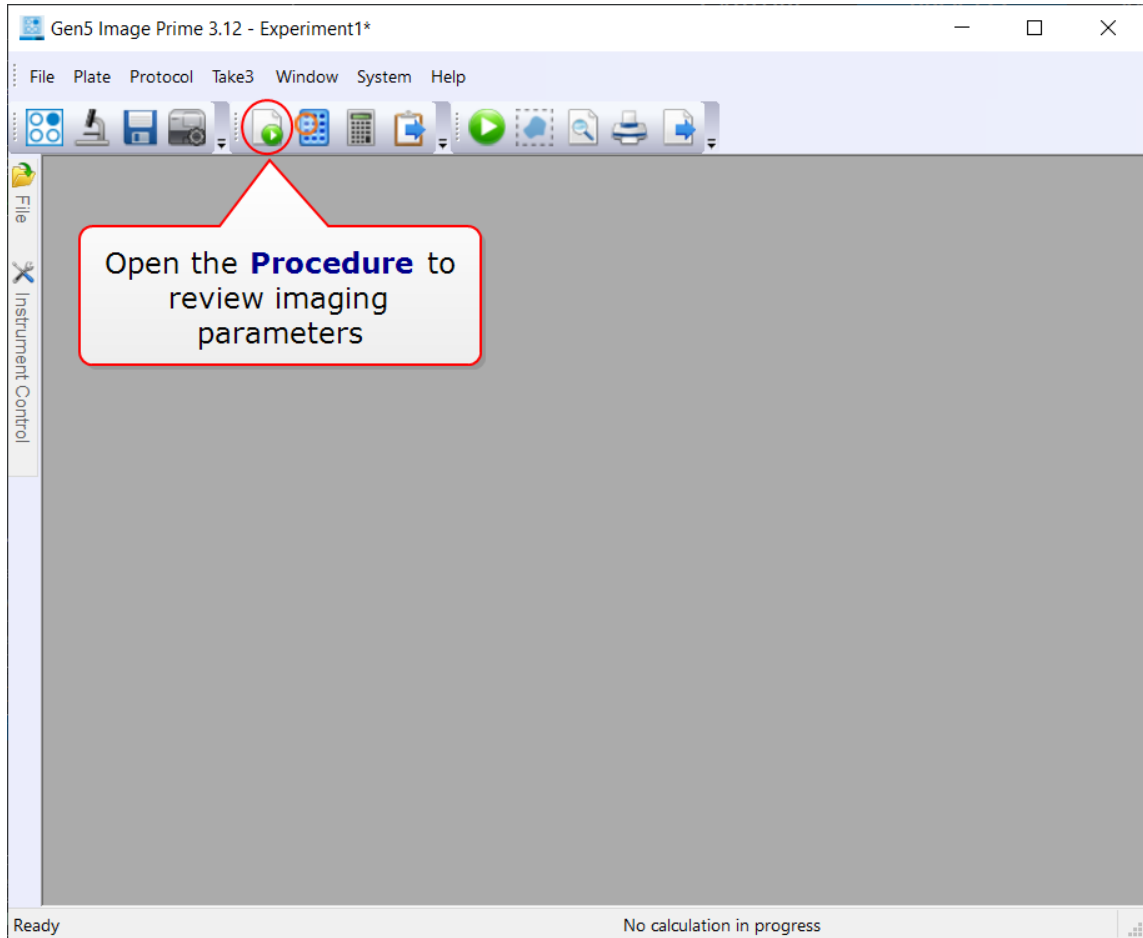


If the protocol has been previously saved, you will be prompted to either "Update", "Save As", or "Ignore" any changes made to the Protocol.



When the protocol file has been saved, you can use the protocol to repeat the same experiment. In the Task Manager, select **Experiments** and **Create using an existing protocol...**

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A new Experiment window will open containing all of the previously defined settings. You may modify settings or begin acquiring data in the new experiment.

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### 1.6 - Example Exercises

Below are example exercises to test your understanding after reviewing the concepts in this introductory chapter and the details in the following chapters. As you think about how to approach performing these exercises, keep in mind the following workflow:

1. Prepare to image:
  1. Know which Gen5 plate type matches your plate. If necessary, make a new Plate Type (copy a plate-type record) before starting, and adjust Bottom Elevation (and other imaging settings).
  2.  Start Manual Mode, select the plate type, and load the plate with your sample into the instrument.
2. Capture an example image:
  1. Focus and adjust the exposure to get a good image. If this is the first time using the plate or if a large change in focus is required ( $> 100 \mu\text{m}$ ) to find the initial image, update the bottom elevation of the plate.
  2. Go through the Augmented Microscopy workflow:
    1. **Capture:** Acquire all of the colors necessary for the experiment. The first color acquired is the focus channel. The focus channel should be bright and present in all of the wells. Common focus channels are nuclear stains (e.g., DAPI, Hoechst, DRAQ5), brightfield, or phase contrast.
      - If a montage and/or z-stack is required, define them in the Manual Mode session to acquire these images.
    2. **Process:** Add processing steps to the example image. Image Preprocessing is used in most experiments. Add Image Stitching or Z-Projection if capturing a montage or z-stack, respectively.
    3. **Analyze:** Add analysis steps to create the desired quantitative data from the image.
3. Create an Experiment from the example image:
  1.  Click the Create Experiment button or right-click on the thumbnail and click "Create experiment from this image set."
  2. Adjust the following settings in the **Procedure** as needed:
    1. Select Wells To Image
    2. Autofocus Settings
    3. Temperature
    4. Kinetic Acquisition
  3.  If prompted, update the necessary **Data Reduction** (analysis) steps, as needed.



## Introduction

4. Save the Protocol to automate future experiments:

1. Protocols are saved using File > Save Protocol As or by closing the Experiment and choosing to "Update" or "Save As" the protocol when prompted.
2. Create a new experiment using a previously created Protocol in the Task Manager by selecting Experiments then "Create using an existing protocol."

\*\*\*\*\*

### Exercise 1: Fluorescent Cell Counting

Goals:

- A) Automate acquisition of a single image of DAPI in a 96 well plate.
- B) Count the number of DAPI labeled cells in a single image.
- C) Estimate the total number of cells in the well based upon the single image.

### Exercise 2: Fluorescent Confluence

Goals:

- A) Automate acquisition of a single image of DAPI & GFP in a 96 well plate.
- B) Measure the confluence of cells in the image based upon the full cell GFP signal.

### Exercise 3: Transfection Efficiency

Goals:

- A) Automate acquisition of a single image of DAPI & GFP in a 96 well plate.
- B) Count the number of DAPI labeled cells in a single image.
- C) Count the number of GFP positive cells in the image.
- D) Calculate the GFP transfection efficiency in each well.

## Agilent BioTek Imaging Training Video Series

In this training video series, you will learn how to acquire and analyze images and plate reading data from our instruments within the Agilent BioTek Gen5 microplate reader and imager software. These training videos are designed to help you capture, process, and analyze the data you have so that you can publish faster.

Click on the link to watch the video.

| Video Name   | Description  |
|--|--|
| <a href="#">Basic Image Analysis Concepts-Part 1</a>           | This training video explains the concept image thresholding, both in image statistics and cellular analysis functions. There is also a demonstration of how to add Image Statistics to your protocols.   |
| <a href="#">Basic Image Analysis Concepts-Part 2</a>           | In this training video, we demonstrate how to determine and add object threshold and sizes for your samples. This video also shows how to adjust advanced detection options such as rolling ball to create cellular analysis and object masking in Gen5.           |
| <a href="#">Basic Image Analysis Concepts-Part 3</a>           | This training video explains how to add Object level metrics from Primary Masks, create plugs and subpopulations in Gen5.  |
| <a href="#">Converting from Manual Mode to Experiment Mode</a> | This tutorial will walk you through creating an experiment from a manual mode session, where to visualize and modify protocol settings, how to run your experiment and visualize your data, and finally, how to save protocols for later use in the Gen5 software. |
| <a href="#">High Contrast Brightfield</a>                      | This video demonstrates how to use high contrast brightfield for label-free cell counting. It covers settings for capturing images in manual mode, as well as process/analysis steps to obtain accurate cells counts.  |

|   |   |
|---|---|
| <a href="#">Kinetics Overview</a>                               | <p>This training video demonstrates how to add continuous and discontinuous kinetics to existing imaging protocols and discuss best use for each type of kinetic workflow.</p>  |
| <a href="#">ROI Feature</a>                                     | <p>This training video reviews the ROI feature in Gen5 and demonstrates how to set up an experiment using this feature.</p>   |
| <a href="#">Gen5 File Types and the Image Library</a>           | <p>This video discusses different data types in Gen5 and explains where the data saved in Gen5 and how to change that. Additionally, it explains how Gen5 uses tiered file organization systems to save the captured images inside the experiment folder.</p>   |
| <a href="#">Montage Image in Manual Mode</a>                    | <p>This video demonstrates how to create a montage image in manual mode. By the end of this tutorial, you will be able to capture multi-channel montage images and learn how to apply image stitching to create a single image from montaged tiles.</p>   |
| <a href="#">Image Exposure Settings</a>                         | <p>A review of the fundamentals in fluorescent imaging and how to adjust acquisition settings to best suit your needs.</p>  |
| <a href="#">Save Color Images in Manual and Experiment Mode</a> | <p>This video covers saving single-color images both in the manual and experiment modes. It also covers the batch image save option in the experiment mode.</p>   |
| <a href="#">Data Management in Gen5</a>                         | <p>This video explains how to relink image folders to the saved experiment files. Additionally, it shows how to create a zipped file for better managing and archiving the data.</p>  |
| <a href="#">Z-Stack Image in Manual Mode</a>                    | <p>This video explains how to create a z-stack image in the manual mode. By the end of this tutorial, you will be able to capture multi-channel z-stack images and learn how to utilize image processing tools in Gen5 to create a final z-projected 2D image for better visualization of the capture 3D image.</p> |

|   |   |
|---|---|
| <a href="#">Image-Based Autofocus</a>           | <p>Gen5 has five different image-based autofocus methods. At the end of this video, you will understand how image-based autofocus works and the default autofocus settings in Gen5.</p>   |
| <a href="#">Laser Autofocus</a>                 | <p>This training video reviews how laser autofocus works, when the laser autofocus accessory should be used, and how you can select to use laser autofocus for your experiment.</p>   |
| <a href="#">Plate Definition</a>                | <p>This tutorial will walk you through the steps to create a cell count in Gen5's manual mode workspace as well as general use of the interface. By the end of this tutorial, you will be able to capture multi-channel images, add preprocessing to your image, and create a basic cell count.</p> |
| <a href="#">Custom Vessel Definitions</a>       | <p>In this training video, you will learn three different ways to create a new plate type or custom labware in Gen5.</p>  |
| <a href="#">Basic Cell Count in Manual Mode</a> | <p>This tutorial will walk you through the steps to create a cell count in Gen5's manual mode workspace as well as general use of the interface. By the end of this tutorial, you will be able to capture multi-channel images, add preprocessing to your image, and create a basic cell count.</p> |

## Introduction

## 2 - Quick Guide to Imaging

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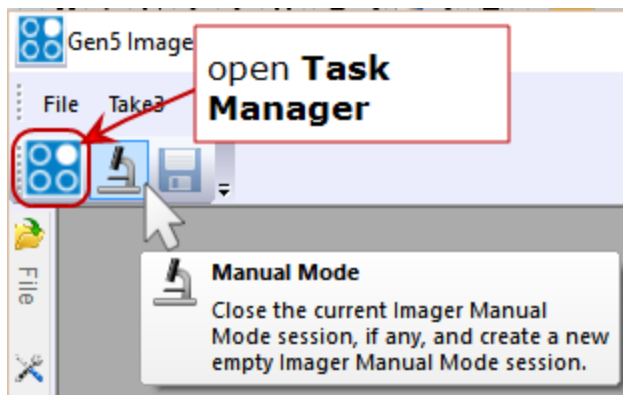
Manual Mode allows you to use your imager as though it were a manual microscope without the oculars. Manual Mode sessions are saved with the filename extension **.imm** and can be opened again at a later time from the **Task Manger**: select **Imager Manual Mode**.

Manual Mode allows you to acquire a **single field at a time** in up to 6 colors. If you keep the same position in focus, i.e., do not change the x-, y-coordinates, when you begin capturing images, Gen5 overlays up to six channels in one image.

*The goal of this section is to help you acquire a single multicolor image.*

|   |           |
|---|-----------|
| <b>2.1 - Acquiring an Image</b> .....             | <b>37</b> |
| <b>2.2 - Image Review and Editing</b> .....       | <b>43</b> |
| <b>2.3 - Image Processing and Analysis</b> .....  | <b>45</b> |
| <b>2.4 - Conversion to Experiment Mode</b> .....  | <b>47</b> |
| <b>2.5 - Additional Acquisition Options</b> ..... | <b>51</b> |
| <b>2.6 - Best Practices</b> .....                 | <b>54</b> |

### Quick Start Instructions



Click the **Manual Mode** button on the main menu toolbar

or

open the **Task Manager** (if necessary)

1. Select **Imager Manual Mode**.
2. Select **Capture now**. The "Load Plate" dialog will open.
3. Put your plate on the carrier/stage and select its **Plate Type** (created in 1.2 - Important Gen5 Imaging Concepts as shown on page 6). The Manual Mode window will open with a live view of your first well.

You may hear the objective turret turn and the stage move— this is normal.

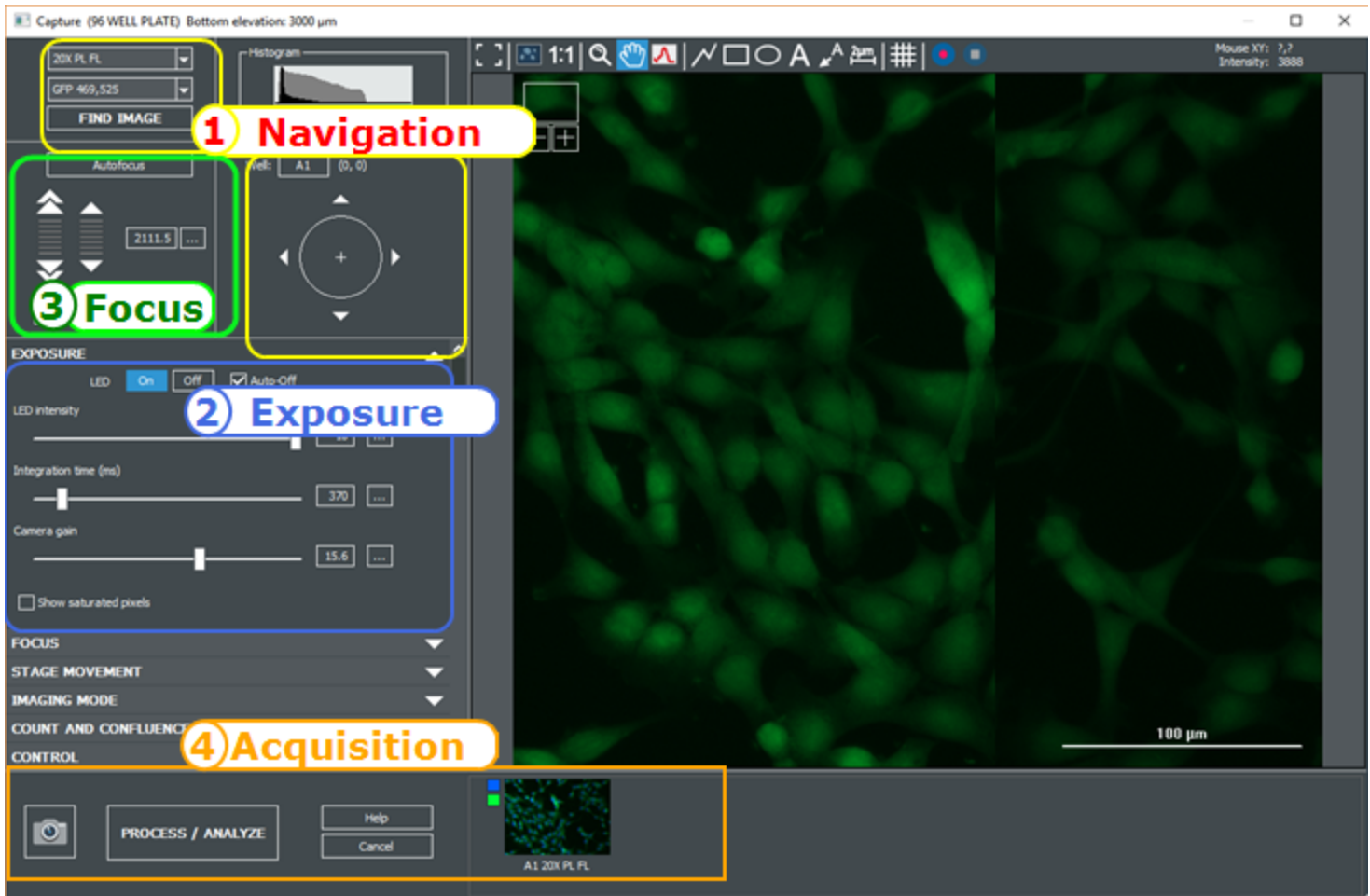
### 2.1 - Acquiring an Image

Manual Mode comprises 4 primary sections:

1. Navigation
2. Exposure
3. Focus
4. Acquisition

**See also 3 - Advanced Acquisition Guide on page 58.**

Gen5 v.3.11 adds confocal imaging capability to support this new imager line. We distinguish imaging methods as "confocal" and "widefield."



## 2 - Quick Guide to Imaging

### Basic Acquisition Steps in Manual Mode

1. Pick the **Magnification** (objective). Confocal imaging requires a 20X, 40X, or 60X objective. Selecting one of these objectives enables the choice of a confocal disk or, alternatively, Wide Field, for widefield imaging.
2. Select the first **Channel** (or light source for the upright imager) to use, and navigate to the area of the plate to capture.
3. Widefield imaging: Click **Find Image**. Gen5 automatically exposes and then focuses on the well. This may take several seconds. **Confocal imaging**: manually set the focus height and exposure settings for faster results.
4. For both methods, if necessary, use the **Auto Expose** and **Autofocus** controls to slightly adjust the settings until you are satisfied with the quality of the image, going back and forth between expose and focus.



5. Click the camera to capture the image. Then, you can change the color/channel and repeat the capture steps.
6. When you have captured the images you want to save or analyze, click **Process/Analyze**.

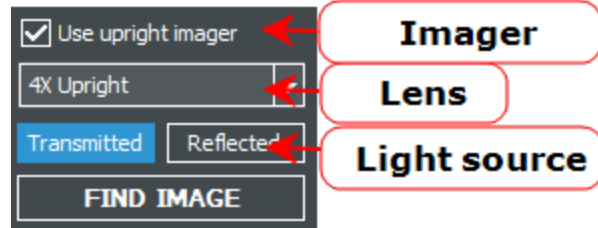


## 2 - Quick Guide to Imaging

### 1. Navigation



**Inverted Imager:** Pick the **Objective** and **Channel** from the objectives and fluorescent cubes installed or Bright Field, Color Bright Field, Phase Contrast, as applicable.



**Upright Imager:** Pick the magnification and light source.

**Find Image** button: Gen5 iteratively applies autofocus and exposure settings when you select Find Image.

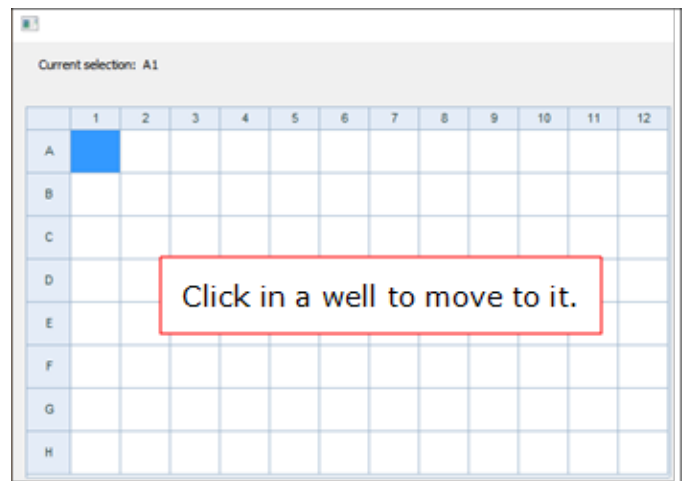
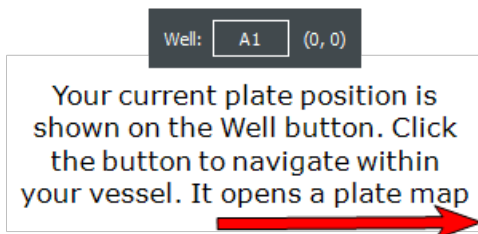
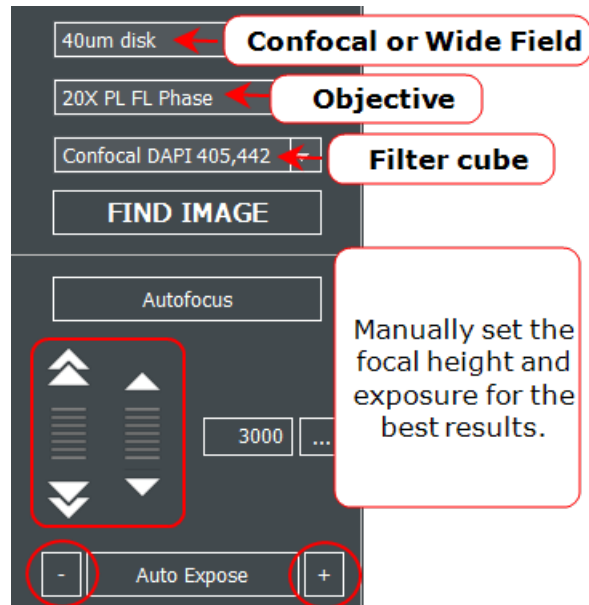
Click "Find Image" in widefield imaging with the inverted and upright imagers. It is not recommended for confocal.

#### Confocal Imager:

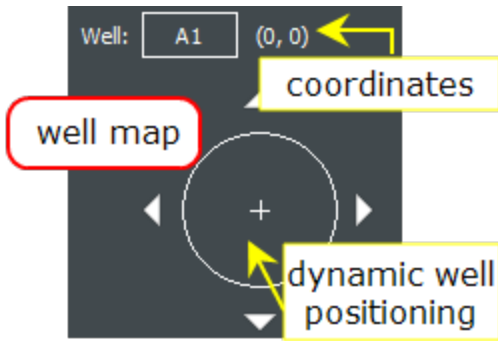
1. Set magnification to 20X, 40X or 60X.
2. Choose a disk, (not Wide Field).
3. Select the confocal filter cube.

Best confocal disk choice (for Cytation C10 "C2" models):

- 60  $\mu\text{m}$  for faster imaging and less photobleaching.
- 40  $\mu\text{m}$  for better z-resolution for 20X/40X objectives (with longer exposure time).



## 2 - Quick Guide to Imaging



You can navigate within the well using:

- the joystick, if equipped,
- the arrow keys,
- your mouse to click directly in the well map (field-of-view) area

### 2. Exposure Controls

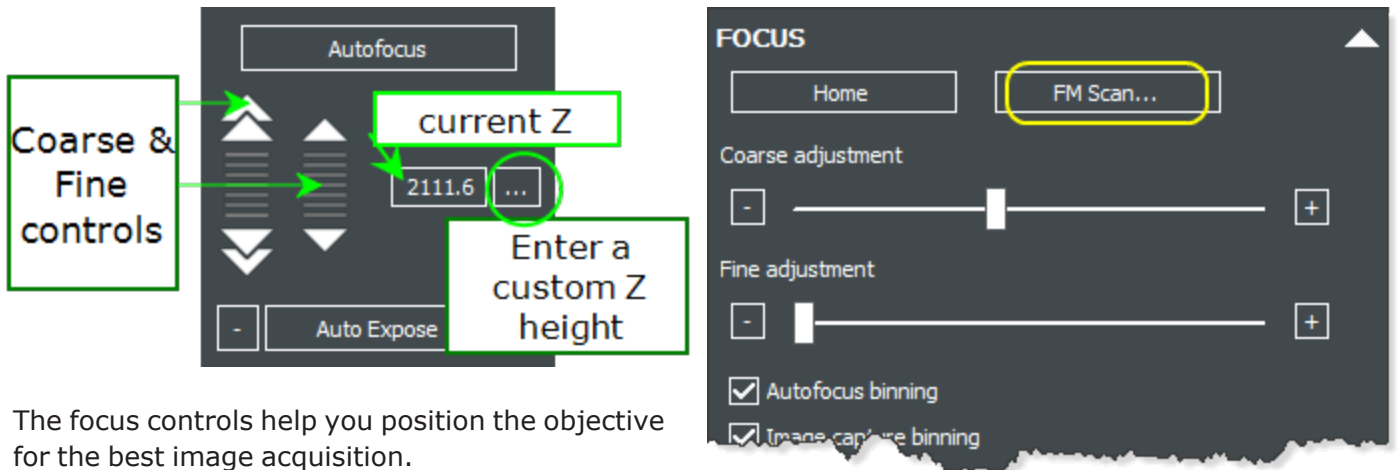
The image shows the 'EXPOSURE' control panel. At the top, there are buttons for 'Auto Expose' (with minus and plus signs) and 'LED' (with 'On' and 'Off' buttons and a checked 'Auto-Off' checkbox). Below these are three sliders: 'LED intensity' (set to 5), 'Integration time (ms)' (set to 50), and 'Camera gain' (set to 0). A checkbox at the bottom is labeled 'Show saturated pixels'. Annotations include: 'You can use Auto Expose (plus or minus buttons) or expand the Exposure panel to access user-controlled sliders.'; 'Affects performance, acquisition speed, and minimum kinetic intervals' pointing to the 'Integration time (ms)' slider; 'How bright?' pointing to the 'LED intensity' slider; 'Exposure time (ms)' pointing to the 'Integration time (ms)' slider; and 'Digital amplification' pointing to the 'Camera gain' slider.



**Best practice:** Capture images with the lowest possible exposure settings for the best analysis results. While human eyes prefer higher exposure images, software prefers lower exposure images. After acquisition, use the Brightness and Contrast control to improve viewing the image.

**Note:** The light source for confocal imaging is the LDI laser accessory. The imager controls the laser's intensity levels, in lieu of a Gen5 control option.

### 3. Focus Controls



The focus controls help you position the objective for the best image acquisition.

- If images are not at the desired focus, try increasing the Exposure settings, and expand the **Focus** panel to run **FM Scan**. When you find a good peak, click the **Set Focus Height to Max FM Ratio** button.
- If necessary, use the **Auto Expose** and **Autofocus** controls to slightly adjust the settings until you are satisfied with the quality of the image, going back and forth between expose and focus.

## 2 - Quick Guide to Imaging

### 4. Acquisition Controls

The screenshot displays the Gen5 software interface. At the top, a toolbar contains various icons for zooming, panning, and measuring. The main area shows a green fluorescence image of cells. A scale bar at the bottom right indicates 100 μm. The bottom panel includes a camera icon, a 'PROCESS / ANALYZE' button, and 'Help' and 'Cancel' buttons. A thumbnail strip at the bottom right shows three image thumbnails labeled 'E5 20X PL FL', 'E8 20X PL FL', and 'C9 20X PL FL'. Mouse coordinates and intensity are shown in the top right corner.

Annotation Toolbar offers zoom, measurement, and annotation tools.

Gen5 remembers the last-used focus and exposure settings for an objective-channel combo!

Click the **camera** to capture the current display.

Gen5 creates a thumbnail of the image, then overlays up to 4 channels for that image. Adding more channels or moving in X/Y generates another image.

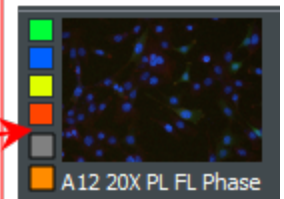
100 μm



The **camera** button captures the image with the current settings.

Click **Process/Analyze** after capturing images. Gen5 will save the collection of thumbnails and present them for processing and analysis as you proceed.

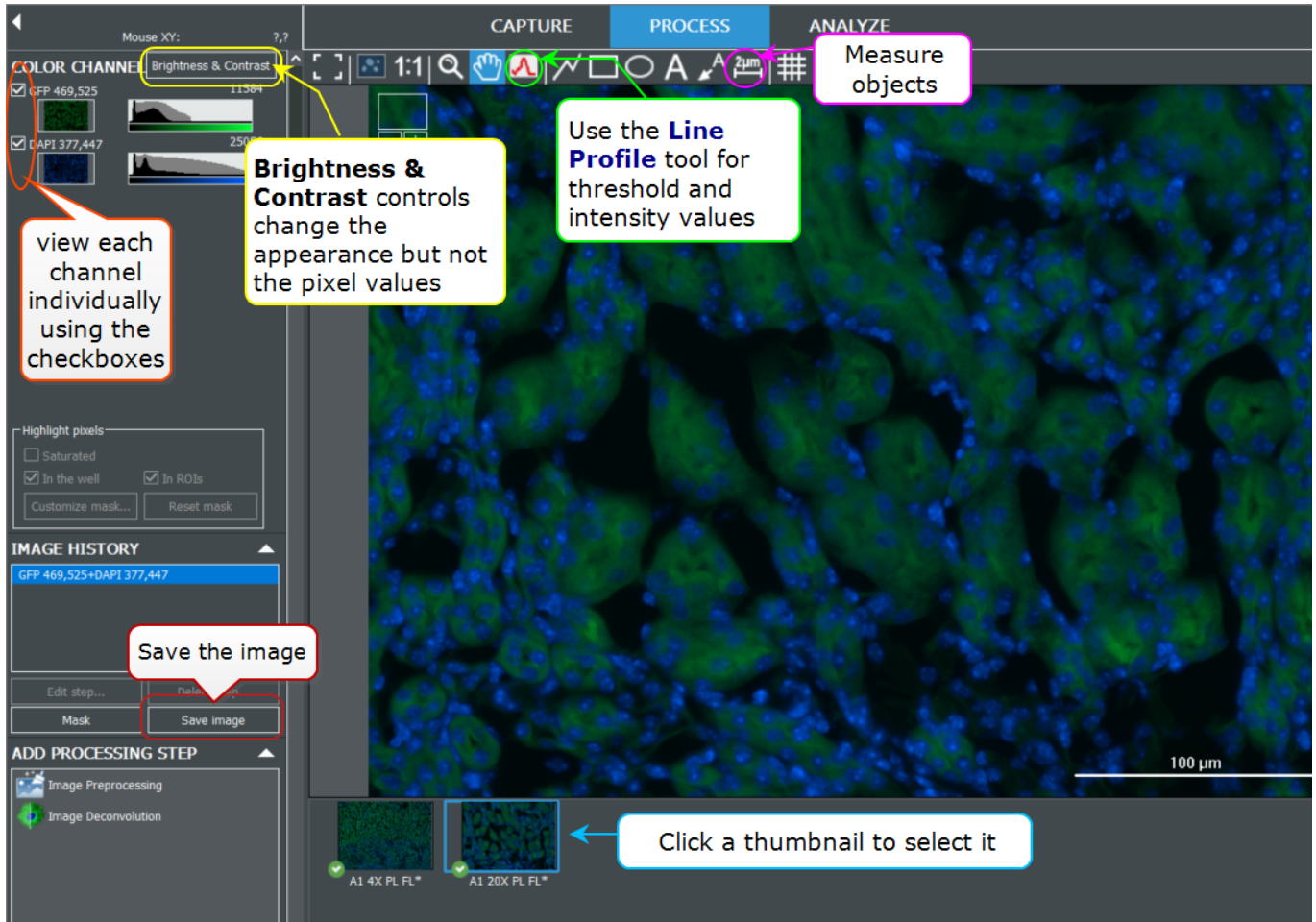
Gen5 overlays up to 6 channels in the same capture (same X & Y coordinates) - color squares show channels acquired.



one image set

## 2.2 - Image Review and Editing

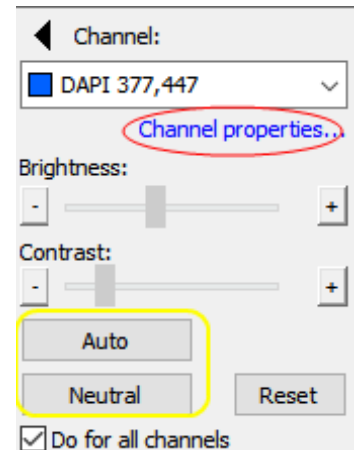
Before processing and analysis, you can review the images, adjust the brightness and contrast to improve their appearance, annotate the image, and save it for presentation. Thumbnails in the bottom panel represent acquired images: click to select them for review.



**Brightness & Contrast (B&C):** Click **Brightness & Contrast** to adjust the brightness and contrast of the image for optimal viewing. Click the Channel arrow in the control to close it. Adjusting B&C does not affect image analysis; it merely improves the display for the computer monitor.

Choose **Auto** to let Gen5 make the adjustments. Select **Neutral** to restore the image to its original state.

**Channel properties:** To apply transparencies and/or adjust color gradients to improve the image, click Channel properties in the B&C control.




## 2 - Quick Guide to Imaging

### Annotation Toolbar



1. Show image in full screen
2. Change zoom to see entire image
3. Zoom to 1 screen pixel per 1 image pixel
4. Zoom tool: draw a box on your image to zoom in; right-click to zoom out
5. Hand scroll tool: click and drag to move your image
6. Line profile tool to draw a line through your image to see relative intensities
7. Common annotation tools to draw polylines, rectangles, and circles, apply text, and call out regions of interest
8. Measure tool to determine size of objects of interest
9. Grid tool to turn custom grid on and off
10. Shift channels to overcome offset and improve overlay
11. Export image or metadata to Excel

 Tip: you can also use your mouse wheel to zoom in and out.

Learn more: [6.1 - Annotation Tools](#) as shown on page 141.

## 2.3 - Image Processing and Analysis

Image processing and analysis in Manual Mode can be performed after image acquisition:



select **Process/Analyze**.

See 7 - Basic Image Analysis on page 148 to learn more.

### PROCESS

#### Image Processing

Gen5 presents context-sensitive processing options. Generally, they work best when applied in the sequence provided.

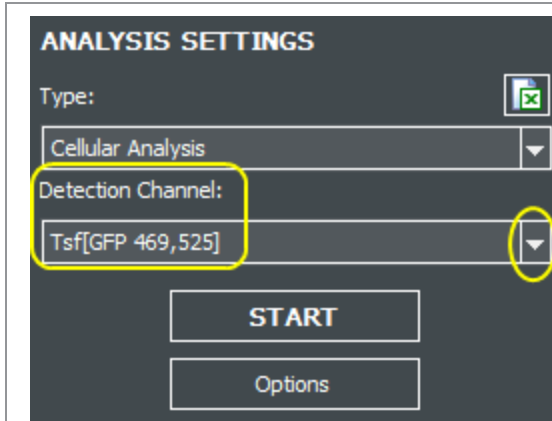
|  |  |
|--|--|
|  | <ol style="list-style-type: none"> <li>1. Click an option in the <b>Add Processing Step</b> box.</li> <li>2. Keep the default settings initially: click <b>OK</b>; you can change the settings later if necessary.</li> <li>3. Use the processed image in <b>Image Analysis</b> (in the next step).</li> </ol> |
|  | <p>Gen5 creates a new data set for the processed image. You can compare the two by selecting them in the <b>Image History</b> box.</p> <p>Double-click the processed data to modify its parameters.</p>  |


### ANALYZE

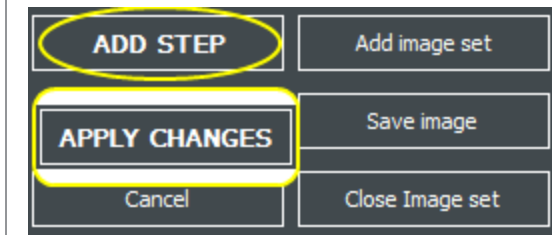
#### Image Analysis

|  |   |
|--|---|
|  | <ol style="list-style-type: none"> <li>1. Click <b>Analyze</b> to move to the analysis step.</li> <li>2. Click <b>Cellular Analysis</b>.</li> </ol> |
|--|---|

## 2 - Quick Guide to Imaging



3. Select your Detection Channel and click **Start**.
4.  Use the Line Profile tool and draw a line to get thresholding and object size parameters.
5. Click **Options** (under the Start button), enter your settings, and click **Apply** (and **OK** to close the dialog).
6. Gen5 returns to the previous window, and updates the results displayed in the **Image results** and **Object details** tables on the right.



7. At the bottom of the screen, click **Add Step** to save the analysis (or **Apply Changes** if the step was previously saved).

**Line Profile tool**

Line Profile

Measure object Channel: Deconvolved[Tsf[DAPI 377,447]]

Bright objects on a dark background

Deconvolved[Tsf[GFP 469,525]] Deconvolved[Tsf[DAPI 377,447]]

PEAK=31509

WIDTH=16.7  $\mu$ m

ELEVATION=30868

25%=7717

BKG=641

line length [ $\mu$ m]

Use this info to edit analysis Options

**Analysis results**

| Name                    | Value    |
|-------------------------|----------|
| Cell Count              | 56       |
| Object Size             | 14.2     |
| Object Area             | 160      |
| Object Sum Area         | 8.93E+03 |
| Object Mean[Deconvol... | 22953    |
| Object Area[Deconvol... | 160      |
| Object Sum Area[Deco... | 8.93E+03 |
| Object Mean[Deconvol... | 16096    |
| Detection Threshold     | 5000     |

Object details:

| #  | Size | Area | Mean[D...] | Area[Deco...] | Mean[D...] |
|----|------|------|------------|---------------|------------|
| 1  | 11.5 | 97.3 | 16224      | 97.3          | 12284      |
| 2  | 15.4 | 185  | 19340      | 185           | 16416      |
| 3  | 16.5 | 213  | 24976      | 213           | 15837      |
| 4  | 15.8 | 196  | 32422      | 196           | 24645      |
| 5  | 15.8 | 193  | 14836      | 193           | 11342      |
| 6  | 16.2 | 203  | 26343      | 203           | 16271      |
| 7  | 12.2 | 117  | 26721      | 117           | 25214      |
| 8  | 15.3 | 184  | 28024      | 184           | 15634      |
| 9  | 11.2 | 99.2 | 31847      | 99.2          | 28450      |
| 10 | 10.7 | 88.8 | 33829      | 88.8          | 23116      |
| 11 | 14.5 | 163  | 23632      | 163           | 17130      |
| 12 | 11.4 | 102  | 26978      | 102           | 18883      |
| 13 | 11.2 | 96.8 | 28247      | 96.8          | 24489      |
| 14 | 11.6 | 105  | 27224      | 105           | 25705      |
| 15 | 15.1 | 173  | 19357      | 173           | 18028      |
| 16 | 11.3 | 100  | 26872      | 100           | 20116      |

**ADD STEP** Add image set

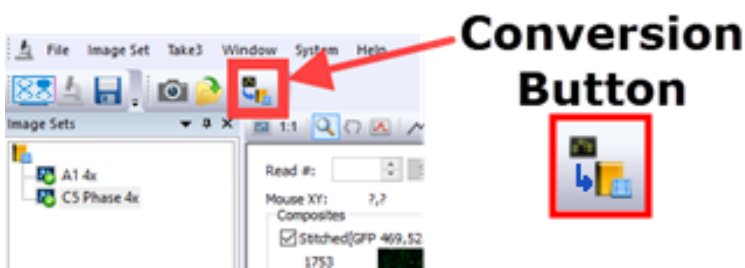
Cancel Close Image set

Be sure to **Add Step** to save the analysis results




### 2.4 - Conversion to Experiment Mode

Once you've acquired an image with optimal settings and applied processing and analysis steps as necessary, you can **easily convert the settings for that image to Experiment Mode**, which automates acquisition of multiple samples, for example, a chamber well slide or multi-well plate.



1. In Manual Mode, select the image of interest.
2. Click **Create experiment from an image set** button.

3.  Then, you can click **Read New** to begin acquisition.

Alternatively, to edit the protocol, see below.

#### To Edit Your Experiment

You can make changes to your experiment after conversion. The most common changes will be:

- A. Selecting wells of interest**
- B. Changing the autofocus method**

## 2 - Quick Guide to Imaging

### A. Selecting wells of interest

After converting your Manual Mode session to an experiment, open the read step to make changes:

The screenshot displays the Gen5 Image Prime software interface. The main window is titled "Procedure - Cytation5 (USB)". The "Actions" list on the left includes "Image", "Read", "Set Temperature", "Shake", and "Dispense". The "Image" step is selected, and its configuration window is open. The "Image (4x): GFP 469,525, DAPI 377,447" step is highlighted in the "Read/Image step" list. The "Imaging Step-Inverted imager" window shows settings for "Step Label", "Magnification", "Image", "Binning", and "Channels". The "Full Plate" button is highlighted in the upper right corner of this window. A "Well Selection" dialog box is open, showing a 12x12 grid of wells. Wells B1 through B12 are highlighted in blue, indicating they are selected for imaging. A callout box explains that clicking "Full Plate" opens this map, and wells highlighted in blue will be imaged. The "Montage" section at the bottom shows options for "Montage" (checked), "Montage (rows x columns): 2 x 2", and "Tile Overlap" (set to "Auto for stitching").

1. Select Procedure.

2. Double-click the image step to edit it.

3. Click Full Plate to select the wells to image.

Click Full Plate in the upper-right corner of the imaging step. A map of your plate will open. Click wells to select or deselect them. Wells highlighted in blue will be imaged.

In addition, if it will improve your assay, you can add activities (steps) to your Procedure by clicking them. For example, add "Set Temperature" to turn on incubation. Drag and drop steps in the list to change their order.

## 2 - Quick Guide to Imaging

### B. Changing the Autofocus Method

Gen5 offers **5** image-based autofocus methods and a 6<sup>th</sup> method, Laser Autofocus, which requires purchase of an optional accessory.

- Image-based autofocus methods take a series of images in a range of z-planes in search of maximum contrast.
- Laser autofocus shines an infrared laser at the bottom of the well to locate the vessel-liquid interface.

By default, Gen5 will use "Autofocus with optional scan" for fluorescent channels and "Bright field autofocus (dual peak)" for bright field and phase contrast.

See [5 - Understanding AutoFocus on page 124](#) for a detailed discussion of Autofocus methods .

## 2 - Quick Guide to Imaging

### To change the autofocus method:

Channel 1: DAPI 377,447

Click Focus Options in the imaging step, and de-select Use default focus method.

Choose a method from the drop-down list.

Focus options...

Use default focus method

Focus method: Autofocus with optional scan

Autofocus with optional scan

Scan and then autofocus

Autofocus without optional scan

Bright field autofocus (dual peak)

User-trained autofocus

Laser autofocus

Auto Exposure Options

Prioritize speed over image-quality

- Advanced options -

Use default integration threshold

Integration threshold (msec): 5

Gain threshold: 24

Target exposure %: 75

Skip %: 0.1

Vibration Detection Options

CV Threshold: 0.01

Images to average: 1

(1=disabled; >1=enabled)

OK Cancel Help

By default, Gen5 uses the focal plane of Channel 1 for Channels 2-4. We therefore recommend making your strongest signal as Channel 1. If you anticipate that your channels will NOT have the same focal plane, click **Focus options** under your subsequent channels. You can either:

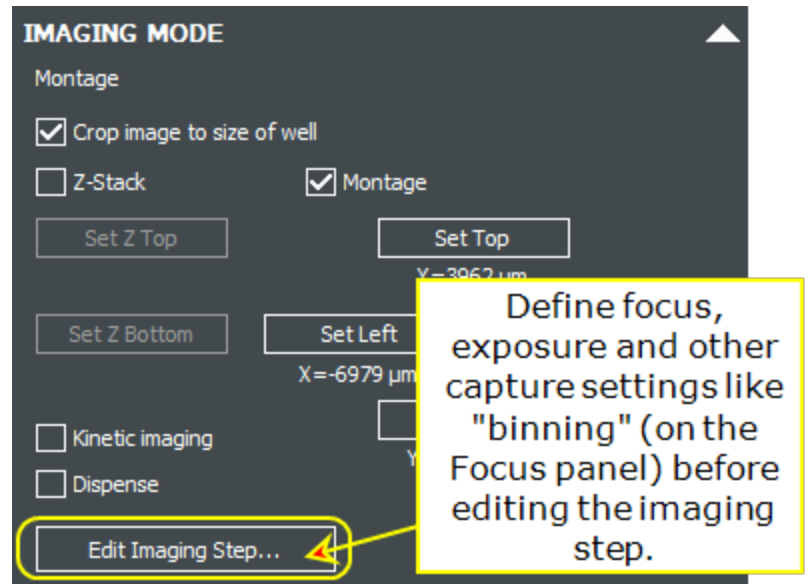
- Build in a defined offset from Channel 1 by typing a number into the **Offset (µm)** field.
- Autofocus separately on your other channels.
- Laser AutoFocus is recommended for most confocal experiments.

▶ Watch this [Manual Mode to Experiment Mode Training Video](#).

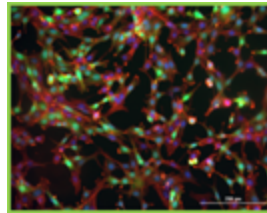
### 2.5 - Additional Acquisition Options

The **Imaging Mode** panel in Manual Mode lets you define additional options for image acquisition, including:

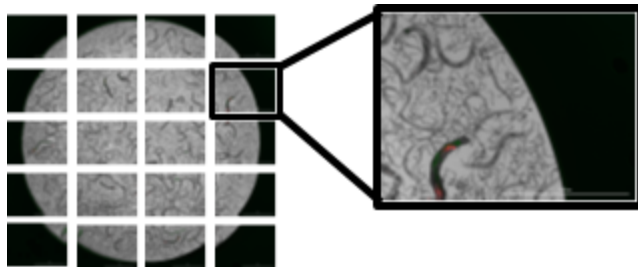
- **Kinetic Imaging:** to capture live biology in real time or set up time-lapse imaging.
- **Dispensing:** if the optional Reagent Injector accessory is set up, dispense steps can be built into your assay.
- **Advanced Imaging:** to acquire single vs multiple images in the x/y (Montage) or z (Z-Stack) plane.
- **Crop Image to Well:** Eliminate edge effects in microplate wells, and enable **Customize mask...** feature.



**Single Image Set** – A single field composed of a single x-, y-, and z-coordinate. Can be captured in up to 4 colors, including phase or bright field.

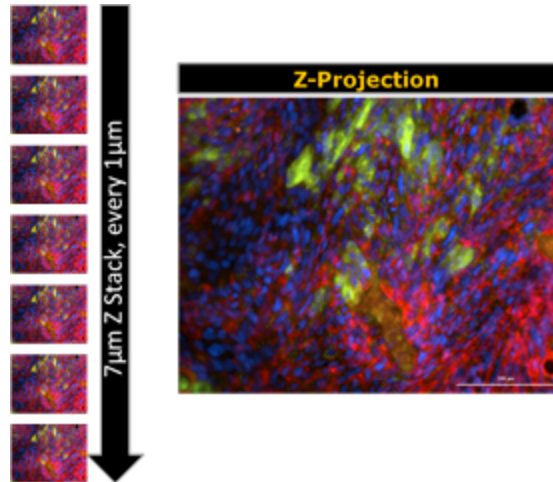


**Image Montage** – A field composed of multiple images (2-2000 images) that can be compiled (stitched) to provide a larger field of view (within a single z-plane). Tiles can be stitched together in Processing.



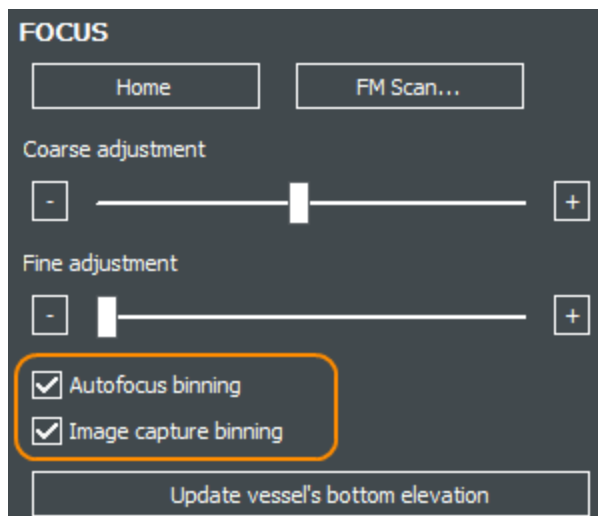
## 2 - Quick Guide to Imaging

**Image Z-Stack** – A field composed of single x-/y-coordinates captured at multiple z-planes (up to 200 slices), down to 0.1  $\mu\text{m}$  thickness per step. A single image, called a Z-Projection, can be created from these images during Image Processing.



See 3 - Advanced Acquisition Guide on page 58 for a detailed discussion.

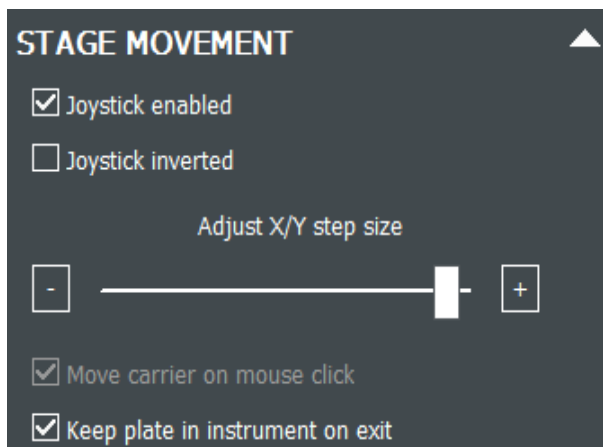
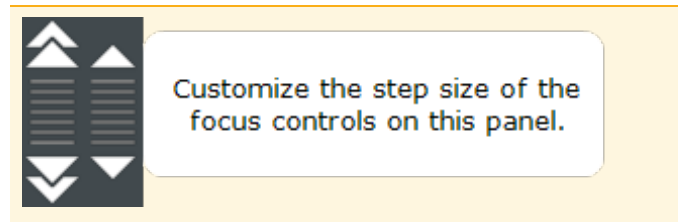
### More Manual Mode Options



#### Binning

Expand the **Focus** panel to apply binning to your image captures.

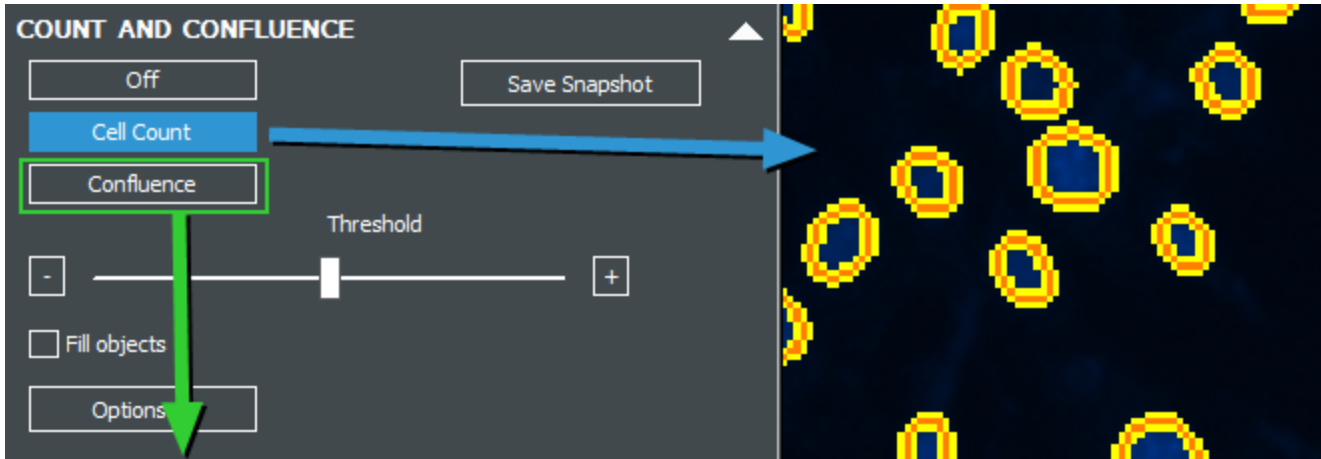
See Binning Options on page 86.



Expand the **Stage Movement** panel to change these default settings:

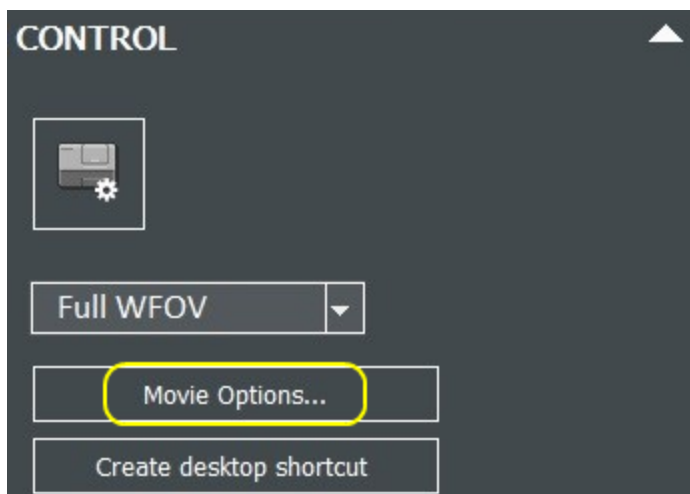
- **Adjust X/Y step size** slider on the Stage Movement panel to customize the degree of movement when using the well map.
- **Move carrier on mouse click**: lets you pick the focus area with a mouse click in the well map or displayed image. Enabling the joystick disables this option.
- **Keep plate in instrument on exit**: overrides Gen5's normal behavior to eject the plate carrier after every read.

## 2 - Quick Guide to Imaging



When you have an in-focus and properly exposed image of the sample, expand the **Count and Confluence** panel to perform an on-the-fly cell count or confluence measurement.

Gen5's "augmented microscopy" gives you instant feedback to help you quickly assess the quality of your sample. Click **Options** to use the same background flattening, image smoothing, and threshold filtering offered in Cellular Analysis to apply a nuclear mask to count cells and/or measure confluence in live mode.



### Movie Maker

Expand the **Control** panel to change default movie-making settings.

### Cytation and Lionheart "W" models:

Available on the **Control** panel: Drop list of options to capture images in Standard-size field-of-view; Crop 75%, or Full WFOV.

### 2.6 - Best Practices

Search for app notes and other resources at:  
[www.biotek.com/applications](http://www.biotek.com/applications)

#### Recommended microplates:

- Agilent 96-well clear 204624-100
- Agilent black with clear bottom 96-well 204626-100 and 384-well 204628-100
- Greiner  $\mu$ Clear TC-treated 655090 96-well & 781091 384-well black polystyrene
- Greiner  $\mu$ Clear cell-repellent 655976 96-well & 781976 384-well black
- Greiner 655892 96-well & 781892 384-well glass bottom
- Corning 4850 96-well half-area and 4851 384-well glass bottom

Get best image quality when using high-power objectives using glass-bottom plates. Oil and low WD objectives require glass vessels.

**Important:** Make sure your Windows "Display setting" is at 100% for the best viewing experience.

Use slower carrier speed

To prevent media from sloshing or spilling, especially for cells in suspension in low-density plates, you can **slow down the plate carrier** to avoid disrupting vessel contents. For instruments that support it:

- In imaging manual mode, set the option on the "load plate" screen.
- In experiment mode, enable the slower speed in the **Procedure>Options** dialog.
- Enable or disable the setting using the **Instrument Control** panel (or via the System menu).

**Setting Exposure for low signal samples:** The best practice for analysis is to capture images with the lowest possible exposure settings. While human eyes prefer higher exposure images, software prefers lower exposure images. With low signal media, it is easy to overexpose the image, especially when using WFOV cameras, where illumination correction may reduce the available intensity signal range at higher exposures.

When using a "W-model" imager, instead of using **Full Wide FOV** to set the exposure settings, use the drop list to select **Standard FOV** or **Crop 75%** for a good way to choose optimal exposure values. You can reset to Full WFOV before capture.

After acquisition, use the **Brightness and Contrast** control to improve the image's appearance.

CONTROL

Carrier out...



In manual mode, expand the Control panel to access FOV drop list. In experiment mode, find the options next to Magnification in the Image Read step.



## 2 - Quick Guide to Imaging

### Fix Focusing Problems

#### Define your vessel's "Bottom Elevation"

See [Setting the Bottom Elevation on page 11](#): Gen5 uses the "bottom elevation" as the starting point for autofocus. It is essential to correctly define the bottom elevation for the vessel and samples you are imaging.

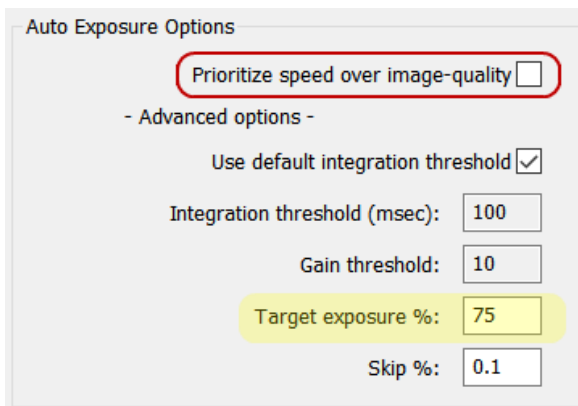
#### Adjust the Correction Collar

See [Adjusting the Correction Collar on page 15](#): when using high-magnification objectives, 40X and 60X (air), make sure the objective's "correction collar" is properly set for the plate type you are using. Likewise, make sure other "plate type" settings, like the "accessible bottom focus region" match the vessels you are using.

#### Try a different Autofocus method

Review the options for autofocus to determine if an alternate method is a better choice for your application. See [5.1 - Autofocus Methods on page 125](#).

#### Adjust Auto Exposure parameters



Auto Exposure Options

Prioritize speed over image-quality

- Advanced options -

Use default integration threshold

Integration threshold (msec):

Gain threshold:

Target exposure %:

Skip %:

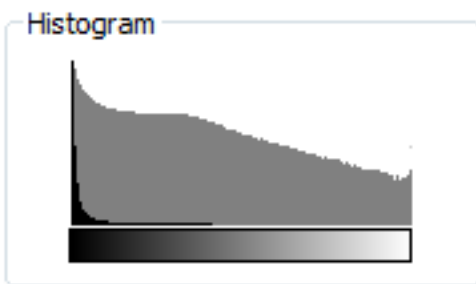
De-select "Prioritize speed over image quality." Integration time will be increased, typically resulting in a better image.

Manual mode: open the Imaging Mode panel, select **Advanced options**. Experiment mode: click **Focus Options**.

#### Still blurry? Change the **Target exposure %**

- If the signal is relatively uniform across the image: decrease to 25-50%.
- If the image intensity is highly variable: increase to 80-100%. This will force the focus algorithm to target dimmer objects and ignore bright objects, which may be impeding focus.

#### Use Dynamic Histogram to Set Exposure



Use Gen5's dynamic histogram during image acquisition to determine the best exposure settings. The histogram updates as you select different exposure settings, giving you instant feedback on the effect of your choices. See [Histograms on page 60](#).

After acquisition, use the Brightness and Contrast (B&C) controls to improve online viewing.

## 2 - Quick Guide to Imaging

### Plating, Incubating & Handling Cell Plates

Find app notes and other useful info at Agilent's:

[www.biotek.com/applications](http://www.biotek.com/applications)

Agilent provides guidelines in an app note titled: *Automated Tissue Culture Cell Fixation and Staining in Microplates*.

Many factors influence fluorescence imaging and image analysis data quality, including one of the most critical factors: good sample preparation. A fluorescent image is the raw data for an experiment, so close attention to the details in sample preparation significantly improves the assay results.

Make sure your cells are properly plated (i.e., 40-70% confluent).

- Cell Plating
  - After plating cells, allow them to sit on the lab bench 15 to 20 minutes prior to incubation to give cells time to settle more evenly, reducing uneven distribution within wells and minimizing edge effects.
- Cell Growth
  - Do not stack plates in an incubator to avoid uneven heat and humidity distribution that increases plate-to-plate variation.
  - Keep plates away from the incubator door, which is often heated, creating additional edge effects in the wells.
- Compound Addition
  - DMSO: Cells can typically handle a final concentration between 0.25-1% DMSO (cell-type dependent). Dilute the solvent and mix thoroughly to avoid variability caused by inadequate mixing.
  - The main source of false positives in many screens involves auto-fluorescent compounds. Visually confirming images in hit wells is recommended to ensure the hits are not caused by auto-fluorescent compounds.
    - Example of red auto-fluorescent compounds: Doxorubicin and Epirubicin.
- Reagent Addition
  - Warm fixatives and reagents prior to additions to decrease cell stress.
- Condensation on Plate Lid/Seal

Gradient:  °C

- Apply a temperature gradient when defining incubation settings to prevent condensation forming.
  - To add a temperature gradient to your Experiment, open the Procedure window and add a **Set Temperature** step. Here you can add a 1-3°C gradient.
- Imaging Microplates
    - Clean the bottom of the microplate or slide surface prior to imaging. Remove any condensation from the bottom of the plate with a lint-free cloth.

## 2 - Quick Guide to Imaging

- When imaging a previously stained plate that is stored at 4°C, allow the plate to warm to room temperature; otherwise, the plate bottom collects condensation that causes blurred images.
- Select the correct microplate in Gen5; selecting the wrong vessel causes autofocus failures due to incorrect Plate Type dimensions.

**See also Speed Up Focusing Process on page 130.**

## 3 - Advanced Acquisition Guide

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### *Tools to Optimize Your Assay*

Once you've become comfortable with basic acquisition in Manual and Experiment Modes, Gen5 provides a variety of ways to improve your settings to best optimize your assay and acquire more complex images. In this section you will find:

|  |           |
|--|-----------|
| <b>3.1 - Optimizing Exposure Settings</b> .....      | <b>59</b> |
| <b>3.2 - High Contrast Bright Field (HCBF)</b> ..... | <b>62</b> |
| <b>3.3 - Image Montage</b> .....                     | <b>64</b> |
| <b>3.4 - Z-Stacking</b> .....                        | <b>72</b> |
| <b>3.5 - Kinetic Image Capture</b> .....             | <b>79</b> |
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### 3 - Advanced Acquisition Guide

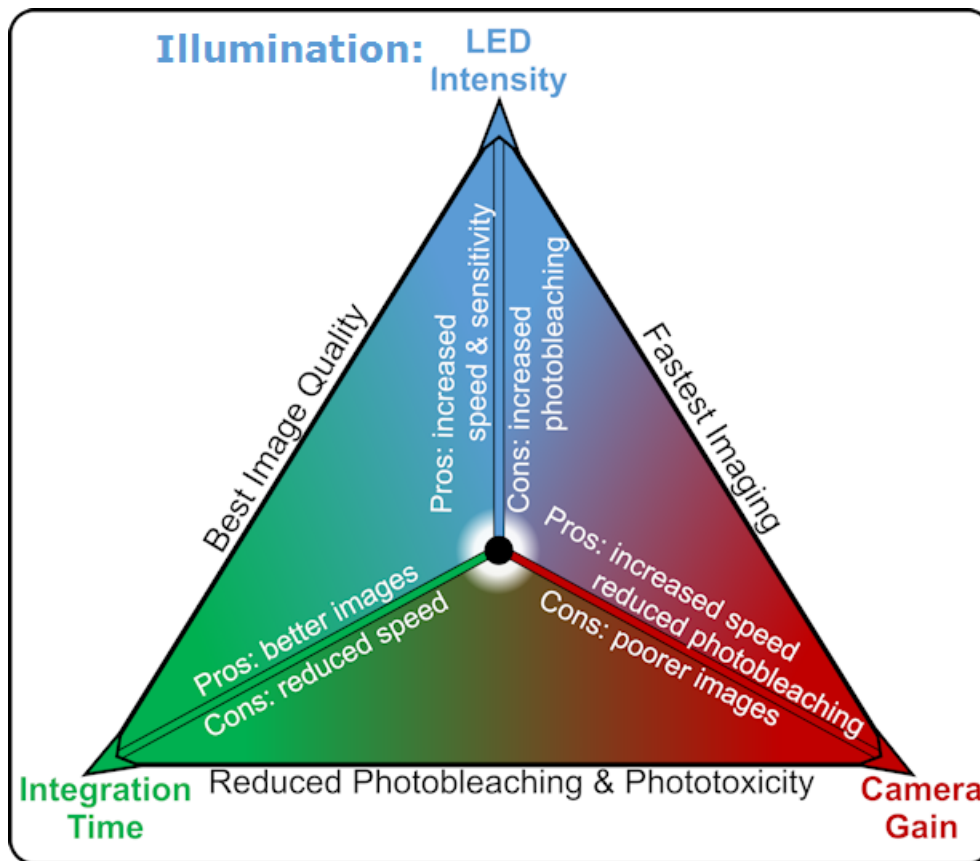
#### 3.1 - Optimizing Exposure Settings

Exposure relies upon **3** variables for widefield imaging: Illumination or LED intensity (light brightness), integration time (length of exposure), and camera gain (digital amplification of signal). For confocal imaging, light is provided by the accessory LDI laser and controlled by the imager. Only integration time and camera gain controls are provided in confocal imaging.

Each has its own benefits and drawbacks, as outlined below:

| Exposure Parameter               | Benefit of high setting | Drawback of high setting           |
|----------------------------------|-------------------------|------------------------------------|
| 1. Illumination (widefield only) | Sensitivity             | Photo-bleaching                    |
| 2. Integration time              | Higher image quality    | Significantly slower imaging speed |
| 3. Camera gain                   | Faster read speed       | Lower image quality                |

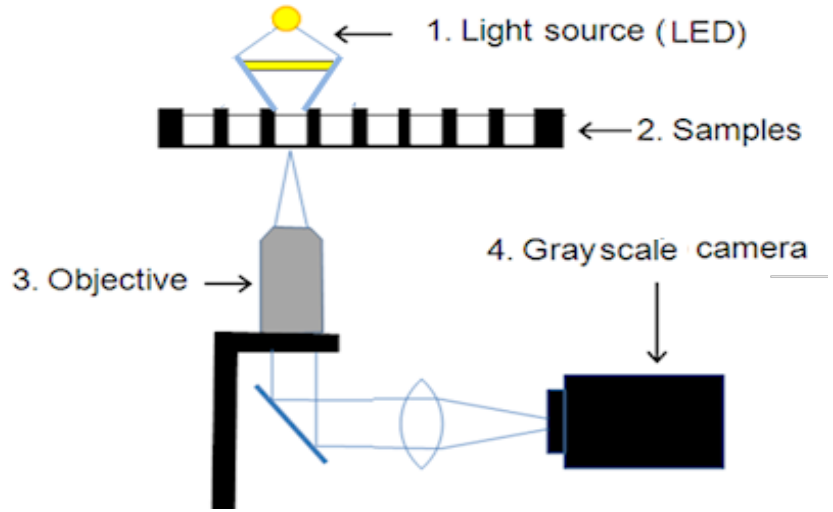
In addition to the Auto Expose options, sliders for the variables allow the user to achieve a well-exposed image while accommodating for the specific requirements of the assay, for example, compensating for a photosensitive reagent by reducing LED intensity and integration time, but increasing gain. These sliders are present for all imaging modes except Color Bright Field.



### 3 - Advanced Acquisition Guide

Bright Field and phase contrast use **transmitted light** to image samples, rather than the LED-Filter cubes used in widefield fluorescence imaging.

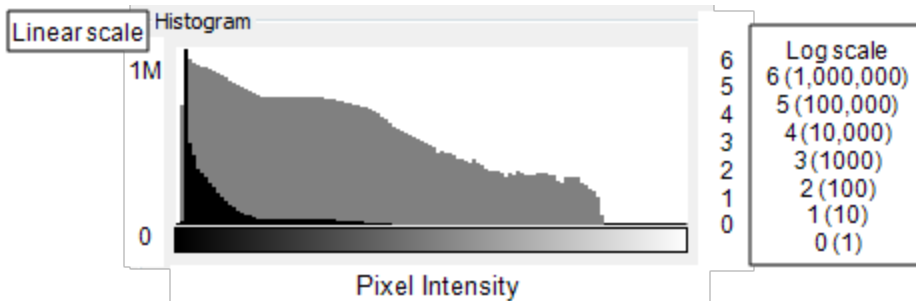
As such, light illuminates the sample from above, making these modes **only compatible with clear lids**.



▶ Watch this [Image Exposure Settings Training Video](#).

## Histograms

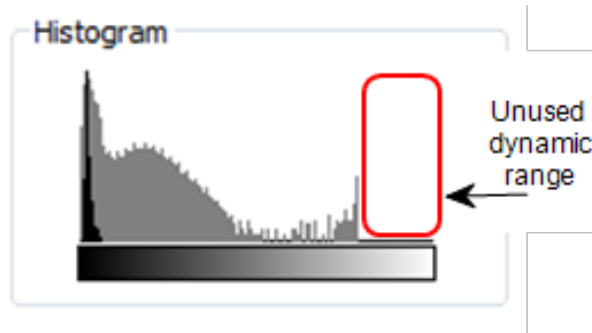
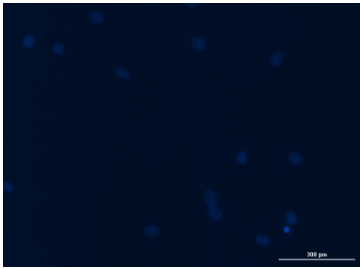
In addition to the visual cues in the live image, the image histogram can provide additional information for fine-tuning exposure settings and maximizing use of the camera's dynamic range. Histograms display the relative quantity of pixels of a given intensity level (from black to white) of all pixels for the current image. The x-axis shows the pixel intensity values, and the y-axis provides a count of pixels at each intensity level. The greater the number of pixels in the image at a specified intensity level, the higher its peak in the histogram.



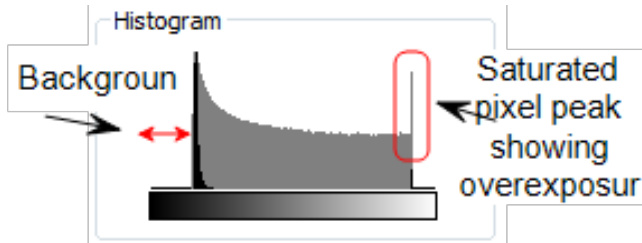
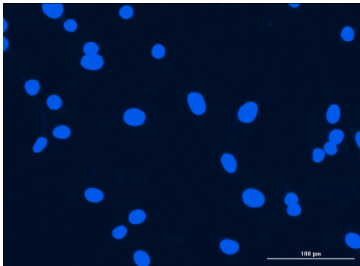
Gen5 displays an image histogram in two y-axis scales, linear and logarithmic. The black (or dark) plot uses a linear scale and the gray (or lighter) plot uses a logarithmic scale.

When capturing an image, the image histogram can verify that the camera's dynamic range is being used effectively. Overexposed images have a peak at the far right, illustrating an accumulation of pixels that have saturated the camera's dynamic range. Underexposed images show the majority of pixels accumulated on the left on the histogram.

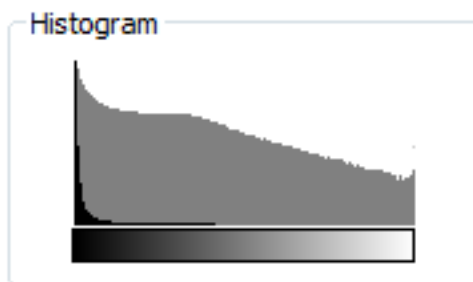
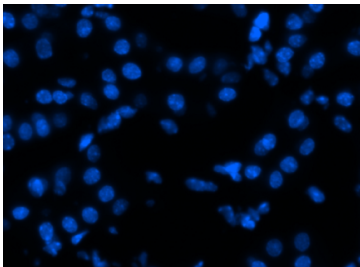
### 3 - Advanced Acquisition Guide



**Underexposed** image and histogram of DAPI stained cell nuclei. The histogram does not use the full dynamic range and there is correspondingly poor contrast in the image.



**Overexposed** image and histogram of DAPI stained cell nuclei. Note the loss of texture detail in the saturated nuclei.



**Properly** exposed image and histogram of DAPI stained cell nuclei. Note the retention of texture detail in the nuclei in the image and use of the full dynamic range in the histogram without over- or under-exposure of pixel intensity.

## 3.2 - High Contrast Bright Field (HCBF)

*Requirements: Lionheart FX, Cytation 1, Cytation C10, Cytation 5 (phase contrast models only: CYT5MPV or CYT5PV), Cytation 7 ("W" models), and Gen5 Image Prime or Image+ software. Not supported on Cytation 3. Lionheart FX and Cytation 5 instruments require the purchase and installation of an optional accessory provided by Agilent. Cytation C10 requires installation of the accessory but it is provided with the imager.*

Significant improvements to cell counting have been seen when using a custom aperture in bright field imaging, for certain instrument models. This simple modification yields high-contrast images of adherent cells that can be easily counted by Gen5. The HCBF aperture needs to be installed and the .xml file for the 4x objective imported before image capture (follow installation instructions provided with the kit or operator's manual).


The custom aperture is not needed for the **Cytation 1** and **Cytation 7** imagers, as these instruments are equipped for high contrast bright field (HCBF). **Cytation 7** imagers have a manual switch to engage HCBF: lift the top door and move the switch to **High Contrast**.

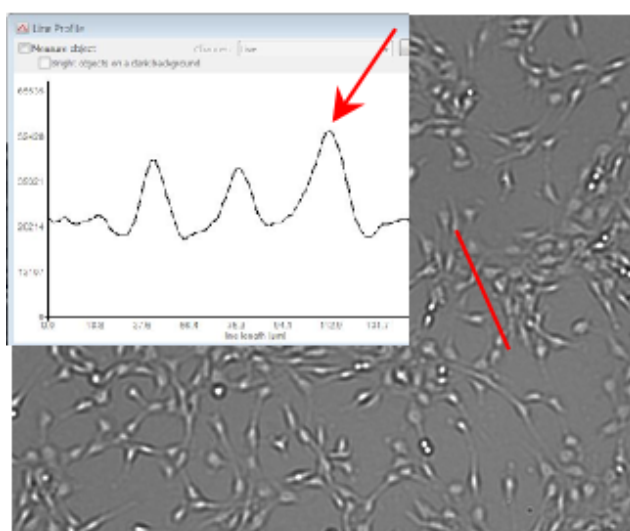
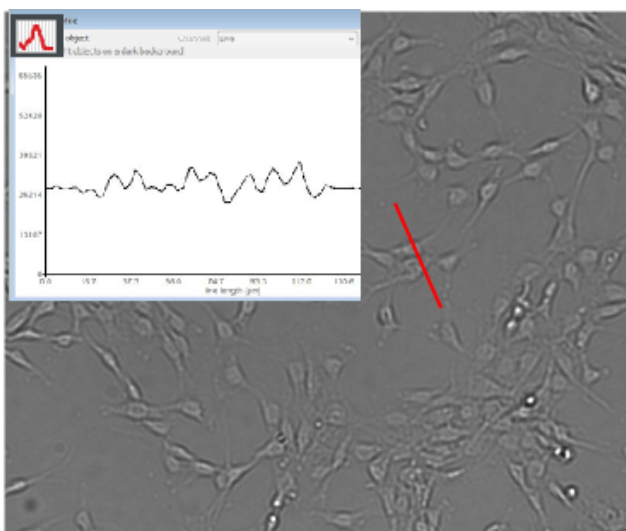
### Image Capture

Images can be captured in either manual mode or experiment mode.

### Defocusing for Better Contrast

Slightly defocusing the bright field image is recommended to improve cell counting. Usually this is an iterative process of trial and error to determine how to achieve the highest contrast while retaining acceptable focus.

1. First, find the optimal focus settings.
2. Then, defocus the image by reducing the "Current position" by a starting point of -250  $\mu\text{m}$  (in manual mode).
3.  Use the **Line Tool** after defocusing to evaluate the setting's effect on separating background and foreground signals. Your goal is to find well-defined intensity peaks for defocused cells.




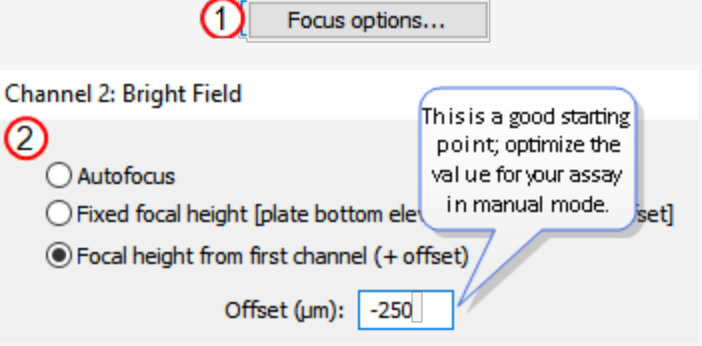


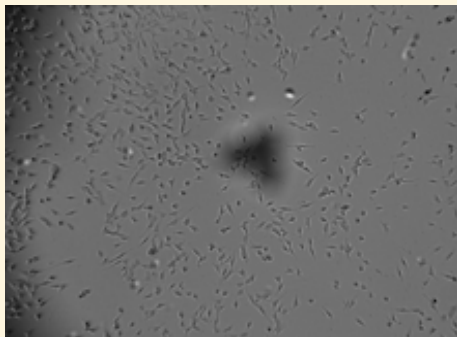


### 3 - Advanced Acquisition Guide

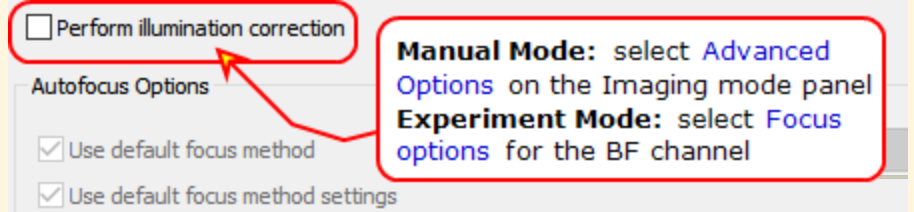
image before defocusing

image after defocusing

|  <b>Manual Mode</b>  |  <b>Experiment Mode</b>   |
|---|--|
|    |    |
| <p>After finding the best focus position, click the position button and subtract 250 µm to "defocus" the image (for example: 3263-250=3013). Evaluate the results of this defocusing and fine-tune the setting as needed.</p> | <p>Perform two Bright Field reads, one "in focus" for the best images and one "defocused" for the best cell counting. For the second channel, select <b>Focus options</b>. Select Focal height from first channel (+offset), and enter a defocusing <b>Offset</b>.</p> |



Better results have been observed in high-contrast cell counting in wells with severe meniscuses by disabling Gen5's "illumination correction" feature.



If this type of meniscus effect occurs, de-select **Perform illumination correction**.

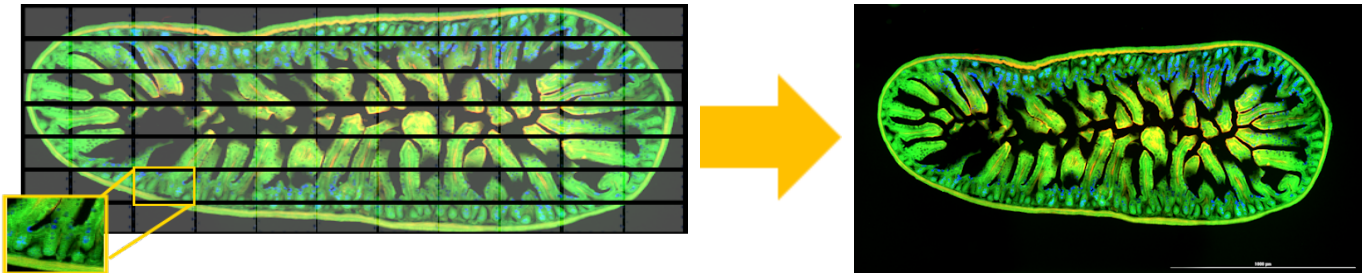
▶ Watch [High Contrast Brightfield Training Video](#)

### 3.3 - Image Montage

Montaging allows you to capture a larger field-of-view while maintaining the resolution of your desired objective. Montage is a good choice when:

- Many cells are required for analysis
- Large objects contain information beyond a single field-of-view

Gen5 supports acquisition of up to 2000 images (tiles) in a single field, which can be processed, stitched, and analyzed individually or as a single final image.

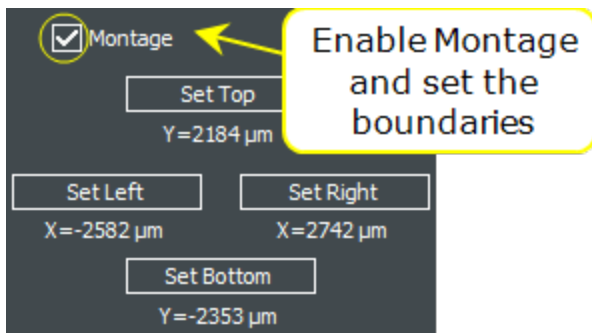


For Image Stitching, *Gen5 Image+* or *Image Prime* software is required.

#### Montage in Manual Mode

IMAGING MODE

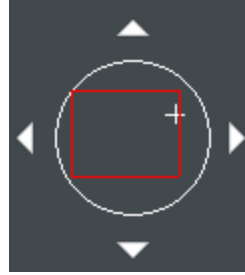
Expand the **Imaging Mode** panel.



Enable **Montage**. Navigate the field-of-view with the software controls or the joystick, and set the boundaries: Set Top, Set Bottom, Set Left and Set Right.

### 3 - Advanced Acquisition Guide

Red lines will appear on the well map as you set boundaries. When finished, you will see a red box indicating the area to be covered by the montage.



After defining the area you want to capture, click the camera button. The default "Auto for stitching" setting gives 10% overlap between tiles.

💡 Best practice: set boundaries as close as possible to the edges of the object of interest.

Edit Imaging Step...

After defining the capture area for a montage in manual mode, you can edit some of the parameters, including the **Tile Overlap** for stitching.

### Montage in Experiment Mode

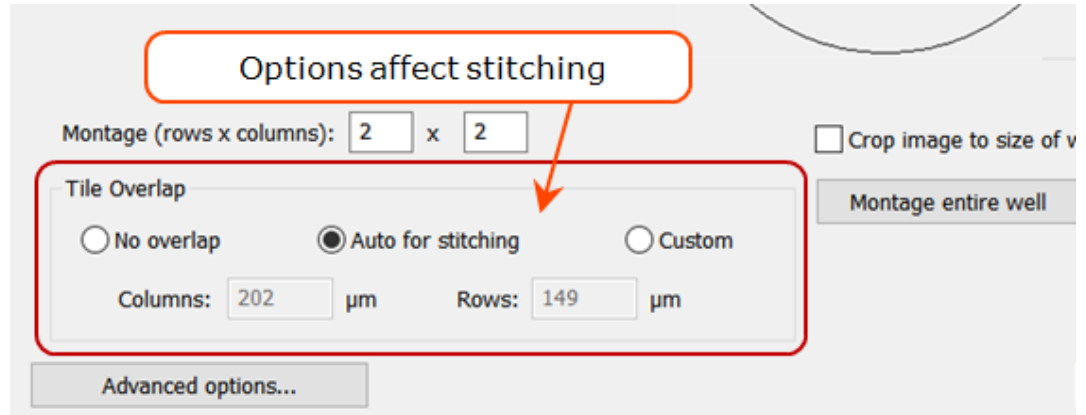
In the Procedure, create an imaging read step and select **Montage**.

Adjust the number of rows and columns to define the capture area, which will be shown in the well map. If the entire well is desired, you can click on **Montage entire well**.

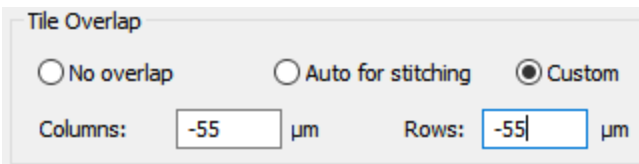
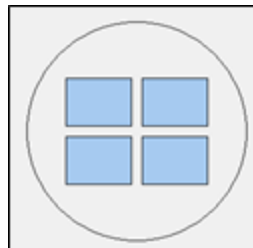
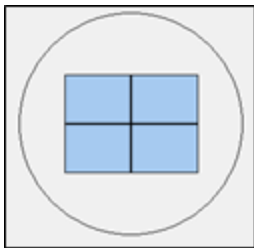
You can also define offsets for the montage center or use beacons (covered in 4.3 - Beacons, ROIs, and Offsets ).

### 3 - Advanced Acquisition Guide

Gen5 offers 3 methods for tile overlap. By default, Gen5 applies "Auto for stitching," which determines the overlap based on objective size. This creates an optimal amount of common information between images (indicated by dotted lines) to create a seamless final stitched image.



Alternative options include:

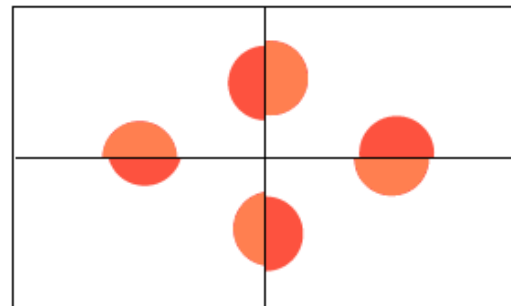


**No Overlap:** Images touch, but have no common areas.

**Custom:** You can input values for column and row separation. Positive values increase the overlap; negative values spread the images out, as shown here.

Objects in a stitched image based on a montage with no overlap may appear jagged and distorted, and a single object may even appear as multiple objects.

For example, in the image at right, Gen5 would count 8 objects:

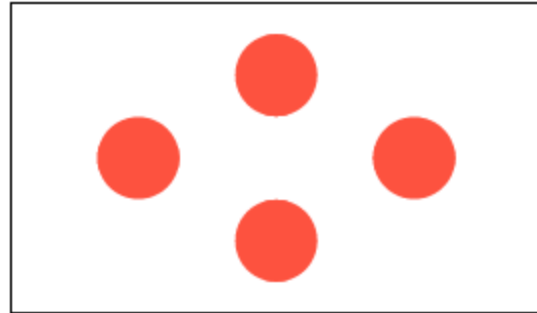


**Gen5 would count 8 cells in this "No overlap" montage.**

### 3 - Advanced Acquisition Guide

Applying an overlap is recommended for higher quality stitched images. Gen5 uses a cross-correlation method to blend individual tiles into one smooth image.

In this case, the final stitched image will look like this image and only 4 objects would be counted:



**Gen5 would count 4 cells in this stitched montage.**

▶ Watch this [Montage in Manual Mode Training Video](#).

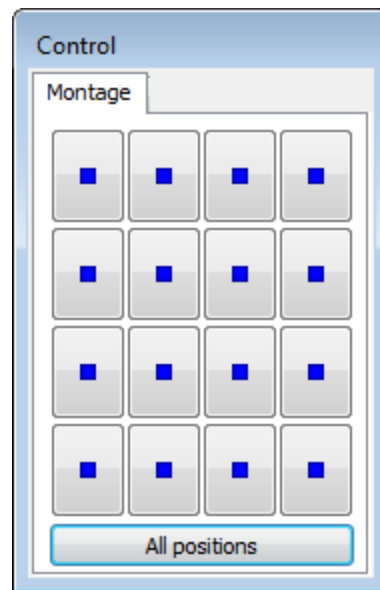
## Viewing Montage and Stitched Image

### Viewing Individual Tiles

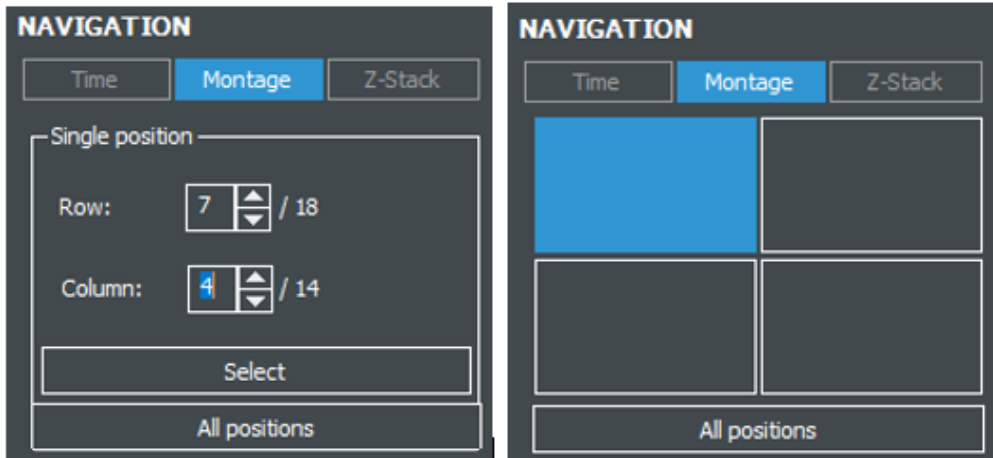
After capturing the montage image, Gen5 presents a special **Control** box to let you view the whole raw image (unstitched) or an individual tile:

- **All positions:** click this button to display the entire montage.
- Click a tile in the control to display that segment of the montage. Each tile represents a tile in the montage.

Note: the Montage Control is only presented when the raw montage is open. It closes when a stitched image is being viewed.



### 3 - Advanced Acquisition Guide



**Navigation** tools: Depending on the number of tiles, the Navigation panel in manual mode, and when processing or analyzing images, will vary from buttons to spinners for selecting tiles.

### 3 - Advanced Acquisition Guide

#### Image Stitching

Image Stitching, or creating a single image from montage tiles, can be found under **Image Processing** in both Manual Mode and Experiment Mode. Refer to section 2.3 - Image Processing and Analysis as shown on page 45 for Manual Mode instructions or section 6 - Image Processing as shown on page 138 for Experiment Mode instructions.

- **Registration channel** is the channel that will be used to determine the tile alignment. Choose the channel with the most signal and coverage, often this is your nuclear stain.
- **Fusion Methods** are algorithms to stitch images from a montage into a single image. Each option refers to a method for resolving the overlapped pixels.
- **Crop stitched image to remove black rectangles on the borders** crops the final montage to align with the area of most coverage. As tiles are aligned into a montage, some require more shifting to generate a seamless overlay with its neighbors. If this option is left unchecked, the remaining area will be filled with a black box.
- **Fill gaps between tiles with local background color** examines the 4 nearest pixels that have a valid intensity and averages these values to estimate the local intensity; and, uses this intensity to fill the gaps.
- **Downsize stitched image** reduces the final image size. A full-resolution image will be (2.2 MB [minimum size of each tile] X number tiles in the montage). Downsizing improves computer performance and storage space, but also reduces the resolution of the resulting montage.

**Important:** The pre-downsized stitched montage must be less than 4096 MB. Stitched images (after downsizing) must be smaller than 1 GB (1024 MB).

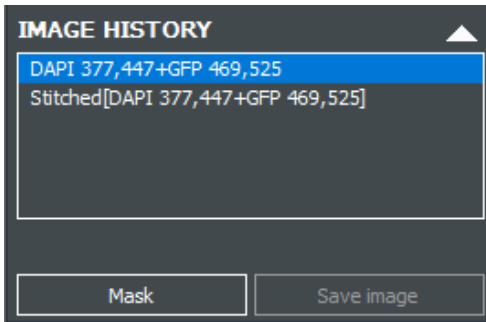
**To test different fusion methods: click **Edit Settings** in Manual Mode or edit the Read step in Experiment Mode.**

**💡** Linear Blend is typically the most effective fusion method, and the default choice. You may want to experiment with the other methods to confirm the best option for your needs.

### 3 - Advanced Acquisition Guide

#### Exclude Unwanted Tiles

"Mask" tiles to exclude unwanted areas from the stitched montage and analysis results.



1. Select the unstitched montage in the Image History box in manual mode, or the raw image set in an experiment.
2. Select the tile you want to mask by clicking in the image or using the Navigation tools.
3. Click **Mask**. Gen5 marks the tile with a red X.



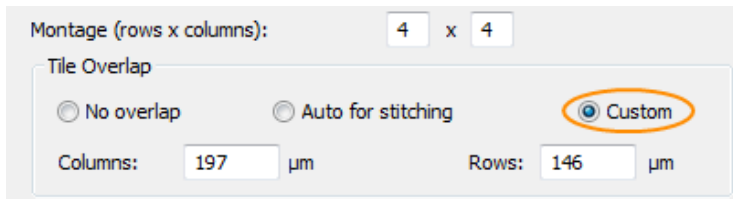
Gen5 prompts for an audit-trail comment when a mask is applied. A "Good Laboratory Practice" (GLP) is keeping notes on the reasons for the mask.

#### Fixing Montage/Stitching Problems

##### Poor Cross-Correlation

To correct "poor cross-correlation" in a stitched image, define a **Custom** tile overlap in the read step. Select:

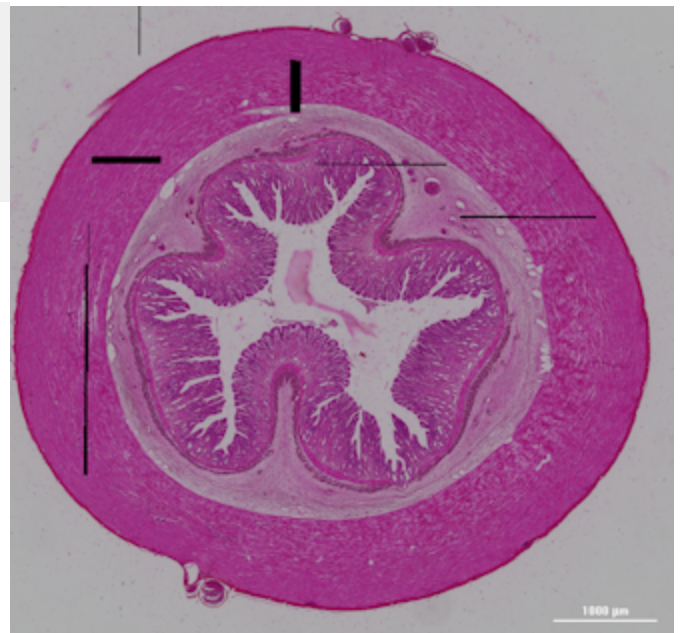
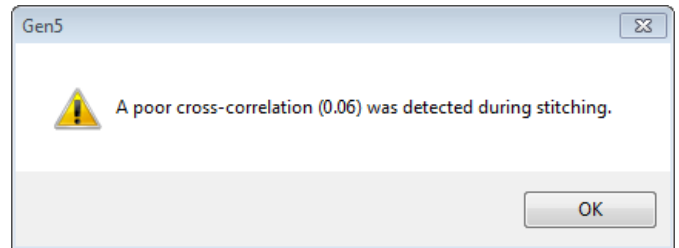
- **Edit Imaging Step** in manual mode,
- **Read Step** in experiment mode.



**Auto for stitching:** by default Gen5 defines a 10% overlap or 75 μm, whichever is greater.

**Custom:** lets you specify the tile overlap. Increase the overlap to 20-30% to avoid poor cross-correlation.

**Note:** Gen5 may display a "poor cross-correlation" message even when stitching is perfect. When Gen5 detects two or more blank tiles with nothing crossing between tiles, it reports poor cross-correlation.



Sample of stitched image with poor cross-correlation



### 3 - Advanced Acquisition Guide



In the stitched image above with the poor cross-correlation results, the montage images (tiles) were taken with a 10% overlap.

When the images were taken with a **Custom** 20% overlap (shown left), Gen5 generated the same stitched image with smoothed transitions between tiles.

#### Montage Takes Too Long?

Click **Advanced options** at the bottom of the Read Step (in Experiment mode) or Edit Imaging Step (in Manual mode):

Image Read Step Options

Delay after plate movement: 300 msec

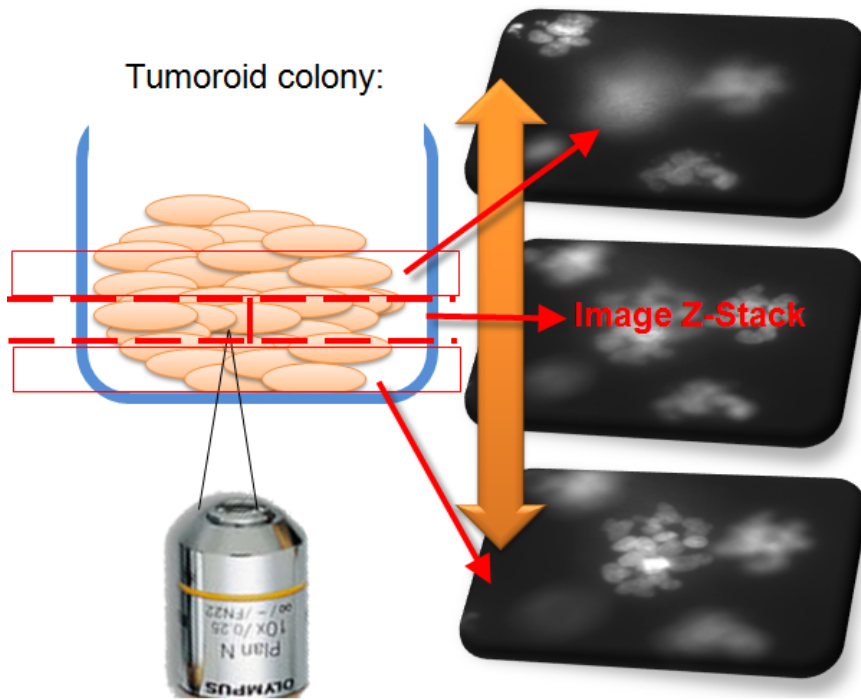
Montage / Beacon / ROI Autofocus Option

- Autofocus all images
- Only autofocus on center of montage, beacon or ROI closest to center of the well
- Autofocus option based on objective size

1. Reduce the Delay after plate movement setting.
2. Turn on Only autofocus on center of montage.

Typically these changes save 30-40 minutes on a 3-hour imaging read.

### 3.4 - Z-Stacking



Z-stacking captures multiple images in multiple z-axis (or focal) planes. Use Z-stacking when analyzing:

- thick samples like spheroids and tumoroid colonies,
- hanging-drop assays,
- whole organisms like zebrafish and C.Elegans,
- assays run in matrigel, like angiogenesis and tube formation.

With these types of samples, multiple images (up to 200 slices) must be acquired by moving the objective in the z-axis with a step size of  $0.1 \mu\text{m}$  or greater. After acquisition, Gen5 lets you view, analyze, and save individual slices (images). Gen5 can also combine the slices (or a subset of slices) into a "Z-Projection." Z-projections can be processed and analyzed as a single image.

**Recommendation:** First use Manual Mode to determine the optimal parameters for acquiring a z-stack of your sample. Then, apply those parameters in an experiment (Experiment Mode).

▶ Watch this [Z-Stack Image in Manual Mode Training Video](#).

### Capturing a Z-Stack

Good to know before you begin:

- In both manual mode and experiment mode, Gen5's starting point for a z-stack acquisition is the **Bottom Elevation** of the selected plate type. Make sure your vessel's bottom elevation is correctly

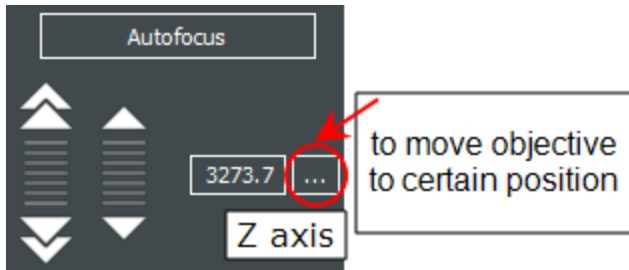
### 3 - Advanced Acquisition Guide

defined: See **Bottom Elevation:** on page 1.

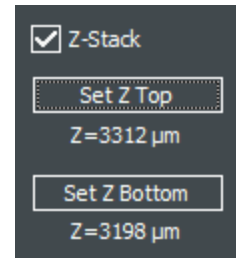
- Gen5 defines the capture height of the z-stack as the "sample thickness."



#### Manual Mode Z-Stack



Focus Controls



When you define the capture height for a z-stack, Gen5 determines the **Sample thickness**.

1. After selecting the magnification and color channels or light source; apply binning, if desired, on the Focus panel; and set the exposure or Auto Expose.
2. Use the **Autofocus** control buttons (shown above) or the joystick (not shown) to find the Top or Bottom z-axis position that best defines the start of the sample area you want to capture.

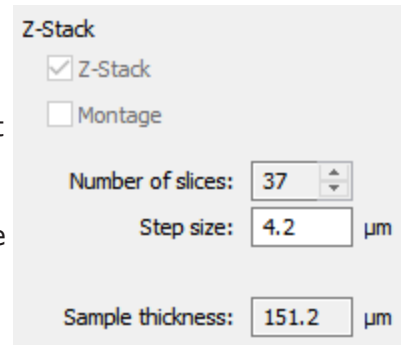
3. **IMAGING MODE** Expand the Imaging Mode panel and enable **Z-Stack**.

- Gen5 always shows the **current Z position** as you move in the z-axis.

4. Click the **Set** button for each position when you have found the lowest and highest in-focus images of the sample.



5. Click the camera button to capture the Z-stack.



Step size is set by the objective.

**Edit Imaging Step...**

Edit the imaging step to review/change the number of slices.



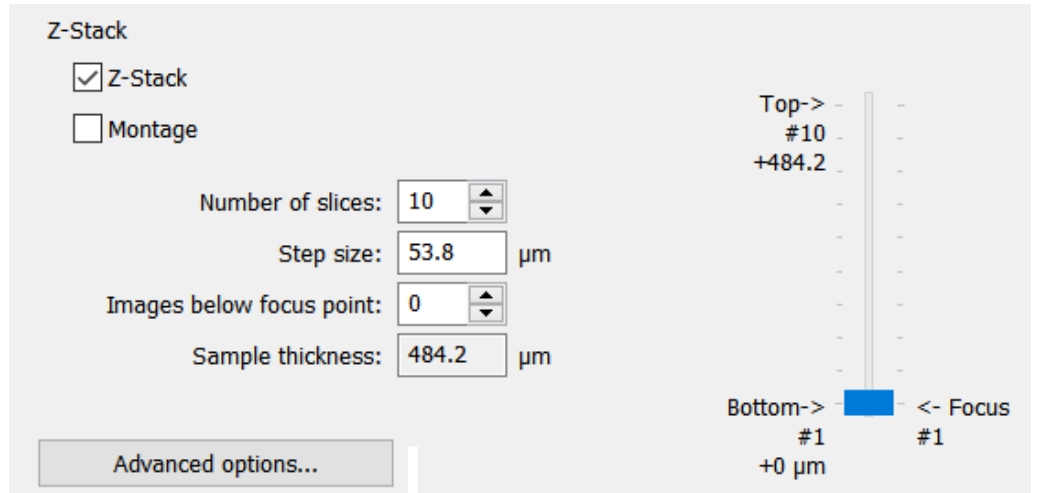
You can use manual mode to determine the best parameters and then save them in an experiment, click **Create an experiment from the image set**.

### 3 - Advanced Acquisition Guide

#### Experiment Mode Z-Stack

To define a Z-stack directly in experiment mode, set:




- **Step size:** defaults to the depth-of-field of the objective; it can be changed if needed;
- **Number of slices:** defines the Sample thickness.
- **Focus point:** by default, the Bottom position is the focus point. You can use the slider or the number of Images below focus point to define an alternative capture range.

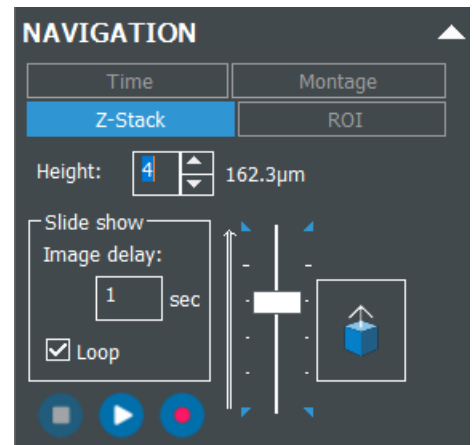


Flexible tools for defining the capture height for a Z-stack

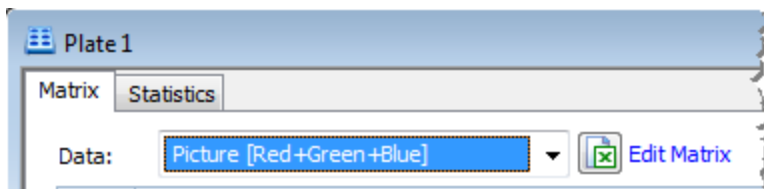
#### Viewing a Z-Stack

After capture, Gen5 presents a Z-Stack Control. You can use:

- the slider or the Height scroll buttons to display one slice at a time in sequence,
- **Z-Stack Slide Show:** to view the slices like a slide show: 
- **Z-Stack Movie:**  click the record button to make a movie.
- **3D Viewer:** click the  to render the z-stack in 3D. See 3D Viewer on page 76.



well zoom or manual mode



In experiment mode, set the plate's Matrix Data to "Picture" without a ZProj label to open the Z-Stack Control and view the individual slices.

### 3 - Advanced Acquisition Guide

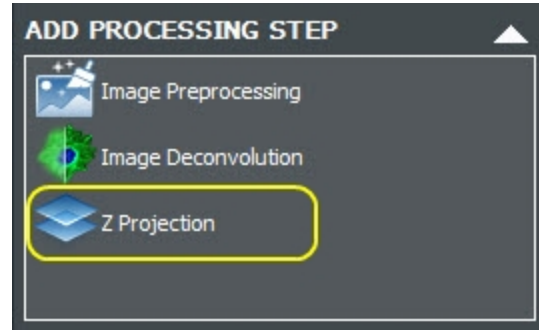
#### Creating a Z-Projection

*Gen5 Image+ or Image Prime software required*

After acquiring a z-stack, Gen5 can combine the slices (images) into a 2D rendering or **Z-Projection**. All or a selected subset of the slices in the z-stack can be included in the projection.

To create a Z-Projection:

- In Manual Mode, click **Z Projection** in the Image Processing area (shown right).
- In Experiment Mode, define a **Data Reduction** step.



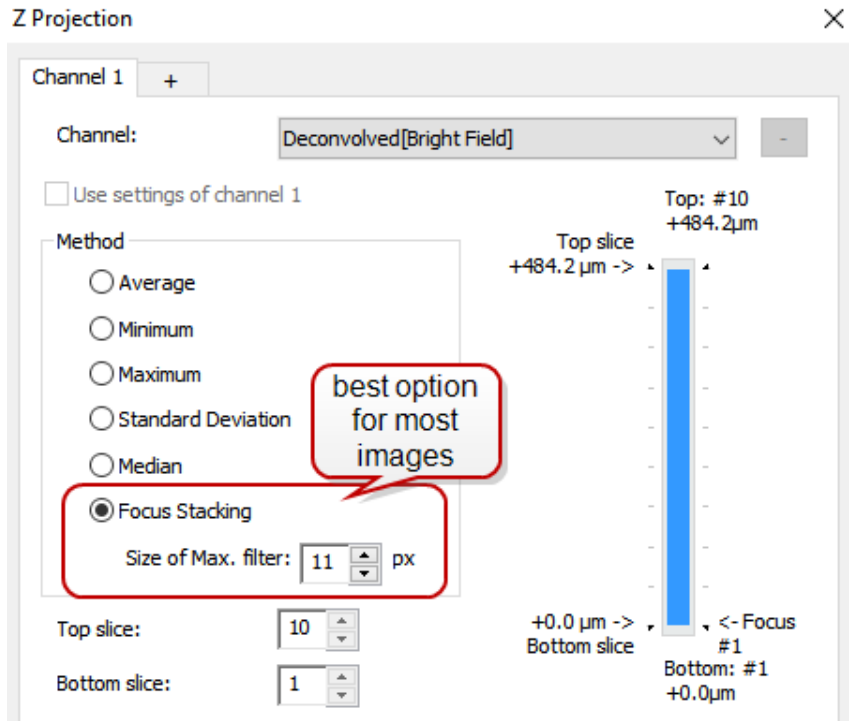
Manual Mode

Before or after defining a Z-Projection, Gen5 lets you try to improve image quality with preprocessing and/or deconvolution. After creating the projection, you can analyze the image.

When combined with a montage, Image Stitching must be done before making a Z-Projection.

#### Specify Method and Slices for Z-Projection

1. In the Z Projection window, select a color Channel. For a multi-channel z-stack, click the + tab for each additional color. Gen5 will supply any subsequent channels. By default, they are set to "Use settings of channel 1," but you can change the settings for each channel individually.
2. Select a Method. For most assays, the **Focus Stacking** method is best. It includes only in-focus images in the projection.
3. Use the Top and Bottom slice scroll buttons or drag the slider to limit the projection to a subset of slices.



💡 Create multiple Z-Projections, each using a different method, to quickly evaluate the results of each method and choose the best one for your samples.

### 3 - Advanced Acquisition Guide

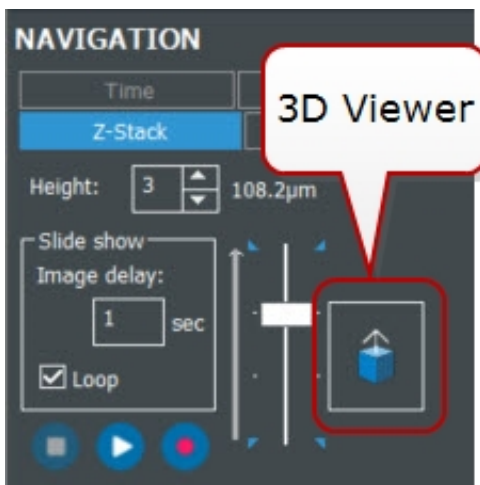
Gen5 can perform image analysis on the Z-Projection in both manual and experiment modes. The Z-Projection is treated like any other single image.

#### Troubleshooting:

- **Z-Projection Focus Stacking:** When using the Focus Stacking method to make a z-projection, you can adjust the "Size of Max. filter" to improve the output. Filter size is sample dependent: generally, the smaller the objects of interest, the smaller the filter size should be, and conversely, the larger the objects the larger the filter size.
- **Acquiring a Z-Stack:** (for inverted imagers) Make sure all your objectives and LED and Filter Cubes are calibrated if you experience problems capturing z-stack images. Select **System>Instrument Configuration**. Double-click the imager item and click **Setup**. Click the **Imaging Configuration** tab, and rerun **Auto Calibration**. This may take several minutes to complete.

### 3D Viewer

Requires Gen5 v.3.11



For z-stacks captured in fluorescent channels, Gen5 provides the 3D Viewer. Click the **3D Viewer** button in the Navigation tools to launch the viewer.

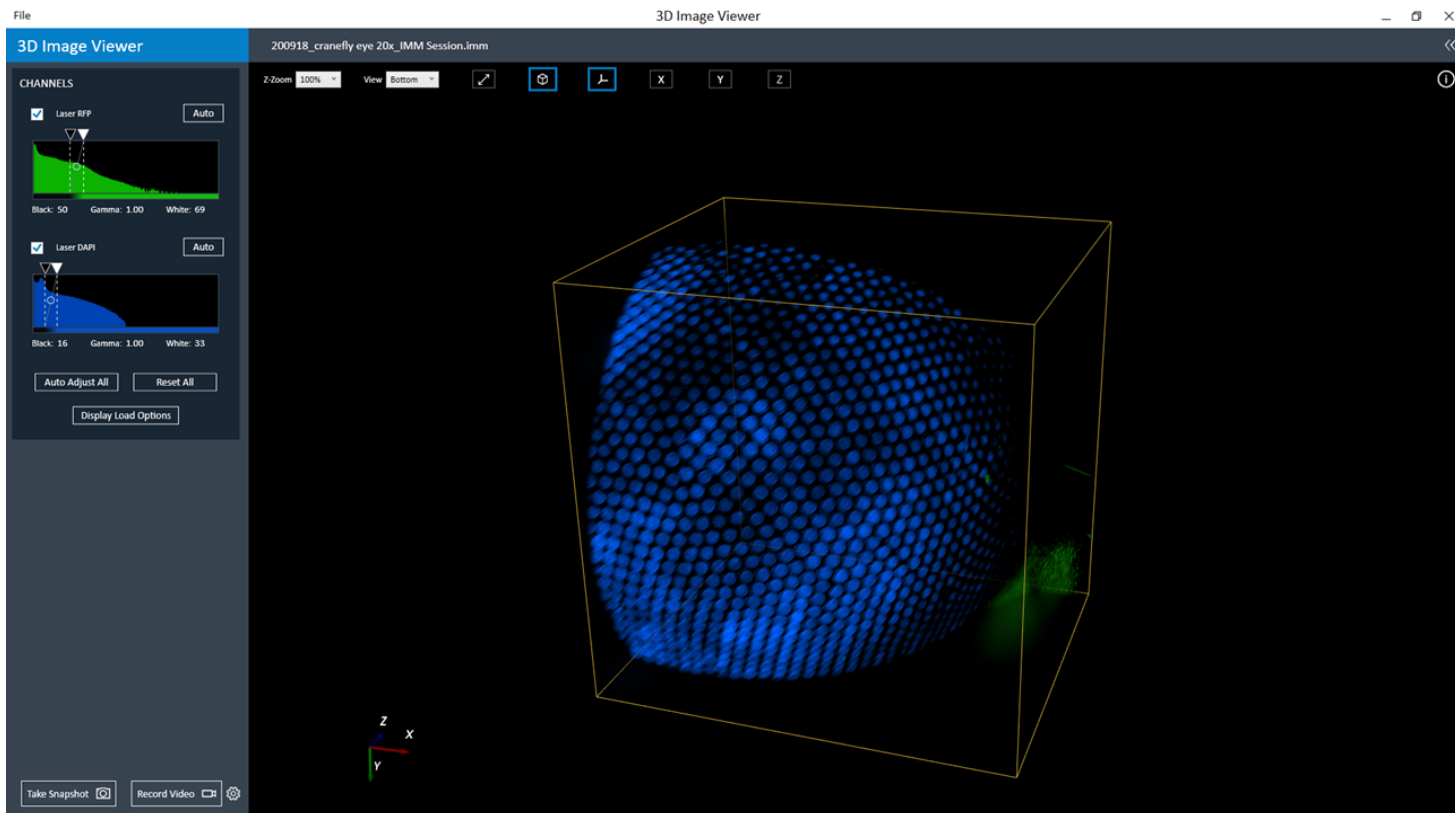
#### Good to Know:

- Transmitted light captured images, e.g., brightfield, are not supported. However, images captured in both fluorescent and transmitted light can be viewed in the fluorescent channels only.
- Z-step size affects rendering. Smaller step sizes produce better images. Rule of thumb: divide Gen5's default step size by 3. However, be conscious of file size when employing the setting, as extremely large file sizes create a data management challenge.



- Dim or low signal channels can sometimes obscure the rendering of bright or high signal channels in multichannel images. If part of the image appears blank, try toggling visibility by de-selecting dark channels to improve the view of other channels in the image.
- High performance computers are recommended for the best experience using the 3D Viewer.

### 3 - Advanced Acquisition Guide



#### Interactive Controls:

Several controls for manipulating the 3D projection are provided. Experiment with them to determine the best rendering of your sample.



Begin by applying the bounding box to enhance the 3D dimensions.



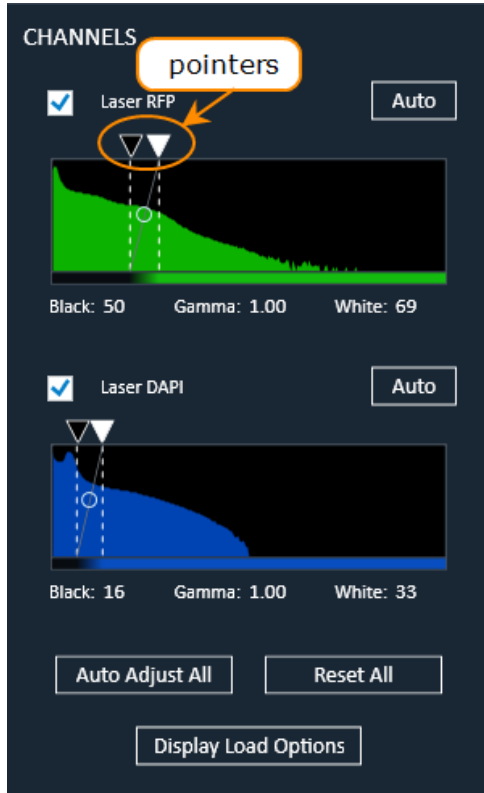
Toggle the X, Y, and Z planes to add these views to the projection, if useful.

In addition to the toolbar buttons, you can use your mouse to:

- Zoom into and out of the projection using the mouse wheel.
- Press and hold Ctrl while using the mouse to rotate the projection on its center axis.
- With an X, Y, or Z plane visible, click and drag the plane to view it in grayscale at various positions in the stack. Right-click and drag on the plane to change its brightness and contrast.

### 3 - Advanced Acquisition Guide

#### Use Histograms to improve projections:



Histograms for each color channel are interactive in the 3D viewer. The X-axis displays intensity, Y-axis displays number of voxels.

De-select a channel to hide it.

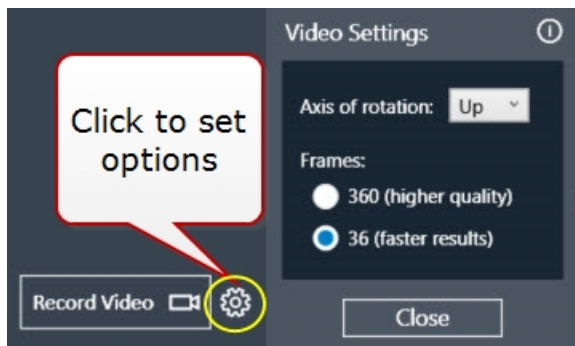
Click and drag the black and white pointers at the top of histogram to adjust the intensity and number of voxels. Click **Auto** or **Auto Adjust All** to restore the pointers to their original positions.

**Reset All** moves the white pointer to its maximum setting and moves the black pointer to its minimum setting.

The bottom bar of the histogram shows the color of voxels in the rendering.

Consider using the **Display Load Options** to improve the projection by reducing the number of slices included. Downsizing may also reduce file size for better performance.

#### 3D Snapshots and Movies:



Use the controls at the bottom of the 3D Viewer to capture snapshots and movies of the current 3D projection. Snapshots can be saved as TIF or JPEG.

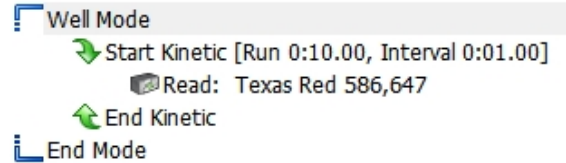
Click the options button to customize the movie settings. This is especially useful for reducing the file size of the movie.



## 3.5 - Kinetic Image Capture

Time-lapse image capture is available in both Manual and Experiment Modes. Several factors affect the interval duration, including:

- Number of detection channels
- Integration time
- Autofocus method
- Binning (See [Binning Options](#) on page 86)
- Advanced imaging modes: Montage and/or Z-Stack

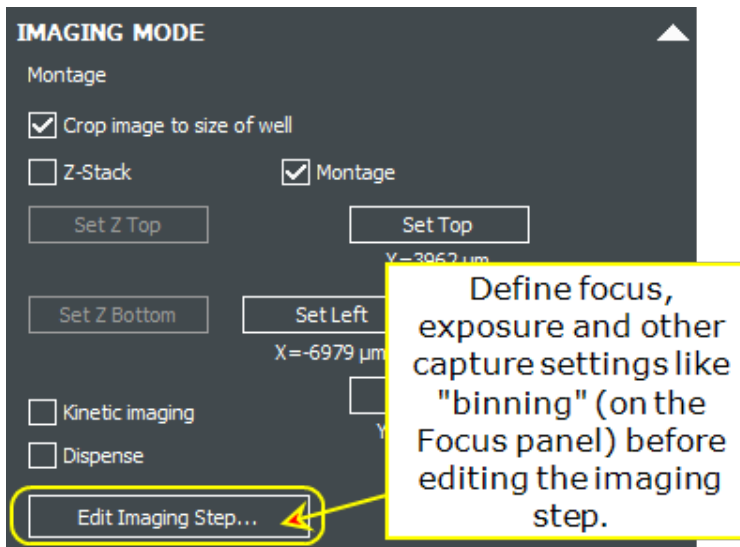


Kinetics requiring a fast frame rate, such as Ion Channel Assays, can be performed when the signal-of-interest has correspondingly rapid integration times. Integration times to achieve 10 frames-per-second (fps) must be set to 50 milliseconds (ms) or lower; integration times to achieve 20 fps must be 20 ms or lower (with binning turned on). Fast kinetics must be performed in Well Mode.

▶ Watch the [Kinetics Overview Training Video](#).

## Kinetic Acquisition

### Manual Mode



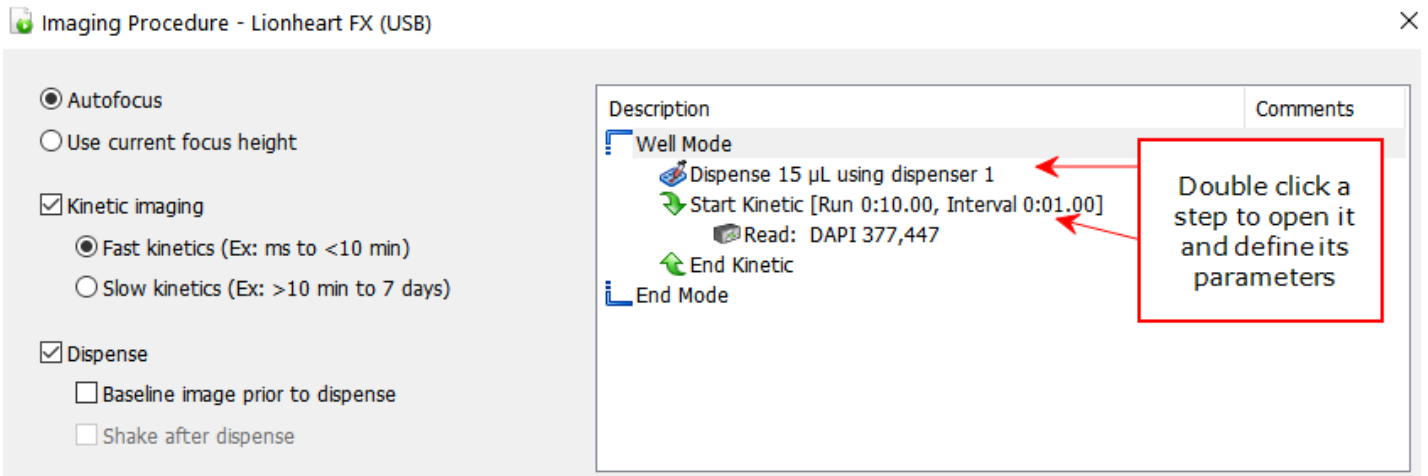
Select **Kinetic imaging** and/or **Dispense** to enable the **Edit Imaging Step** button:

1. Expand the Imaging Mode panel, (Z-Stack and Montage can be performed in a Kinetic loop) and check **Kinetic imaging** and **Dispense**, if applicable.
2. Click **Edit Imaging Step**.
3. Within the Well Mode loop, double-click the Dispense and **Start Kinetic** steps to define the dispense, interval (the time between imaging steps), and total run time.



4. Click  to execute the run.

### 3 - Advanced Acquisition Guide



Imaging Procedure - Lionheart FX (USB)

Autofocus  
 Use current focus height

Kinetic imaging  
 Fast kinetics (Ex: ms to <10 min)  
 Slow kinetics (Ex: >10 min to 7 days)

Dispense  
 Baseline image prior to dispense  
 Shake after dispense

| Description                                   | Comments |
|---|----------|
| Well Mode                                     |          |
| Dispense 15 µL using dispenser 1              |          |
| Start Kinetic [Run 0:10.00, Interval 0:01.00] |          |
| Read: DAPI 377,447                            |          |
| End Kinetic                                   |          |
| End Mode                                      |          |

Double click a step to open it and define its parameters

**Important:** The kinetic interval must be long enough to acquire the images.

**Burst Rate:** 10 frames per second (fps) at full resolution, 20 fps at 2x2 binning.



Save your manual mode settings to create an experiment. Otherwise, define a kinetic read step as usual in Gen5's Procedure dialog.

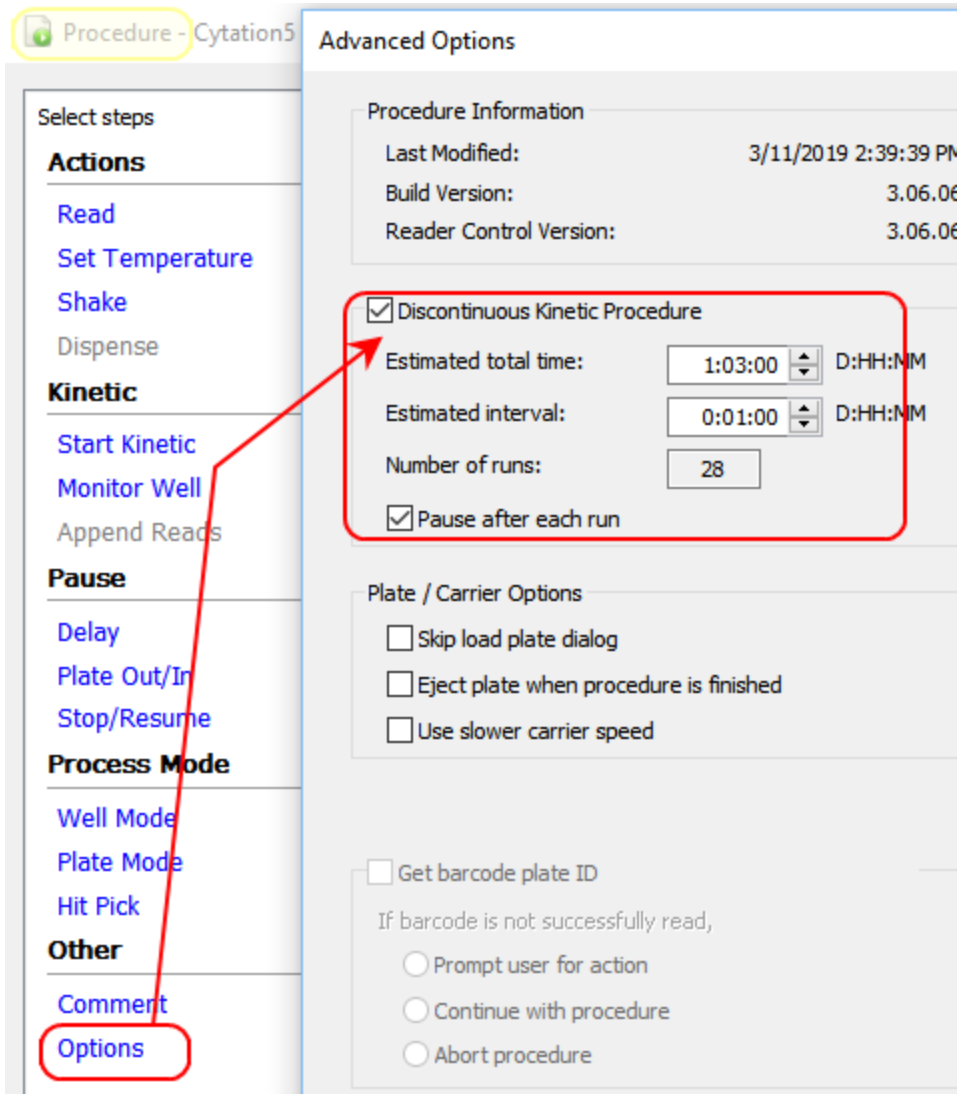
#### Discontinuous Kinetics

Gen5's most flexible kinetics option "discontinuous kinetics," frees up the instrument so it can be used in other experiments, during incubation or rest periods. Discontinuous kinetics also gives you the opportunity to add reagents, or otherwise manipulate the plate/vessel in between reads (image acquisition). Gen5 aggregates the measurements/images obtained from the multiple sessions, combining them into one result set. It gives users full control of image acquisition, rather than relying on the automated or programmed intervals of standard kinetic image capture. Discontinuous kinetics provides all the kinetic analysis options of the standard kinetics, e.g., kinetic curves, Max V.


**Note:** Discontinuous Kinetics is required for BioSpa OnDemand protocols.

### 3 - Advanced Acquisition Guide

#### Set Up a Discontinuous Kinetic Experiment:



1. From the Task Manager, select **Protocols > Create New** (or create a new experiment).

2.  When defining the procedure, select **Options**, and select **Discontinuous Kinetic Procedure**.


3. Enter "estimated" time points in Days:Hours:Minutes: Estimated total time and Estimated interval. This is just an estimate and will not interfere with actual experiment activity. Gen5 uses your estimated time lines to set up the data views and formulas with placeholders until the actual data is captured. When in doubt about the required time period and intervals, it is best to overestimate them.

4. Define the Read step(s) as usual, and save the protocol.

5. Create an experiment based on the protocol, and  read the plate.

6. Save the experiment (and close the file, if desired).

7. Remove the plate and process (e.g., add reagent, incubate, etc.) until it is time for the next reading.

8. Open the experiment, put the plate on the carrier, click  **Read New**, and select "Continue reading."

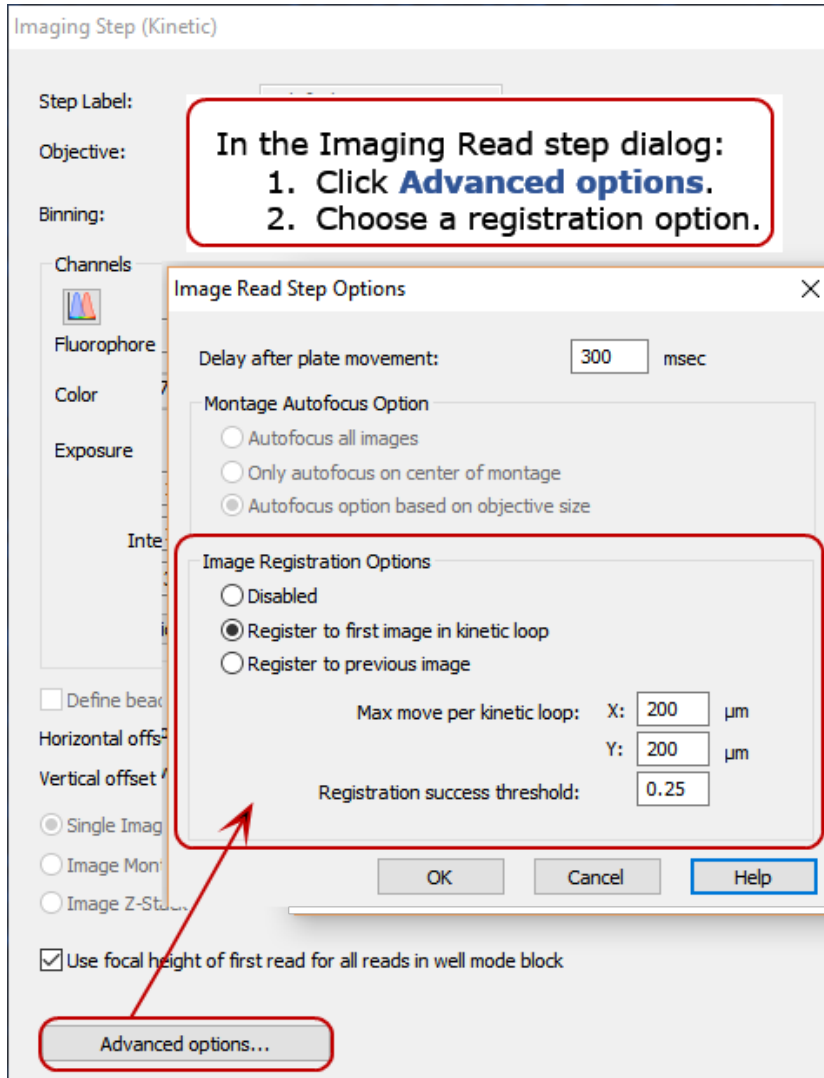


Gen5 compiles all the reads into a kinetic data set, and performs Data Reduction as defined in the protocol.

### 3 - Advanced Acquisition Guide

#### Kinetic Image Registration Options

**Note:** For fast kinetic assays, Kinetic Frame Alignment as shown on the next page is a better option for improving image acquisition. Kinetic Image Registration slows down the process, capturing up to 5 additional images which could add as much as 20 seconds per frame.



Gen5 provides this feature to correct some of the effects of incubation on plastic plates and improve tracking of cellular activity during time-lapse assays. **Image Registration** performs X/Y carrier adjustments between kinetic image acquisitions. Registration compares before and after images to determine drift in either the x- or y-axis. Then, corrects for any drift detected prior to image capture by repositioning the carrier.

Choose the reference image: either the first image captured in the kinetic time span or the previous image captured.

- **Register to first image:** If you expect little change in the sample biology over the course of the experiment.
- **Register to previous image:** if the biology is changing quickly, e.g., cell growth or phenotypic changes.
- **Registration success threshold:** Enter a value between 0 and 1 (default is 0.25). This value represents the threshold that the image registration must meet to be considered successful, with 1 being a perfect match and 0 being no match.

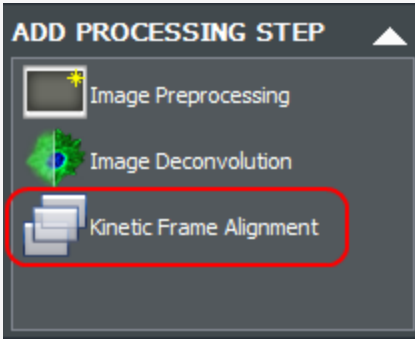
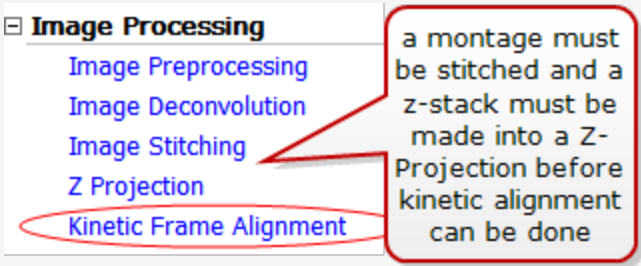
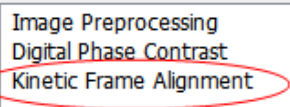
### 3 - Advanced Acquisition Guide

If the normalized cross-correlation factor between the test and the reference image is below the registration success threshold, the registration move is not made. In cases where image registration is adjusting the position of the sample incorrectly, it may help to raise the threshold.

- **Max move per kinetic loop:** Optionally, you can specify the maximum distance the alignment can move during each kinetic read.

#### Kinetic Frame Alignment

After executing a kinetic read, Gen5 offers an image processing step to improve the quality of a movie made from the images. Kinetic Frame Alignment removes the X-Y movement between frames that occurs naturally during image capture.

| Manual Mode   | Experiment Mode   |
|---|---|
| <p>In Processing pane:</p>  | <p>Add a Data Reduction step:</p>  <p>or in a well zoom (from plate matrix), click <b>Process</b>:</p> <p>Process Tool</p> <p><input type="radio"/> I want to edit the Image Processing step that produced this image</p> <p><input checked="" type="radio"/> I want to setup a new Image Processing step to apply to this image</p> <p>Select:</p>  |

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#### Kinetic Frame Alignment

Alignment channel:

Perform alignment on entire image

Alignment

Reference image:

First valid image in the series

Previous valid image in the series

Maximum offset value:   $\mu\text{m}$

Image background

Background is dark

Evaluate background on:  % pixels

Aligned Images

Crop to keep the area common to all images

Extend to keep the information of all images

Before making a movie, perform this frame alignment step, choosing options that produce the best effect for your assay. Some experimentation may be needed.

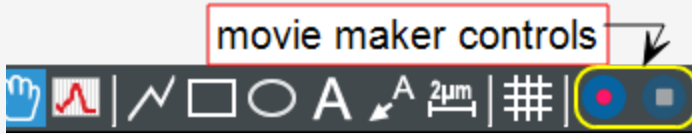
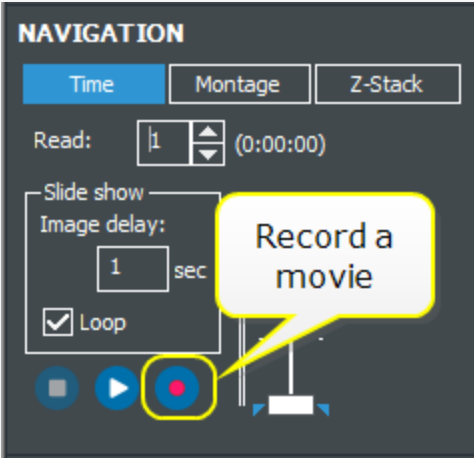
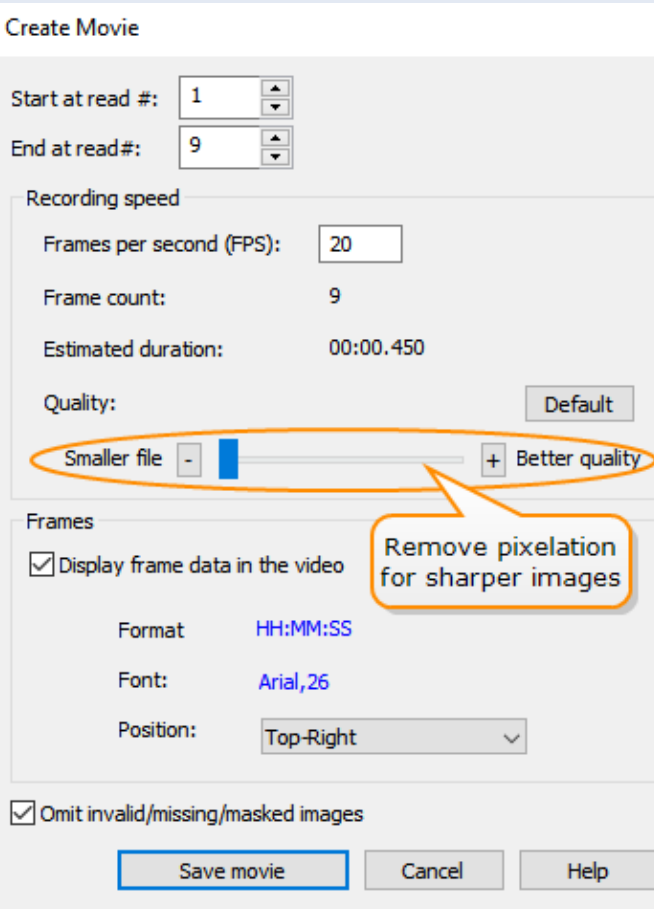
Then, choose the "**Aligned(detection channel)**" data set as your movie source:

See Recording and Saving Time-Lapse Movies below.

### Recording and Saving Time-Lapse Movies

Gen5 3.00 (and higher) offers movie maker controls in both manual and experiment mode. Generally, a kinetic or time-lapse acquisition produces the best results, but Z-stack and manual manipulation of the imaging process can also be captured in a movie.

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| Manual Mode  | Experiment Mode   |
|--|---|
| <p>Click record during the session.</p>  <p>Click stop button to end recording.<br/>Select <b>Movie Options</b> from the <b>Control</b> panel to customize settings:</p>  | <p>After you define and execute a kinetic read step in Gen5, drill into a well zoom:</p>  |
| <ul style="list-style-type: none"> <li>• Use the <b>Quality</b> slider to make the images sharper or conversely to save disc space by compressing the file size.</li> <li>• Annotations made to the images will be captured and saved in the movie.</li> <li>• Kinetic acquisition is supported in manual mode.</li> <li>• Masked frames can be excluded from the movie.</li> <li>• <b>Kinetic Frame Alignment:</b> a special image processing step can be applied to improve the quality of movies made from a kinetic read.</li> </ul> | <p>Click the record button to open the controls:</p>                                     |

### 3 - Advanced Acquisition Guide

#### Saving Movie Files

|               |                                       |
|---------------|---------------------------------------|
| File name:    | unique filename for my movie          |
| Save as type: | MPEG-4/H.264 video file (*.mp4)       |
|               | WMV3 Windows Media video file (*.wmv) |
|               | MPEG-4/H.264 video file (*.mp4)       |
|               | All Files (*.*)                       |

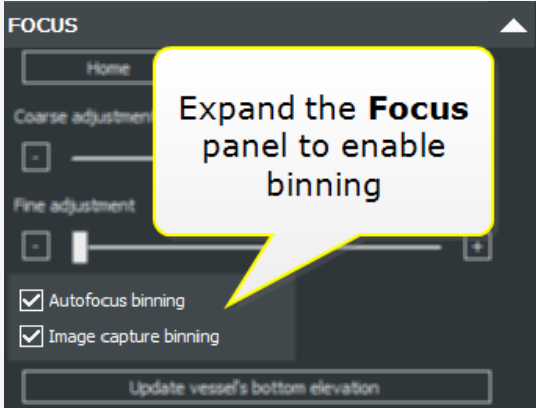
Gen5 offers two file formats and several other options for saving your movie files:

- .wmv is Windows default video file format
- .mp4 is an industry standard video file format

**Note:** Stitched Montage Movies: MP4 is limited to 9.4 megapixel images. WMV is limited to 12.3 megapixel images. If you try to record a video with a larger image resolution, like a stitched montage, you may get an error message or the video may not be viewable.

#### Binning Options

Image binning can improve focus and acquisition speed. Binning is a method that combines intensities from a 2x2 pixel grid into a single pixel. This reduces the image size and storage down to 25% the original size. It also can reduce the integration time needed for exposure by a factor of up to 1/4 because the intensities of each pixel in the 2x2 grid are summed into the final pixel. Binning affects exposure settings: binned images are brighter than non-binned images with the same exposure settings because pixel intensities are doubled.

| Manual Mode   | Experiment Mode  |
|---|--|
|  | <p>Objective: 4x Image: 1973 x 145</p> <p>Binning: <input checked="" type="checkbox"/> Auto focus binning<br/><input type="checkbox"/> Capture binning (affects exposure)</p> <p>Define in the Procedure's Read step</p> |

Gen5 offers two options for binning images: Autofocus and Capture, but binning must be "on" for Autofocus to apply it to acquisition. You can test the binning effects in manual mode by enabling and disabling the option and viewing the difference.

| Advantages of Binning                      | Disadvantages of Binning              |
|--|---------------------------------------|
| smaller image file size                    | reduced spatial resolution            |
| potentially improved signal-to-noise ratio | reduced image size (lower resolution) |



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| Advantages of Binning                       | Disadvantages of Binning |
|---|--------------------------|
| faster performance - smaller integration    |                          |
| higher sensitivity, especially for red dyes |                          |

- **Autofocus:** Binning affects the exposure setting during autofocus. Gen5 adjusts the exposure automatically to get the most intensity possible.
- **Capture:** Gen5 stores the resizing information (height and width number of pixels) with the image. Generally images will be 1/4 size compared to non-binning capture.

💡 Recommended practice when using binning: check and set the exposure settings in each channel with binning enabled before acquisition.

### Kinetic Z-Stack Montage

In Gen5 3.04 or higher you can combine the advanced acquisition methods. Combining time-lapse imaging with z-stack and montage captures may be useful for multicellular assays, spheroid or zebrafish imaging, for example. Such a complex image compilation will produce significantly larger data files depending on the number of color channels, z-stack slices, montage tiles, and kinetic intervals. Consider your file management needs before beginning this type of acquisition, e.g., can you save the files to an external hard drive?

Gen5 supports up to 2000 images/channel/well or up to 8000 images/well. Keep this limit in mind when defining the number of slices in the z-stack and number of tiles in the montage.

**Important:** The pre-downsized stitched montage must be less than 4096 MB. Stitched images (after downsizing) must be smaller than 1 GB (1024 MB).

| Manual Mode  | Experiment Mode   |
|--|---|
| <p><b>IMAGING MODE</b></p> <p>Z-Stack Montage</p> <p><input checked="" type="checkbox"/> Z-Stack      <input checked="" type="checkbox"/> Montage</p> <p>Set Z Top      Set Top<br/>Z = 3000 <math>\mu\text{m}</math>      Y = 1648 <math>\mu\text{m}</math></p> <p>Set Z Bottom      Set Left      Set Right<br/>Z = 2500 <math>\mu\text{m}</math>      X = -1934 <math>\mu\text{m}</math>      X = 1527 <math>\mu\text{m}</math></p> <p><input checked="" type="checkbox"/> Kinetic imaging      Set Bottom<br/><input checked="" type="checkbox"/> Dispense      Y = -937 <math>\mu\text{m}</math></p> <p>Edit Imaging Step... </p> | <p>Z-Stack Montage</p> <p><input checked="" type="checkbox"/> Z-Stack</p> <p><input checked="" type="checkbox"/> Montage</p> <p>Number of slices: 32</p> <p>Step size: 4.2 <math>\mu\text{m}</math></p> <p>Images below focus point: 0</p> <p>Sample thickness: 130.2 <math>\mu\text{m}</math></p> <p>Montage (rows x columns): 2 x 2</p> |

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#### Copy & Paste to add a Channel

In manual mode, Gen5 copies the live session settings for a color channel and lets you paste them into the read step. To add another channel to the kinetic, z-stack, and/or montage read:

1. Click **Edit Imaging Step**. Open the **Read** step (inside the kinetic loop, if applicable).
2. Add a channel in the imaging step dialog and click OK to close the window.
3. Back in the **Capture** mode, select the additional channel and define its focus and exposure settings. Note: Gen5 remembers the last parameters used for the channel.



4. When settings are defined, click the **Copy** button, and then select **Edit Imaging Step**.



5. Click the **Clipboard** button for the channel to paste the settings. When all the channels are defined, click OK to close the Read step. Click OK to close the procedure window.



6. Click to start the procedure.

## 3.6 - High Power and Oil Objectives

### Focusing Suggestions for High Power Objectives

- **Joystick:** if available, use the joystick for the most precise controls for navigation and focus.
- **Exposure controls:** See 3.1 - Optimizing Exposure Settings on page 59 for more details. Except for setting LED intensity, to gain more precise control of exposure settings, click on the slider and then use your keypad's arrow keys or your mouse wheel to more gradually increase or decrease the settings.
- **Reduce the Integration Time** and increase **Camera Gain**, at least initially, when trying to find the object of interest. Then, select **Auto Exposure**.

**Important:** See 1.5 - High-Power Objectives for important setup guidelines for high-power objectives.

### 40X, 60X, and 100X Objectives: Limitation

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| A | X | X | X | X | X | X | X | X | X | X  | X  | X  |
| B | o |   |   |   |   |   |   |   |   |    |    | o  |
| C | o |   |   |   |   |   |   |   |   |    |    | o  |
| D | o |   |   |   |   |   |   |   |   |    |    | o  |
| E | o |   |   |   |   |   |   |   |   |    |    | o  |
| F | o |   |   |   |   |   |   |   |   |    |    | o  |
| G | o |   |   |   |   |   |   |   |   |    |    | o  |
| H | X | X | X | X | X | X | X | X | X | X  | X  | X  |

|   | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | o | o | o | o | o | o |
| B | o |   |   |   |   | o |
| C | o |   |   |   |   | o |
| D | o | o | o | o | o | o |

96- and 24-well plate maps when using a high-power objective

Depending on the vessel, high-power objectives may not be able to capture images of all wells or sections of the vessel. Keep this in mind when you are plating your samples. This limitation is visible when selecting the wells to read in a read step (click the **Full Plate** button). On the contrary, the limitation is not obvious when defining the Plate Layout.

Gen5 identifies this limitation in the plate map:

- X to show when no part of the well can be imaged;
- O to show when only a portion of the well can be imaged.

To view the plate map in manual mode, click **Well:**

Well:  (0, 0)

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To view the plate map in experiment mode:

1. Create a **Procedure** and select the **Plate Type**.
2. Click **Read**, and set **Image** as the detection method.
3. Set the **Objective** to 40X or 60X (phase/standard) and click **Full Plate**.

#### Oil Objectives

##### *Lionheart only*

The Lionheart supports the use of oil immersion objectives and Gen5 guides users through the process of applying and removing the oil. The oil immersion objectives are optimized for viewing through cover-slip thick glass, 0.17 mm, and do not have a correction collar to override this preference.

Olympus oil immersion objectives are available in magnifications of either 60X or 100X. The transparent Olympus® Immersion Oil Type-F supplied with your objective has a high viscosity that produces an uninterrupted liquid column between the two surfaces. It is not recommended to use other oil types. Moreover, the oil is optically similar to glass with a refraction index of 1.515.

#### Requirements

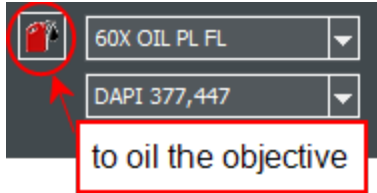
- Use **glass vessels**:
  - cover slips
  - slides
  - glass-bottom microplates and Petri dishes
- **Manual Mode Only**: oil objectives can only be used in live or manual-mode sessions, not experiments.
- **One well at a time**: best practice to avoid spreading the oil on the vessel, image one well or slide at a time.
- **A single drop of oil**: use as little oil as possible, typically a single drop is sufficient. You can reapply oil as needed.
- **Clean the oil after each application**: clean the objective with lens paper after each image capture. This prevents oil seeping into and damaging substage components. Also, clean the vessel bottom.
- **Note**: Autofocus is not supported and Gen5 does not calibrate oil objectives. Likewise, "Color Bright Field" imaging is not supported.

#### Procedure: Using Oil Immersion Objectives with the Lionheart

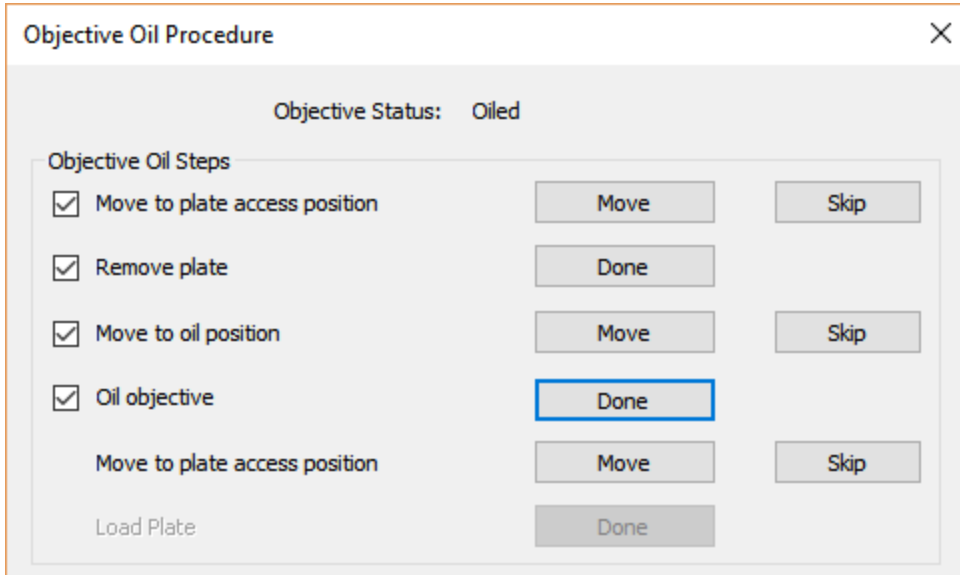
Instructions for installing oil immersion objectives can be found in the operator's manual.

Oil loading and cleaning procedures are stepwise. You can execute them by using Gen5's instrument control panel or you can respond to prompts when you select an oil objective in manual mode. Best practice is to follow the sequence as prompted to load oil and then clean the objective before switching back to a non-oil objective.

### 3 - Advanced Acquisition Guide



**Control** panel: When an oil immersion objective is installed, you can open the **Clean Objective** wizard via Instrument Control panel.



1. Select an oil objective.
2. Click the oil can to open the wizard.
3. Follow the prompts, clicking the buttons to tell Gen5 which step you are on.

Select **Skip** when applicable to advance to the next step. For example, if there isn't a plate on the carrier when you begin, click the first Skip button.

4. Be sure to clean the objective after use.

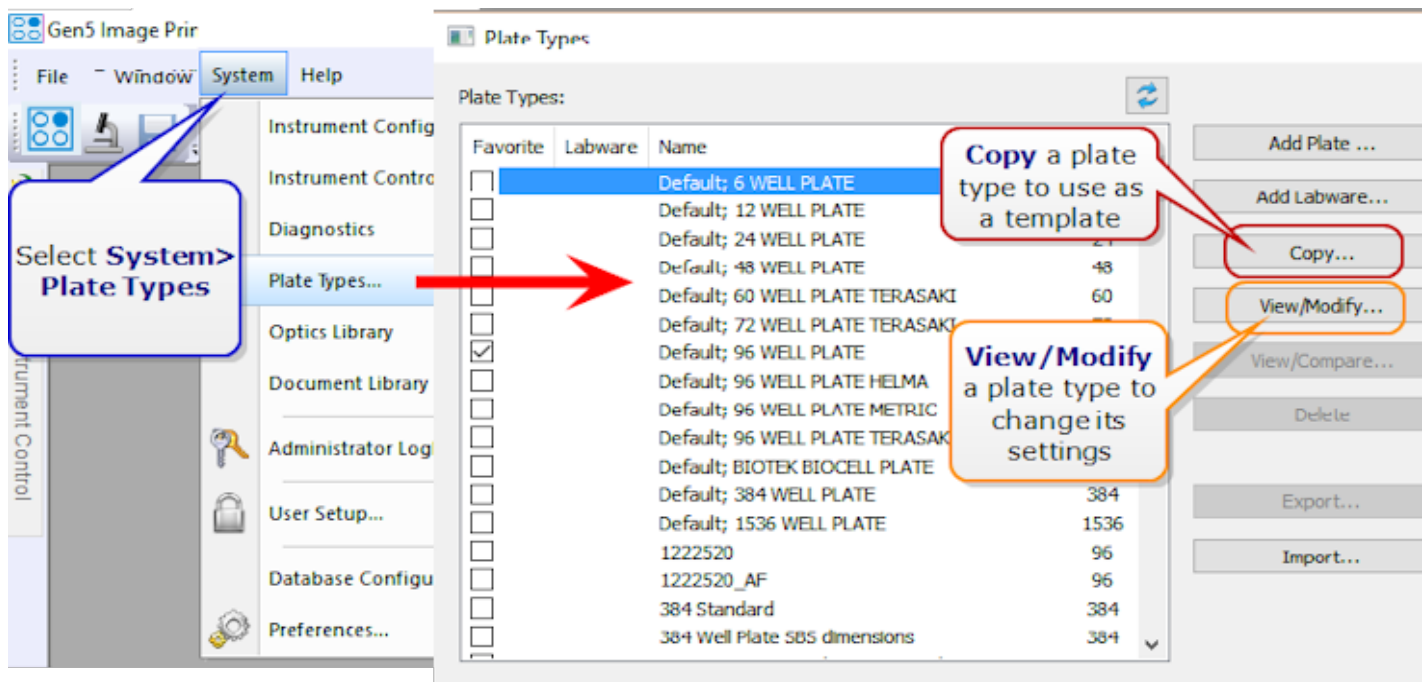
## 3.7 - Plate Definition and Labware

Choice of cell culture vessel significantly affects image quality and assay success, especially with higher power objectives. If possible, choose the thinnest, flattest medium possible. Get the best image quality when using high-power objectives by using glass-bottom plates. Oil and low WD objectives require glass vessels. Best choice: glass or optical-bottom plates, like these:

### Recommended microplates:

- Greiner® µClear TC-treated 655090 96-well & 781091 384-well black polystyrene
- Greiner µClear cell-repellent 655976 96-well & 781976 384-well black
- Greiner 655892 96-well & 781892 384-well glass bottom
- Corning® 4850 96-well half-area and 4851 384-well glass bottom
- Corning 4520 ULA microplates for spheroids

The proper plate definition for your vessel is also crucial to achieving the best possible results. At its most complex, you can create custom layouts, including wells within wells (see Custom Labware in this section); however even the most basic plate definition requires properly defining the **Bottom Elevation** and **Bottom Thickness** (as described on page 1).

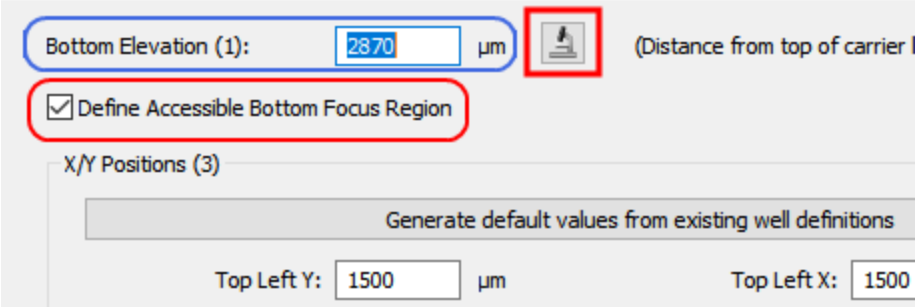



1. Select **System>Plate Types**.
2. Find the Plate Type you are using and click **View/Modify**. "Default plate types" cannot be modified: you must **Copy** them and create a new plate definition with a unique name.
3. Imaging Parameters... Click **Imaging Parameters**.
4. In the Plate Description – Imaging Parameters window: make sure that **Define Bottom Focus Region** is checked to establish exclusion zones and prevent objective collisions with the carrier, and

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click .

#### Plate Description - Imaging Parameters



Bottom Elevation (1):  μm  (Distance from top of carrier)

Define Accessible Bottom Focus Region

X/Y Positions (3)

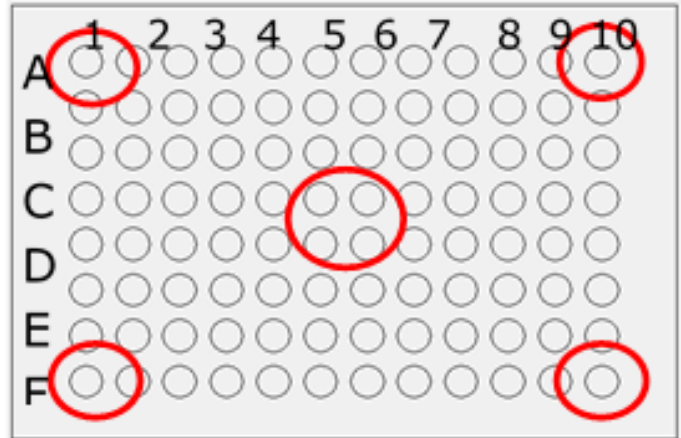
Top Left Y:  μm      Top Left X:  μm

- At the prompt, place the vessel on the imager's carrier or stage and click OK.
- Select your objective and LED filter cube. Use the strongest wavelength for your samples, (i.e., GFP or DAPI).
- Accept the default well selected, **A1**, or choose another well to take measurements at various parts of the plate (as described below).
- Run **Auto Expose** and then **FM Scan**. When you get a defined peak: click **Set Focus Height to Max FM Ratio**.
- Click **Save Settings**. Gen5 will update the **Bottom Elevation** value.

#### Correct for Variability Across the Plate

Plastic tends to warp especially when placed in an incubator; this may cause a high amount of variability across a microplate. For example, a 200-1000 μm difference between A1 and D6 in a 96-well plate is possible. Lower density vessels (e.g., 6-well plates) also have a large amount of variability across the bottom of the plate.

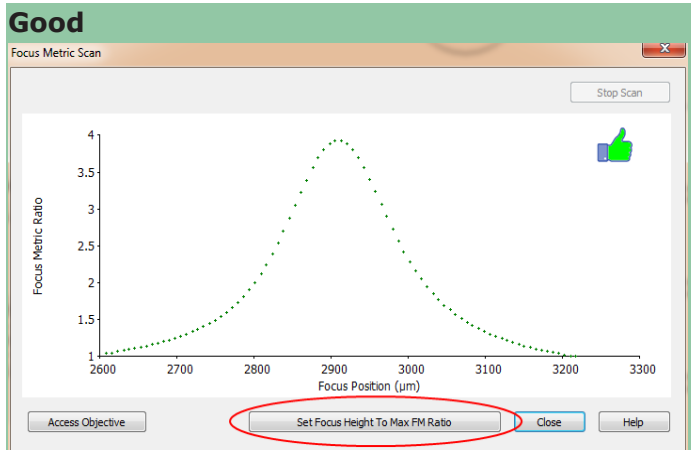
If you are seeing an image focusing problem across your plate, take 2-4 more measurements in the corners and the center. (In step 7 above select each of the four corners: e.g., A1, A10, D6, H1, H12. ) Run the **FM Scan** for each well (step 8 above) and record the Bottom Elevation for each well. Average the values to obtain the recommended Bottom Elevation for the plate. Enter the average value into the **Bottom Elevation** field.



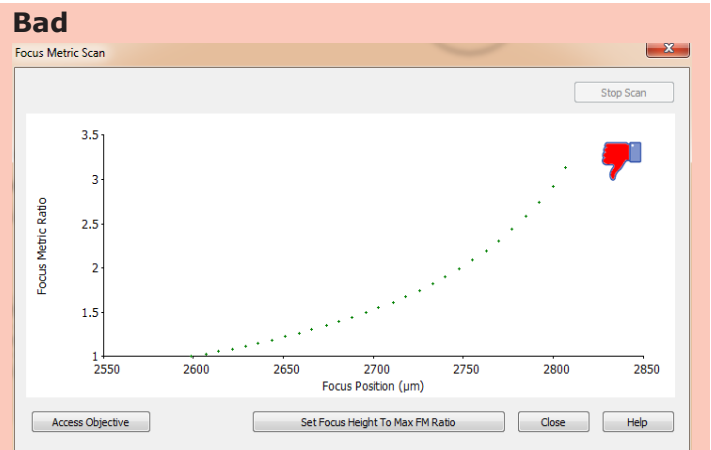
**Note: When using Laser Auto Focus (LAF) this process is not required.**

### 3 - Advanced Acquisition Guide

#### FM Scans



Good results: a well-defined peak. Save these settings for your plate type.

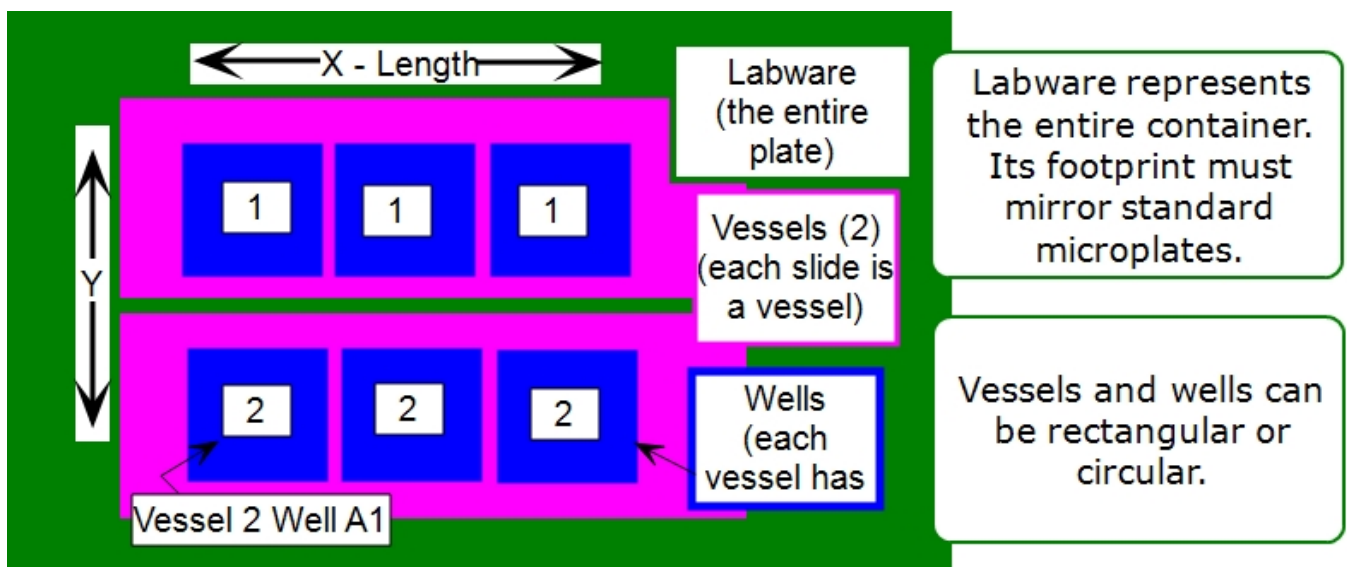


Bad results: insufficient range; peak not found. To get better results, expand the **Scan Distance** under **Advanced Options** and rerun the FM Scan.

#### Defining Custom Labware

Gen5 offers tools for creating your own "plate type" or "labware." If your imaging assays are performed in chamber slides or other non-standard microplates, you can define custom labware. For best results, use calipers to measure the vessel's dimensions, and/or obtain specifications from the manufacturer.

Best practice: Begin with an existing plate type. When creating new plate types and labware, it is easier to start with the dimensions of a predefined vessel similar to the new one.





Some rules apply:



### 3 - Advanced Acquisition Guide

- Vessels must be the same size
- Wells must be the same size
- Each vessel must contain the same number of wells

To speed up the process, take a photo of the vessel to use as a template:

| Create a labware record:   | Follow the prompts when loading a photo of the labware:   |
|--|---|
| <ol style="list-style-type: none"><li>1. Select <b>System &gt; Plate Type</b>.</li><li>2. Click <input type="button" value="Add Labware..."/>.</li><li>3. Choose an existing plate type as a guide, if possible.</li><li>4. <input type="button" value="Load Labware Picture..."/> If possible, upload a photo of the vessel to use as a guide.</li><li>5. Follow the on-screen prompts:</li></ol> | <p> Gen5 considers that the plate outline uses correct length and width dimensions. You now need to overlay the picture of your plate with the drawing of the defined plate type.</p> <p>First, please click the top left corner of the plate in the picture.</p> <p> Then, please click the top right corner of the plate in the picture</p> <p>Click on each edge of the labware when prompted.</p> |

### 3 - Advanced Acquisition Guide

Labware Description

Name:  Catalogue #:

Manufacturer:

Plate Width:   $\mu\text{m}$  Plate Length:   $\mu\text{m}$

Plate Height:   $\mu\text{m}$  Stacked Height:   $\mu\text{m}$

Plate Lid adds:   $\mu\text{m}$   Include Lid Parameters

Vessels

Vessel Name:  Number of Vessels:

Vessel Shape:  Circle  Rectangle

Vessel Length:   $\mu\text{m}$  Vessel Width:   $\mu\text{m}$

Vessel1 (45300,11000)  
Vessel2 (44700,45500)

Well Positions (per vessel)

Number of Rows:  Number of Columns:

Top Left Y:   $\mu\text{m}$  Top Left X:   $\mu\text{m}$

Bottom Right Y:   $\mu\text{m}$  Bottom Right X:   $\mu\text{m}$

Well Shape:  Circle  Rectangle

Well Length:   $\mu\text{m}$  Well Width:   $\mu\text{m}$

OK  
Cancel  
Help  
Lid Parameters...  
Insert...  
Modify...  
Delete  
Imaging Parameters...  
Auto Map...

take photo exactly vertical from above

Labware (the entire plate)

Vessels (2) (each slide is a vessel)

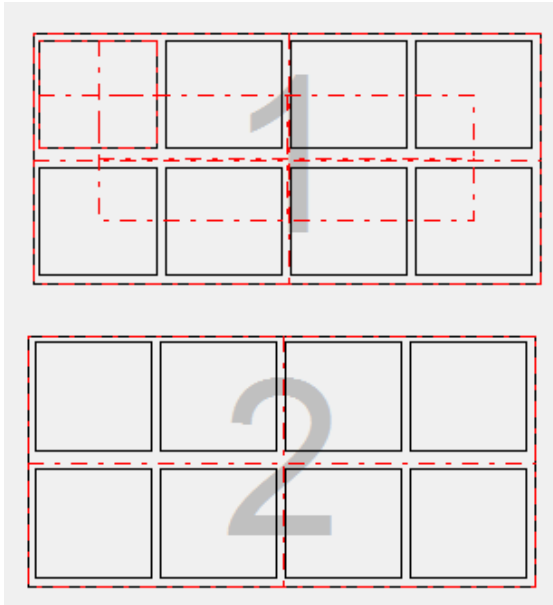
Drawing tool to define the wells

Wells (each vessel has 8 wells)

Show 2.5 mm grid  Hide picture  Load Labware Picture...

Use a combination of "clicking and dragging" the drawing tool and filling in the dimension fields with approximate values to define the new labware. Note: the imported picture is used for reference only and is not retained.

### 3 - Advanced Acquisition Guide



Hide picture

Experiment with the controls to determine which work best for you. Often, hiding the picture makes it easier to fine-tune the well sizing.

Gen5 marks your finished labware record as a "Favorite" automatically, making it easy to select when defining a session.

## 4 - Experiment Mode Imaging

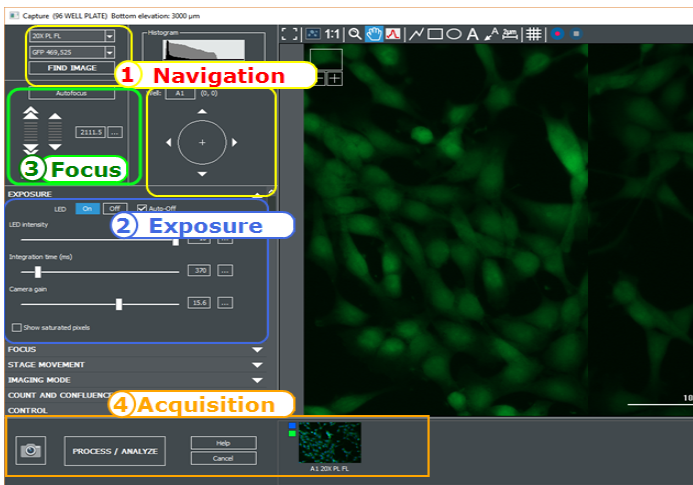
### Automating Your Assay

#### Best choice: Manual Mode or Experiment Mode?

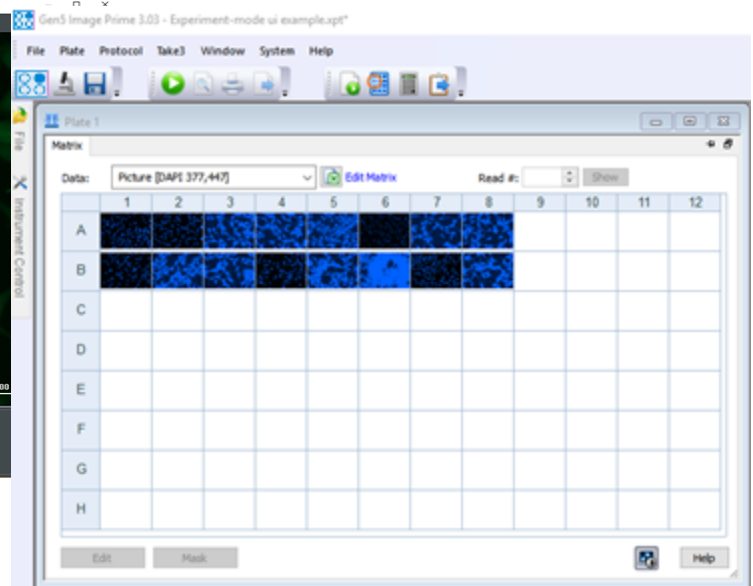
Choose the imaging mode that best fits your needs:

- **Manual Mode**, (2.1 - Acquiring an Image as shown on page 37), is a good starting point to fine-tune focusing and determine the best parameters to apply in an experiment. Manual mode is sufficient to capture a small number of images, low throughput, or for analysis with either Gen5 or an external software program. Choose manual mode to make movies or capture images for presentations. Gen5 file type: .IMM
- Oil Immersion and High NA Objectives are only supported in manual mode.
- The Upright imager Finder Scope is only available in manual mode.
- **Experiment Mode** is the best choice to capture multiple images and analyze them with Gen5 and for high-throughput multi-plate processing and analysis. Finding and imaging ROIs can only be done in experiment mode. Gen5 file type: .XPT
- Some features are only available in experiment mode: Laser Autofocus, ROIs, Beacons, etc.

Manual Mode Image Capture




Experiment Mode

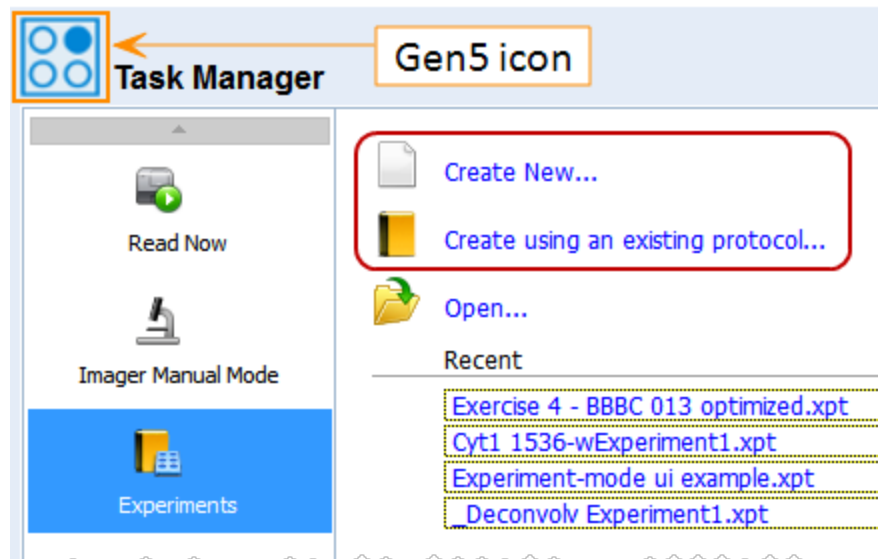


|  |            |
|--|------------|
| <b>4.1 - Creating an Imaging Experiment</b> .....                  | <b>99</b>  |
| <b>4.2 - Difference Between an Experiment and a Protocol</b> ..... | <b>103</b> |
| <b>4.3 - ROIs and Beacons</b> .....                                | <b>104</b> |
| <b>4.3.2 – Auto ROI Add-On Module</b> .....                        | <b>109</b> |
| <b>4.3.3– Add Beacons to Mark ROIs</b> .....                       | <b>118</b> |
| <b>4.3.4 – ROI Manager for Batch Processing</b> .....              | <b>121</b> |

### 4.1 - Creating an Imaging Experiment

Three ways to create an imaging experiment:

1.  From a Manual Mode (.IMM) session (See 2.4 - Conversion to Experiment Mode on page 47).
2. Create a new experiment from scratch from the Task Manager: select **Experiments>Create New** and opt for a Standard protocol.
3. Create a new experiment based on a protocol from the Task Manager. (Learn about Gen5's Differences between Protocols and Experiments **page 103**.)
  - Click the Gen5 icon in the toolbar to open the Task Manager:

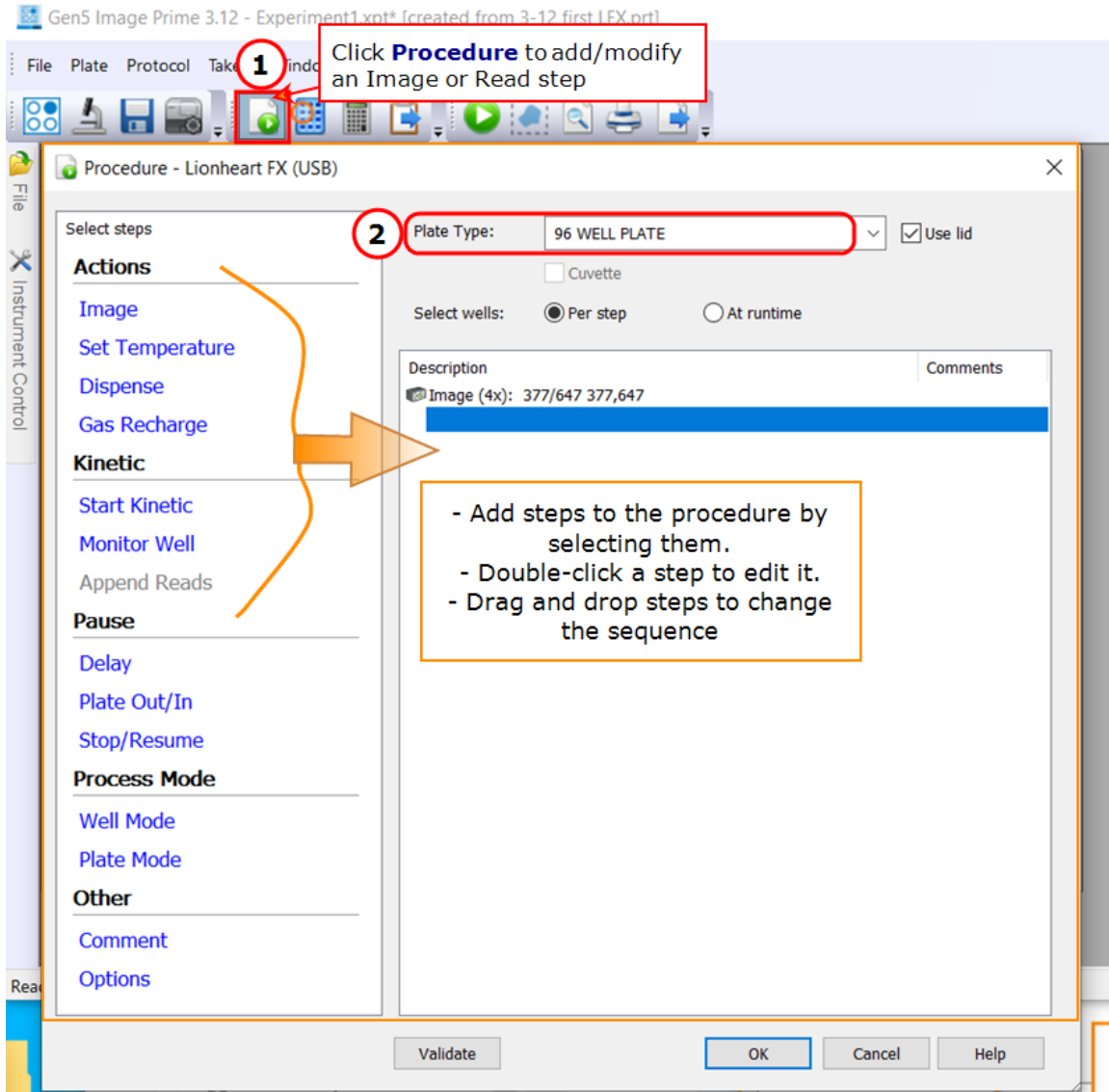



## 4 - Experiment Mode Imaging



### Modifying an Experiment

After opening an experiment file, here are steps to enhance it:



1. Click  **Procedure**.
2. **Plate Type**: Pick your pre-made plate definition from the Plate Type drop down list. Check "Use Lid" if applicable. (Make sure your plate definition is optimized with your samples (*as described on page 92*.)

## 4 - Experiment Mode Imaging

- To add a step, click **Image**.
- Tell Gen5 if you want to find ROIs and, if applicable, how.

See 4.3 - ROIs and Beacons on page 104.

**Note:** You can search for ROIs and then image them using any imager and any magnification.

**Image Step Definition**

Find regions of interest (ROIs)

Lower magnification for ROI Discovery

Upright imager  
 Inverted imager

Higher magnification for ROI Imaging

Upright imager  
 Inverted imager

- Select the **Magnification** (Objective) to use.
- Define a plate map or keep the Full Plate, if applicable.
- Depending on the imager, choose the light source, e.g., **Color** LED cube and filter cube for up to 4 channels.
- Exposure: To use Auto Exposure, check Auto and click the **Wells**

Wells:  button.

|   | 1        | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|----------|---|---|---|---|---|---|---|---|----|----|----|
| A | positive |   |   |   |   |   |   |   |   |    |    |    |
| B |          |   |   |   |   |   |   |   |   |    |    |    |
| C |          |   |   |   |   |   |   |   |   |    |    |    |
| D |          |   |   |   |   |   |   |   |   |    |    |    |
| E |          |   |   |   |   |   |   |   |   |    |    |    |
| F |          |   |   |   |   |   |   |   |   |    |    |    |
| G | negative |   |   |   |   |   |   |   |   |    |    |    |
| H |          |   |   |   |   |   |   |   |   |    |    |    |

Choose at least one positive and one negative well for Auto Exposure

Select both positive and negative wells (highest and lowest intensity) for the best auto exposure results.

Channels

Fluorophore

Color: DAPI 377,447

Exposure:  Auto

LED:

Integration time:


Gain:

De-select Auto and click microscope to manually define focus settings.


- Plate map shortcut: Instead of choosing one well at a time, click a column or row header to choose that entire block.
- Alternatively, de-select Auto and click to launch manual mode and choose optimal exposure settings for each channel.

- Now, you can process all your sample vessels/plates using the optimal settings. Click OK to close the Read step.

## 4 - Experiment Mode Imaging

10.  Click the **Read** button.

**Note:** If your samples are not in focus, you may need to check the focal options and optimize them (See 5 - Understanding AutoFocus on page 124).

 **Upright Imager** experiments: **Fixed focal height** is typically a good choice when using 2X magnification. Image capture results are equivalent to Autofocus and fixed focus is significantly faster. Click **Focus Options** to enable this feature.


Laser AutoFocus is recommended for most confocal experiments.



## 4 - Experiment Mode Imaging

### 4.2 - Difference Between an Experiment and a Protocol

Gen5 uses two common terms to define distinct elements of its toolkit.

| Protocol (*.prt)  | Experiment (*.xpt)  |
|---|---|
| A protocol is a set of instructions designed to capture, transform, and report and/or export data.  | An experiment has a copy of the protocol and at least one plate. It executes the instructions provided by the protocol to produce results.  |
| Protocols are created and saved as stand-alone files. They function as a template; an unlimited number of experiments can be based on one protocol.   | Although an experiment is created using an existing protocol, that protocol can be modified within the experiment.  |
| A protocol consists of reading requirements, like detection method and wavelength, and reading-related actions, like shaking and incubation (procedure), plate layout, data reduction, and data viewing, reporting, and exporting parameters. | Running an experiment is the only way to process a protocol.<br> The <b>Read Now</b> button is available after a read step has been defined in a protocol; Using it creates an experiment based on the protocol. |
| A protocol can be used repeatedly (as is or modified) within experiments. By itself, a protocol does not produce results. Protocols do not have plates associated with them.  | Multiple plates can be processed in an experiment, each one considered a unique assay with independently reported or exported results.  |
| .prt is the protocol's file name extension.   | .xpt is the experiment's file name extension.   |
| Changes made to a stand-alone protocol are not reflected in any previously created experiments based on that protocol. A new experiment must be created to apply the revised protocol.  | Within an experiment, you can select <b>Save Protocol As</b> to capture the current details of the protocol and save them as either a new protocol or as an overwrite of the original protocol.   |

Learn more in the Gen5 Help system.

## 4 - Experiment Mode Imaging

### 4.3 - ROIs and Beacons

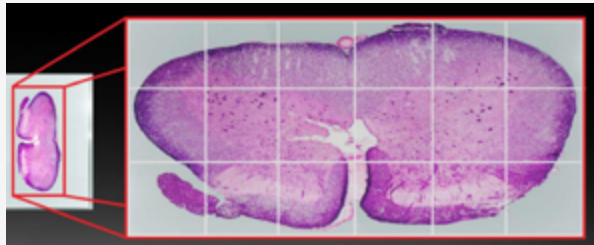
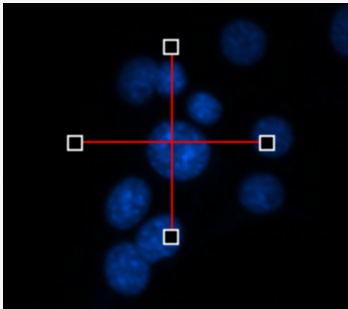
Two distinct methods are available for identifying and imaging Regions of Interest (ROIs) in an experiment:

1. **ROI Identification Experiments:** Discover ROIs using low magnification, image ROIs using high magnification. (Gen5 v.3.09 or higher required.) See [Identifying ROIs in Gen5](#) on the next page.
2. **Add Beacons to Mark ROIs:** Place a beacon on each ROI in every well individually when creating the Gen5 experiment. (Gen5 v.3.08 or higher required.) See [4.3.3— Add Beacons to Mark ROIs](#) on page 118. With Gen5 v3.12 or higher, a pattern of beacons can be assigned to multiple wells simultaneously: See [Using a Grid to Define Beacons](#) on page 120.

Gen5 also provides tools for automating ROI processing:

- **ROI Batch Processing:** Using a BioStack plate stacker to process multiple slides or microplates containing ROIs. (Gen5 v.3.10 or higher required.) See [4.3.4 — ROI Manager for Batch Processing](#) on page 121.
- **Auto ROI Add-On Module:** After purchasing and activating the Auto ROI add-on module, Gen5 will find ROIs automatically based on user-defined criteria. (Gen5 v.3.11 or higher required.) See [4.3.2 — Auto ROI Add-On Module](#) on page 109.

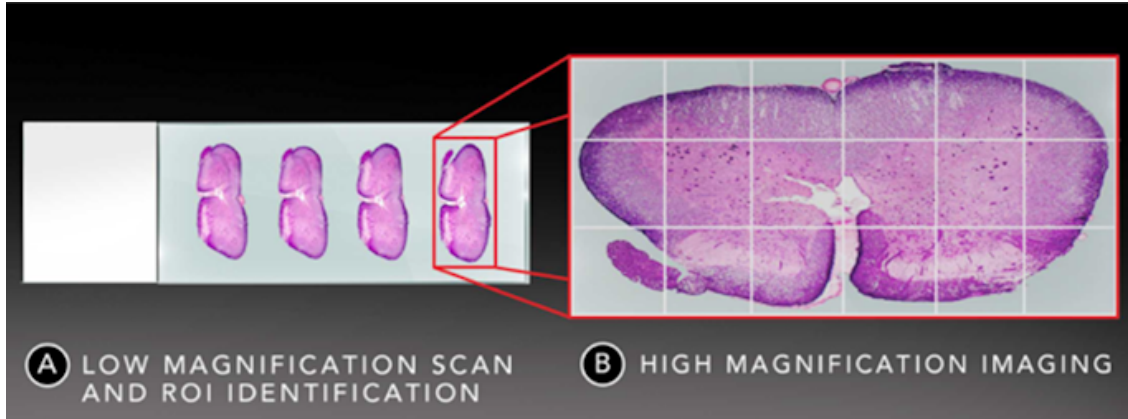
#### Advantages of ROI imaging options

| ROIs  | Beacons  |
|---|--|
|  |   |
| Interactive discovery step to find ROIs gives users ultimate control                | Easy one-step ROI definition (after navigating to each well or by applying a grid of beacons to multiple wells simultaneously) |
| Presentation of the whole plate makes it easier to identify wells with ROIs         | All ROIs are the same size and shape   |
| Custom sizes and shapes   | Both wells with and without ROIs are imaged  |
| See <a href="#">Identifying ROIs in Gen5</a> on the next page                       | See <a href="#">4.3.3— Add Beacons to Mark ROIs</a> on page 118  |


## 4 - Experiment Mode Imaging

▶ Watch this [ROI Feature Training Video](#).

### Identifying ROIs in Gen5



In Gen5 versions 3.09 and higher, Gen5's Regions of Interest (ROIs) feature is a three step process:

1. **Discovery step:** scans a sample using low magnification to let you see the entire sample area.
2. **ROI definition:** the user draws a rectangle or polyline shape to define the ROIs in each well and/or defines criteria for Auto ROI identification.
3. **ROI captured:** Gen5 images the defined ROIs using high magnification (typically). Only wells with a defined ROI are imaged; blank wells are skipped.
  - Follow these **Step by Step ROI Imaging** instructions.
  - If the add-on module is installed, follow these **Auto ROI Step by Step** instructions to automatically detect ROIs.
  -  **ROI Manager for Batch Processing:** For slide scanning assays and other multi-plate experiments. Especially useful when using a BioStack to capture ROIs.

### Step by Step ROI Imaging

1.  **Find regions of interest (ROIs)** Create a new experiment with an imaging step and tell Gen5 to find ROIs.
2. Choose the imagers, if applicable, to perform the discovery and imaging steps.
3. First, in the discovery step, define the entire potential field for the ROIs.

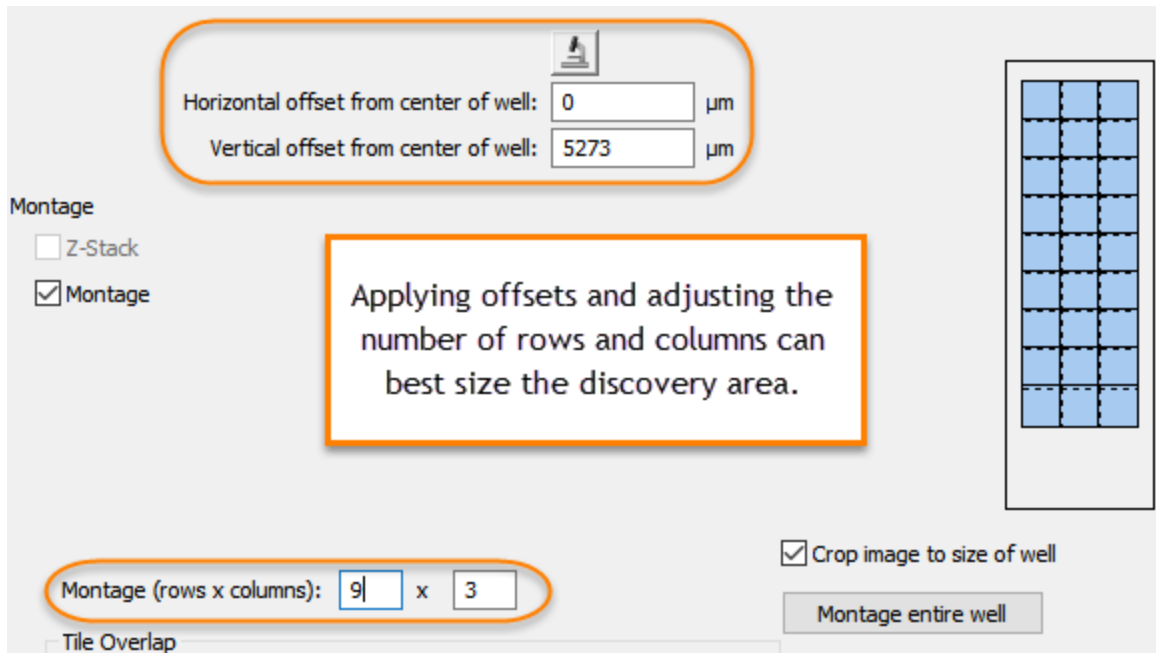
**Montage entire well**

Use the **Montage entire well** shortcut to cover the entire well, if useful.

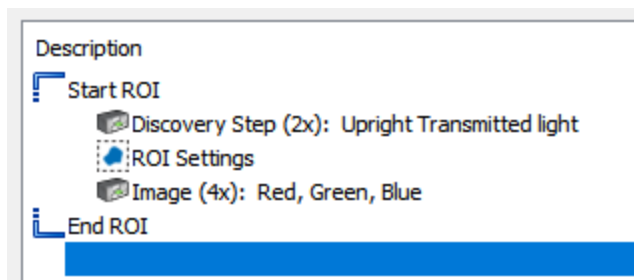
**Full Plate**

Click **Full Plate** to limit the wells to be imaged, if applicable. This setting will be fixed for ROI imaging, also, except that only wells with one or more defined ROIs are imaged.

## 4 - Experiment Mode Imaging



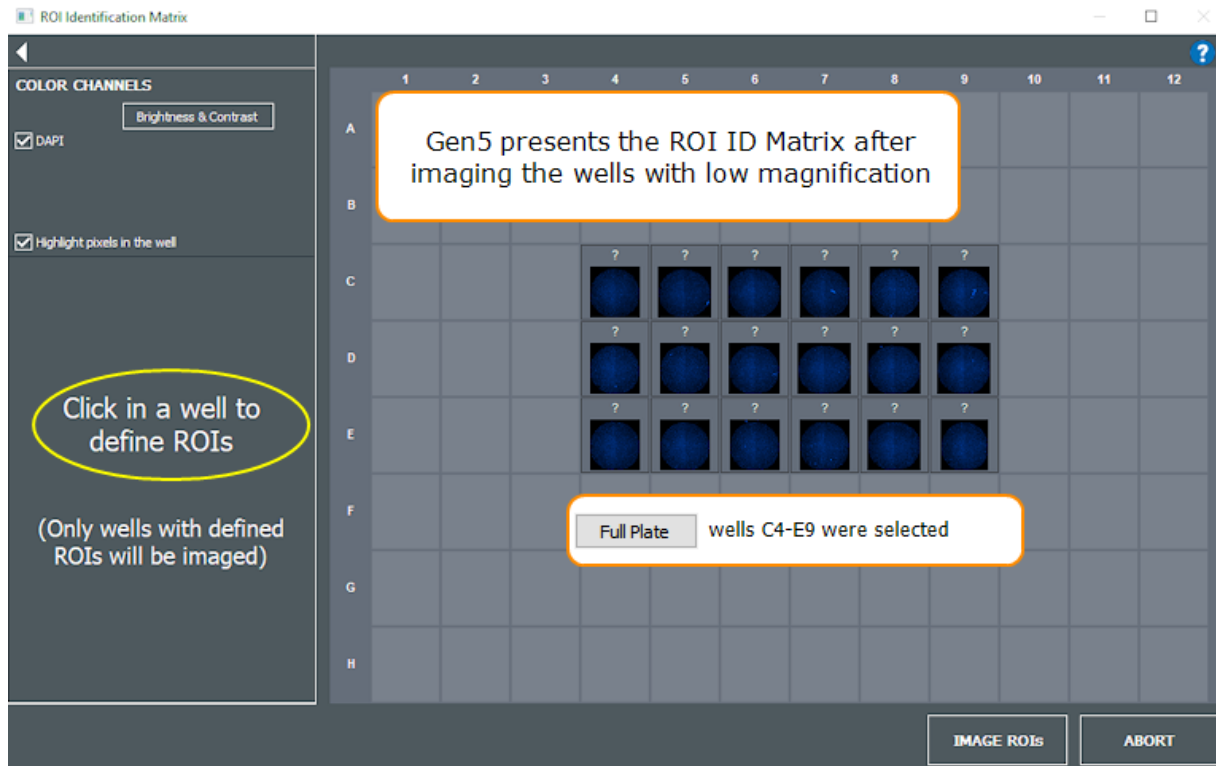
4. Retain the default settings in the ROI Setting dialog, at least initially. You may want to adjust the ROI margin after gaining some experience with the process.
5. Define the image acquisition step with the desired magnification and color channels. Click **Validate** to make sure the procedure can be performed as defined.



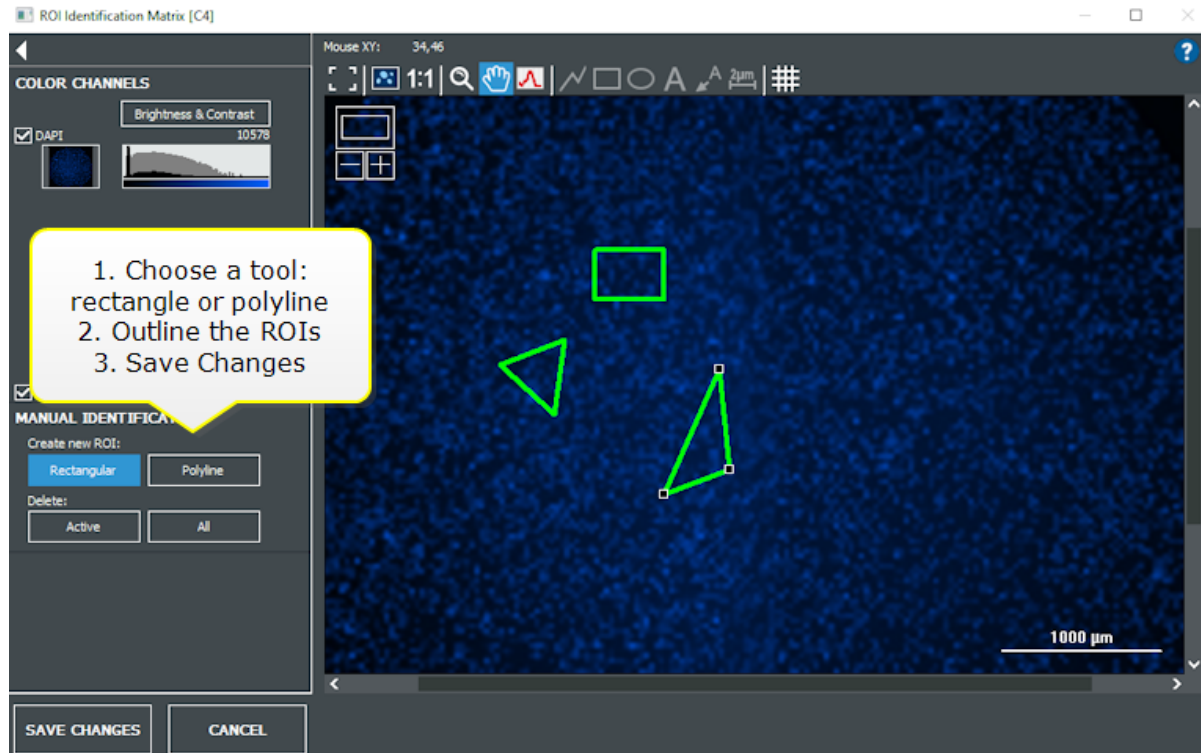
6.  Read the plate.

## 4 - Experiment Mode Imaging

### ROI Discovery Step

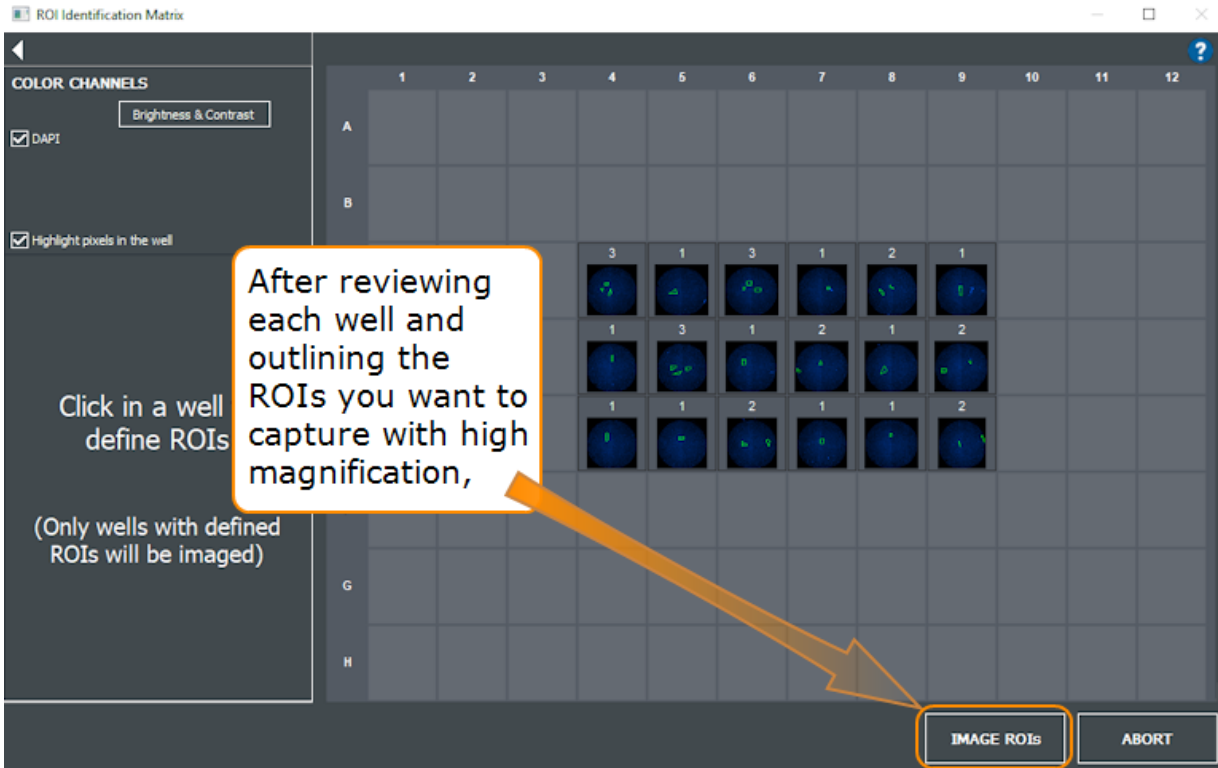


1. At the ROI Identification Matrix, click in a well to define its ROIs.



## 4 - Experiment Mode Imaging

2. **Save Changes** for each individual well.
3. When all ROIs have been defined, click **Image ROIs**.

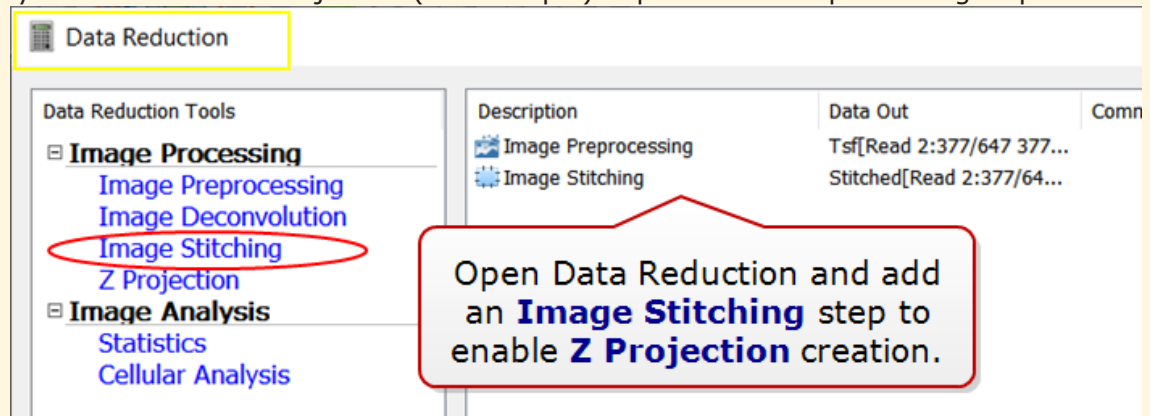


See Viewing ROIs on page 122.

## ROI - FAQs

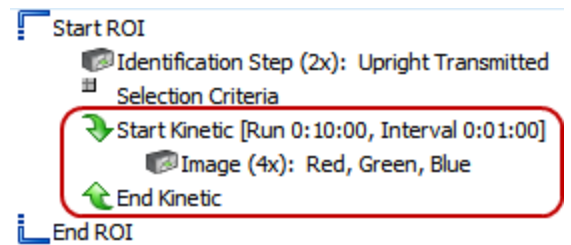
- Gen5 only images wells/vessels that have a defined ROI; wells or slides that do not have an ROI are skipped.

**Important:** Gen5 expects that ROIs will be captured in a montage. Thus, even when the ROI requires only a single tile, you must manually add an image stitching step (under Data Reduction) before you can create a Z-Projection (for example) or perform other processing steps.




## 4 - Experiment Mode Imaging

- The initial image step in the process, the ROI Discovery image (usually captured with low magnification for the largest field-of-view) is saved with the experiment. It can be viewed, reported, and exported.
- Gen5 applies its standard naming convention to ROIs: Well Name-ROI:ROI-index.
- Image statistics can be reported for an individual ROI and/or the sum/average of **All ROIs** in the well. However, cellular analysis object detail data sets are not supported. When it's available, "Each ROI" is a nice timesaver for reporting all ROIs individually.
- Timestamps: As it does for montage and z-stack captures, Gen5 will retain one timestamp for all images/ROIs in the well. This time will be used to perform kinetic analysis. Likewise with temperature data points, Gen5 will keep one per well.
- **Autofocus** can be performed on each ROI or limited to the ROI closest to the center of the well.
- **Kinetic** time lapse imaging: a kinetic loop can be added to the ROI procedure after the imaging step has been defined.



- **Image Statistics** in an image zoom: the highlighted data points are reported from image metadata not from measuring the current ROI. The height and width are based on the .tiff itself, not the ROI dimensions.
- Slide scanning assays using a BioStack are supported with an [ROI Manager](#).

Image-Level results:  Edit

| Image Statistics[Read 2:Red]      | Value             |
|-----------------------------------|-------------------|
| Well                              | A3                |
| Well ID                           |                   |
| Name                              |                   |
| Min [Read 2:Red]                  | 8103              |
| Mean [Read 2:Red]                 | 42865             |
| Max [Read 2:Red]                  | 65504             |
| Std Dev [Read 2:Red]              | 5738              |
| CV [Read 2:Red]                   | 13.387            |
| Saturated Pixels Pct [Read 2:Red] | 0.001             |
| Focal Height [Read 2:Red]         | 1526.000          |
| FM Ratio [Read 2:Red]             | -1.000            |
| Total Intensity [Read 2:Red]      | 1387473126773.000 |
| Total Area [Read 2:Red]           | 3928795.030       |
| Confluence [Read 2:Red]           | 100.000           |
| Width [Read 2:Red]                | 694               |
| Height [Read 2:Red]               | 694               |

### 4.3.2 – Auto ROI Add-On Module

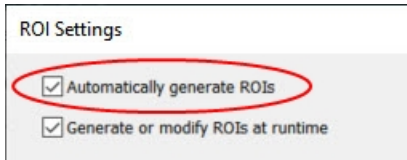
*Requires Gen5 v.3.11 and the AutoROI Add-On Module*

To automatically identify and capture ROIs, the Auto ROI Add-On module must be purchased and its license activated.

Auto ROI is especially useful for capture and analysis of core sample slides. In combination with a BioStack, slide scanning applications are made fast, easy and reliable.

**Good to Know:**

## 4 - Experiment Mode Imaging



### MANUAL IDENTIFICATION

Create new ROI:

Rectangular

Polyline

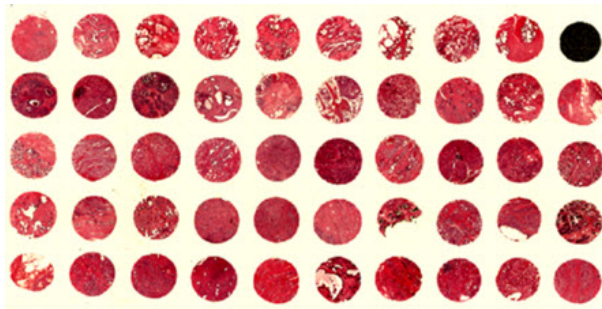
- Auto ROI and manual ROI tools: Gen5 lets you use both manual and auto ROI tools in the Auto ROI identification step. This option is recommended for best results.
- **Important:** always define and run the Auto ROI Selection Criteria **before** using the manual ROI tools (rectangle or polyline) to identify objects missed by the Auto ROI criteria.

See also ROI - FAQs on page 108.

## Auto ROI Step by Step

*Auto ROI Add-On module required.*

Doing a preliminary manual mode session to evaluate your samples to measure object sizes and distances may be helpful for customizing the Auto ROI parameters.



1. Set up the ROI experiment similar to the manual method: [Step by Step ROI Imaging](#) as shown on page 105.

The ROI Discovery Step was defined this way, using a Cytation 7, to accommodate our core sample slide:



## 4 - Experiment Mode Imaging


ROI Discovery Step-Upright imager

Step Label:

Magnification:  Image: 7065 x 7065  $\mu\text{m}$   Use Wide FOV

Binning:  Autofocus binning  Capture binning (affects exposure)

Bright Field light source  
 Transmitted light  
 Reflected light


Exposure  Auto   
Wells:

Horizontal offset from center of well:   $\mu\text{m}$   
Vertical offset from center of well:   $\mu\text{m}$

Montage  
 Z-Stack  
 Montage

Montage (rows x columns):  x   Crop image to size of well

Tile Overlap  
 No overlap  Auto for stitching  Custom  
Columns:   $\mu\text{m}$  Rows:   $\mu\text{m}$

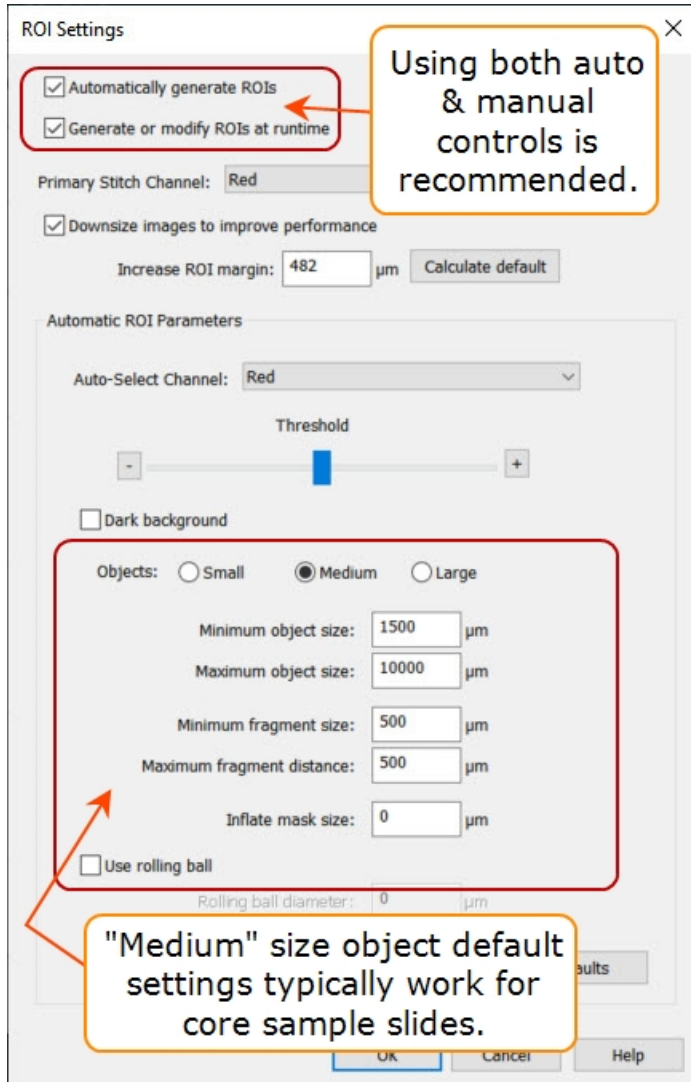


Notice the offset defined to focus on the slide section containing the samples.

Montage defined to capture all the samples on the slide.

2. In the ROI Settings, enable both Auto and manual ROI options:

## 4 - Experiment Mode Imaging



Using both auto and manual ROI identification options adds a useful review step to the automated process. See Auto ROI Parameters on page 114.

Adjust the Auto ROI parameters as needed for your samples. (You will have a chance to optimize the parameters later.)

3. After the discovery step, click on a well to fine-tune the ROI definitions in the ROI Identification Matrix. The automatically generated ROIs will be visible. If the ROIs are not optimal, revise the parameters.

You can use both the Selection Criteria measurement values and the manual ID tools.

## 4 - Experiment Mode Imaging

**SELECTION CRITERIA**

Channel:

Dark background

Threshold:

Rolling ball size:

Minimum size:

Maximum size:

Max. fragment distance:

Min. Fragment size:

Inflate ROI mask:

Highlight threshold outliers

**MANUAL IDENTIFICATION**

Create new ROI:

Delete:

Click on the ruler next to a value to measure the desired ROIs in the image. Then, click **Run selection process** to see the affects of your changes.

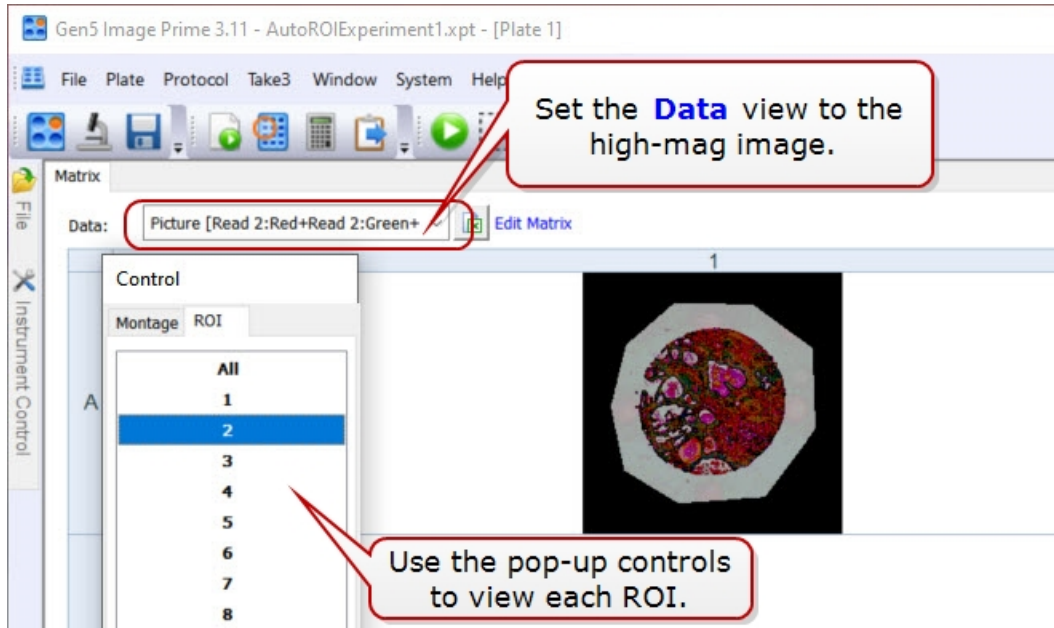
Apply the auto criteria FIRST, then use manual tools to identify more ROIs, if needed.

**Note:** You can delete both manual and auto-generated ROIs, too.

When you are happy with the selected ROIs: **Save Changes** and **Image ROIs**. Gen5 will capture all the ROIs that match the criteria.

4. After the ROIs are captured, you can view each one, and perform analysis on all of them simultaneously.

## 4 - Experiment Mode Imaging



See also [Auto ROI - Fragmented Samples on the next page.](#)

### Auto ROI Parameters

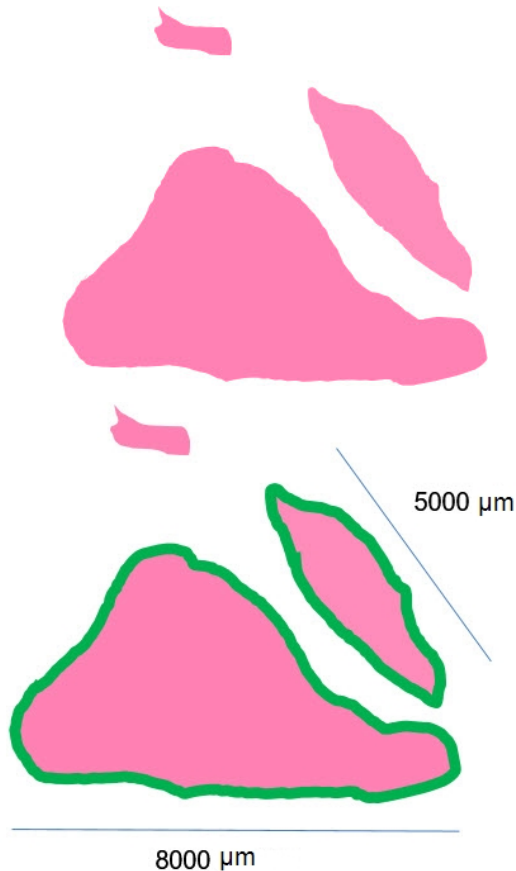
|                           |   |
|---------------------------|---|
| Auto-Select Channel       | Channel used for running the Auto Thresholding for ROI detection. This can be any of the channels defined in the Discovery (low-mag) scan.                              |
| Threshold                 | Same dynamic threshold algorithm used in Gen5 Cellular Analysis.  |
| Dark background           | Toggle for either Brightfield or Fluorescence images. Dark background: Fluorescence Images (and sometimes Phase & HCBF); Light Background: Transmitted Light, BF & CBF. |
| Minimum object size       | Smaller objects will be ignored.  |
| Maximum object size       | Larger objects will be ignored.   |
| Minimum fragment size     | Smallest object of interest.  |
| Maximum fragment distance | See <a href="#">Auto ROI - Fragmented Samples on the next page.</a>   |

## 4 - Experiment Mode Imaging

|  |  |
|--|--|
| Inflate mask size                        | To include some background (or signal below the threshold) around each ROI. Inflating the mask size is recommended to accommodate growth of the ROIs during kinetic analysis.  |
| Use rolling ball - Rolling ball diameter | Reduces the background, potentially eliminating elements that may be erroneously considered ROIs. Rolling ball size is typically less than or equal to the minimum object size. Too small a rolling ball size will erase valuable foreground signal. |

### Auto ROI - Fragmented Samples

Tissue samples often fragment when fixed to a slide. Gen5 provides Auto ROI parameters to let you define the fragments as either individual ROIs or combined into one ROI.



Here is an example of an H&E liver section slide.

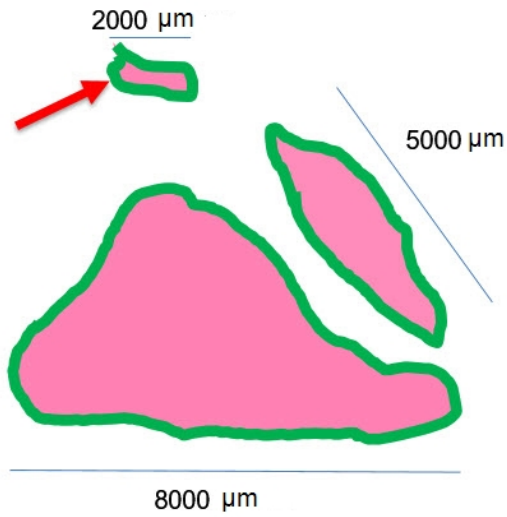
The liver section can be defined as 1, 2, or 3 ROIs depending on the values assigned to:

- Minimum fragment size
- Maximum fragment distance
- Min and Max object sizes

|                            |                                   |    |
|----------------------------|-----------------------------------|----|
| Minimum object size:       | <input type="text" value="4500"/> | μm |
| Maximum object size:       | <input type="text" value="8500"/> | μm |
| Minimum fragment size:     | <input type="text"/>              | μm |
| Maximum fragment distance: | <input type="text"/>              | μm |

Let's consider the two largest segments as our min and max object sizes. The small segment will be a fragment.

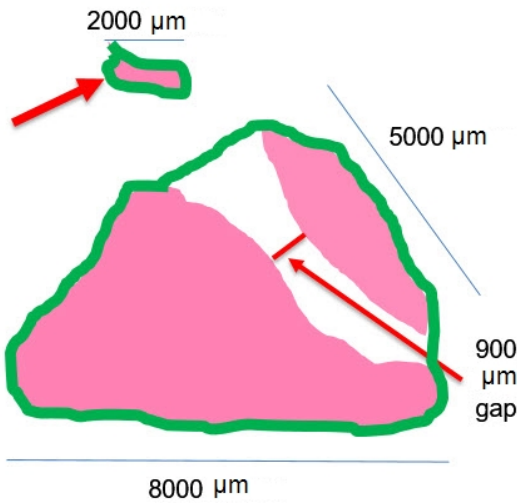
## 4 - Experiment Mode Imaging



|                            |                                   |    |
|----------------------------|-----------------------------------|----|
| Minimum object size:       | <input type="text" value="4500"/> | µm |
| Maximum object size:       | <input type="text" value="8500"/> | µm |
| Minimum fragment size:     | <input type="text" value="2000"/> | µm |
| Maximum fragment distance: | <input type="text"/>              | µm |

The minimum fragment size will be 2000 µm, telling Gen5 to treat it as an object of interest.

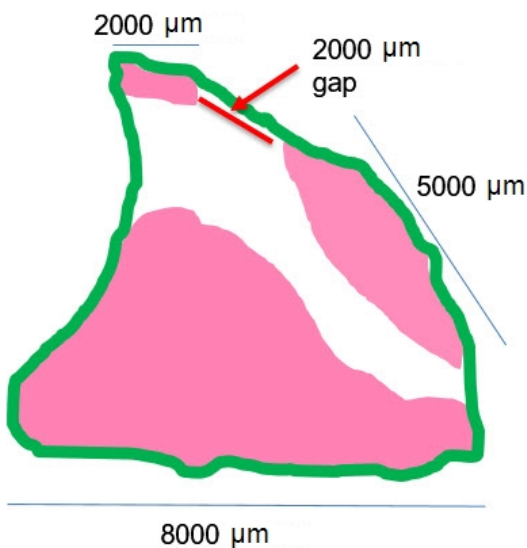
Note: to define each segment as its own ROI, make the maximum fragment distance less than the 900 µm, shown below.



To create two ROIs, one combining the two largest segments; and making the fragment the second ROI, we would set the maximum fragment distance to 900 or 1000 µm.

|                            |                                   |    |
|----------------------------|-----------------------------------|----|
| Minimum object size:       | <input type="text" value="4500"/> | µm |
| Maximum object size:       | <input type="text" value="8500"/> | µm |
| Minimum fragment size:     | <input type="text" value="2000"/> | µm |
| Maximum fragment distance: | <input type="text" value="1000"/> | µm |

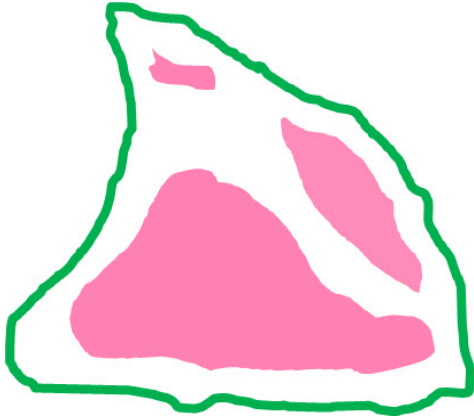
(the fragment is further away)



To create one ROI that combines all three segments, we would set the maximum fragment distance to exceed the size of the gaps between the segments.

|                            |                                   |    |
|----------------------------|-----------------------------------|----|
| Minimum object size:       | <input type="text" value="4500"/> | µm |
| Maximum object size:       | <input type="text" value="8500"/> | µm |
| Minimum fragment size:     | <input type="text" value="2000"/> | µm |
| Maximum fragment distance: | <input type="text" value="2200"/> | µm |

#### 4 - Experiment Mode Imaging



Depending on your samples, you may want to **Inflate mask size** to include some background (or signal below the threshold) around each ROI.

Inflating the mask size is recommended to accommodate growth of the ROIs during kinetic analysis.

## 4 - Experiment Mode Imaging

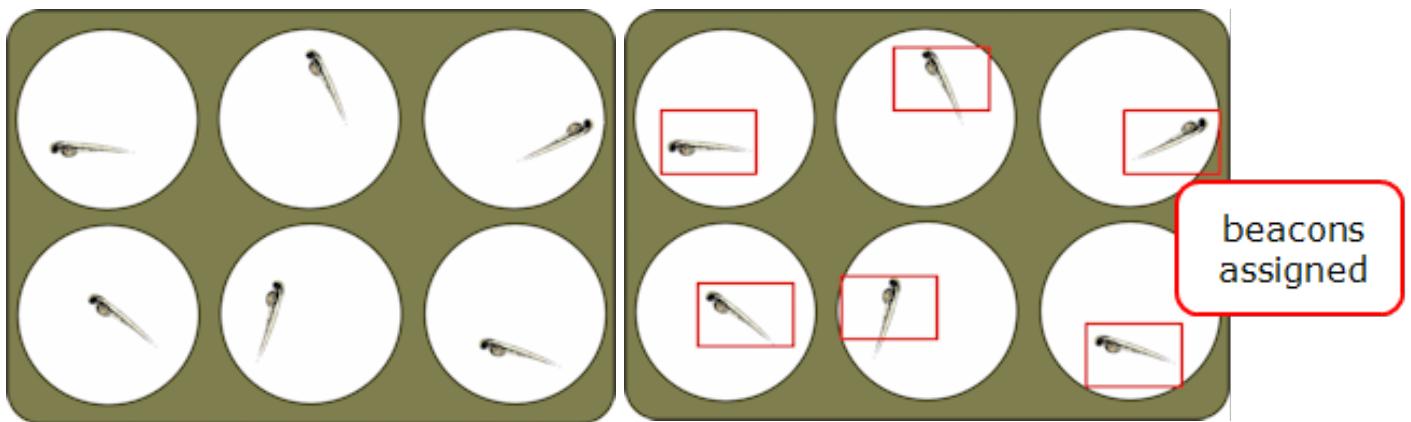
### 4.3.3— Add Beacons to Mark ROIs

Requires Gen5 version 3.12 or higher for all features described


By default Gen5 captures images at the center of the well. Two ways to change the X/Y position of the capture area in an experiment are:

- Beacons: to identify regions of interest, when the ROIs vary from well to well. Beacons may be useful in kinetic assays when cells have moved away from center, for example.
- Horizontal and vertical offsets: When the region of interest is not in the well center but occurs in the same area (X/Y coordinates) of every well.

💡 Beacons or offsets can be used in scratch assays when the scratch is off-center.



### Defining Beacons - ROIs

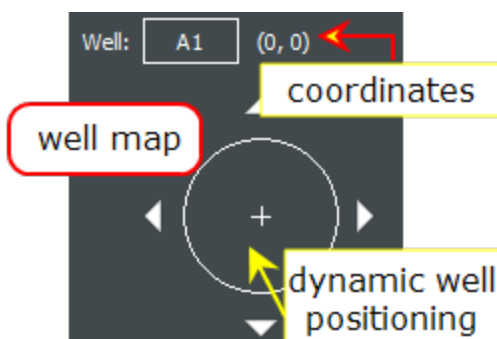
Define beacons 

Horizontal offset from center of well ( $\mu\text{m}$ ):


Vertical offset from center of well ( $\mu\text{m}$ ):

Instead of capturing the center of a well, you can capture one or more ROIs in the vessel by marking the ROI with a beacon. The beacon specifies a horizontal and vertical (x/y) offset in the well.

**Note:** Also see: Using a Grid to Define Beacons on page 120.




1. In the imaging Read Step dialog, set the **Objective** and

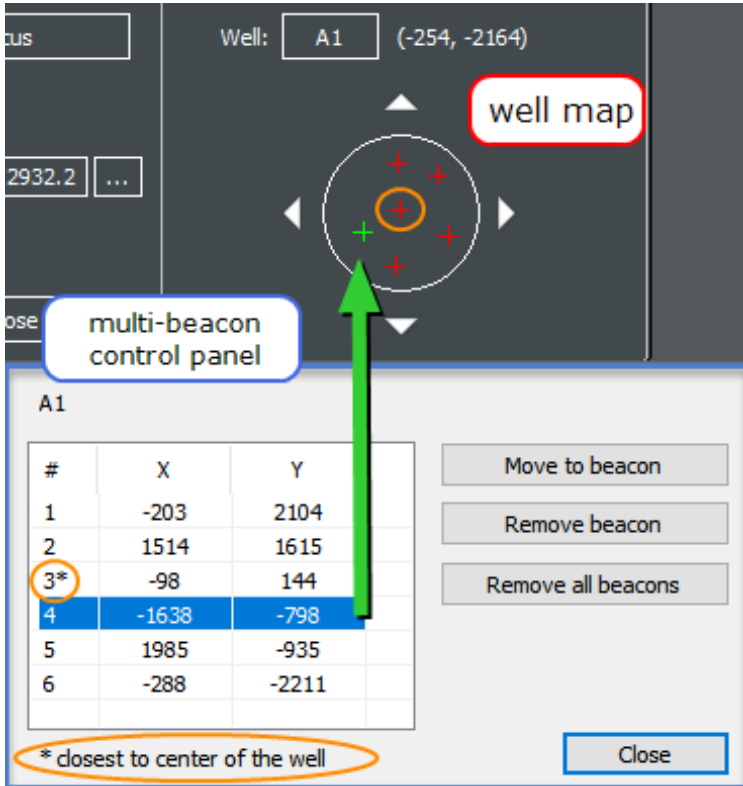
**Color** and select **Define beacons**, then click  to define the beacons. Gen5 launches a special manual mode session to let you define beacons. Use the focus and exposure tools as needed.



## 4 - Experiment Mode Imaging

 20X PL FL You can change the magnification (lower or higher power) and/or the color when identifying beacons, if desired.

2. Click the **Well** button to select the well in which you want to define beacons.
3. Move to the ROI using the navigation tools. For example, using the well map, click inside the well schematic to move to the corresponding location in the well, then, **click the image itself to place the beacon.** + A red cross in the image itself and a red cross in the well map marks the spot of the beacon.

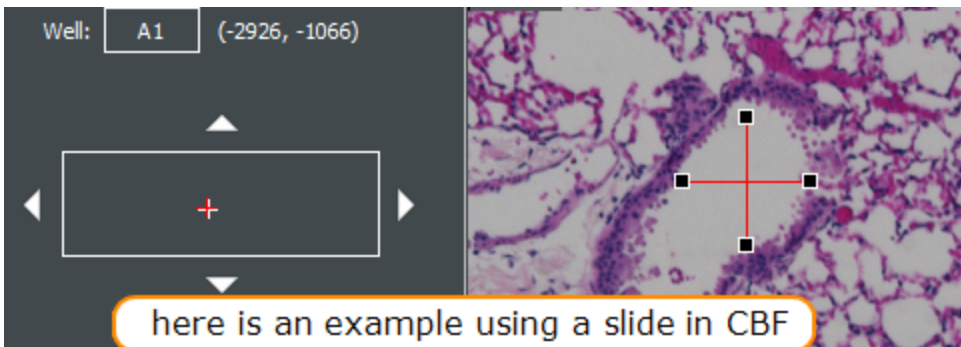


The screenshot shows the Gen5 software interface. At the top, it displays "Well: A1 (-254, -2164)". Below this is a "well map" showing a circular well with several red crosses representing beacons. A green arrow points from the "multi-beacon control panel" to the well map. The control panel is a table with columns for "#", "X", and "Y". The row for beacon 3 is circled in orange, and the row for beacon 4 is highlighted in blue. Below the table, there is a note: "\* closest to center of the well". To the right of the table are buttons for "Move to beacon", "Remove beacon", and "Remove all beacons". At the bottom right of the control panel is a "Close" button.

| #  | X     | Y     |
|----|-------|-------|
| 1  | -203  | 2104  |
| 2  | 1514  | 1615  |
| 3* | -98   | 144   |
| 4  | -1638 | -798  |
| 5  | 1985  | -935  |
| 6  | -288  | -2211 |

Multiple ways to assign and manage beacons:

- Click inside the image itself to identify the ROI/beacon.
  - Click in the well map to move to that area of the well. Gen5 updates the coordinates to identify your location.
  - Use the arrow buttons around the well map to move in that direction.
4. Gen5 activates the multi-beacon controls once beacons are defined. Use the top control to evaluate and modify beacon selection.
  5. To define beacons in another well, repeat steps 2 through 4.
  6. When you are finished defining beacons on that plate, click **Save settings**.



The screenshot shows the Gen5 software interface with a slide in CBF. The well is labeled "Well: A1 (-2926, -1066)". The slide image shows a cross-section of tissue with a red crosshair indicating the location of a beacon. A text box at the bottom says "here is an example using a slide in CBF".

## 4 - Experiment Mode Imaging

### Full Plate

In the read step, click the plate button to see where beacons are defined. Hover the mouse over the well to see its offsets.

|   | 1 | 2 | 3      | 4 | 5 | 6 |
|---|---|---|--------|---|---|---|
| A |   |   |        |   |   |   |
| B |   |   | beacon |   |   |   |
| C |   |   | B      |   |   |   |
| D |   |   |        |   |   |   |
| E |   |   | B      |   |   |   |

### Good to know:

- Image statistics can be reported for an individual ROI and/or the sum/average of **All ROIs** in the well. However, object-level metrics are not supported for multiple beacons/ROIs. When it's available, the "Each ROI" option is a nice timesaver for reporting all ROIs individually.
- Gen5 applies its standard naming convention to ROIs: Well Name-ROI:ROI-index
- Max number of beacons is 255.
- When beacons are not assigned to a well, Gen5 captures an image for that well as normal, in the center of the well.
- Kinetic image registration: the beacon closest to the center of the well will be used when this option is enabled.
- Timestamps: As it does for montage and z-stack captures, Gen5 will retain one timestamp for all images/ROIs in the well. This time will be used to perform kinetic analysis. Likewise with temperature data points, Gen5 will keep one per well.
- Autofocus can be performed once per well on the center-most beacon or for every captured image in a well.

## Using a Grid to Define Beacons

Requires Gen5 version 3.12 or higher

When defining beacons, Gen5 lets you assign multiple beacons in a grid pattern. Using a grid in combination with "Copy beacons to all wells in vessel" is a fast way to apply beacons to multiple wells.

Here are some details about using the grid:

|             |         |
|-------------|---------|
| Rows        | Columns |
| 3           | x 4     |
| Create grid |         |

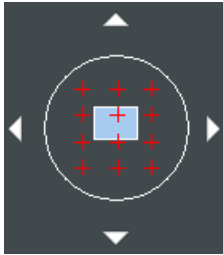
Clear beacons in current well

Remove all beacons on vessel

- To apply a grid to a well, enter the number of Rows and Columns and click **Create grid**. After the grid applies the beacons, they behave normally. You can add, move, delete, and manage the beacons as usual.
- To change the grid dimensions, first clear beacons from either the current well or the entire plate, as needed.

## 4 - Experiment Mode Imaging

Copy beacons to all wells in vessel



- Use the copy-to-all button to repeat the placement of beacons in a well to all wells on the plate. Gen5 offers to erase all existing beacons before copying the current pattern to all wells.
- Beacons are evenly spaced in the well when a grid is applied. For circular wells: well width/height = diameter/ $\sqrt{2}$ . For rectangular wells: well width/height.

Gen5 does not remember the grid, it is simply a tool to quickly assign beacons.

### Copy beacons to all wells in vessel

When defining beacons, Gen5 offers this copy-to button to speedily assign beacons to all wells in the plate.

Copy beacons to all wells in vessel

If the "regions of interest" in each well are somewhat uniform in distribution, you can assign beacons in one well and copy that distribution pattern to all other wells. You can start with a grid and then manually move, delete, or add beacons in the well to be identify ROIs. Gen5 will copy that beacon configuration to all wells when you click **Copy beacons to all wells in vessel**.

**Note:** Gen5 prompts you to delete any existing beacons before executing the copy function. Choose the behavior that will work best for your vessel.

### 4.3.4 – ROI Manager for Batch Processing

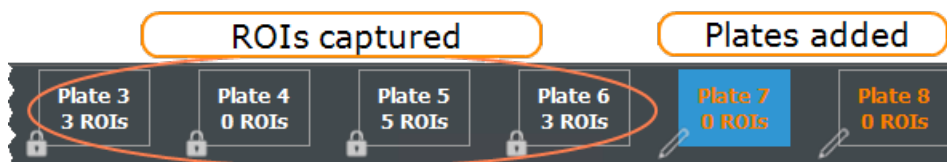
Requires Gen5 version 3.10 or higher



Especially useful for slide scanning and similar assays using the BioStack plate stacker, the ROI Manager saves the original discovery images and the captured ROIs with the experiment.

When using the BioStack, all plates in the original batch are imaged once in the discovery step. Then, the ROI Manager presents each plate for ROI identification. When all ROIs have been identified, the ROIs are captured in the image step, one plate after another.

#### Good to Know



- You can add new plates to the batch: When you process additional plates in an experiment, Gen5 adds them to the current batch, restarting the process just for those added plates.
- During the run, click **Details** in the status window to track progress.

## 4 - Experiment Mode Imaging

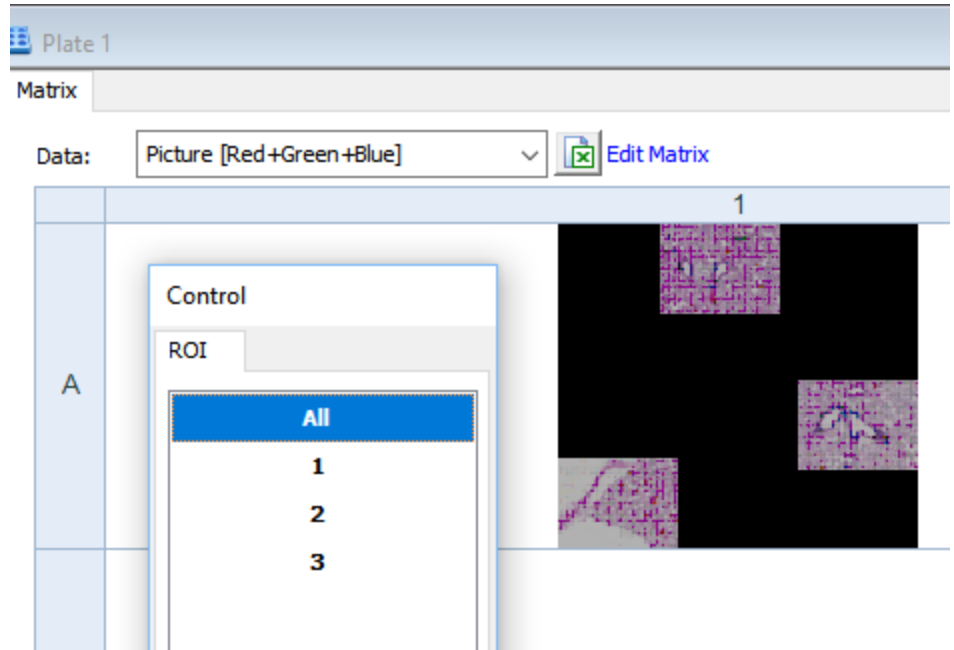
- Slides/wells and plates without defined ROIs are not imaged. **Gen5 skips plates in the batch that do not have ROIs** identified after the discovery step. A message confirming this action is displayed at the end of the run.

### Viewing ROIs

ROIs are handled/treated like other multi-image captures, e.g., montage, kinetic.

Gen5 opens an ROI control window after capture and processing so you can view each individual ROI.

Like other multi-index data sets, individual images can be viewed and analyzed, but certain functions can only be performed on "All" ROIs.



Gen5 expects an ROI captured with high magnification to be a montage, and thus always presents the Image Stitching processing option. Montages must be stitched before Gen5 can analyze them.

## 4 - Experiment Mode Imaging

## 5 - Understanding AutoFocus

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### *How to Choose the Correct Method for Your Assay*

Gen5 offers two different types of autofocus: image (software)-based autofocus and laser-based autofocus. Choosing the correct setting depends on the configuration of your system and the biology in your wells. For ease of use, the default image-based settings can be used without change.

Choice of autofocus method has a significant impact on your assay, both on the reliability of acquiring a focused image, but also on the total assay time. Image-based and user-trained autofocus options come standard with all instruments. Laser Autofocus requires an additional hardware accessory.

|                                      |            |
|--------------------------------------|------------|
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| <b>5.2 - Laser AutoFocus</b> .....   | <b>132</b> |

## 5 - Understanding AutoFocus

### 5.1 - Autofocus Methods

Gen5 offers 5 different algorithms for image-based autofocus, outlined below. For most imaging assays, the default settings work well, except when certain circumstances require additional optimization. Gen5 applies default settings for an imaging read step based on the objective's magnification.

| Manual Mode  | Experiment Mode   |
|--|---|
| <p>In manual mode, expand <b>IMAGING MODE</b> and select <b>Advanced Options</b> when capturing a one-dimension image:</p> <p>Single Image <b>Advanced options...</b></p> <p>or click <b>Edit Imaging Step...</b> for multi-dimension images, e.g., kinetic.</p> | <p>Color: DAPI 377,447</p> <p>Exposure: <input type="checkbox"/> Auto, LED: 5, Integration time: 100, Gain: 0</p> <p><b>Focus options...</b></p> <p>in the Read step, click the <b>Focus options</b> button for the detection channel</p> |

#### Autofocus Options

Use default focus method → Focus method: Autofocus without optional scan

Use default focus method settings

Minimum focus metric ratio: 3

Experiment Mode only

- Autofocus without optional scan
- Autofocus with optional scan
- Scan and then autofocus
- Autofocus without optional scan
- Bright field autofocus (dual peak)
- User-trained autofocus
- Laser autofocus

**Note:**  **Use default focus method** and  **Use default focus method settings:** Default settings are based on the selected objective. If you change the objective, be sure to reset these checkboxes to reapply the default settings for the current objective.

By default, Gen5 uses Channel 1 in your Read step as the focus channel and applies that focal plane to all other channels; therefore, it is important to select your most robust signal for Channel 1. When possible, a nuclear counterstain (DAPI, Hoechst, Draq5) works best or, alternately, the channel with the strongest signal and the least well-to-well variability.

## 5 - Understanding AutoFocus

Channel 1: DAPI 377,447

Autofocus  
 Fixed focal height [plate bottom elevation (3000  $\mu\text{m}$ ) + offset]  
 Focal height from first channel (+ offset)

Offset ( $\mu\text{m}$ ):  (Fixed height: 3000  $\mu\text{m}$ )

Perform illumination correction

Autofocus Options

### Experiment Mode Only

When your assay involves signals in each channel that do not have the same focal plane as Channel 1, you can either apply a predetermined offset to each well or perform a separate autofocus step for each channel.

Set this up by clicking **Options** under each channel and using the controls at the top of the dialog.

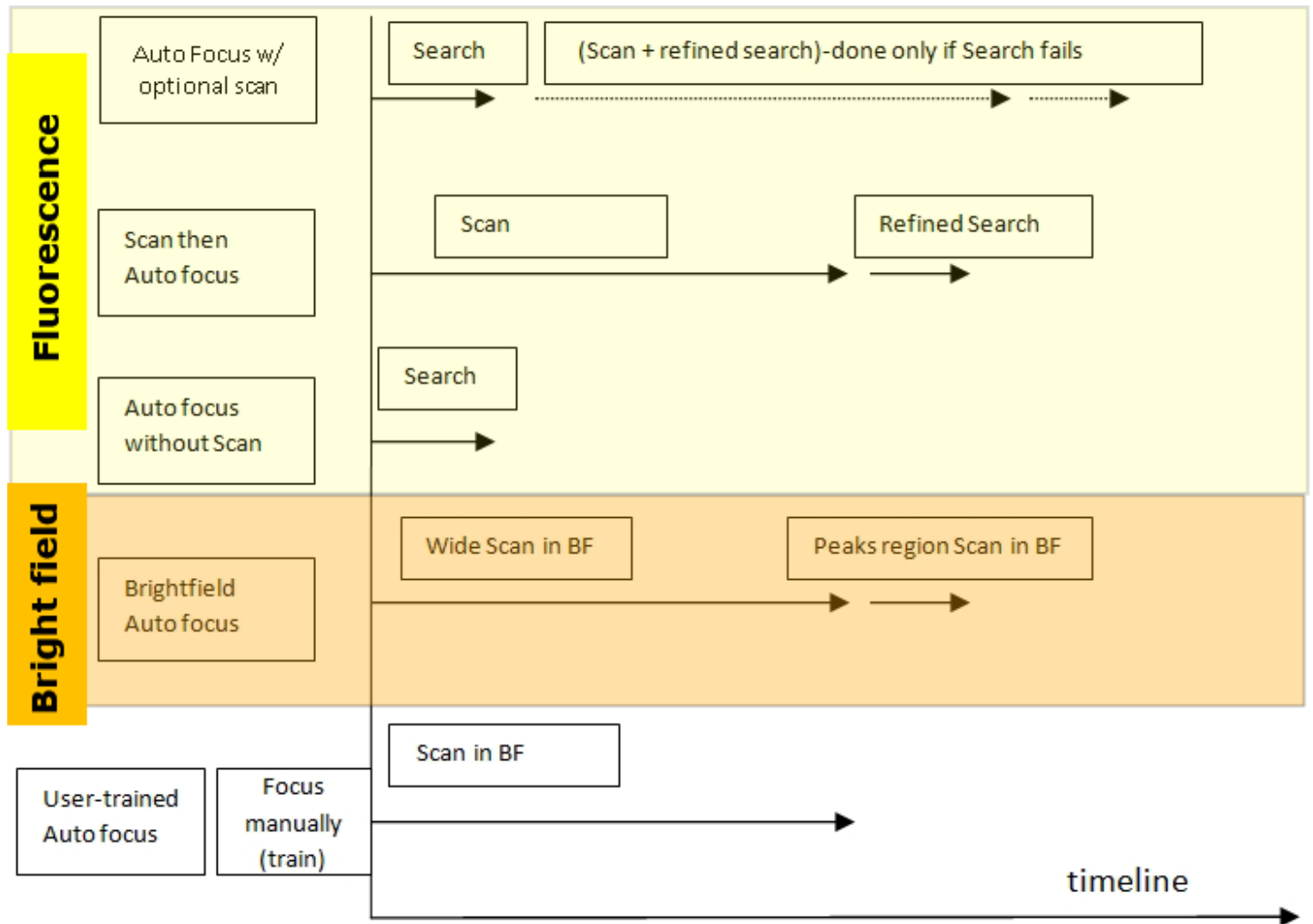
### Image-based Autofocus Methods

| AutoFocus Method |                              | Quick Description   |
|------------------|------------------------------|---|
| 1                | AutoFocus with optional scan | <b>Gen5's default method for fluorescence:</b> Searches a small area, broadening to a larger scan if optimal FM ratio is not achieved.  |
| 2                | Scan then AF                 | Scans a larger area then performs autofocus in the peak region. This is the most time consuming but can be most accurate for complex or unusual systems.  |
| 3                | AF without Scan              | Fastest method when focus settings are already proven to produce good images. This is a good option when wells are sparsely populated.  |
| 4                | Brightfield AF               | <b>Gen5's default method for brightfield:</b> Works like "Autofocus with optional scan" but is optimized for the dual peak found in brightfield and phase contrast images.                                |
| 5                | User-Trained AF              | Based upon a reference image, Gen5 is "trained" to recognize the preferred focal plane in reference to the scan peak. This method takes the longest. (Learn about User-Trained Autofocus in Gen5's Help.) |



## 5 - Understanding AutoFocus

### Autofocus Timeline



▶ Watch this [Image-Based Autofocus Training Video](#).

See also 5.2 - Laser AutoFocus on page 132.

## 5 - Understanding AutoFocus

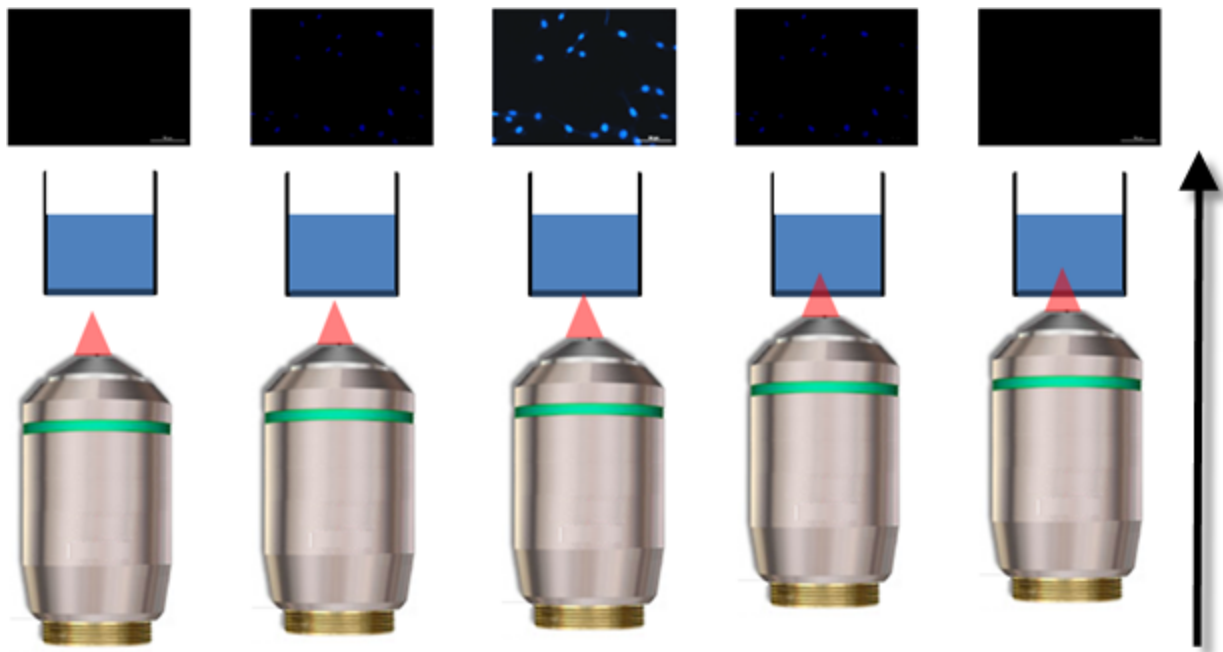
### Image-Based Autofocus Explained

Image-based autofocus methods work by taking a series of images through a range of Z-planes to determine the position with the highest "focus metric" or "FM." Focus metric is a number used to evaluate how well an optical system was focused when an image was taken (or how sharp are the individual elements of an image).

During image-based autofocus, Gen5 moves the objective along the z-axis, beginning at the plate's bottom elevation, and takes three images. The FM ratio is calculated for each image. From this information, Gen5 determines where the focus metric will reach maximum (best image), moves the objective to that position and takes the next image. The process repeats until no significant FM improvement can be achieved. A final image is taken at that position. This is referred to as a "Search" or simply "Autofocus."

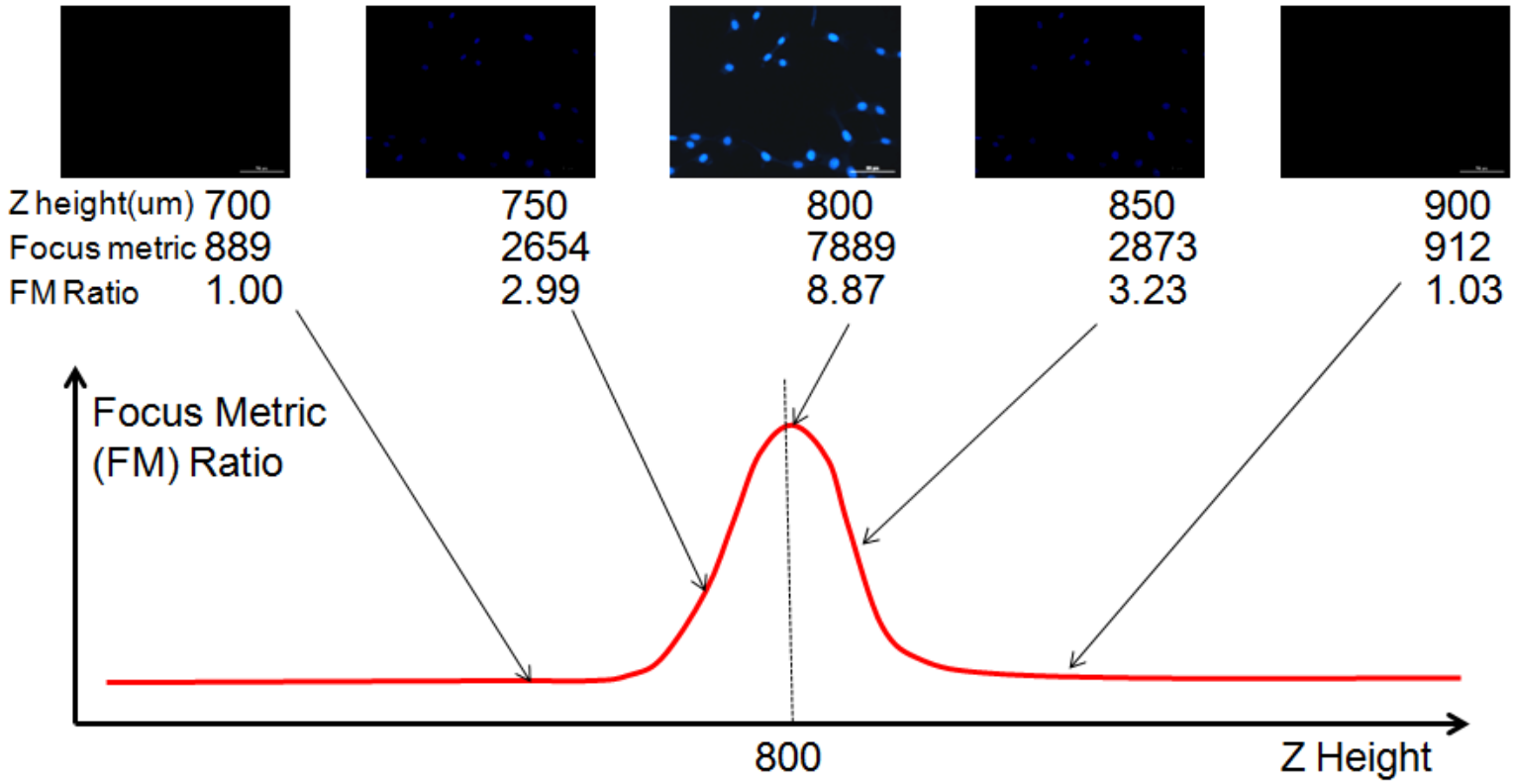
Sometimes the initial estimate for the autofocus starting point may be wrong and the "Search" fails. In this case, a wide focus metric scan can be performed to find a z-location where the focus metric reaches its maximum. This process is called a "Scan."

Additional parameters can also be adjusted in your autofocus method, including Scan Distance and Scan Increment, Auto Exposure Options, and Minimum FM Ratio. These parameters can be adjusted to optimize speed or adapt to difficult biology. To optimize speed, and to practice adjusting these parameters, try the exercise below in comparison with default settings.



Objectives move in the Z-axis towards optimal focus.

### 5 - Understanding AutoFocus



## 5 - Understanding AutoFocus

### Speed Up Focusing Process

Channel 1: DAPI 377,447

Autofocus  
 Fixed focal height [plate bottom elevation (3000  $\mu\text{m}$ ) + offset] **4**  
 Focal height from first channel (+ offset) ← To apply the settings to other channels, select "Focal height from first channel (+offset)".

Perform illumination correction

**1** Autofocus Options  
 Use default focus method  
 Use default focus method settings  
Tip: re-select the checkboxes to restore default settings for the current objective.

Focus method: **2** Autofocus without optional scan

Minimum focus metric ratio: 3

Minimum focus delta ( $\mu\text{m}$ ): 8  
**3** % of capture exposure for focus: 50  
Offset from bottom of well ( $\mu\text{m}$ ): 0  
Mark as out-of-focus if less than min FM   
Search width: 350

**3** Auto Exposure Options  
Target exposure %: 45  
Skip %: 0.1  
Use default integration threshold   
Integration threshold (msec): 50

Vibration Detection Options  
CV Threshold: 0.01  
Images to average: 1  
(1=disabled; >1=enabled)

OK Cancel Help

💡 When using low magnification with both upright and inverted imagers, e.g., 1.25X or 2.5X, with large depths of field, setting a "Fixed focal height" (at the top of the screen) is the best way to speed up AF.

Save time by speeding up the focusing process in your experiments. First, open the "Read" step in your experiment and click **Focus options** for the first channel, (DAPI in above image):

1. De-select both Use default focus method and settings.
2. Change Focus method to "Auto focus without optional scan."
3. Change the % of capture exposure for focus to 50.

## 5 - Understanding AutoFocus

4. Under **Auto-Exposure Options**, reduce the **Target exposure %** to between 35-45. This will slightly underexpose images but retain sufficient dynamic range.
5. De-select the **Use default integration threshold**, and set the **Integration threshold (msec)** to 50. This saves acquisition time by first forcing increased camera Gain and LED intensity.
6. Apply these settings to other channels by setting them to: **Focal height from first channel (+ offset)** at the top of the screen.

### 5.2 - Laser AutoFocus

Agilent offers an optional laser-based autofocus accessory — a commonly-used alternative to image-based autofocus. Especially in Bright Field, laser autofocus is typically more reliable, with better repeatability, making it the recommended option when making movies. Unlike image-based autofocus methods that need to take and compare multiple images, laser autofocus usually takes only one image per well to determine optimal focus.

Advantages compared to the image-based autofocus methods include:

- Speed
- Lower phototoxicity
- Kinetic assay support with better reproducibility
- Repeatability

Also important to know:

- Laser autofocus is only available in **experiment mode**.
- Laser autofocus is supported with 4X-60X phase and standard objectives. Short-working-distance objectives are not supported (i.e., oil immersion, some APO, etc.)
- Not recommended for moving biology: if cells are moving in the Z direction, this method could miss your desired focal plane.
- Plate bottoms must be perfectly clean.
- Do not use plastic microplates with a 10X power objective. Variances in plate thickness cause enough variances in reflected light to interrupt the autofocus process. Use glass plates for best results especially when using a 10X objective.
- Laser AutoFocus is recommended for most confocal experiments.

Prerequisite: Install the Laser Autofocus Cube as shown on page 229.

▶ Watch this [Laser Autofocus Training Video](#).

#### How it works - Laser Autofocus

|   |                          |  |
|---|--------------------------|--|
| 1 | Capture Reference Scan   | The user finds the desired focus for their sample. Gen5 activates the laser cube to scan the surrounding Z height and marks the position of desired focus.   |
| 2 | Gen5 Compares and Aligns | During the plate read, the laser marks the spot and an image is taken. Gen5 compares the image to the reference scan and moves the objective up or down (z-axis) as needed to exactly match the focus of the reference scan. |
| 3 | Repeat                   | Focus process is repeated in each well of the plate.   |

## 5 - Understanding AutoFocus

## 5 - Understanding AutoFocus

### How to use Laser Autofocus

1. Create a new experiment with an imaging read step.
2. Select the objective and color channel.
3.  Find bottom elevation with laser
4. Recommended: manually determine optimal Exposure settings (as usual).
5. Click **Focus options**.

Channels

Fluorophore

Color: DAPI 377,447

Exposure:  Auto

LED: 8

Integration time: 100

Gain: 4.1

Focus options...

5. In the Autofocus window, de-select Use default focus method.
6. Set Focus method to "Laser autofocus."
7. Follow the Gen5 prompt to capture a reference scan now (or click the button to perform this step later).

Use default focus method → Focus method: Laser autofocus

Use default focus method settings

8. Select a **Well** with the best signal, and focus on the sample as you would normally.
9. When you are satisfied with the image quality, click **Save Settings** to "Capture the Reference Scan."

Capture Reference Scan

The button is framed in red before a scan is captured. To recapture a scan, click the button again.

Well: A1

click to select a well

Save settings

This defines the objective's optimal focus position for reading the plate.

For best results, always capture or re-capture a reference scan for each experiment to ensure precise positioning, especially with high-power objectives. From batch to batch, microplates can have different bottom-thickness tolerances.



Proceed as normal with the Gen5 experiment, e.g. Read the plate. Gen5 will determine and apply the offset of the objective to the sample for every well in the plate.



## 5 - Understanding AutoFocus

### Troubleshooting Laser Autofocus

**Important:** Do not use plastic microplates with a **10X power objective**. Use glass plates for best results.

Gen5 provides options for fine-tuning laser autofocus when the default settings do not produce satisfactory images.

Use default focus method      Focus method: Laser autofocus

Use default focus method settings

Scan distance (μm): 600

Scan increment (μm): 50

Offset from bottom of well (μm): 0

Override laser exposure settings

LED intensity: 10

Integration time: 5

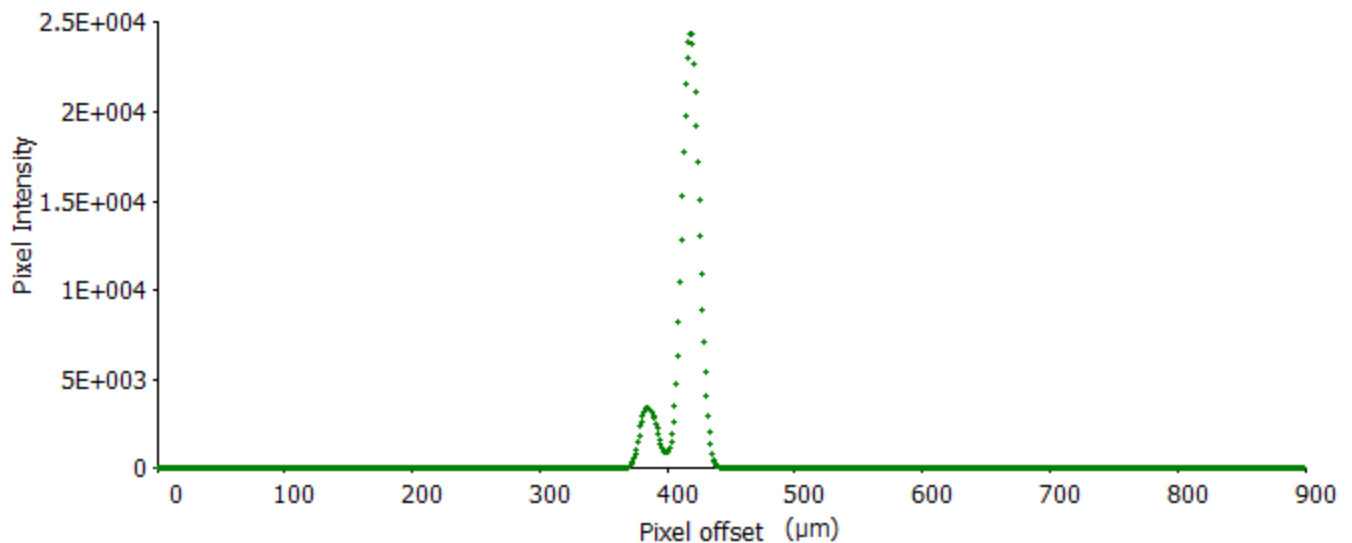
Camera Gain: 0

Disable use focal plane from previous well

Capture Reference Scan

View reference scan

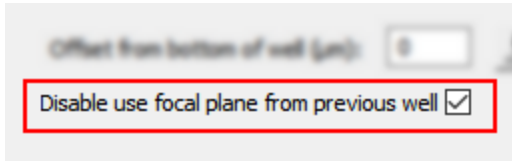
First, **view the reference** scan (example below) to help identify the problem. Depending on the objective magnification, you should see one or two well-defined peaks in the middle of the scan sensor range (x-axis); one peak of high pixel intensity, and a second peak of lower intensity.



- If any peak's pixel intensity is greater than 50,000, the sample may be overexposed. Re-capture the scan and, as needed, **Override laser exposure settings** to manually adjust them.
- No peaks, may indicate under exposure, which in any case requires refocusing: re-capture a reference scan. If problem persists, increase exposure settings.
- Some microplates do not reflect the laser light and cannot be used in this application.

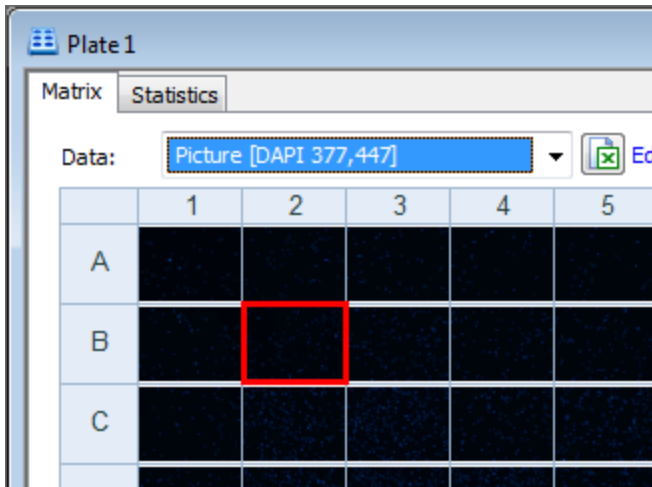
## 5 - Understanding AutoFocus

### Poor or inconsistent image capture results:



"Disable use focal plane from previous well" is provided to prevent a bad well from distorting focus values for all subsequent wells. Laser autofocus normally references the previous well for its focal starting point. If, for example, contamination on the plate bottom confuses the laser, this option limits the problem to one well. It tells Gen5 to rely on the Bottom Elevation for its starting point.

### Out-of-Focus Images



For all autofocus methods, when all attempts to capture images that match the current settings fail, Gen5 takes the best or closest image possible. Thus, Gen5's autofocus methods may acquire out-of-focus images or images that do not meet criteria. When this happens, Gen5 marks the well with a red border in the plate view matrix.

## 5 - Understanding AutoFocus

## 6 - Image Processing

---

### *Optimizing Your Image*

Image processing refers to the mathematical modification of images to improve their quality. Agilent gives you full control of your images and data, thus, the **images presented immediately after acquisition are truly raw**. It's your choice to apply any of several processing methods to improve the image for aesthetics and/or analysis. Depending on the type of image acquisition, these methods include:

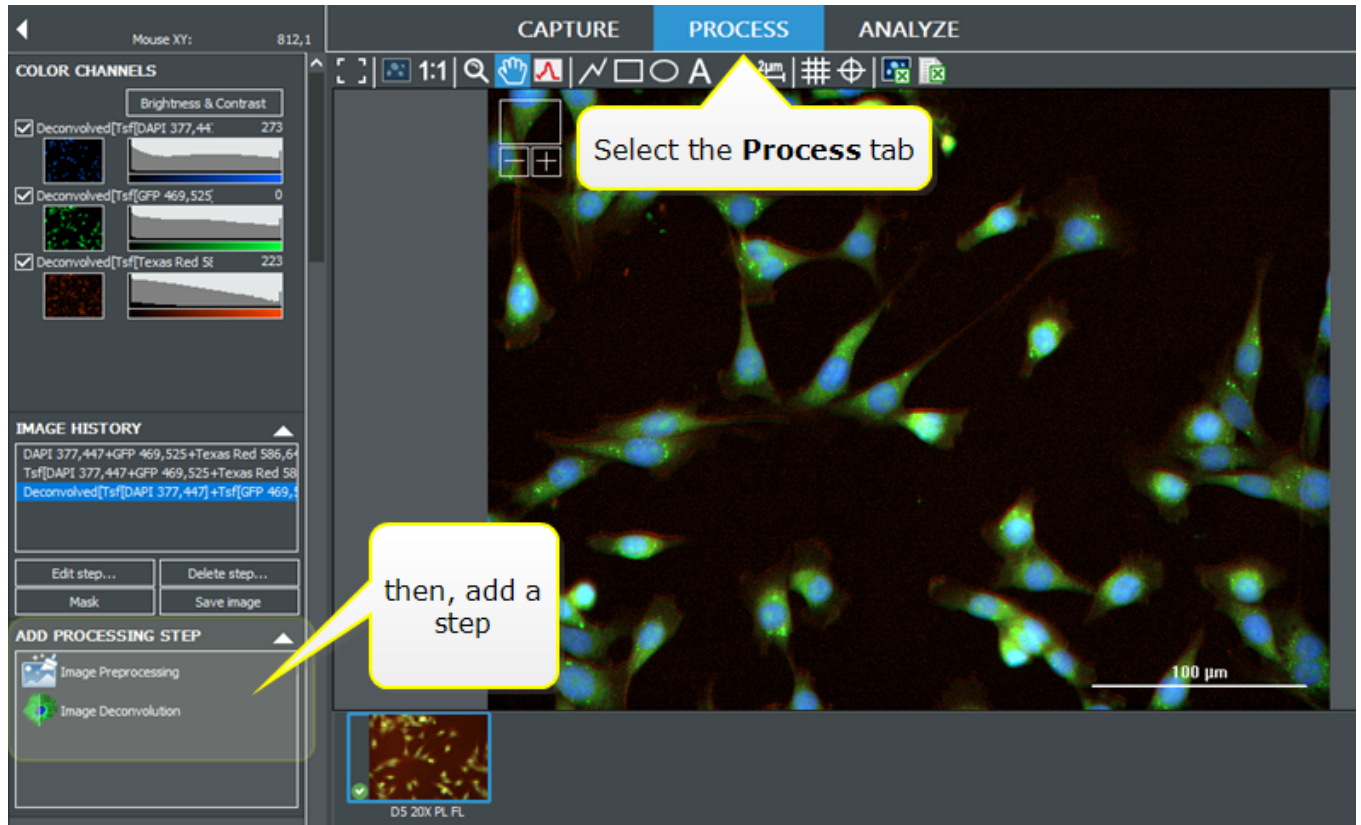
- Image Preprocessing (Background Flattening and Smoothing)
- Deconvolution
- Digital Phase Contrast
- Image Stitching (covered in 3.3 - Image Montage as shown on page 64)
- Z-Projection (covered in 3.4 - Z-Stacking as shown on page 72)
- Kinetic Frame Alignment (covered in 3.5 - Kinetic Image Capture as shown on page 79)

After acquisition in Manual or Experiment Mode, the Brightness and Contrast (B&C) Control window is immediately available. B&C modifications do not alter your raw data in any way. To add other processing steps, select **Process**. Processing options relevant to your acquired images are presented.

|   |            |
|---|------------|
| <b>6.1 - Annotation Tools</b> .....       | <b>141</b> |
| <b>6.2 - Image Preprocessing</b> .....    | <b>142</b> |
| <b>6.3 - Digital Phase Contrast</b> ..... | <b>143</b> |
| <b>6.4 - Deconvolution</b> .....          | <b>146</b> |

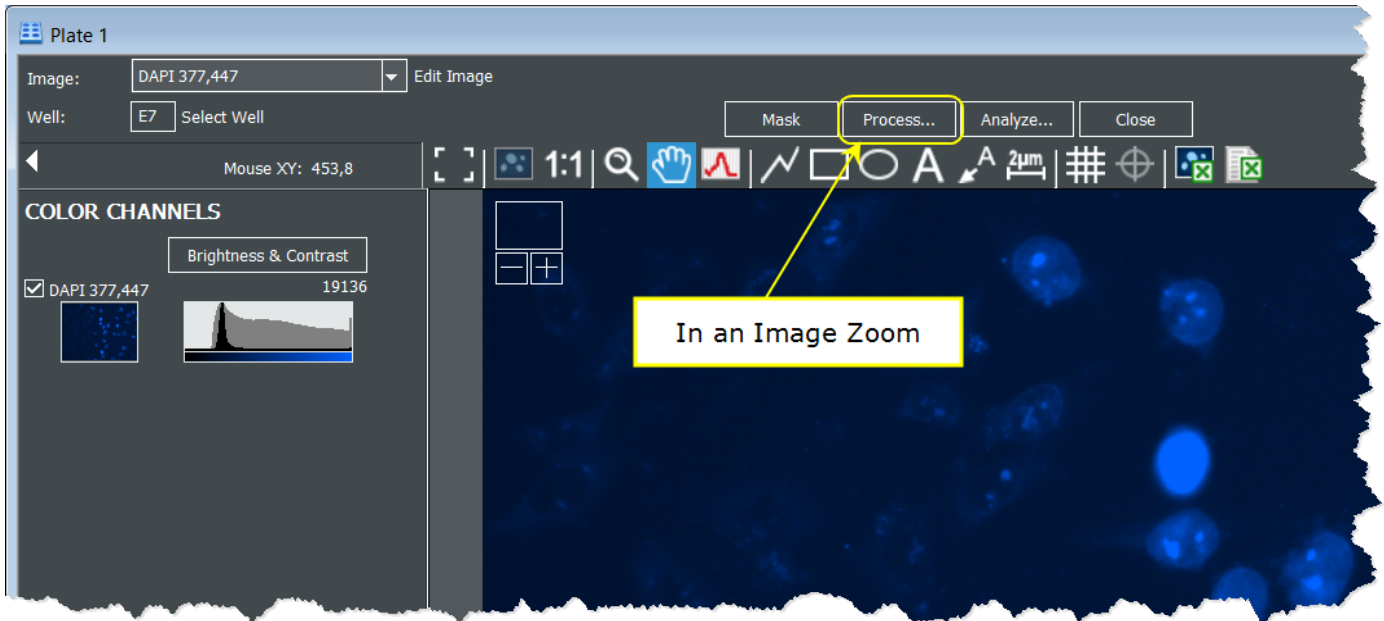
## 6 - Image Processing

### Manual Mode



## 6 - Image Processing

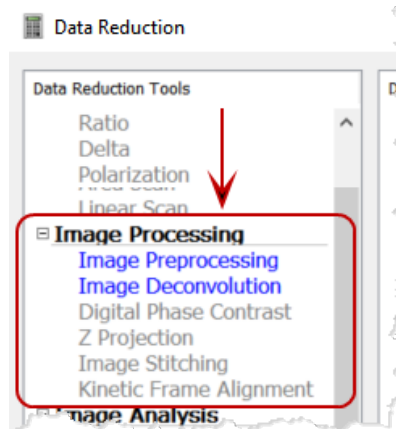
### Experiment Mode



Or:



Add a **Data Reduction** step to the experiment (or protocol):



### 6.1 - Annotation Tools

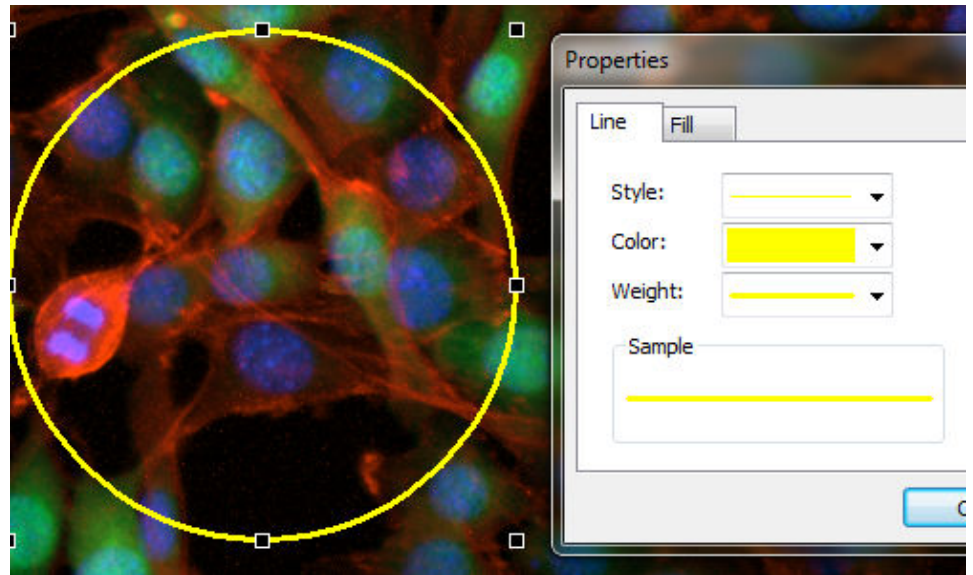
Common software tools for adding notes to images are offered after you have captured an image. They can also be used to annotate graphs and other elements of the Gen5 dataset.



Whenever the toolbar is present, you can add notes to draw attention to areas of interest in the image or chart. Apply the grid to better identify objects. Show the size of objects with the "Measure" tool, etc. These tools can enhance your reports and results analysis.

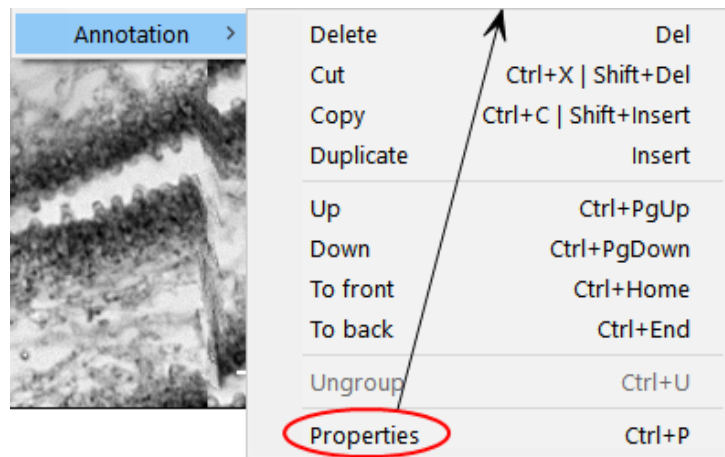
To use the annotation tools:

1. Click and drag across the image.
2. Grab the element's nodes to resize it.
3. Hover for a positioning tool, to move it.
4. Double-click an item to change its "**Properties**."
5. Right-click for a context-sensitive menu.



Select **Properties** to change the color and other elements of the item.

From the context Annotation menu, you can "**Group**" multiple annotations to move or copy them.

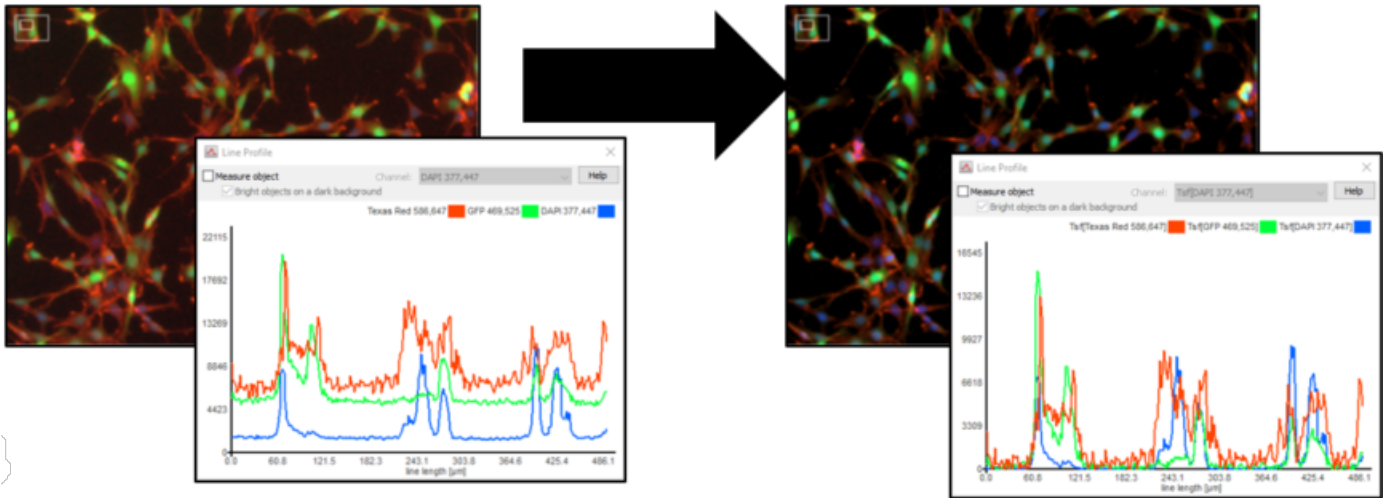


After using a tool to add an item to the image (like the ellipse shown above), right-click on the element and select **Properties** to change its appearance.

Annotations are saved with the images that are saved for presentation; in movies made from kinetic reads; and in reports and exports.

## 6.2 - Image Preprocessing

Gen5's image preprocessing step flattens the background and smooths objects' edges to create a cleaner, sharper image. Background subtraction uses a rolling ball algorithm in which an area of defined size (the "rolling ball") moves through the image. Within the area, all pixel intensities are averaged, and this mean value is subtracted from each pixel. Unlike Brightness and Contrast changes, Image Preprocessing **alters** your data.



Use the Line Profile tool to estimate size and show comparative pixel intensities. It is useful for determining the optimal rolling ball diameter.

### Background:

With Image Preprocessing, weak signal, such as background, is eliminated, creating a dark background. However, with a poorly chosen rolling ball size, legitimate signal can also be removed. Default settings are approximately 3 times the size of the most common objects. Edit the Image Preprocessing step, and de-select "Auto" to customize the rolling ball size.

**Smoothing:** a method of removing noise at the perimeter of an object, can also be added in this step. In smoothing, each pixel is replaced by an average of 9 pixels (a 3x3 pattern), a process that can be repeated until perimeter lines more evenly follow the true shape of the object.



### 6.3 - Digital Phase Contrast

*Gen5 Image+ or Image Prime software required*

Brightfield images may be improved by **Digital Phase Contrast** processing, especially when using 96- or 384-well plates.

Digital phase contrast eliminates the "meniscus effect" that occurs in transmitted-light imaging (e.g. Phase Contrast and Bright Field) in high-density plates. Digital phase contrast corrects the uneven illumination and halo effect that is otherwise observed.

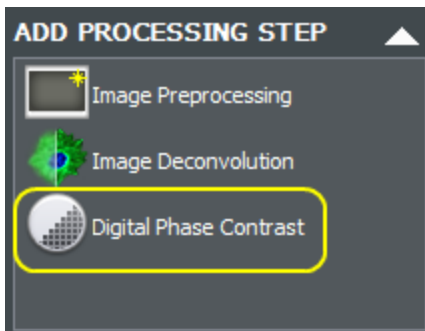
Gen5 performs three steps to apply digital phase contrast to acquired images:

1. Inverts the brightfield image (making it look like a phase contrast image).
2. Filters the image using a "Structuring Element Size."
3. Adjusts the image contrast.

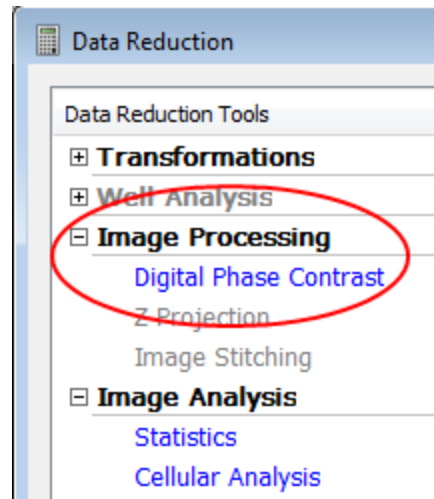
When using 96- or 384-well plates, you may get better results by acquiring Bright Field images and processing them with digital phase contrast than acquiring images in standard Phase Contrast mode.

#### How to apply Digital Phase Contrast:

- In Manual Mode, click **Add step** in the Image Processing area (shown below).
- In Experiment Mode, define a **Data Reduction** step (shown right).

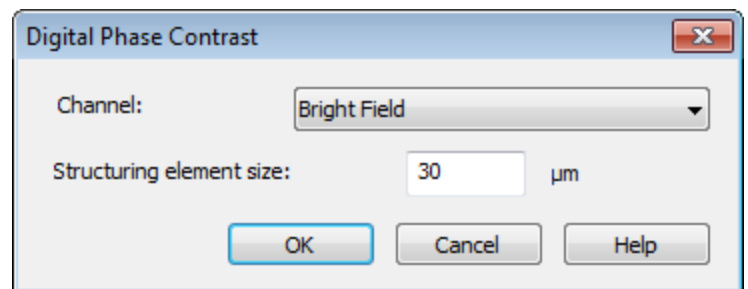


Manual Mode



Experiment Mode

After adding a processing step, reselect the image in the **Image History** and select **Edit step** to alter the element size.



## 6 - Image Processing

### Set the Structuring Element Size

Set the **Structuring element size** to match the size of the objects of interest in the image.

- Too large = geometric artifacts will distort the image.
- Too small = true signal in the sample will be lost.

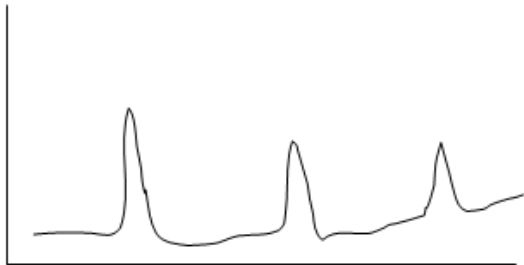
**Note:** the bigger the element size the longer processing takes.

### Digital Phase Contrast and Structuring Element Size Illustrated

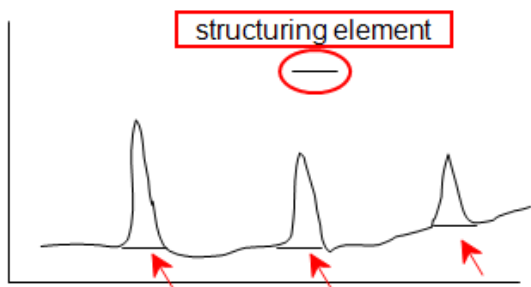
Below is an illustrated explanation of how the 'Structuring Element' process improves Digital Phase Contrast images. While we are showing the concept using 2D images, in the actual application, Gen5 uses 3D surfaces where image intensity is on the Z-axis. Gen5 scales the image to cover the full dynamic range.



1. Original image:  
dark objects on a light background.

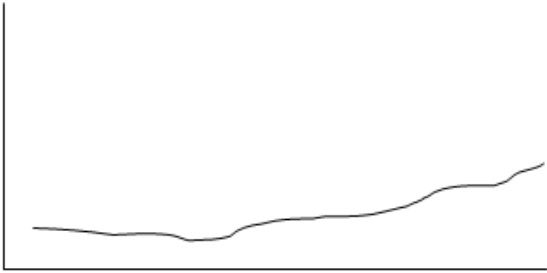


2. Inverted image:  
light objects on dark background

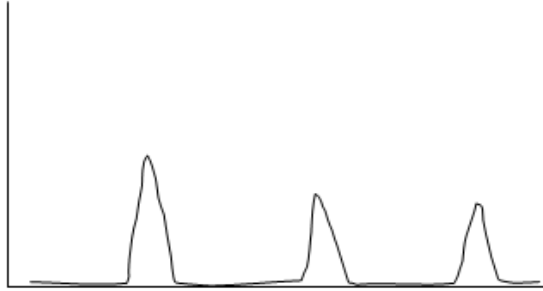


3. Structuring element (SE) slides below image profile to establish background (horizontal lines in this 2D image)

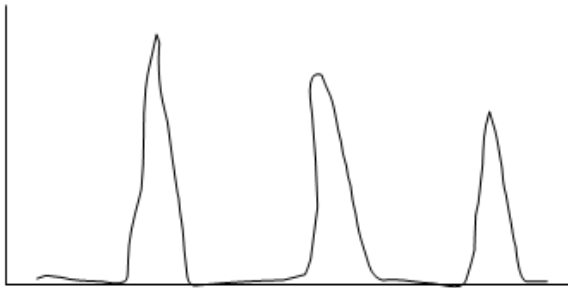
## 6 - Image Processing



4. Background found by previous step.  
(Objects smaller than the SE were removed.)



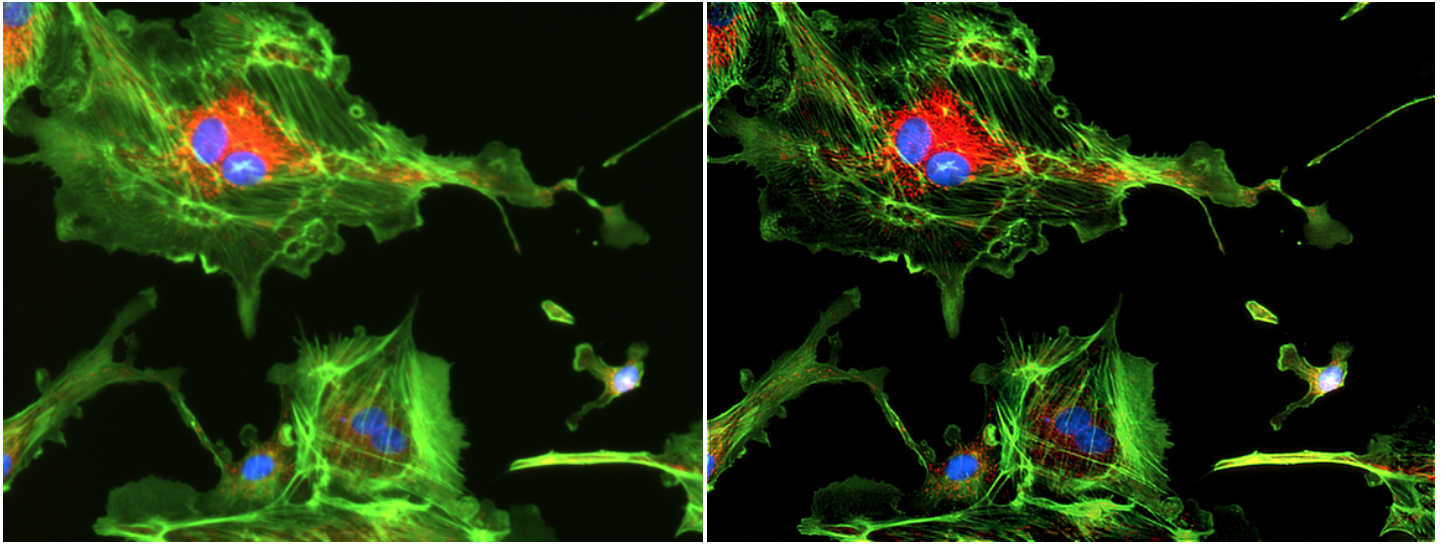
5. With background subtracted, objects of interest are light on a dark background.



In the final step, Gen5 scales the image to cover the full intensity range from zero (total dark) to maximum intensity.

### 6.4 - Deconvolution

Deconvolution (Richardson-Lucy) removes a “blur” error caused by the optics system and is defined by a Point Spread Function (PSF). Gen5 performs the deconvolution on a single image (2-D) using objective-specific values. (Each Objective’s XML file stores its sigma value for the Gaussian Kernel standard deviation.) Kernel will increase the overall area step being sharpened and Stdev distinguishes how much sharpening over that area.



Before deconvolution

After deconvolution

Auto, based on objective

For most Gen5 users, the “Auto” option is the best choice. BioTek’s imagers are reliably stable over time, so the factory-determined correction factor for each objective is a safe choice.

Alternatively, you can experiment with the correction values applied to the image to try to improve it.

## 6 - Image Processing

## 7 - Basic Image Analysis

### Generating Quantitative Data from Your Images

Gen5 Image+ and Image Prime support these features

Basic image analysis helps you identify objects of interest, report desired metrics, and group them into meaningful subpopulations.

|  |            |
|--|------------|
| <b>7.1 - Primary Mask and Count</b>    | <b>152</b> |
| <b>7.2 - Calculated Metrics</b>        | <b>154</b> |
| <b>7.3 - Cellular Analysis Details</b> | <b>157</b> |
| <b>7.4 - Subpopulation Analysis</b>    | <b>161</b> |
| <b>7.5 - Define an Image Plug</b>      | <b>165</b> |

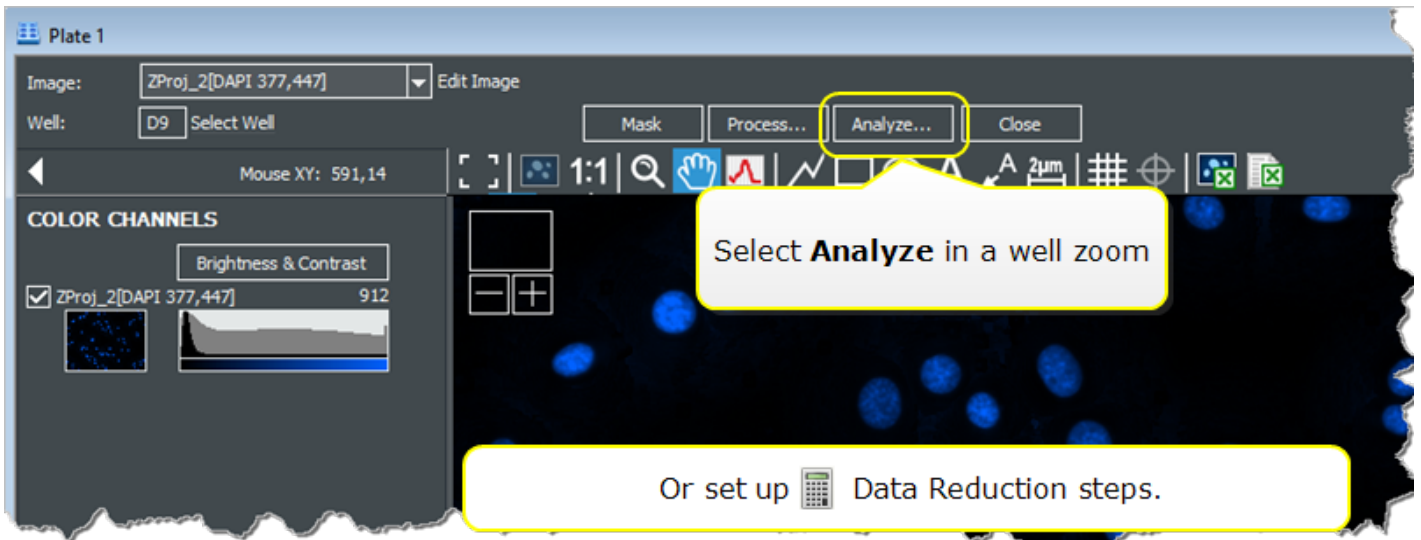
To get started, click **Analyze** in **Manual Mode**:

The screenshot displays the Gen5 software interface in Manual Mode, with the ANALYZE tab selected. The interface includes a top toolbar with icons for capture, process, and analyze. On the left, there are panels for COLOR CHANNELS, IMAGE HISTORY, ADD ANALYSIS STEP, and NAVIGATION. The main window shows a fluorescence image of cells with a 100 µm scale bar. Callouts provide the following instructions:

- 1** After selecting the ANALYZE tab, use the checkboxes in the COLOR CHANNELS panel to show or hide the channel in the current image.
- 2** Highlight the data set to use for the analysis, then click **START**.
- Select the captured image to analyze.

## 7 - Basic Image Analysis

In an image zoom in **Experiment Mode**:



## 7 - Basic Image Analysis

### Analyze Tool

The screenshot shows the 'Analyze Tool' window. On the left, the 'COLOR CHANNELS' panel has 'ZProj\_2[DAPI 377,447]' selected. The 'ANALYSIS SETTINGS' panel shows 'Type: Cellular Analysis' and 'Detection Channel: ZProj\_2[DAPI 377,447]'. A 'START' button is visible. A yellow callout box with three numbered steps is overlaid on the interface:

- 1 Select Cellular Analysis
- 2 Choose the channel that represents the object to be counted
- 3 Click Start

The main image area shows a field of blue-stained cells. On the right, the 'Image-Level results' table is empty, and the 'Object details' section shows 'All objects'.

The screenshot shows the 'Analyze Tool' window after analysis. The 'ANALYZE' tab is active. The main image area shows the same field of cells, but now with yellow outlines around each cell. A yellow callout box with a green arrow points to the 'Edit step...' button in the 'Object details' panel:

To reopen the Analyze Tool to modify analysis settings

The 'Image-Level results' table is now populated with data:

| Cell Count                      | Value    |
|---------------------------------|----------|
| 59                              |          |
| Object Size                     | 14.3     |
| Object Area                     | 161      |
| Object Sum Area                 | 9.48E+03 |
| Object Mean[Tsrf[DAPI 377,447]] | 17499    |

The 'Object details' table is also populated:

| #  | Size | Area | Mean[Tsrf[DAPI 377,447]] | Area[Tsrf[DAPI 377,447]] | Mean[Tsrf[GFP 469,525]] |
|----|------|------|--------------------------|--------------------------|-------------------------|
| 1  | 12.9 | 130  | 18724                    | 130                      | 15329                   |
| 2  | 12.7 | 127  | 22031                    | 127                      | 22429                   |
| 3  | 15.6 | 190  | 18602                    | 190                      | 12093                   |
| 4  | 17.0 | 226  | 15358                    | 226                      | 9994                    |
| 5  | 15.8 | 194  | 12987                    | 194                      | 8460                    |
| 6  | 14.4 | 162  | 23538                    | 162                      | 17648                   |
| 7  | 12.3 | 118  | 19168                    | 118                      | 14392                   |
| 8  | 14.2 | 157  | 18666                    | 157                      | 14833                   |
| 9  | 11.6 | 103  | 24614                    | 103                      | 23105                   |
| 10 | 17.2 | 224  | 20885                    | 224                      | 13554                   |
| 11 | 12.2 | 117  | 19297                    | 117                      | 16127                   |

The 'Object details' panel also shows 'Highlight objects' settings with 'Channel: Tsrf[DAPI 377,447]' and 'Mask: Primary' selected.



## 7 - Basic Image Analysis

**Analyze Tool**

Mouse XY: 7,7

**COLOR CHANNELS**

Brightness & Contrast

ZProj\_2[DAPI 377,447] 608

**ANALYSIS SETTINGS**

Type: Cellular Analysis

Detection Channel: ZProj\_2[DAPI 377,447]

START

Options

ADD STEP

Add image s

Save imag

Cancel

Close Image set

Image-level metrics, including total object count

Objects identified by settings highlighted

Default metrics for all objects

Easily apply analysis to entire experiment or compatible "Manual Mode" images

Export data directly to Excel

|                         |          |
|-------------------------|----------|
| Cell Count              | 64       |
| Object Size             | 13.3     |
| Object Area             | 138      |
| Object Sum Area         | 8.83E+03 |
| Object Mean[ZProj_2,... | 16919    |
| Detection Threshold     | 5072     |
| <Execution time>        | 656      |

Object details:

All objects

| # | Size | Area | Mean[Z Proj_2... |
|---|------|------|------------------|
| 1 | 11.8 | 110  | 13584            |
| 2 | 13.7 | 146  | 19132            |
| 3 | 17.9 | 251  | 15930            |
| 4 | 14.8 | 172  | 13001            |
| 5 | 13.3 | 138  | 11381            |
| 6 | 13.5 | 143  | 19123            |
| 7 | 15.2 | 183  | 22669            |
| 8 | 15.4 | 186  | 16727            |
| 9 | 12.4 | 120  | 13139            |

Highlight pixels

Saturated pixels

Pixels in the Plug  Threshold outliers

Highlight objects

Channel: ZProj\_2[DAPI 377,447]

Mask:  Primary  Secondary

Spots:  Contour  Threshold outliers

Show index  Fill objects

Settings...

If the default settings do not identify objects sufficiently, or you want to add metrics, or create a more detailed analysis, click **Options** (under the Start button). Additionally, if you wish to compare your analysis across multiple wells, you can "Add Image Set," at bottom of window to select additional wells (for example, your positive and negative controls). "Options" opens the Cellular Analysis tools:

Cellular Analysis

Primary Mask and Count Secondary Mask Calculated Metrics Subpopulation Analysis

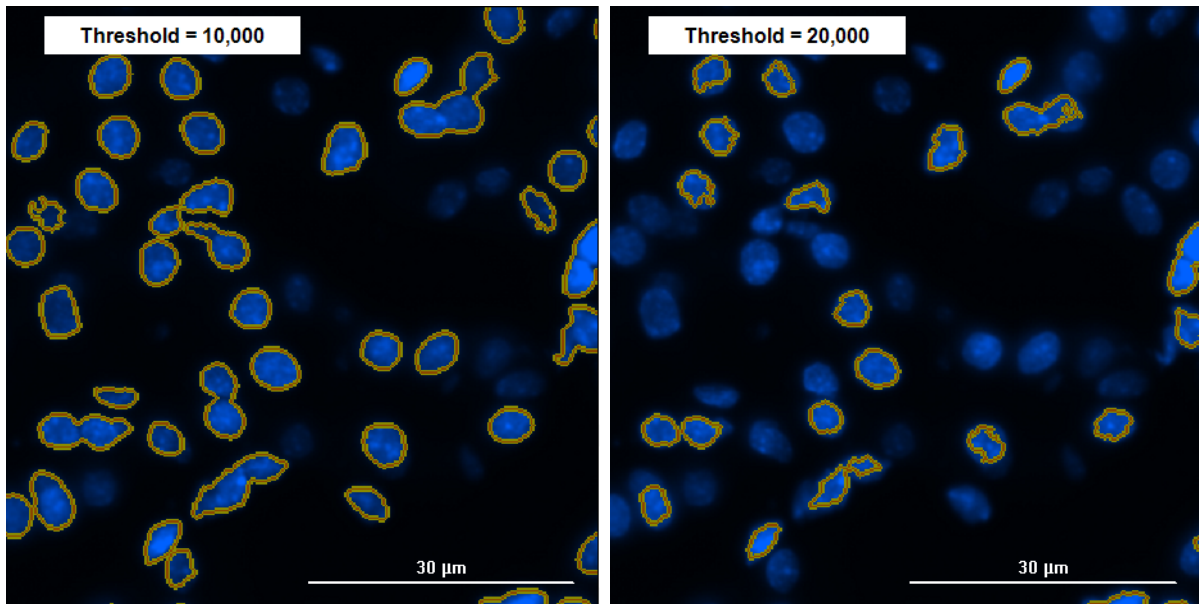
Channel: Tsf[DAPI 377,447]

Threshold

1. **Primary Mask and Count** provides the tools for setting the broadest definition of your object, for example nuclei in mammalian cells. Start by changing the Threshold and object size if the default settings do not correctly identify your objects.
2. **Secondary Mask** is only available if you have purchased **Gen5 Image Prime** and allows for more detailed, high content analysis of your images.
3. **Calculated Metrics** allows you to select the readouts that will generate meaningful data for your assay.
4. **Subpopulation Analysis** allows you to define sub-groups of your objects using AND/OR operators and choose the data you want to generate for these groups.

### 7.1 - Primary Mask and Count


In Cellular Analysis, images can be analyzed for cell level details, like object size, intensity, and circularity. Gen5 reports a total count for all identified objects in the image, defining them with a Primary Mask. The most critical parameter to identifying all of your objects is Threshold. **A threshold sets the minimum pixel intensity value for the signal of interest.** The threshold defines which pixels to include in an object mask. When a threshold of 10,000 is applied, for example, only objects with a pixel intensity from 10,000 to the max 65,520 are identified and circled with a mask. If the threshold were increased to 20,000, the size of the object mask will shrink or disappear since only pixels from 20,000-65,520 are included. The images below illustrate the masking behavior at two thresholds:



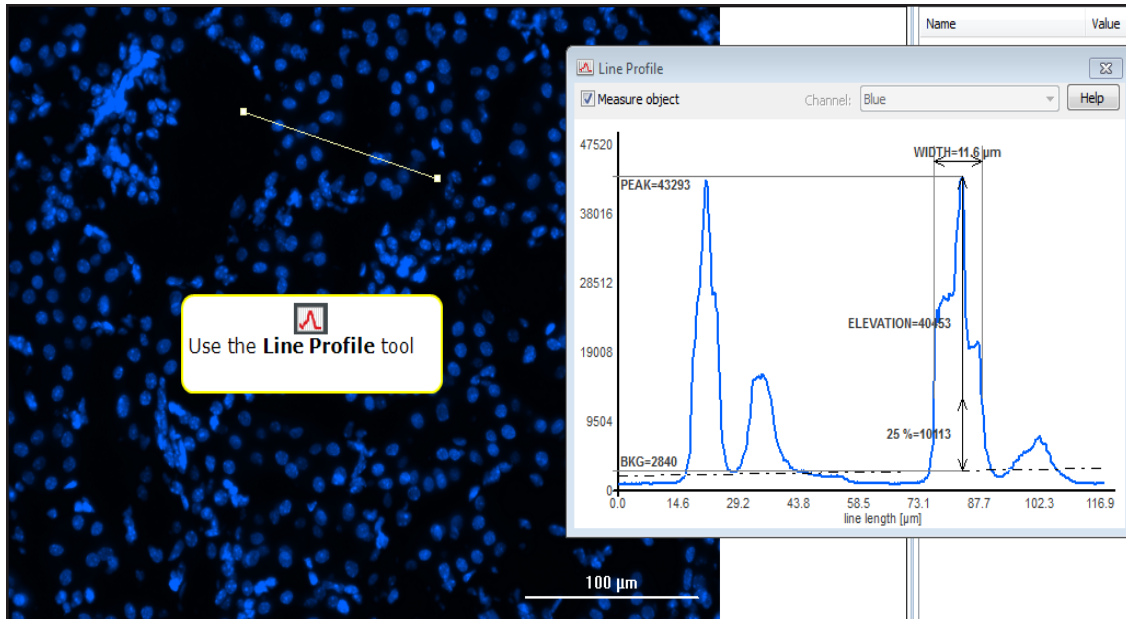
You can clearly see that when the threshold is increased, fewer objects are included in the count.

Any fluorescently-labeled image can be the detection channel and used to set the threshold of the detection mask for measurement purposes. The mask set in one detection channel is cloned to all downstream channels so that intensity measurements can be made. Typically, a nuclear DNA-binding dye such as Hoechst or DAPI is the primary detection channel for a nuclear mask (like the above examples). However, in some applications a mask based on cytoplasmic or whole-cell stains is required.

#### To Determine a Threshold:

Use the Line Profile tool  to trace several representative regions of the image to determine a starting threshold value. The Line Profile tool suggests a "25% = value" as a starting threshold value. When multiple images or image sets are analyzed (in both manual and experiment modes), the same threshold value is applied to all analyzed images so that data can be compared across images. Use the Line Tool across several of the images that represent the dynamic range of the samples or assay, such as positive and negative controls. Since the same threshold will be applied to all images, optimizing the threshold based on the maximum intensity of the images typically offers the best analytical results.

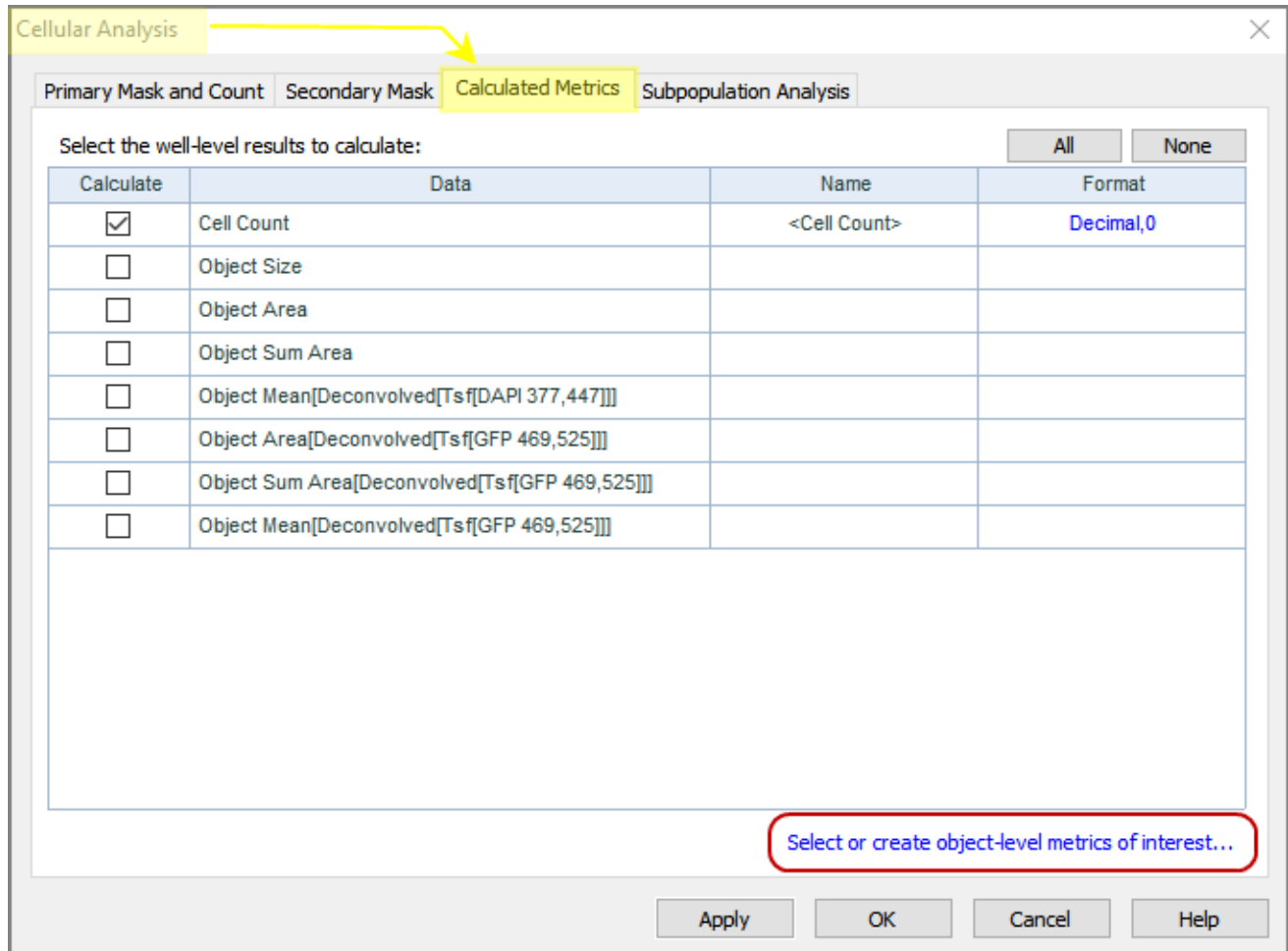
## 7 - Basic Image Analysis



### Best Practice Tips:

- The **Split Touching Objects** feature is recommended in applications where object number is important, such as proliferation, cytotoxicity, or migration studies to separate two or more objects whose masks are touching. The Split Touching Objects feature may not be useful or recommended in applications where the mask is set by cytoplasmic dyes or by multi-cellular structures.
- Using **Include edge objects** in cellular analysis is application dependant. For applications involving object counting, the user must decide whether to include or exclude edge objects; Gen5 applies the rule across all image sets. When Area measurements are important, excluding edge objects is recommended as the cells on the edge of the image are not complete and may corrupt the analytical results.

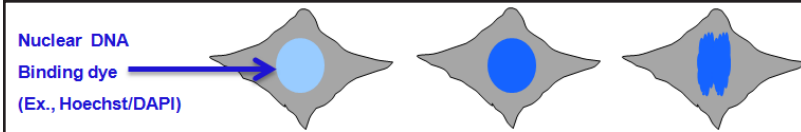
## 7.2 - Calculated Metrics



Gen5 can generate a wide breadth of quantitative data from your images. Select the **Calculated Metrics** tab to choose which metrics are relevant to you. Click "Select or create object level metrics of interest" for additional choices, particularly if you are a **Gen5 Image Prime** user.

## 7 - Basic Image Analysis

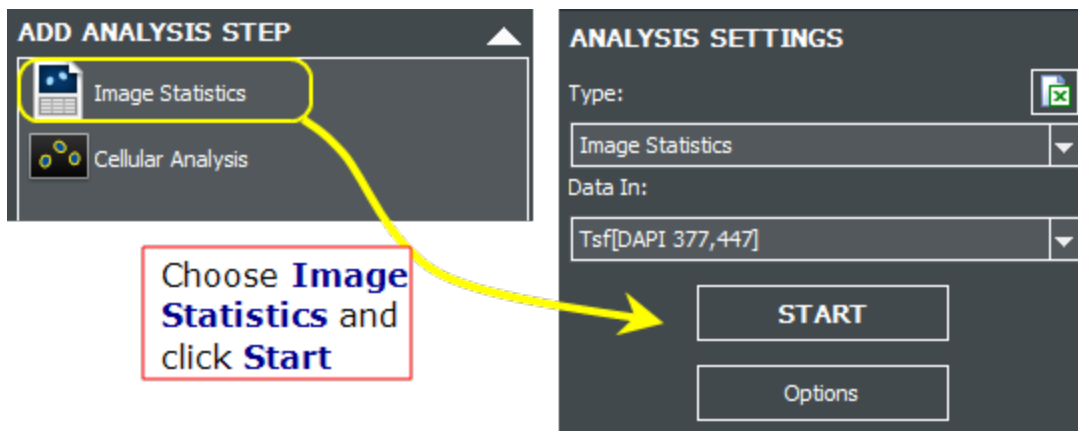
New users often find it challenging to apply the available measurements in Cellular Analysis to actual applications. Here is an example application to illustrate **how measurements of nuclear morphology can be used to determine a cell's stage in the Cell Cycle using a mask defined by a DNA-binding dye** such as Hoechst or DAPI. For a cell in  $G_{0/1}$  phase, the nuclear dye reflects 2N DNA content and will have normal intensity and normal size and shape. Once a cell reaches G2, it has replicated its DNA; at 4N DNA content there will be a measurable doubling of nuclear fluorescent intensity. This can be seen at the cellular level using the Mean, Peak, and Integral nuclear Intensities.



| Measure            | $G_{0/1}$ | G2       | M                   |
|--------------------|-----------|----------|---------------------|
| Object #           | Stable    | Stable   | Stable              |
| Object Size        | Normal    | Normal   | Skewed aspect ratio |
| Object Circularity | Normal    | Normal   | Skewed aspect ratio |
| Object Area        | Normal    | Normal   | Unclear             |
| Object Perimeter   | Normal    | Normal   | Skewed aspect ratio |
| Mean Intensity     | Normal    | Brighter | Brighter            |
| Peak Intensity     | Normal    | Brighter | Brighter            |
| Integral Intensity | Normal    | Brighter | Brighter            |

When a cell reaches the M phase, it will likely have the same intensity as a cell in G2 phase as no additional DNA replication has occurred; however it will have skewed aspect ratios due to spindle formation in advance of cell mitosis. If only one measurement were made per cell it would be impossible to determine the stage of the cell cycle, but in combination these measurements allow for advanced morphological detection of the cell-cycle stages.

## Image Statistics



ADD ANALYSIS STEP

- Image Statistics
- Cellular Analysis

ANALYSIS SETTINGS

Type: Image Statistics

Data In: Tsf[DAPI 377,447]

START

Options

Choose **Image Statistics** and click **Start**

## 7 - Basic Image Analysis

In the Analyze pane, select **Image Statistics** and click **Start** to generate the following data:

| <b>Image Statistics</b>  |  |
|--------------------------|--|
| Mean (per color channel) | Mean intensity of pixels in the object.  |
| Min                      | Minimum intensity value of pixels in the object.   |
| Max                      | Maximum intensity value of pixels in the object.   |
| STD DEV                  | Calculated using the standard deviation (n-1) of the intensity of all pixels located inside or at the perimeter of an object.  |
| CV                       | Calculates standard deviation /Mean.   |
| Saturated Pixels %       | Calculated by counting the number of pixels that have a value greater than or equal to the saturation limit defined for the instrument's camera divided by the number of pixels in the image. Refer to instruments specifications for saturation level and image resolution. |
| Focal Height             | Position of the objective in the Z axis (reported in microns).   |
| FM Ratio                 | FM Ratio is a ratio reported by the Autofocus algorithm.   |
| Total Intensity          | Sum of pixel intensities above or below the threshold.   |
| Total Area               | Sum of area of pixels above or below the threshold (reported in microns).  |
| Confluence               | Number of pixels above or below the threshold divided by the total pixels in the image * 100.  |
| Width                    | Image width is extracted from the image metadata; dimensions in $\mu\text{m}$ .  |
| Height                   | Image height is extracted from the image metadata; dimensions in $\mu\text{m}$ .   |

### 7.3 - Cellular Analysis Details

#### Creating Object Masks for Cellular Analysis

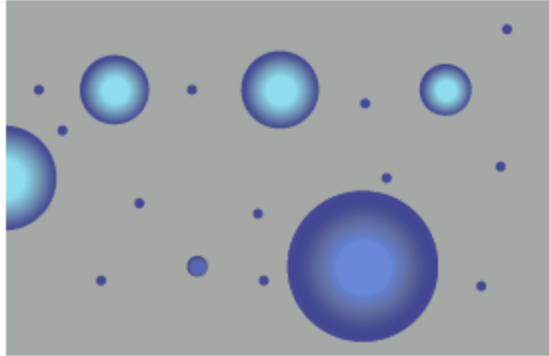
Process for identifying cells or "Primary Mask and Count."

- 1 Gen5 displays the acquired image as a 2D picture.

Each pixel on the screen corresponds to a pixel captured by the camera.

The **intensity** of each pixel is proportional to the signal level received by the camera.

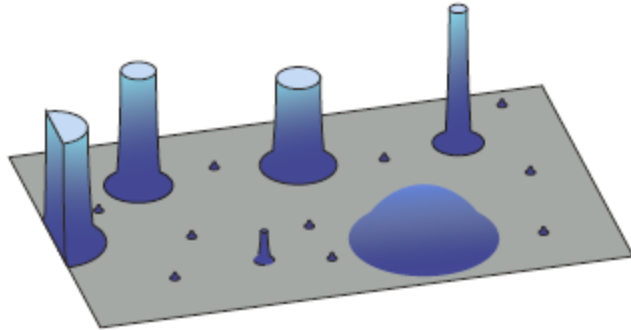
The pixel intensity is reported as a number proportional to the level of light falling onto the pixel: 0 for totally dark to 65,520 for max intensity.



- 2 The goal of the cell identification process (mask creation) is to determine which objects in the image to consider as valid cells or objects-of-interest, and what shape to assign to each object.

(The process is done mathematically, but these illustrations provide a way to visualize the steps of the process.)

The 2D image is transformed into 3D space with X-Y plane representing pixel location and Z axis representing pixel intensity. In this 3D space, an object's height is proportional to its pixel intensity in the image. Bright objects are tall and dim objects are short.

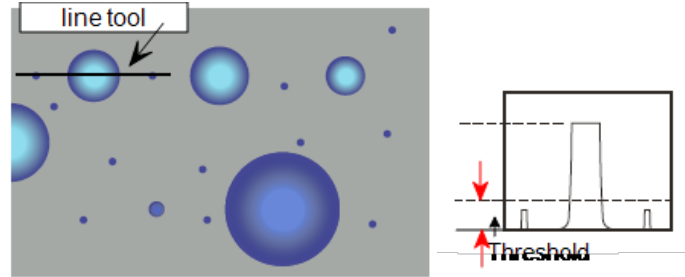


## 7 - Basic Image Analysis

3



Gen5 provides a **line tool** to measure the intensity profile of an image. The user draws a line in the 2D image, and Gen5 generates a vertical plane of this line. The picture of this vertical plane and how it sliced the 3D objects is displayed next to the 2D image.



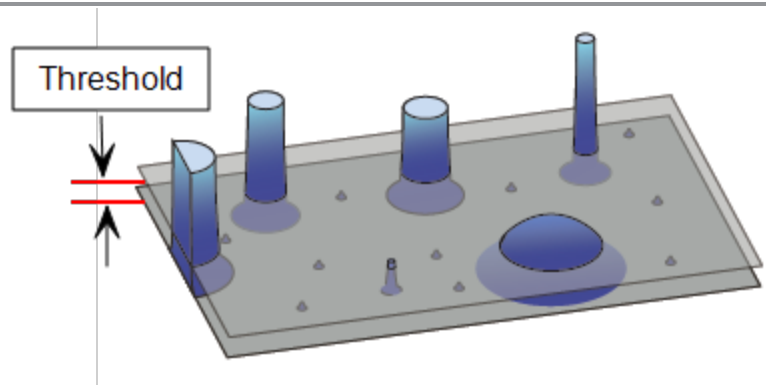
**25%:** When using the line tool, Gen5 displays the 25% threshold value, which is often the optimal setting.

The user can decide which objects are too low in height (intensity) to count as cells and at what intensity level to start counting the objects as valid. This intensity level is called **Threshold** for cell identification.

4

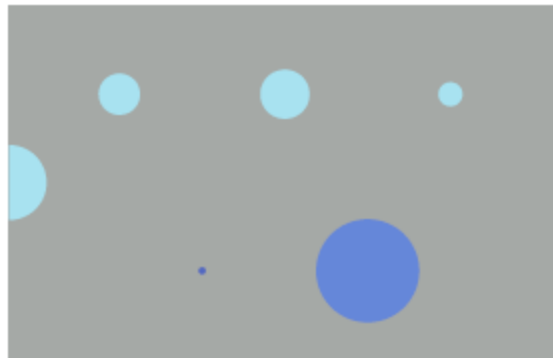
In 3D space, a horizontal plane is mathematically generated at the intensity level equal to the Threshold.

This Threshold plane will slice through 3D objects whose intensity exceeds the Threshold. Dim objects of low intensity will stay below this plane.



5

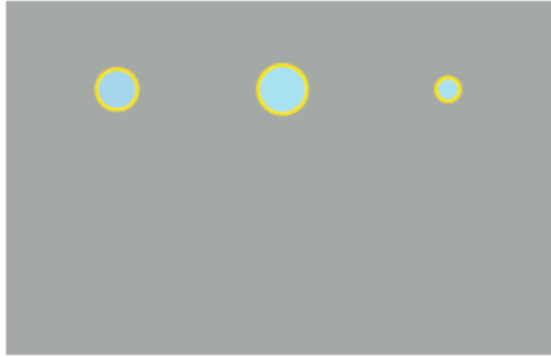
If we now look at the Threshold plane in 2D, it will contain contours where the plane sliced 3D objects whose intensity exceeded the Threshold. These objects met the intensity criteria for a valid cell.





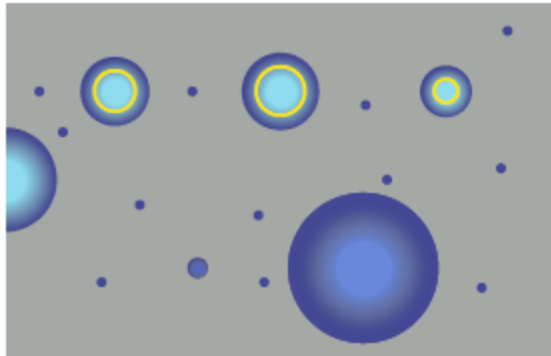
## 7 - Basic Image Analysis

- 6** Next, the X-Y size of each object can be compared to the minimum and maximum object size specified by the user. Objects that do not fit the MIN, MAX criteria are ignored. Objects that fall on the edges of the image (not totally contained in the X-Y area of the image) can be either included or excluded from analysis.



- 7** Gen5 applies masks around the valid objects that correspond to the user-set criteria. Only the valid objects are considered the "population" of interest.

Each mask outlines an area of the image to form individual objects on the image (cells). Cell analysis is run on these objects (e.g., count them, evaluate area, measure perimeter, etc.).



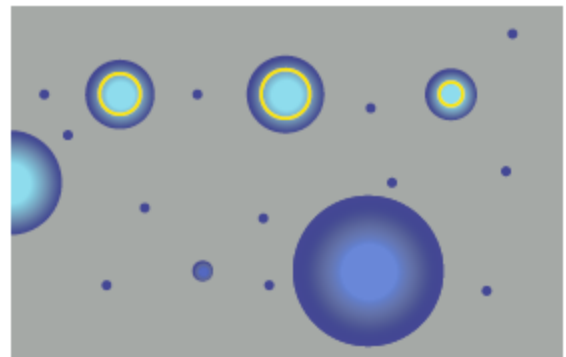
**Note:** The intensity of each object (cell) is not affected by the cell identification process.

### Subpopulation Analysis

Gen5 provides further cellular analysis capabilities by defining and evaluating subpopulations. Subpopulation analysis answers the question: which objects from a total population fit certain criteria.

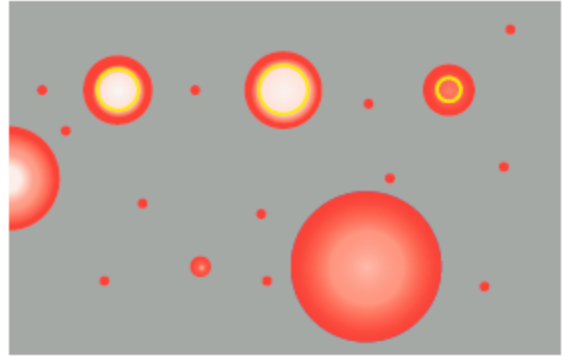
The total population of cells was established as described above using a Threshold and size filters. This process was done using Color Channel 1 (DAPI in our example).

The "population," i.e., total number of objects, is 3 defined by yellow masks.



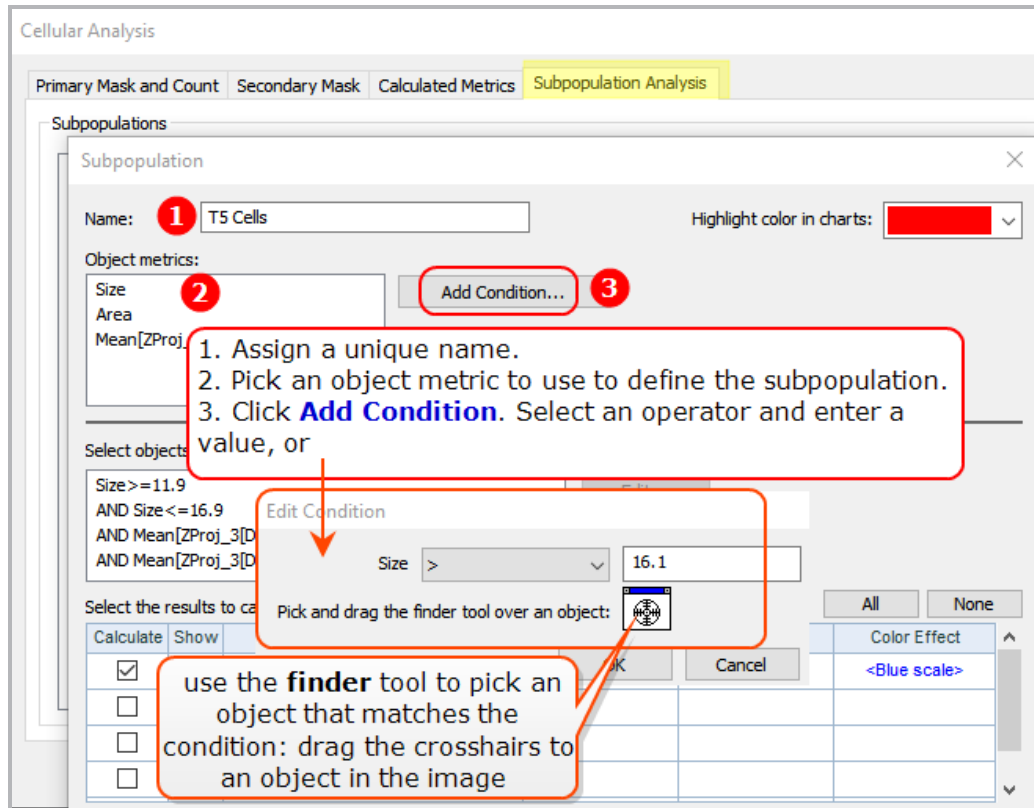
## 7 - Basic Image Analysis

Intensity measurements in Color channels 2-4 can also be used for analysis. Gen5 reports intensities in these channels only on the objects defined by masks developed in channel 1. We can call these measurements "Red on Blue" (Red for intensity of Red channel and Blue for the primary channel used to identify the population or object masks).



In this example, if we define a subpopulation as objects with a certain Red intensity value, the total number of objects would be 2. (The third cell is dimmer than specified.)

7.4 - Subpopulation Analysis

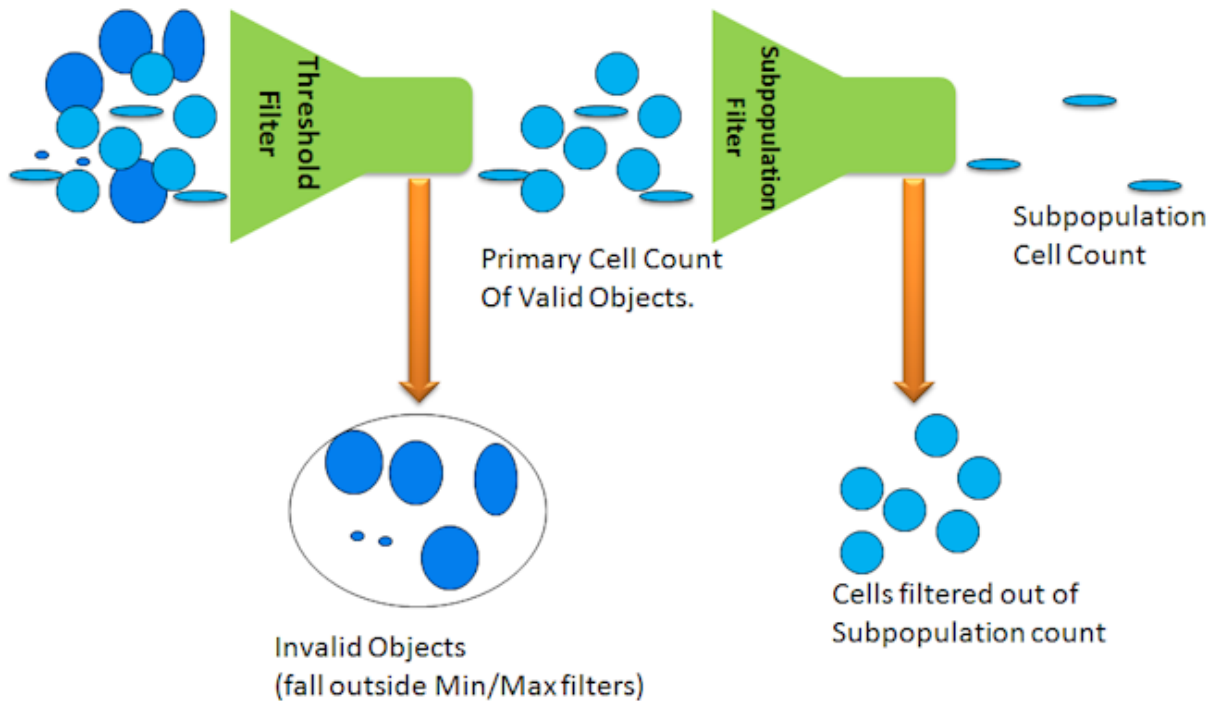


Adding subpopulations allows you to identify sub-groups within a primary population. To continue our cell cycle example, three useful subpopulations might be cells in G0/G1, S, and G2/M. Gen5 lets you use as many of your chosen metrics as desired to make the definition as specific as possible. You can delimit them further by choosing whether "All Conditions" or "At Least One Condition" must be met in order for an object to be counted. Finally, you can select the desired metrics for each subpopulation and stack them to create a custom subpopulations. **See also Define a Subpopulation using a Graph on page 164.**

## 7 - Basic Image Analysis

### Subpopulation Analysis: Result Options

*Gen5 Image+ or Image Prime software required*



#### Object Details:

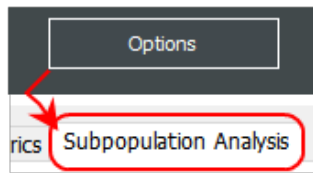
In the Analyze Tool, after applying the primary mask, determine useful conditions for analysis using the Object details panel. It works both ways: you can highlight a cell in the image or a row in the table to pinpoint results.

- click on a cell in the image, or,
- click on a row in the table.
- click a column header in the table to change its sort order to that value.
- select multiple rows in the table to highlight those cells in the image (press Ctrl to choose nonadjacent rows).

The screenshot shows the software interface with a cell image on the left and a table on the right. The table is titled "Object Details Panel" and contains the following data:

| #  | Size | Circularity | Area | Perimeter | Mean [DAPI] | StdDev [DAPI] | Peak [DAPI] | Integral [DAPI] |
|----|------|-------------|------|-----------|-------------|---------------|-------------|-----------------|
| 52 | 18.1 | 0.49        | 249  | 66.9      | 12948       | 4674          | 21450       | 31100162        |
| 53 | 18.6 | 0.564       | 267  | 67.9      | 18975       | 6289          | 33434       | 48804024        |
| 54 | 18.5 | 0.607       | 264  | 62.2      | 16307       | 5805          | 26208       | 41484036        |
| 55 | 32.9 | 0.644       | 842  | 133.6     | 21693       | 6848          | 34864       | 176194168       |
| 56 | 34.8 | 0.44        | 913  | 140.2     | 24202       | 9096          | 52314       | 213244903       |
| 57 | 22.3 | 0.501       | 380  | 93.3      | 15260       | 4908          | 24602       | 56033088        |
| 58 | 5.1  | 0.517       | 19.8 | 24.0      | 8372        | 167           | 8838        | 1599040         |
| 59 | 20.5 | 0.653       | 325  | 80.6      | 17169       | 6528          | 28278       | 53824558        |
| 60 | 19.8 | 0.366       | 290  | 76.2      | 20691       | 7248          | 38794       | 57933844        |
| 61 | 19.5 | 0.75        | 296  | 71.1      | 15230       | 5455          | 25971       | 43543116        |
| 62 | 18.9 | 0.878       | 280  | 65.2      | 14247       | 4895          | 23162       | 38465735        |
| 63 | 20.1 | 0.711       | 314  | 72.0      | 15433       | 4637          | 25194       | 46763388        |
| 64 | 18.4 | 0.527       | 258  | 68.6      | 21837       | 7863          | 33904       | 54396255        |

## 7 - Basic Image Analysis

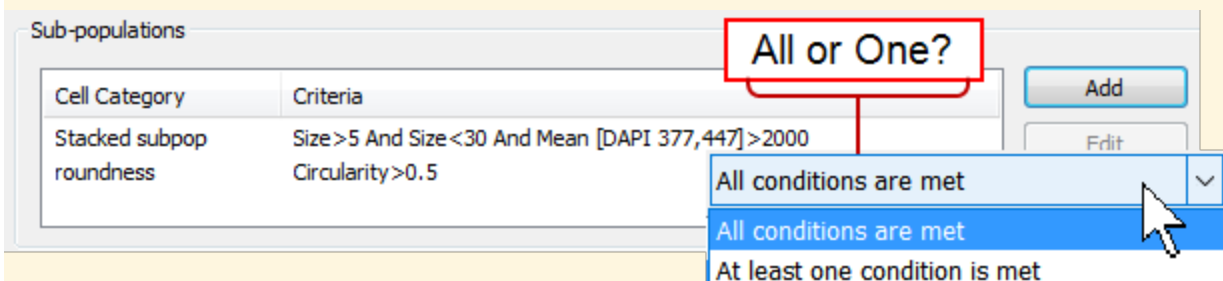


In Cellular Analysis mode, click **Options** (under the Start button), and select the **Subpopulation Analysis** tab. Choose a previously defined population to edit or click Add to create a new one.

1. In the **Object metrics** field, highlight the element to evaluate and click **Add Condition**.
2. In the **Edit Condition** window, use the drop-down to select the function and enter a value or limit; enter whole numbers only with no commas. Click OK.
3. In Cellular Analysis mode, click the **Start** button to rerun analysis and report the results of the condition.

**Important:** Keep in mind the difference between using **Parameters** to apply the nuclear mask to the image (e.g., Threshold) and analyzing subpopulations based on certain conditions found in the analysis results. For example, you can use the Line Profile tool or a Scatter plot to determine an optimal intensity threshold (to ignore objects that are not of interest).

**Stacked Subpopulation Criteria:** When multiple conditions can be used to generate useful results, for example, objects of interest must be a certain size **and/or** within a certain shape, you can Add Conditions for each criterion.

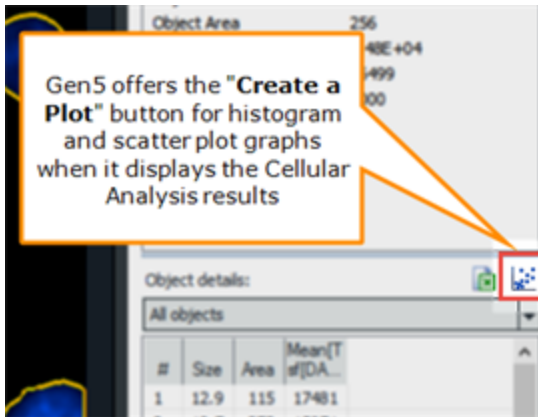


All or One: then you can require objects to meet all or just one condition.

**See also Define a Subpopulation using a Graph on the facing page.**

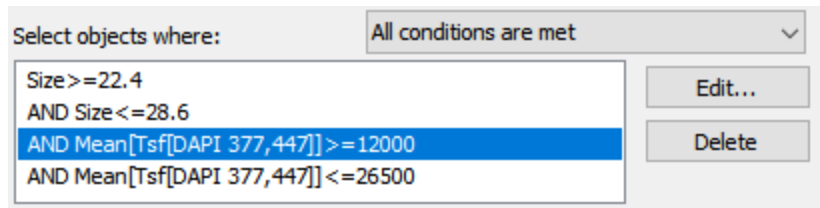
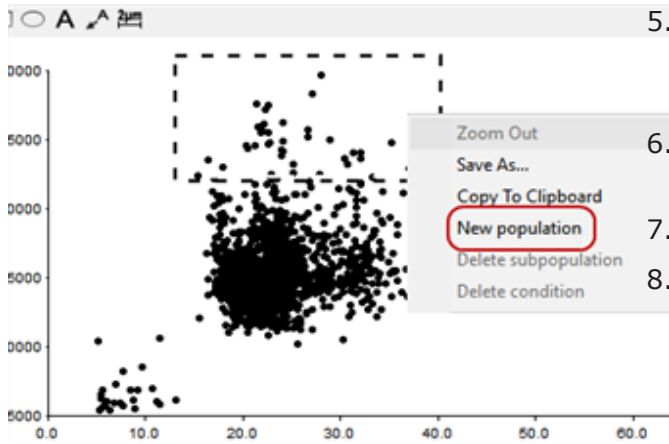
## 7 - Basic Image Analysis

### Define a Subpopulation using a Graph

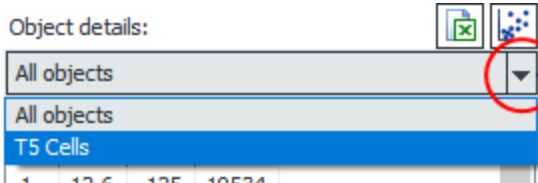


Scatter plots and histogram graphs provide a way to visually define a subpopulation.

1. In Manual Mode, and after image acquisition and processing, proceed to the **Analyze Tool** and select **Cellular Analysis**, then click **Start**.
2. As needed, click **Options** to fine-tune the settings, add a Secondary Mask, etc.
3. Click Create a Plot and choose Histogram or Scatter X, Y plot.
4. Define the **Data** to plot on the X and Y axis and select OK.
5. In the histogram or scatter plot, click and drag to create a box around the points or bars that will define your subpopulation.
6. Select **New population** in the context menu that opens.
7. Assign a unique **Name** for the new population.
8. As needed, choose the All/Or conditions criteria. And, highlight a condition and click Edit to modify its value.



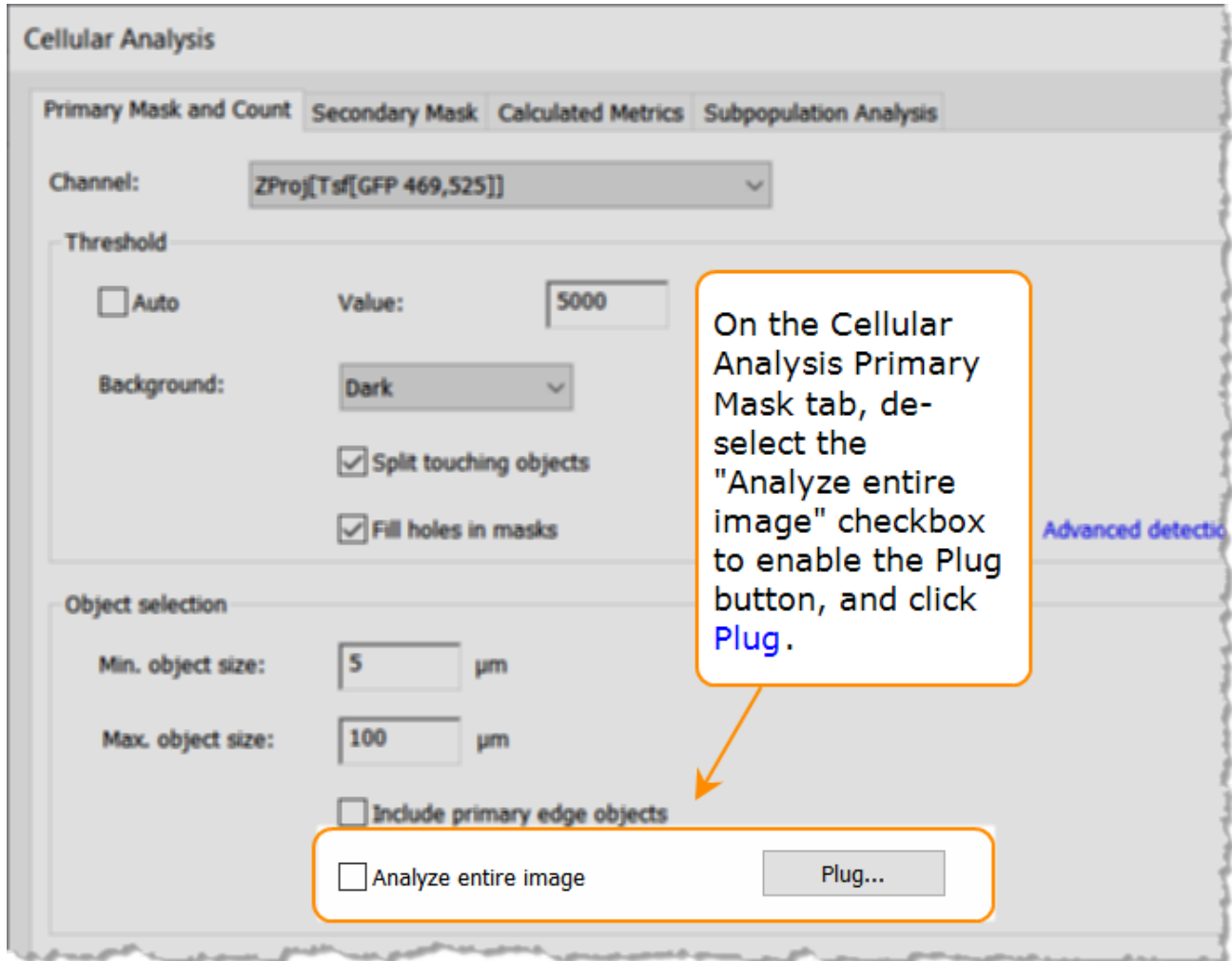
9. Click **OK** to create the subpopulation.



Pick the subpopulation in the Object details table to highlight them in the image.

### 7.5 - Define an Image Plug

For some assays, limiting the analysis region in the image improves the statistical and cellular analysis results. Applying a plug lets you exclude unimportant information. To apply a plug:



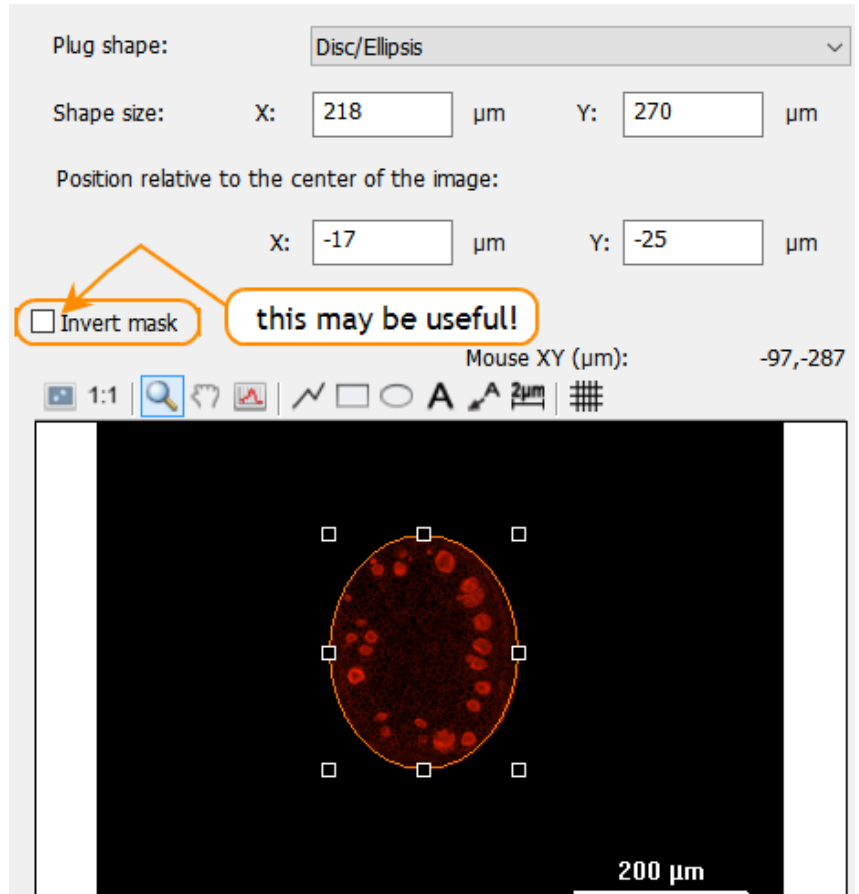
## 7 - Basic Image Analysis

Use the tools to define the plug:

1. First choose the **Plug shape**.
2. Then, draw the area of the plug on the image.
3. Click and drag the plug to resize or move it or use the numeric X and Y values to position and size it.
4. If applicable, you can **Invert** the mask.
5. Or, especially when defining an experiment, **Load a Test Image**.

Note:

- the same plug is applied to all wells in an experiment;
- the plug can be toggled on and off in manual mode;
- using a plug may replace the need to define complex subpopulation filters.





## 7 - Basic Image Analysis

## 8 - Advanced Analysis

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### ***High-Content Analysis with Image Prime***

BioTek's **Gen5 Image Prime** contains specially licensed high-content analysis capabilities, as highlighted in the table below. This section is relevant only to customers who have purchased Image Prime.

Gen5 Image Prime takes image analysis to advanced levels including powerful analysis tools like nuclear mask expansion and reduction; secondary mask for cytoplasm or whole cell identification; custom object-level measurements for translocation and subpopulation analysis. Additionally, a Spot Counting module is available for purchase, i.e., the Spot Counting module requires a unique user license.

|   |            |
|---|------------|
| <b>8.1 - High Content Analysis with Image Prime</b> ..... | <b>171</b> |
| <b>8.2 - Secondary Mask</b> .....                         | <b>176</b> |
| <b>8.3 - Create a Scatter Plot or Histogram</b> .....     | <b>181</b> |
| <b>8.4 - Spot Counting</b> .....                          | <b>185</b> |
| <b>8.5 - Create a Custom Object Metric</b> .....          | <b>190</b> |

## 8 - Advanced Analysis

### Gen5 3.12 Editions Feature Comparison

| Imaging Features/Analysis  | Gen5 | Gen5 Secure/IVD | Gen5 Image+ | Gen5 ImagePrime |
|--|------|-----------------|-------------|-----------------|
| 1.25X to 60X support <sup>1</sup>  | X    | X               | X           | X               |
| Phase contrast <sup>2</sup> (4X to 40X)  | X    | X               | X           | X               |
| Montage  | X    | X               | X           | X               |
| Z-stacking   | X    | X               | X           | X               |
| Z-projection   |      |                 | X           | X               |
| Digital phase contrast   |      |                 | X           | X               |
| Image stitching  |      |                 | X           | X               |
| Cell counting (via primary mask)   | X    | X               | X           | X               |
| Subpopulation Analysis   |      |                 | X           | X               |
| ROI Discovery experiments  |      |                 | X           | X               |
| Hit-picking  |      |                 | X           | X               |
| Imaging and other steps defined for different sections of plate in one procedure |      |                 | X           | X               |
| Image binning to improve focus and acquisition speed                             | X    | X               | X           | X               |
| Custom labware support   | X    | X               | X           | X               |
| Movie maker: live mode recording; kinetic acquisition w/frame alignment          |      |                 | X           | X               |
| Burst-rate acquisition: up to 10 fps at full resolution, 20 fps with binning     |      |                 | X           | X               |
| Cellular analysis and statistics in user-defined plug                            |      |                 | X           | X               |
| Annotations supported in images, graphs, movies                                  |      |                 | X           | X               |
| Advanced Analysis Features:  |      |                 |             |                 |
| Secondary mask to measure cytoplasm/whole cell                                   |      |                 |             | X               |
| Custom object-level metrics  |      |                 |             | X               |

<sup>1</sup>Lionheart FX also supports 60X and 100X oil objectives

<sup>2</sup>except Cytation 1, Cytation 3, Cytation 7

## 8 - Advanced Analysis

| <b>Imaging Features/Analysis</b>                         | <b>Gen5</b> | <b>Gen5<br/>Secure/IVD</b> | <b>Gen5<br/>Image+</b> | <b>Gen5<br/>ImagePrime</b> |
|--|-------------|----------------------------|------------------------|----------------------------|
| Object-level metric graphs: scatter plots and histograms |             |                            |                        | X                          |
| Dynamic thresholding for cellular masks                  |             |                            |                        | X                          |
| Subpopulation analysis using custom metrics              |             |                            |                        | X                          |
| Spot Counting add-on module                              |             |                            |                        | X                          |
| Auto ROI add-on module                                   |             |                            | X                      | X                          |
| # Installations per serial number                        | 5           | 25                         | 2                      | 2                          |

### 8.1 - High Content Analysis with Image Prime



#### Cellular Analysis Tools

1. In the Analyze Tool, select **Cellular Analysis** and choose the Detection Channel, if necessary.
2. Then, click **Options** to customize parameters to best identify or segment the objects of interest in your sample.
3. **Threshold**: Typically, the default "Auto" or dynamic thresholding is most effective at distinguishing items of interest based on their pixel intensity. But, a fixed threshold works better when intensity varies greatly across the image or cell toxicity is a common occurrence in the images.
4. Segment objects of interest based on their size by setting the minimum and maximum object size to best match your analysis goals.
5. Click **Start** to generate results.

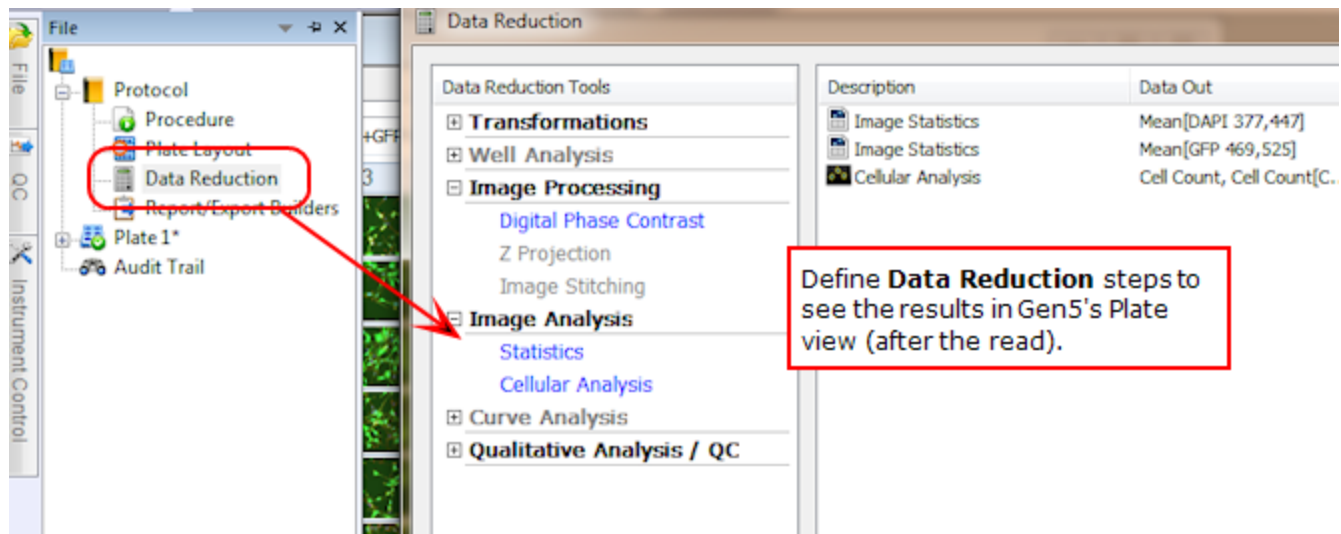
The screenshot displays the Gen5 Image Prime software interface. On the left, the 'COLOR CHANNELS' panel shows two channels: 'Stitched[Tsf[DAPI 377,447]]' (135) and 'Stitched[Tsf[GFP 469,525]]' (481). Below this is the 'ANALYSIS SETTINGS' panel, where 'Cellular Analysis' is selected as the 'Type' and 'Stitched[Tsf[DAPI 377,447]]' is the 'Detection Channel'. A red box highlights the 'START' and 'Options' buttons, with a callout stating 'click **Start** to see results; click **Options** to modify settings'. The main 'Cellular Analysis' dialog box is open, showing the 'Primary Mask and Count' tab. The 'Channel' is set to 'Stitched[Tsf[DAPI 377,447]]'. The 'Threshold' section has 'Auto' checked, with a callout 'Dynamic thresholding'. The 'Background' is set to 'Dark'. Under 'Object selection', 'Min. object size' is 5  $\mu\text{m}$  and 'Max. object size' is 100  $\mu\text{m}$ . A callout 'To better identify objects by size' points to these sliders. The 'Advanced detection options...' button is also visible. A yellow box at the bottom right states 'Gen5 Image Prime options are shown'. The 'START' button is highlighted with a red box, and a callout points to it with the text 'click **Start** to see results; click **Options** to modify settings'. A blue callout 'Advanced detection options...' points to the 'Plug...' button. Another blue callout 'To better identify objects by size' points to the object size sliders. A green callout 'Dynamic thresholding' points to the 'Auto' checkbox. A blue callout 'data in = preprocessed image' points to the channel selection dropdown.

## 8 - Advanced Analysis

💡 After reviewing the results, and using the analysis controls, like the **Line Profile** to determine the optimal threshold and object size values, you can change the parameters and rerun the analysis: click **Start** again to apply a nuclear mask to the image.

- Use the **Advanced detection options** to further identify objects of interest image based on the object size.
-   You can use the quick export buttons to save an image and/or its analysis results to Excel.

**Experiment Mode:** Define Data Reduction steps to perform this analysis in an experiment. Gen5 will report the analysis for all the wells in your sample vessel.



| Description       | Data Out                   |
|-------------------|----------------------------|
| Image Statistics  | Mean[DAPI 377,447]         |
| Image Statistics  | Mean[GFP 469,525]          |
| Cellular Analysis | Cell Count, Cell Count[C.. |

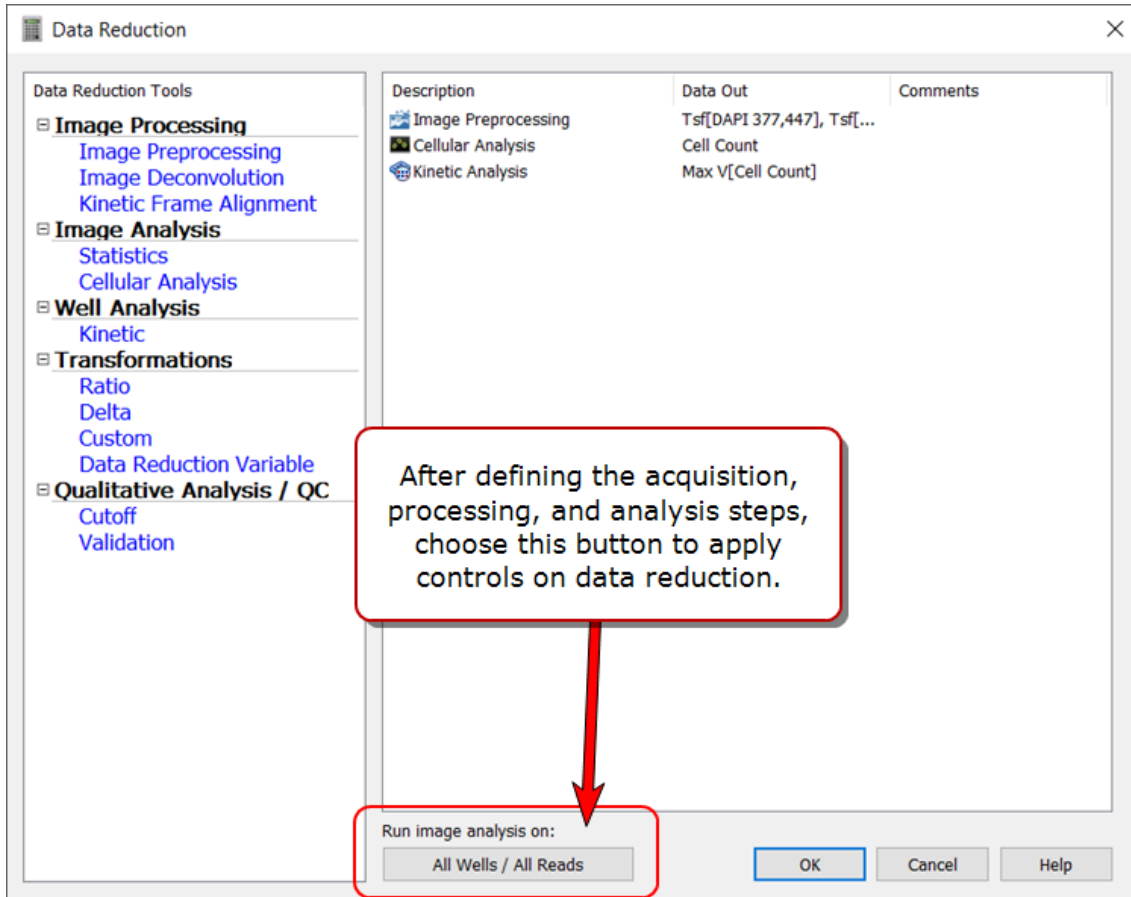
## 8 - Advanced Analysis

### Image Processing and Analysis Optimization Control



Access via button at bottom of Data Reduction dialog

As some imaging experiments can take hours to complete, Gen5 provides these controls to let investigators assess their image processing and analysis settings on a subset of the samples rather than waiting for all the samples to be completed.



High throughput experiments, when using a BioStack, BioSpa or another robot, can also benefit from use of these controls to free up resources for image acquisition. When all images are captured, the control can be released to let Gen5 process and analyze them.

## 8 - Advanced Analysis

Select wells (and kinetic reads) to process and

All  
 None (Image Processing and Analysis OFF)  
 Custom

- Wells:

|   | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A |   |   |   |   |   |   |
| B |   |   |   |   |   |   |
| C |   |   |   |   |   |   |
| D |   |   |   |   |   |   |

In this example, only 2 wells and 2 reads will be processed and analyzed with the current settings.

- Kinetic reads:

All  
 Range


1 to 2

Run image analysis on:

2/24 Wells / Reads 1 to 2

When the controls are set the button label changes to indicate it.

Recommended work flow:

- HTS - for high throughput screening: set to **None** to delay processing and analysis until all images are captured. Remember to reset to **All** when acquisition is completed.
- Kinetic and other complex experiments:
  1. Select **Custom** and choose Wells to evaluate.
  2. For Kinetic runs, select **Range** and set the number of reads to perform.
  3.  Read the plate.
  4. Do a well zoom on a processed well to evaluate the affect of current settings.

| Settings Need Editing  | Settings Are Correct  |
|--|---|
| If processed images do not meet your expectations, use an iterative process to revise the Image Processing and/or Analysis steps, reevaluate and repeat. When the parameters are optimal, release the control: set to <b>All</b> . | If the images are satisfactory, release the control (set to <b>All</b> ) to apply processing and analysis to all samples and reads. |



## 8 - Advanced Analysis

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**Note:** When a Kinetic Frame Alignment step has been defined, the kinetic range cannot be limited.

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### 8.2 - Secondary Mask

#### Gen5 Image Prime required

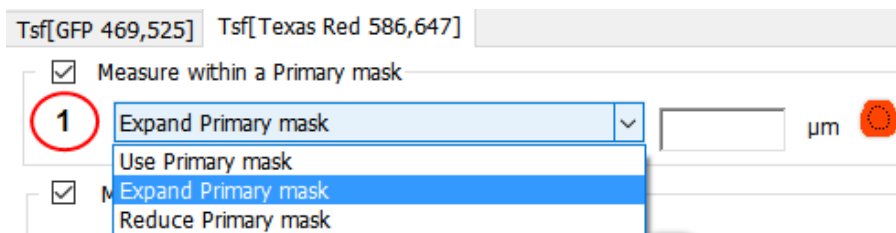
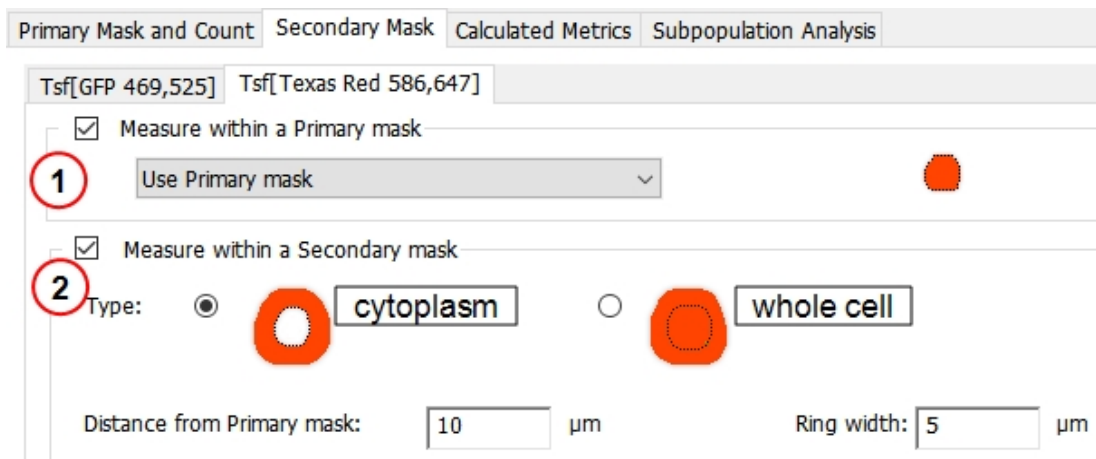
Based on the primary mask, which is typically used to identify the cell nucleus, Gen5 Image Prime lets you define a secondary mask to measure cytoplasm or the whole cell. Some examples of when a secondary mask is useful include:

- Translocation assays: measure signal change between nucleus and cytoplasm
- Cell morphology: measure shape change in cytoplasm or whole cell
- Phenotypic assays: screening strategy for drug discovery

The secondary mask generates distinct data sets, which can be used in concert with those generated by the primary mask: doubling your toolset for complex analysis with subpopulation filters and custom object metrics.

#### Create a Secondary Mask

After defining the primary mask, Gen5 gives you several options for defining the area of the second mask:



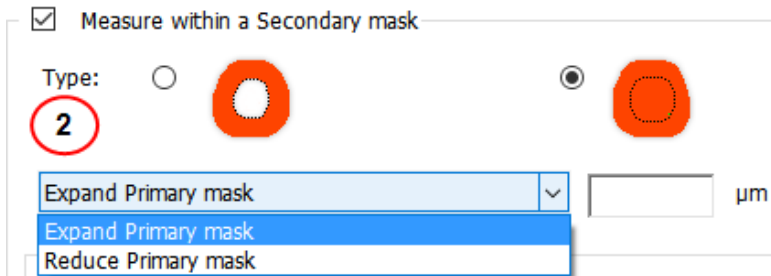
- First, choose to include or exclude the primary mask measurements in your data set, i.e. de-select the checkbox to exclude the primary mask from analysis.

- If included, you can Expand or Reduce the size of the mask, if useful.

## 8 - Advanced Analysis

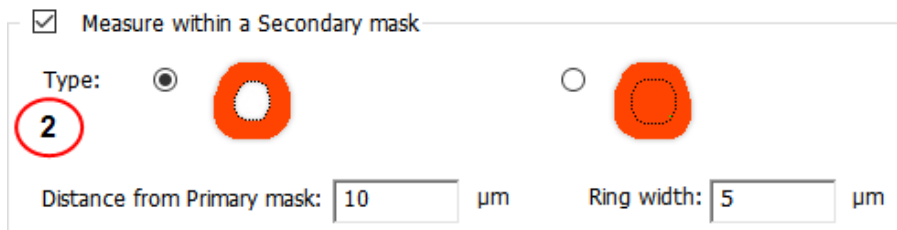
Examples:

- De-select the Primary mask measure to exclude the DAPI signal and measure GFP only in whole-cell measurements. When measuring changes in cell size, apply a propagate mask threshold.
- Consider **Expanding** the mask to cover the perinuclear region if signal is observed there.
- Conversely, for translocation assays, you may want to **Reduce** the primary mask to prevent double counting the intensity signal in the transition zone around the nucleus.



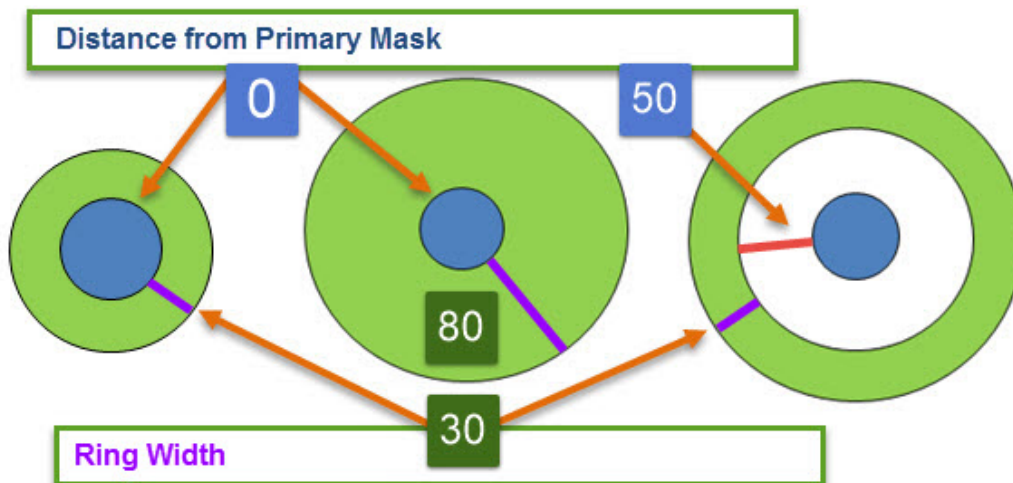
For whole-cell measurements, choose to **Expand** or **Reduce** the size of the primary mask by the specified microns.

💡 Use the line tool to determine the optimal size.



For cytoplasm measurements, specify the size of the secondary mask based on:

- Distance from the Primary mask
- Ring width



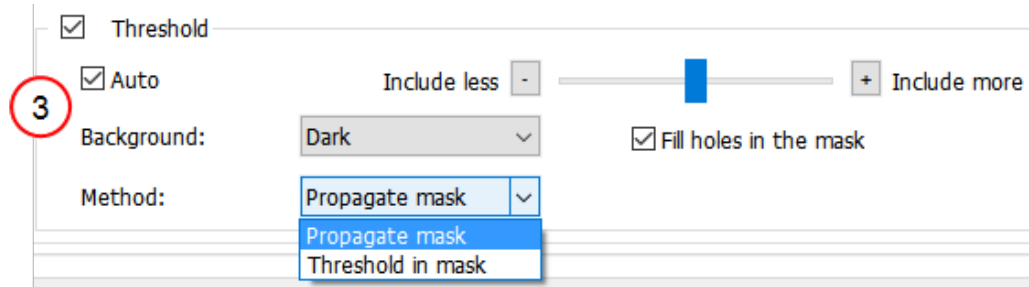
Measurement areas are defined by setting the optimal distance from the nucleus or primary mask and the size of the ring around the nucleus.

Analysis can be restricted to defined ring, or include the whole cell, with or without the signal in the primary mask.

### Secondary Mask Threshold Methods

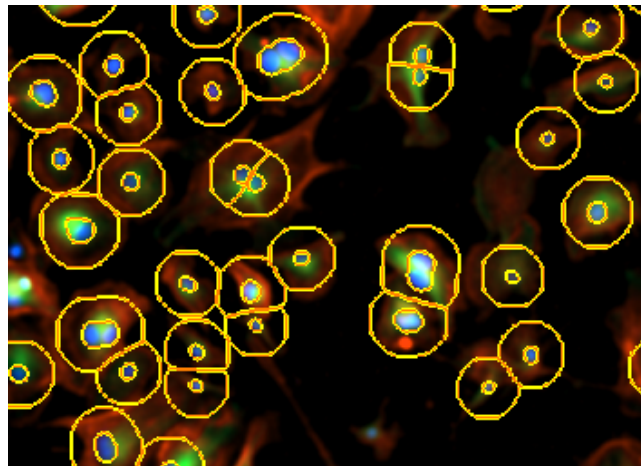
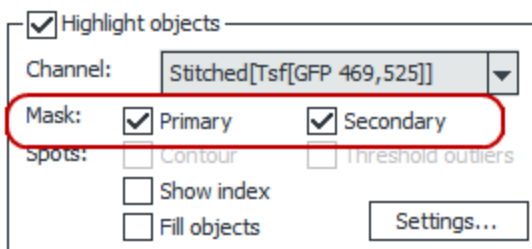
When measuring cytoplasmic intensity is required, applying a threshold improves the results. Two threshold methods are available: Propagate Mask and Threshold in Mask. Both methods use the primary mask as a starting point for the secondary mask, but grow differently.

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| Propagate Mask Method   | Threshold in Mask Method  |
|---|---|
| When the signal is high around the primary mask. Applies threshold <b>as it grows</b> . | When objects are well separated or globally circular. Applies threshold <b>after it grows</b> . |
| To measure variation in shape.  | To measure variation in intensity.  |

### View the Masks On-Screen



After defining a secondary mask, choose the "Channel" and fill the **Mask** checkboxes to view them (in the bottom right corner of the window).

### Gen5 Data Set Names

Naming the resulting data sets:

- Measurements in Channels 2-4 for Primary Mask show measurement and channel. Example:
  - Object Mean (GFP)
  - Object Mean (Texas Red)
- Measurements in Channels 2-4 for Secondary Mask show measurement\_2 and channel. Examples:
  - Object Mean\_2 (GFP)
  - Object Mean\_2 (Texas Red)


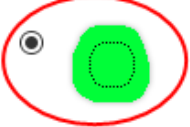
**Note:** Data sets produced by Image Preprocessing, e.g. flattened background, have a **Tsf** prefix (short for transformation).

### Secondary Mask Options

How to apply a secondary mask depends on the measurement goals; here are some examples:

#### Whole cell mask with threshold

Measure within a Secondary mask

Type:    

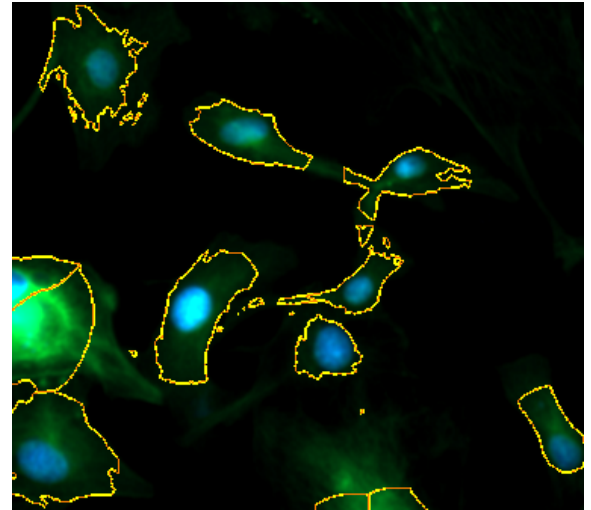
Expand Primary mask:   $\mu\text{m}$

Threshold

Auto      Include less

Background:        Fill holes in the mask



Method:



Application: whole cell signaling; measuring cell area; confluence; cytoskeletal changes.

#### Cytoplasm and nuclear masks with threshold

Measure within a Secondary mask

Type:    

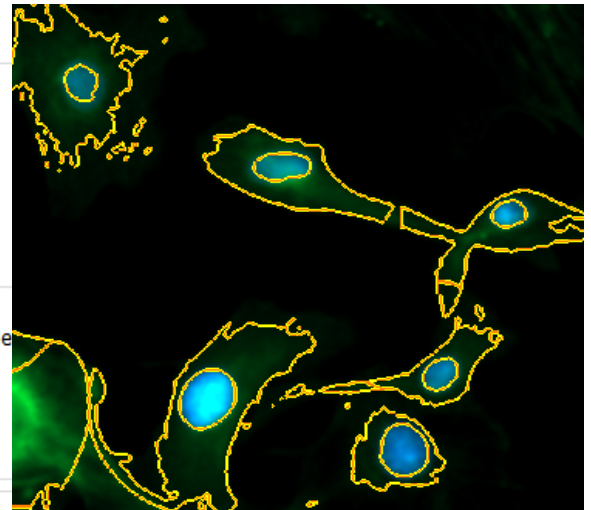
Distance from Primary mask:   $\mu\text{m}$       Ring width:   $\mu\text{m}$

Threshold

Auto      Include less       + Include

Background:        Fill holes in the mask

Method:



Application: cell shape changes like hypertrophy, cytoskeletal changes.

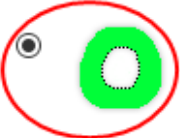

## 8 - Advanced Analysis

### Excluding the perinuclear area, measuring cytoplasm and nucleus (without threshold)

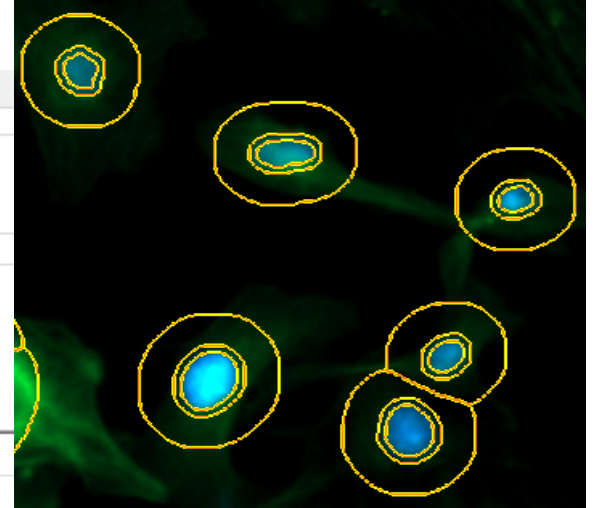
Tsf[GFP 469,525]

Measure within a Primary mask  
Use Primary mask

Measure within a Secondary mask

Type:    

Distance from Primary mask:   $\mu\text{m}$  Ring width:





Application: translocation. No threshold is applied because when the signal moves (translocates) the mask would disappear.

### Excluding the perinuclear area, measuring cytoplasm and nucleus (with threshold)

Measure within a Primary mask  
Use Primary mask

Measure within a Secondary mask

Type:    

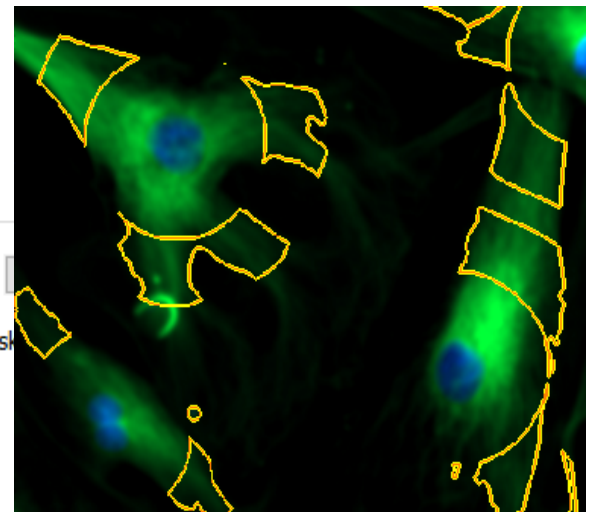
Distance from Primary mask:   $\mu\text{m}$  Ring width:

Threshold

Auto Include less

Background:   Fill holes in the mask

Method:



Application: HCS

💡 Experiment with various settings and values, evaluating the differences by viewing the effects to determine the optimal parameters for your assay.

### 8.3 - Create a Scatter Plot or Histogram

Gen5 Image Prime required



A scatter plot or histogram can quickly inform you about trends in your assays. These graphs can be very useful for:

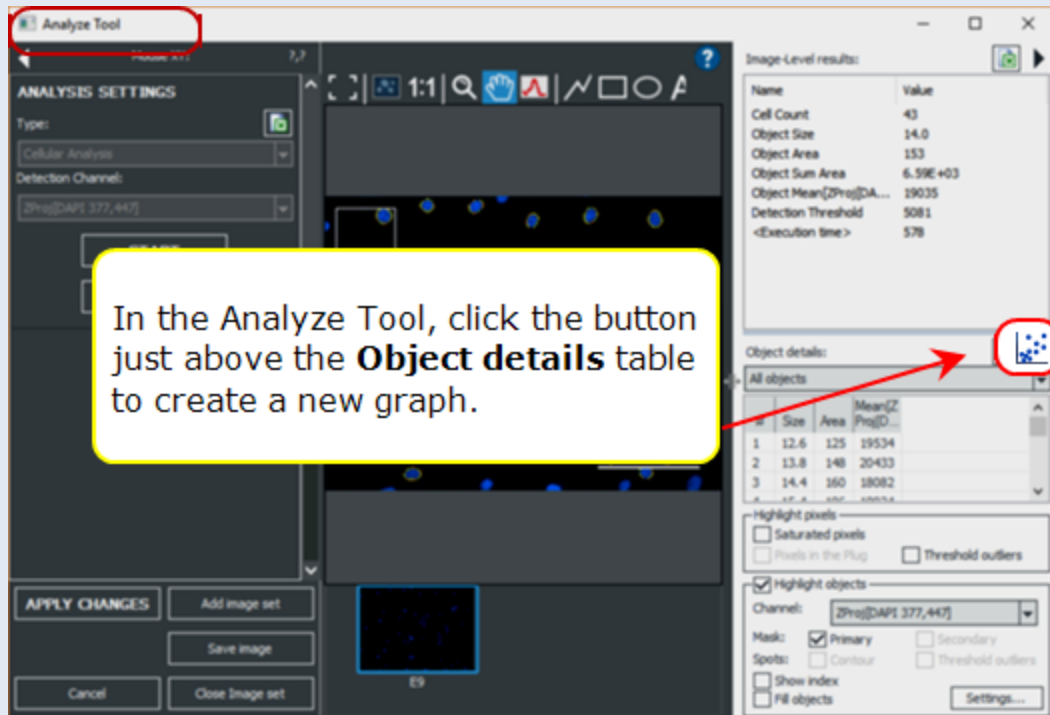
- assessing the quality of the assay (e.g. too many outliers?) and how to optimize it;
- identifying cutoff values to use to filter subpopulations;
- identifying maximum and minimum values, etc.

## 8 - Advanced Analysis

### Multiple ways to create a scatter plot or histogram:

#### Manual Mode

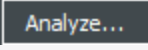


After an initial round of **Cellular Analysis**, in the "Object details" pane click 



See also **Cellular Analysis: Manage Object-Level Metrics** on page 195.

#### Experiment Mode

Two methods:

1. Drill into a well in the plate matrix(well zoom), click , create or edit an Image Analysis step, in the "Object details" pane click  (similar to Manual Mode).
2.  In a **Cellular Analysis** Data Reduction step, select **Calculated Metrics**:



## 8 - Advanced Analysis

**Experiment Mode**

Secondary Mask **Calculated Metrics** Subpopulation Analysis

Results to calculate: All None

| Data            | Name         | Format      | Color Effect |
|-----------------|--------------|-------------|--------------|
| Cell Count      | <Cell Count> | <Decimal,0> | <Blue scale> |
| Object Size     |              |             |              |
| Object Area     |              |             |              |
| Object Sum Area |              |             |              |
| Object Metrics  |              |             |              |

3. Click the link at the bottom of **Calculated Metrics** tab, then select "**Object Metrics of Interest**," and click **Manage Graphs**.

Select or create object-level metrics of interest...

1. Select **Histogram** or **Scatter Plot**. A dialog opens, in which you can define various settings for the graph.
2. Define the x-axis settings:
  - a. Select data to display from the list. Any object-level metric displayed in the object details pane is available.
  - b. Define the granularity of the histogram by setting the range. The range must be greater than zero.
3. For histograms, the y-axis displays the cell count percentage for the selected metric; you can choose the data sets for a scatter plot.
  - a. Using an Auto range means Gen5 sets the y-axis values from 0 to 100% of the population. If your distribution is large, you can decrease the range so that the highest value is closer to the highest end of the range.



[Edit Graph](#) When viewing a previously defined scatter plot or histogram, click **Edit Graph** to alter its appearance.

## 8 - Advanced Analysis

Range

Auto  
 Fixed

Min:  Max:

Scale:  Linear  Log


Scatter Plots and Histograms (Y-axis) can be scaled to Log (or Linear). Data is not transformed, only displayed according to selection.

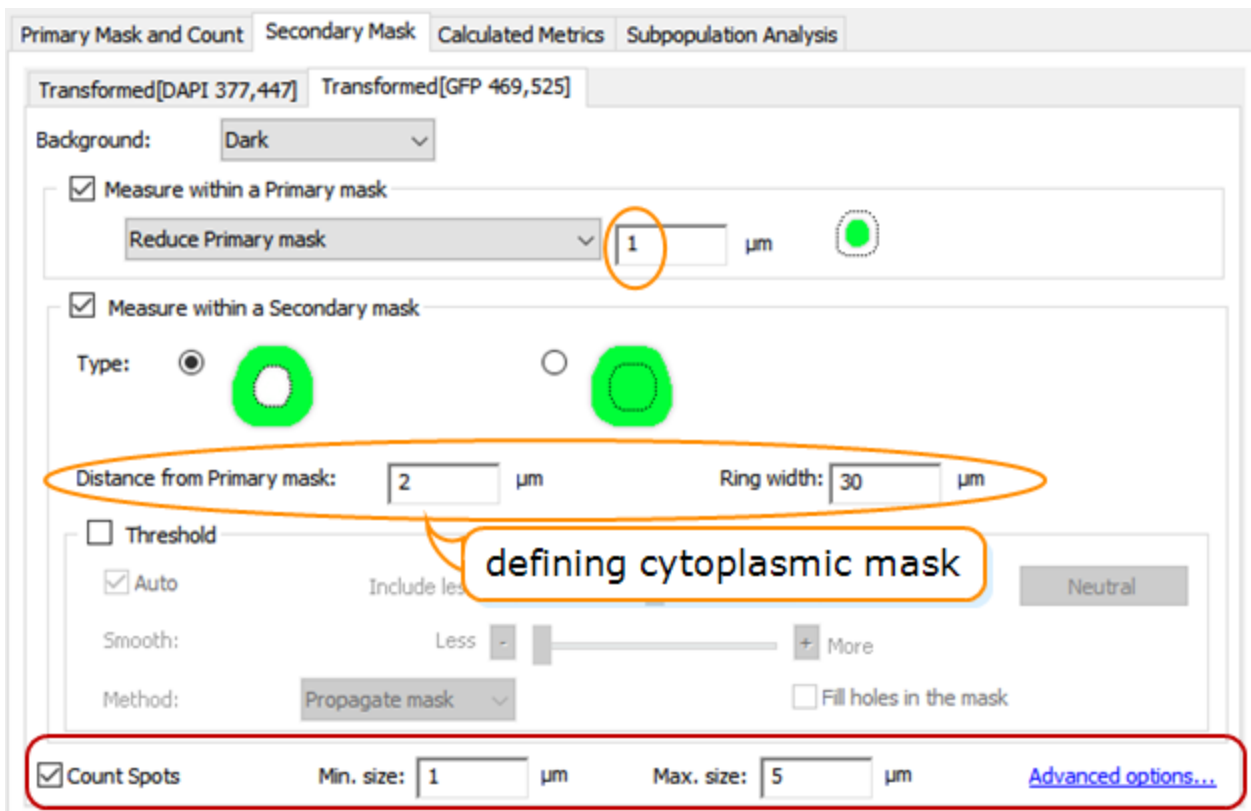
## 8 - Advanced Analysis

### 8.4 - Spot Counting

Gen5 Image Prime "Spot Counting" add-on module required

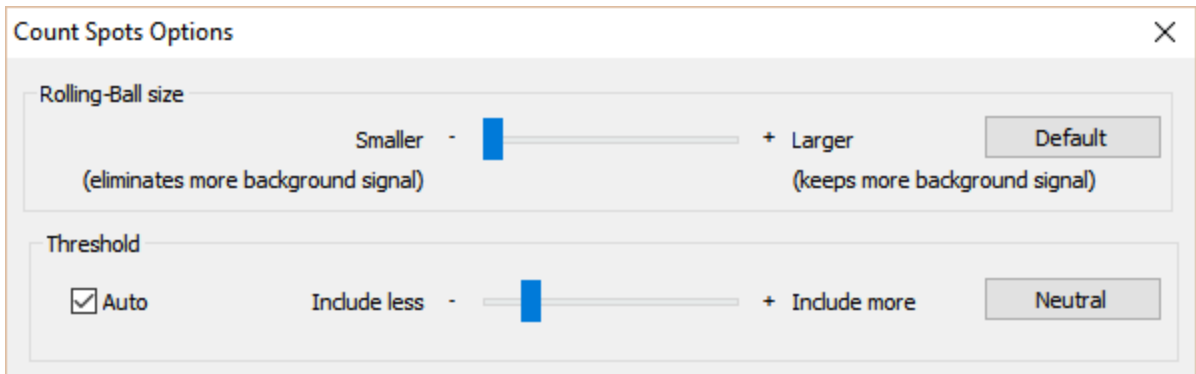
For assays that require measurements of intracellular small objects ("spots") like steatosis, autophagosomes, liposomes, micronuclei, viral infection, etc. Review the list of available measurements: Spot Counting Calculations on page 189.

**Best Practice:** After applying a Primary or Secondary mask, view a representative well and use the  Line Tool to determine likely spot size.



1. Fill the **Count Spots** checkbox.
2. Set the Minimum and Maximum size values to ignore spots outside of the expected range.
3. Click **Advanced options** to refine the image for spot counting:

## 8 - Advanced Analysis



Optimal settings for the rolling-ball size and threshold depend on the sample under study, but you can experiment with different values, evaluate the effect, and fine-tune them until the best results are achieved.

**Note:** Spot Counting is available only when you have defined either a Primary or Secondary mask.

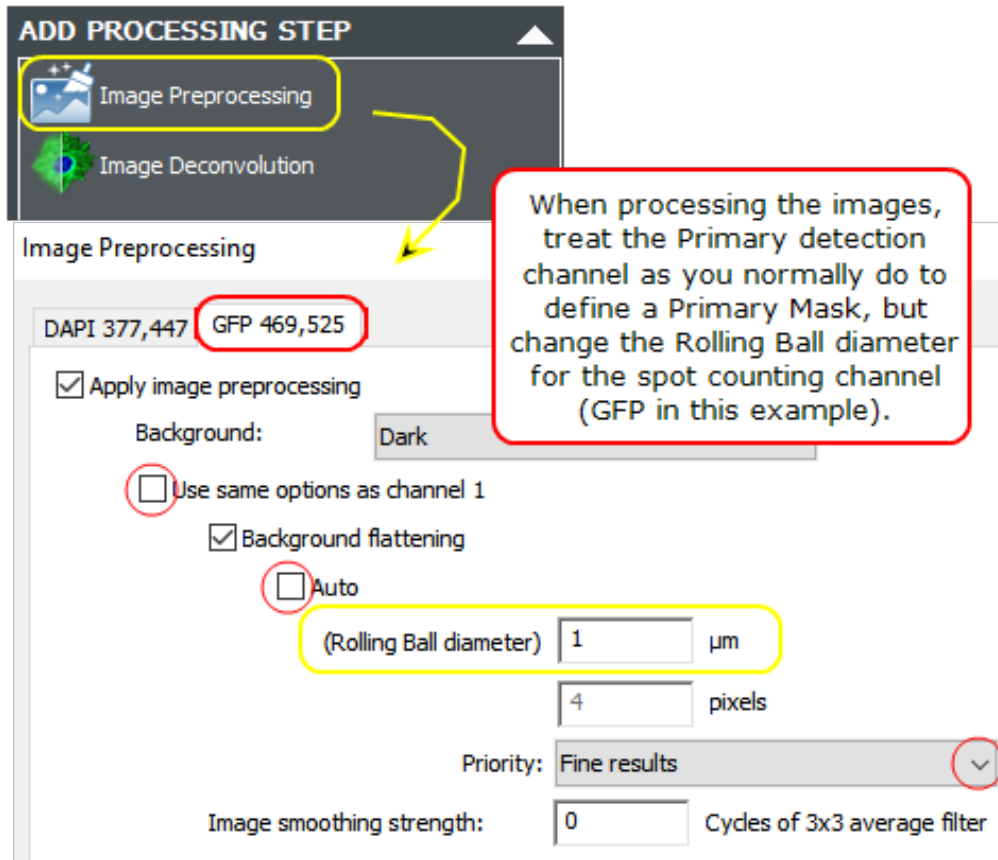
### Spot Counting Tips

**Prerequisite:** The Spot Counting module must have been purchased and its special license registered with BioTek.

Enable spot counting and define its parameters on the **Secondary Mask** tab of Cellular Analysis as described earlier. The following suggestions may improve your results.

While your sample may dictate different settings, often a good starting point for spot counting is **aggressive preprocessing** to remove as much background as possible, making the spots easier to detect. (You can edit an **Image Preprocessing** step that has already been defined.)

## 8 - Advanced Analysis



ADD PROCESSING STEP

Image Preprocessing

Image Deconvolution

Image Preprocessing

DAPI 377,447 GFP 469,525

Apply image preprocessing

Background: Dark

Use same options as channel 1

Background flattening

Auto

(Rolling Ball diameter) 1 μm


4 pixels

Priority: Fine results

Image smoothing strength: 0 Cycles of 3x3 average filter

When processing the images, treat the Primary detection channel as you normally do to define a Primary Mask, but change the Rolling Ball diameter for the spot counting channel (GFP in this example).

1. Open **Image Preprocessing**.
2. Select the secondary channel tab (GFP in this example) and de-select "use same options as channel 1."
3. De-select **Auto** and set the **Rolling ball diameter** to **1 μm**.
4. Change **Priority** to **Fine Results**.

 **Primary Channel:** Keep default or apply preprocessing settings for the primary channel as you normally do for identifying nuclei, counting cells, etc. Aggressive background flattening only needs to be done on the spot counting channel.

## 8 - Advanced Analysis

Count Spots Options

**Advanced Options...**

Rolling-Ball size

Smaller - [Slider] + Larger  
(eliminates more background signal) (keeps mo

Threshold

Auto Value:

Experiment with different values to determine the best settings for your samples. Small adjustments to the rolling-ball size and threshold can make a significant difference in spot counting.

Tsf[API 377,447]

Tsf[GFP 469,525]

De-select a channel to gain more control in the **Highlight objects** panel

Highlight objects

Channel: Tsf[GFP 469,525]

Mask:  Primary  Secondary

Spots:  Contour  Threshold outliers

Show index  
 Fill objects

Settings...

- After performing the calculations, evaluate them in the **Object Results** table. Modify the analysis settings, experimenting with different values to determine which give you the best results.
- To view the spots, use the controls in the **Highlight objects** panel (under the **Object Results** table). View one channel at a time to enable all of the controls.

### Spot Counting Calculations

Gen5 supports the following object-level metrics. The **highlighted** items are selected and calculated by default. On the Calculated Metrics tab, you can choose additional metrics to calculate and report:

Dimension measurements:

- **Size**
- **Area**
- Perimeter
- Circularity

Gen5 calculates these metrics using the same formulas as [Image Analysis - Data Reduction Definitions](#).

**Primary mask intensity measurements:**

- **Area[<Channel>]**
- **Mean[<Channel>]**
- StdDev[<Channel>]
- Peak[Channel]
- Integral[Channel]
- **Spots Count[Channel]**
- Spots Size[Channel]
- Spots Area[Channel]
- Spots Sum Area[Channel]
- Spots Mean[Channel]
- Spots StdDev[Channel]
- Spots Int[Channel]
- Spots Sum Int[Channel]

**Secondary mask intensity measurements:**

- **Area\_2[<Channel>]**
- **Mean\_2[<Channel>]**
- StdDev\_2[<Channel>]
- Peak\_2[Channel]
- Integral\_2[Channel]
- **Spots Count\_2[Channel]**
- Spots Size\_2 [Channel]
- Spots Area\_2 [Channel]
- Spots Sum Area\_2 [Channel]
- Spots Mean\_2 [Channel]
- Spots StdDev\_2 [Channel]
- Spots Int\_2 [Channel]
- Spots Sum Int\_2 [Channel]

## 8 - Advanced Analysis

### 8.5 - Create a Custom Object Metric

*Gen5 Image Prime required*

Gen5 lets you create your own "custom object metric" to measure cellular activity that is not currently calculated in the default results. Similar to other Gen5 data reduction operations, you choose the data sets and apply a formula to calculate the desired results.


Primary Mask and Count | Secondary Mask | **Calculated Metrics** | Subpopulation Analysis

Select the well-level results to calculate: All None

| Calculate                           | Show                                | Data                         | Name                  | Format       | Color Effect |
|-------------------------------------|-------------------------------------|------------------------------|-----------------------|--------------|--------------|
| <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | Cell Count                   | <Cell Count>          | <Decimal,0>  | <Blue scale> |
| <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | Object Size                  | <Object Size>         | <Decimal,1>  | <Blue scale> |
| <input type="checkbox"/>            | <input type="checkbox"/>            | Object Area                  |                       |              |              |
| <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | Object Sum Area              | <Object Sum Area>     | <Best Fit,3> | <Blue scale> |
| <input type="checkbox"/>            | <input type="checkbox"/>            | Object Perimeter             |                       |              |              |
| <input type="checkbox"/>            | <input type="checkbox"/>            | Object Circularity           |                       |              |              |
| <input type="checkbox"/>            | <input type="checkbox"/>            | Object Mean[Bright Field]    |                       |              |              |
| <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | Object StdDev[Bright Field]  | <Object StdDev[Brigh  | <Decimal,0>  | <Blue scale> |
| <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | Object Peak[Bright Field]    | <Object Peak[Bright F | <Decimal,0>  | <Blue scale> |
| <input type="checkbox"/>            | <input type="checkbox"/>            | Object Int[Bright Field]     |                       |              |              |
| <input type="checkbox"/>            | <input type="checkbox"/>            | Object Sum Int[Bright Field] |                       |              |              |
| <input type="checkbox"/>            | <input type="checkbox"/>            | Object Area[GFP 469,525]     |                       |              |              |

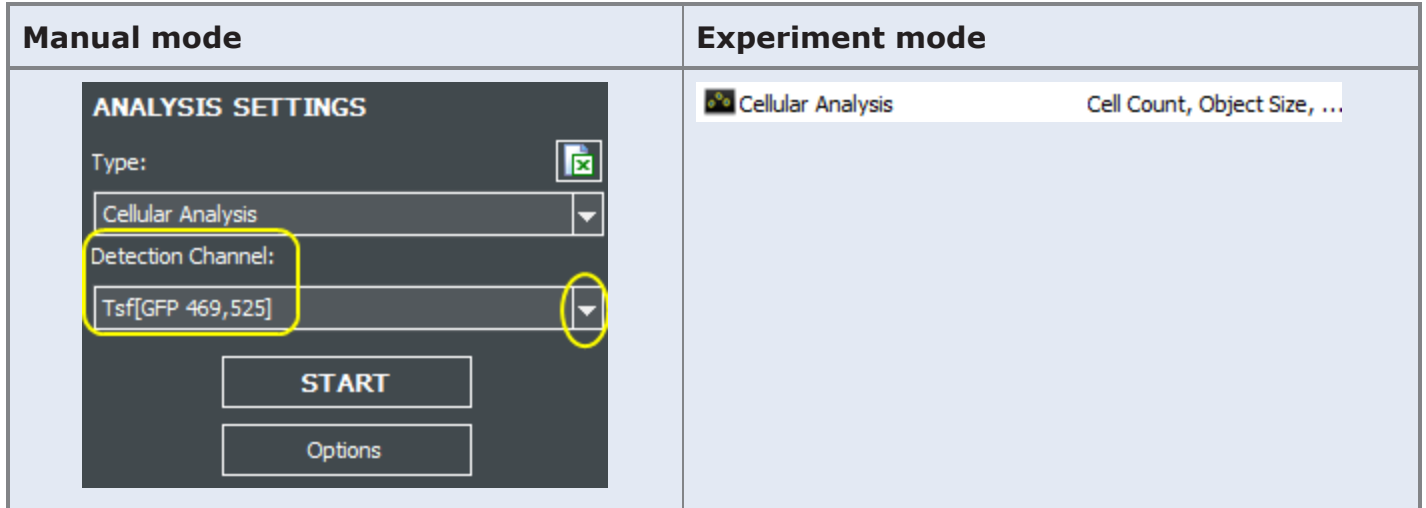
Select or create object-level metrics of interest...


Open the **Cellular Analysis** options in either manual or experiment mode:

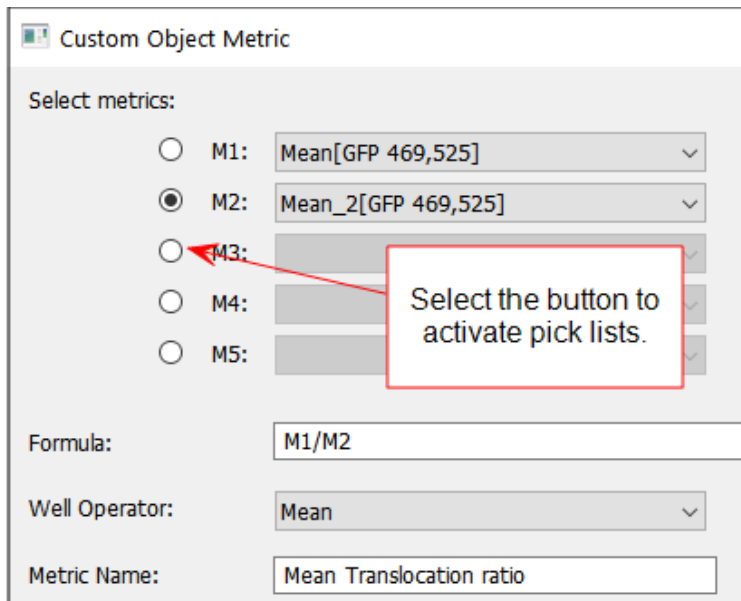
| Manual mode   | Experiment mode  |
|---|--|
| <ol style="list-style-type: none"> <li>After acquiring an image, add a <b>Cellular Analysis</b> step.</li> <li>Click <b>Options</b>.</li> </ol> |  <b>Data Reduction&gt;Cellular Analysis</b> |



## 8 - Advanced Analysis



1. Select the **Calculated Metrics** tab.
2. Click the "**Select or create object-level metrics of interest**" link
3. Click .
4. Select up to five variables to use in your custom measurement:



5. Write a formula using the tokens, e.g. M1, that represent each variable and pick a Well Operator.
6. Give the new custom object metric a unique name.

You can include the custom metric in reports and use it to define subpopulations and other data reduction steps.

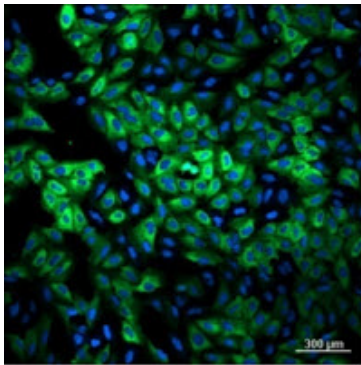
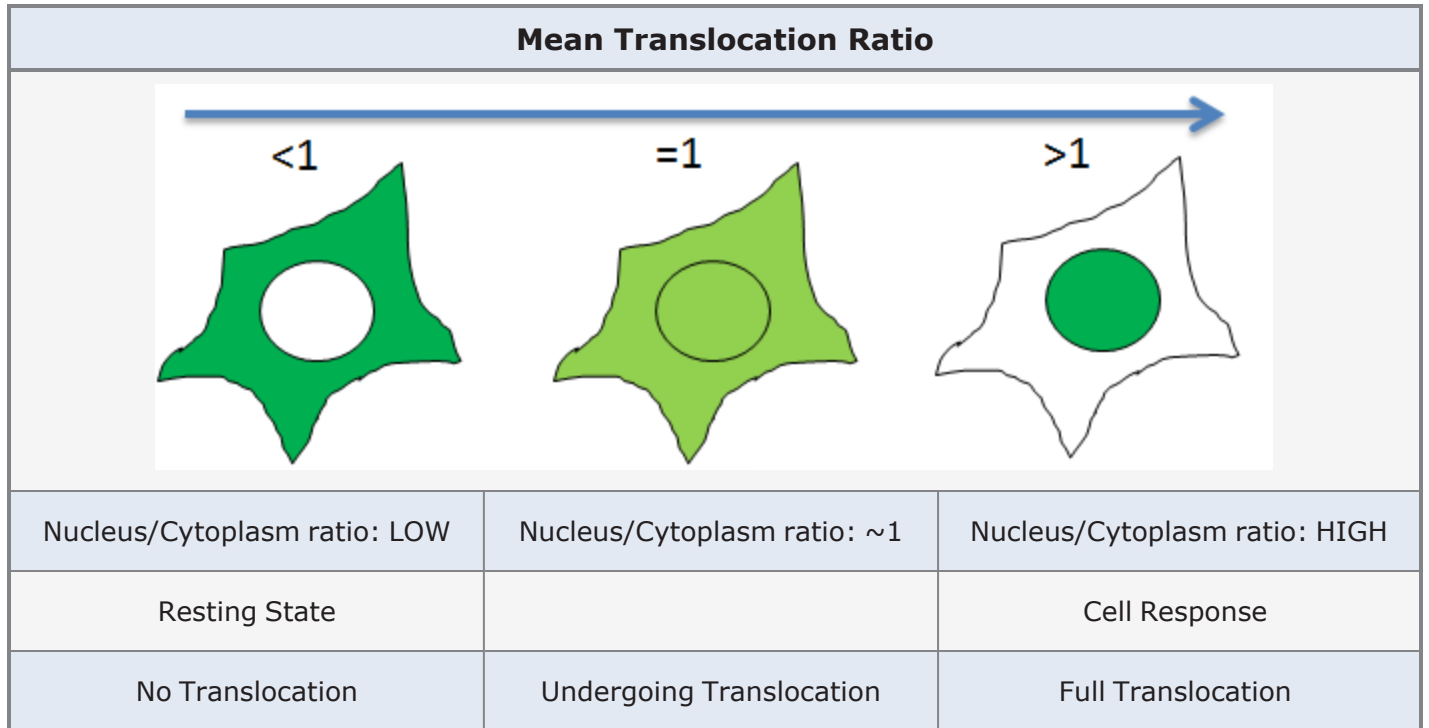
See Custom Object Metric: Example Application on the facing page.

## 8 - Advanced Analysis

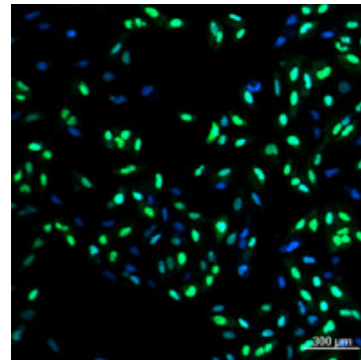
### Custom Object Metric: Example Application

*Gen5 Image Prime required*

Measuring nuclear translocation is commonly used to determine cellular response to a compound of interest. In this example, we'll create a custom object metric and apply it in a Subpopulation Analysis. Our custom object metric will be a measure of the ratio between cytoplasm and nuclear signal. A single value shows how signal is moving in both compartments.



Resting state: Before translocation cytoplasm is clearly visible



Translocation: after translocation, cytoplasm has moved into nucleus

After an "Image Preprocessing" step (called 'Transformed GFP#' in this example) to reduce the background and improve the images for analysis, we will use the Cellular Analysis tools to create a custom metric: Mean Translocation ratio.

## 8 - Advanced Analysis

Primary Mask and Count Secondary Mask Calculated Metrics Subpopulat

Channel: Transformed[DAPI 377,447]

Threshold

Auto Value: 5000

Background: Dark

Split touching objects

Fill holes in masks

1

Apply a **Primary Mask** to measure the nucleus.

Primary Mask and Count Secondary Mask Calculated Metrics Subpopulation Analysis

Transformed[GFP 469,525]

Measure within a Primary mask

Reduce Primary mask 1  $\mu\text{m}$

Measure within a Secondary mask

Type:

Distance from Primary mask: 2  $\mu\text{m}$  Ring width: 30  $\mu\text{m}$

Threshold

2

Apply a **Secondary Mask** to measure cytoplasm.

### Custom Object Metric

Select metrics:

- M1: Mean[GFP 469,525]
- M2: Mean\_2[GFP 469,525]
- M3:
- M4:
- M5:

Formula:

M1/M2

Well Operator:

Mean

Metric Name:

Mean Translocation ratio

3

Follow the step-by-step instructions to open Gen5's controls: See 8.5 - Create a Custom Object Metric on page 190.

Choose the primary & secondary data sets :

- Mean[Transformed[GFP#]]
- Mean\_2[Transformed[GFP#]]

for the metric named "Mean Translocation ratio"

## 8 - Advanced Analysis

### Subpopulation

Name:

Object metrics:

- Size
- Mean [DAPI 377,447]
- Mean\_2[GFP 469,525]
- Mean Translocation ratio

Add Condition...

---

Select objects where:

- Mean Translocation ratio > 1

Edit... Delete

4 See Subpopulation Analysis: Result Options on page 162.

Create a subpopulation definition: Mean translocation cells

### Subpopulations

| Cell Category           | Criteria                     |
|-------------------------|------------------------------|
| Mean translocated cells | Mean Translocation ratio > 1 |

Define the criteria to fit your assay.

5 View results in the plate matrix:

| Data: | Mean translocation ratio |       |       |       |      |      |      |      |      |      |      |       |
|-------|--------------------------|-------|-------|-------|------|------|------|------|------|------|------|-------|
|       | 1                        | 2     | 3     | 4     | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12    |
| A     | 0.791                    | 0.769 | 1.08  | 0.828 | 1.28 | 1.79 | 4    | 5.58 | 5.39 | 5.35 | 4.77 | 5.77  |
| B     | 0.671                    | 0.842 | 0.867 | 0.898 | 1.24 | 2.12 | 4.04 | 5.39 | 5    | 5.14 | 5.5  | 5.28  |
| C     | 0.738                    | 0.773 | 0.922 | 0.941 | 1.38 | 2.17 | 4.37 | 5.08 | 5.07 | 4.87 | 5.28 | 5.47  |
| D     | 0.815                    | 0.886 | 0.92  | 0.989 | 1.27 | 2.47 | 4.54 | 5.01 | 4.77 | 5.19 | 4.57 | 5.12  |
| E     | 5.73                     |       |       |       |      |      |      |      |      |      |      | 0.905 |
| F     | 4.98                     |       |       |       |      |      |      |      |      |      |      | 0.869 |
| G     | 4.97                     |       |       |       |      |      |      |      |      |      |      | 0.973 |
| H     | 6.38                     |       |       |       |      |      |      |      |      |      |      | 0.89  |

ratio < 1 = no translocation  
 ratio between 1-3 = transition zone  
 ratio > 4 = full translocation

6 Create additional data reduction steps:

| Description         | Data Out  |
|---------------------|---|
| Image Preprocessing | Transformed[DAPI 377,447], Transformed[GFP 469,525] |
| Cellular Analysis   | Cell Count, Translocation ratio                     |
| Transformation      | % Translocated cells - Mean                         |
| ZPrime              | ZPrime - Mean                                       |
| Dose Response Curve | DRCurve - Mean                                      |

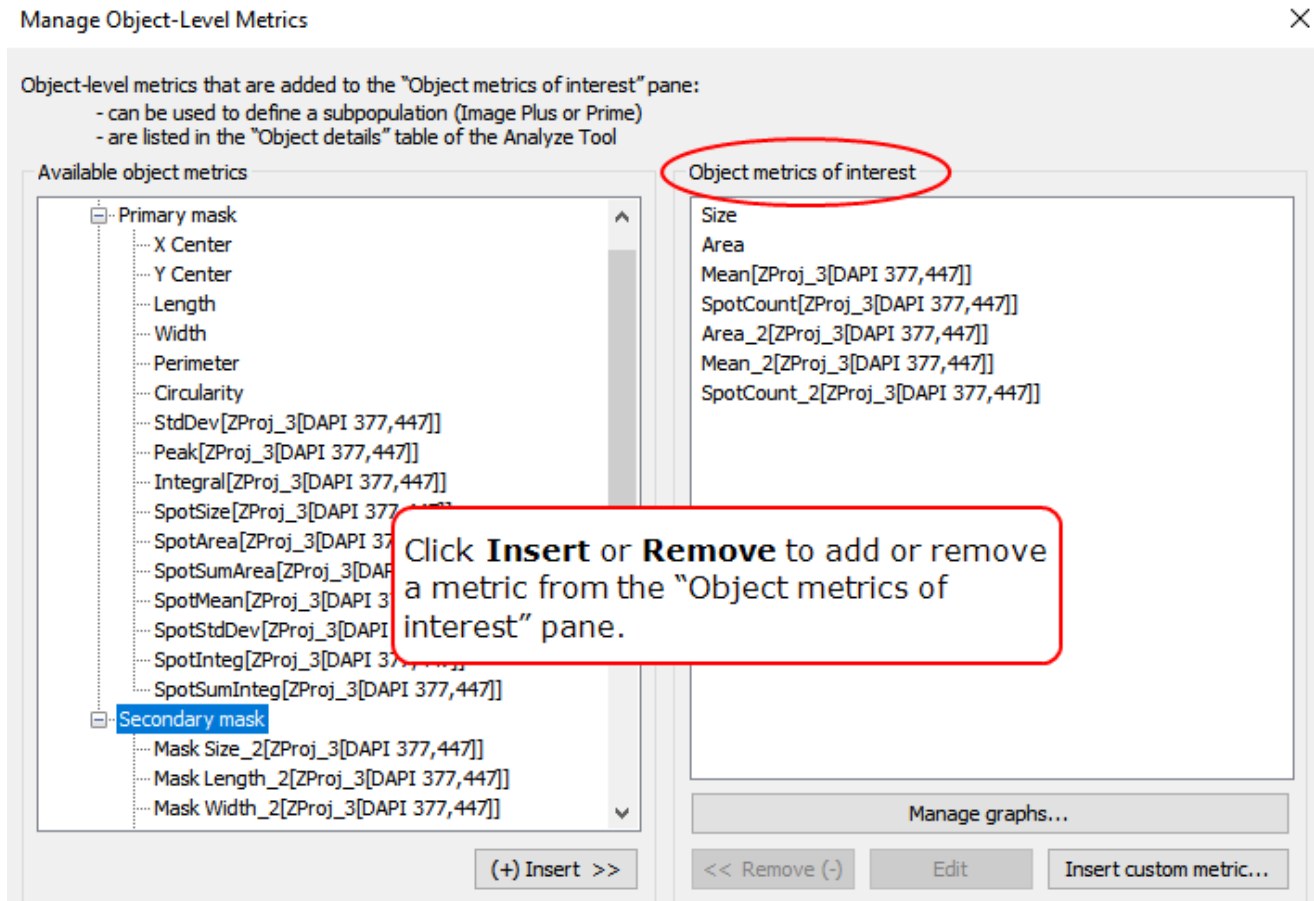
Finally, to create useful reports, charts, and calculations, the "custom object metrics" can be used in additional data reduction steps. In this example, we created a Transformation to use in Z-Prime and curve generation.

## 8 - Advanced Analysis

### Cellular Analysis: Manage Object-Level Metrics

In the Data Reduction window, create a Cellular Analysis step>Select the "Calculated Metrics" tab>Click link at bottom of window.

In the Manage Object-Level Metrics dialog, you can add or remove metrics from your view to refine the results you want to see, and most importantly, to save the data with the experiment. Any object-level metric can be used to generate a well-level metric.



### Create/Edit a Scatter plot or Histogram

**Manage graphs...** Click **Manage graphs** to add a scatter plot or histogram to the experiment data.



In Gen5 Image Prime, the metrics in the "Object metrics of interest" pane are also available in an experiment created from a manual mode capture and can be used to plot a chart or create a table in the online view or report/export file.

**See also 8.5 - Create a Custom Object Metric on page 190.**

## 9 - Saving Files

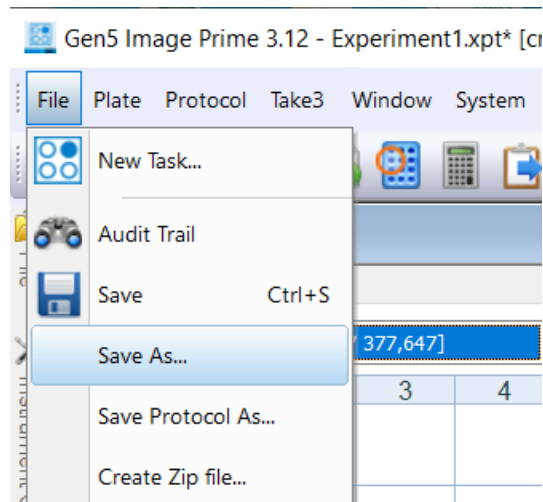
---

### *Protect Your Data*

|   |            |
|---|------------|
| <b>9.1 - Saving Images</b> .....                            | <b>197</b> |
| <b>9.1.1 - Recommendations for Saving Your Images</b> ..... | <b>198</b> |
| <b>9.1.2 - Strategies for Data Management</b> .....         | <b>201</b> |
| <b>9.1.3 - Back End Data Management Tools</b> .....         | <b>203</b> |

### Gen5 Saving Options

Gen5 offers several ways to save images and other file types. Files can be stored locally or on a server/network. A zipping feature compiles the entire content of an experiment file or a manual mode session, including any images, movies, data and analysis into one single file. Zip files are especially useful for sharing and moving experimental files and data.

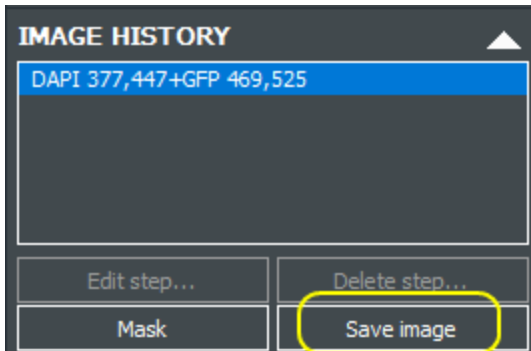


### 9.1 - Saving Images

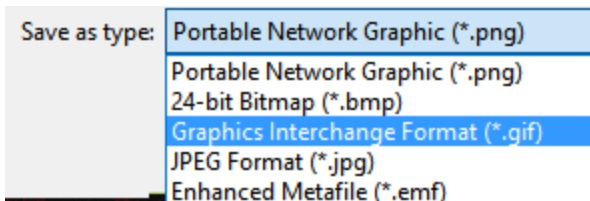
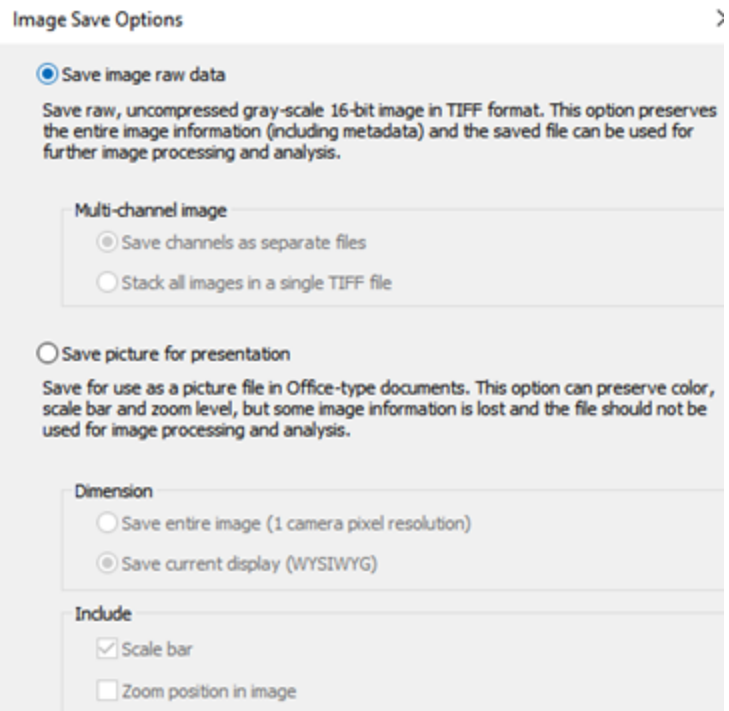
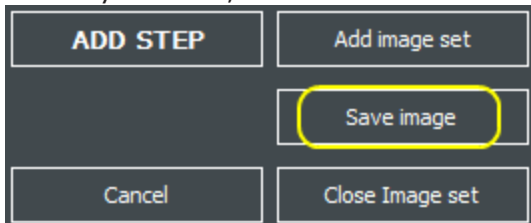
**Note:** Agilent BioTek imagers can create big data sets. Live cell kinetic imaging and large scanning assays are likely to generate data sizes that require management strategies. See 9.1.2 - Strategies for Data Management on page 201 for options to consider employing to reduce image file sizes.

When a Gen5 session is saved, all acquired images are automatically saved in Gen5's Image Library. Raw images are automatically saved as 16-bit TIFFs in native grayscale. TIFF is commonly considered the best file type for saving the highest quality image for use in image analysis software and for re-analysis with Gen5. To save colorized overlays with the scale bar or to re-save your raw data in an alternative format, you can click as indicated below or simply right click the image.

In addition, acquired images can be saved in various file formats by manually invoking the **Save** function.



In Analyze mode, bottom left corner:



- Save Image Raw Data saves the image in **16 bit TIF** format. Multi-channel images can be saved as separate files per image or as stacked images in a single TIF file.
- Save Picture for Presentation saves the image or current display in color, rather than grayscale. The scale bar and zoom position in the image can be included. Several image file options are available, including: TIF, JPEG, PNG, GIF, BMP, and EMF.

### 9.1.1 - Recommendations for Saving Your Images

In addition to 9.1.3 - Back End Data Management Tools on page 203, here are some suggestions to consider for protecting your data.

**Important:** Consult with your IT (information technology) department to design and implement a file storage strategy. Issues to consider include storage location, file-naming conventions, backup frequency, and user access.

#### Agilent Recommendations:

- **Keep All Files Local:** If it is compatible with your work flow and work rules, store all your experiment and image files locally on your computer's hard drive using either the Windows file system or Gen5 database. Make sure the hard drive has the capacity to store all the images, including a 20 GB virtual memory reserve. Consider attaching an auxiliary storage device to your computer to expand its capacity. With your IT department, develop a plan to backup (or archive) and/or share the files by transferring them to a network or external drive (see guidelines below).
- **On a Secure Network Drive:** If you have a reliable system network and multiple users need to access the Gen5 experiment files and images, put them on a shared drive. Use Gen5's database (SharedDB), which is required for compliance with the FDA's 21 CFR Part 11 regulation, to store your experiment and protocol files. And create the Gen5 Image Library on the same network drive as the shared database. (Select **System>Preferences>File Storage** to specify storage method.)


**Important:** If your network is not trouble-free, i.e. the network crashes or errors occur with some regularity, then "Keep All Files Local" on a robust PC. This is the best way to avoid data losses.

- **Turn off some automated features:** In both cases, network or local, turn off the computer's auto-update routines, virus scans, or other power settings that can interrupt a Gen5 experiment. This is especially important when running kinetic or time-elapse assays.
- Follow instructions shipped with the imager re: Gen5's Computer Requirements to "**Expand the Virtual Memory**" to optimize memory paging size.

#### Set Up the Gen5 Image Library

Gen5 provides two ways to define the file naming and location of generated files:

- Global setting: to apply the naming convention and file storage locations to all image files by default, select **System>Preferences>Image Save Options** (under Initial Protocol Settings).
- Per Protocol setting (Protocol>Protocol Options>Image Save Options): to override the global setting, apply distinct save options to image files, or to reconnect an experiment with its images.

 More info: search for "Image Save Options" in Gen5's Help for additional information.

#### File Storage

Image file sizes are significantly larger than other data files.



## 9 - Saving Files

Widefield (standard field-of-view) image files are at least 2 MB each.

A 4x4 montage per well of a 96-well plate will require **3327 MB of storage space**.

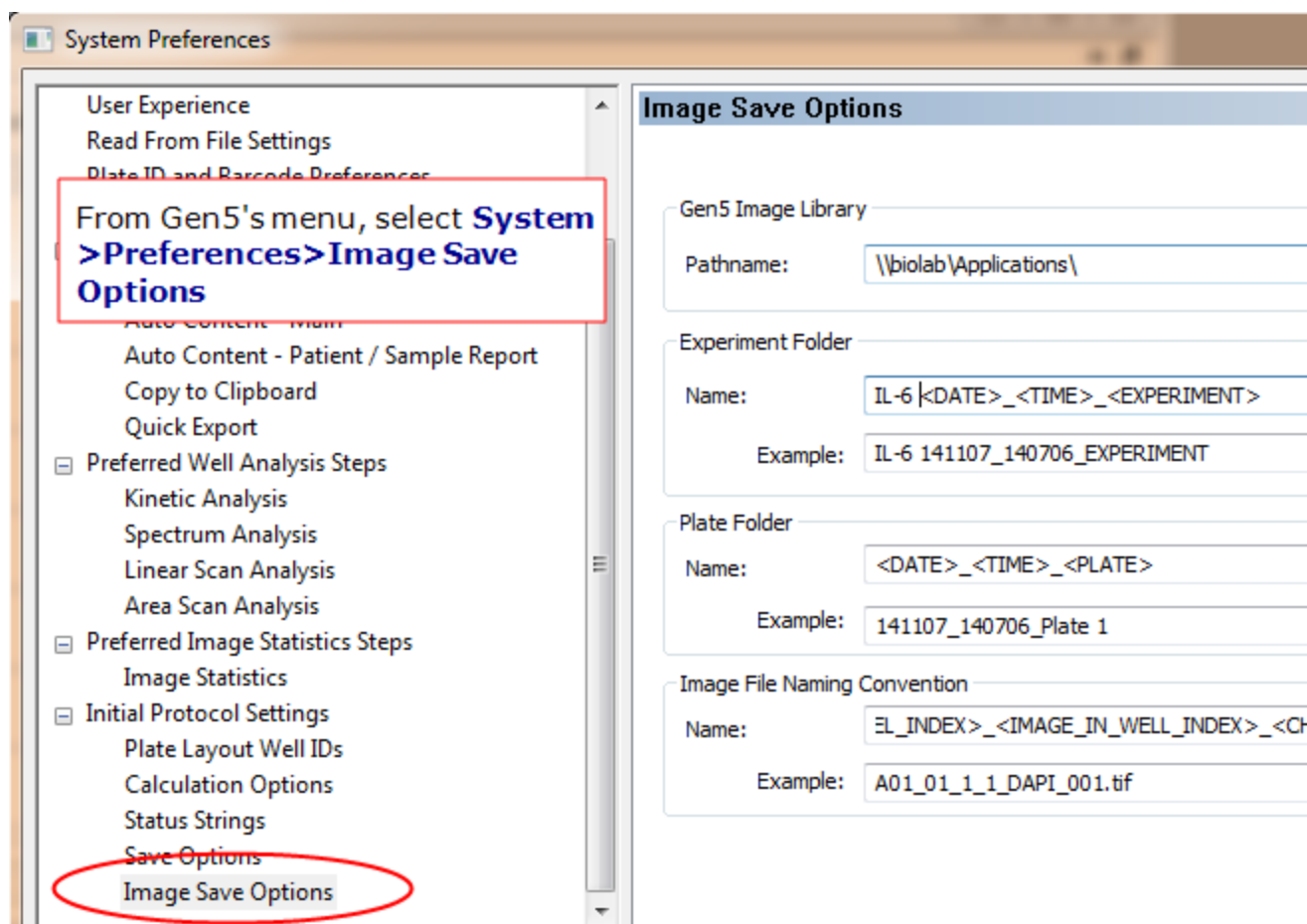
Confocal and Wide Field of View (WFOV) camera imaging create files around 7.57 MB.

A 4x4 montage per well of a 96-well plate will require **11.6 GB of storage space**.

Using an external hard drive or another form of additional storage for images is strongly recommended. However, storing images on a cloud drive is **not** recommended.

### File Management System:

Gen5 stores the image files in a separate folder from the Experiment, Protocol, and Imager Manual Mode (IMM) files. The image file location (Gen5 Image Library) is user defined. The setting is global and all new experiments or manual mode captures will default to this system preference.



Apply global file naming and storage locations to all captured images.

### Determine where Gen5 files will be stored: on a local computer or a secure network?

- Keep files local, if possible
- Except for Gen5 Secure for FDA's 21CFR Part 11 Compliance: use a network

## 9 - Saving Files

### File Storage

Storage option for Experiment and Protocol files:

- Gen5 Database
- Windows File System

Because image files are very large compared to other Gen5 data files, Gen5 handles them differently.

Except for image files, Gen5 experiment files can be stored either in the [Gen5 Database](#) or in the [Windows File System](#).

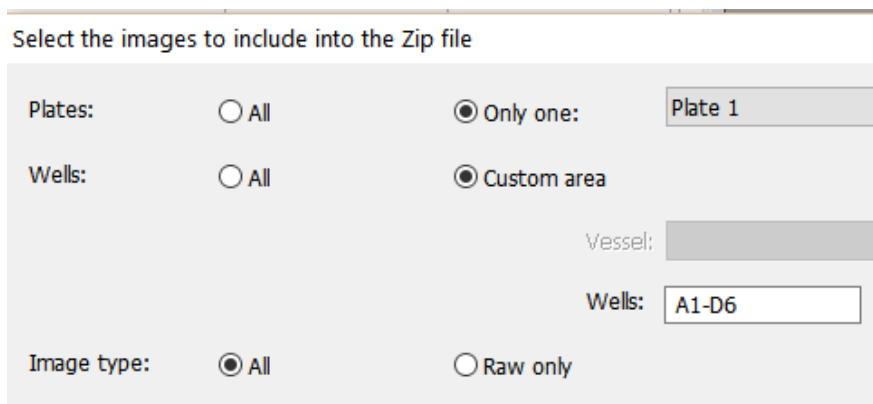
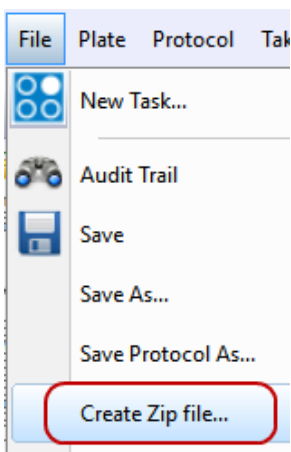
Image files are too big to store in the Gen5 Database. Image files are stored in the **Gen5 Image Library** in a user-specified location in the [Windows File System](#) or on an external hard disk drive.

During the installation of the imager and Gen5 software, the save location for image files was defined because this is required to run the imager. To change the location or determine where the image files are saved, click **System>Preferences>Image Save Options**. This is a global setting for all image files captured in Experiments and Image Manual Mode (IMM) files.

### Image storage and file format options

- .IMM for imaging manual mode session files
- .XPT for Gen5 experiment files
- Image raw data (TIF file) for analysis
- Saving pictures for a presentation
- TIF, JPEG, PNG, GIF, BMP and EMF file formats
- Movie Maker (mp4, wmv)
- Review automated "Image File Naming Convention"
- Review [Save Options: "File Naming Convention"](#)

### Gen5's Zip File Feature: Exporting image files



Select "Create Zip file" from the File menu to neatly package your manual imaging session or experiment. This will zip your images, protocol, and your analysis into one easily transferrable file.

**Note:** You must be in imager manual mode or have the experiment file open to use the Zip feature.

## 9 - Saving Files

**Note:** Experiment files and image files can be moved to other locations. But, never rename the image file folder: renaming the image folder breaks the link to the experiment and Gen5 will not be able to link to it. Image file folders should be a sub-folder under the experiment folder when moved so that the experiment and image folders can automatically relink.

### 9.1.2 - Strategies for Data Management

Here are some suggestions to consider for most efficiently capturing the information you need, with the aim of reducing file storage demands without compromising analysis capabilities. **See also 9.1.3 - Back End Data Management Tools on page 203.**

#### 1. Is stitching necessary?

- a. Full well coverage and objects that spread greater than the field of view (e.g., neuronal projections and large 3D biology) may require stitching montage tiles to a single image. However, often this is not necessary and can double your data size. Typically, 2D cell cultures at lower magnifications (4-20x) do not require a stitched image to analyze each individual image separately.
- b. Opening a montage (negative values) or the using ROI/Beacons sampling tools will enable you to take patterns of images to sample across a well. This will automate the larger sample size, give data on the well level, and not require the processing and storage of large-stitched images.

## 9 - Saving Files

Open Montage with negative values ( $\geq -50 \mu\text{m}$ ):

Imaging Step-Inverted imager

Step Label: <default>

Magnification: 4X PL FL Image: 2017 x 1489  $\mu\text{m}$

Binning:  Autofocus binning  Capture binning (affects exposure)

Channels

Fluorophore: 1 2 3 4 5

Color: DAPI 377,447 GFP 469,525

Exposure:  Auto Wells: 2/96  Auto Wells: A1

Focus options...

Define beacons:  Horizontal offset from center of well: 0  $\mu\text{m}$  Vertical offset from center of well: 0  $\mu\text{m}$

Montage

Z-Stack  Montage

Open the montage by selecting Custom for the Tile Overlap and entering negative values.

Montage (rows x columns): 2 x 2

Tile Overlap

No overlap  Auto for stitching  Custom

Columns: -50  $\mu\text{m}$  Rows: -50  $\mu\text{m}$

Crop image to size of well

Montage entire well

Advanced options... OK

### 2. Is Z-stacking necessary?

- For spheroid work, when there is only one object per round bottom well, an autofocus step can take you to the central focal plane where size can be measured. User-trained autofocus may be the best option:
  - Pros: much less data and faster.
  - Cons: it is possible to lose fluorescent information for probes and expressed fluorophores at different heights in the spheroid.
- If not doing confocal work, do not decrease the recommended z-step size in Gen5 (minimum number of z-slices necessary).

### 9.1.3 - Back End Data Management Tools

Investing in hardware solutions for storing data will give you peace of mind that your data is protected. It is best to set this up before running any analysis. Your organization's IT team should play an integral role in determining, installing and maintaining these systems.

1. **Install a  $\geq$  10Tb hard disk drive (HDD)** for any BioSpa or large ROI scanning system.
  - a. This is an easy solution. Agilent is happy to provide purchasing suggestions.
  - b. The size of the HDD is determined by use and desired time to run sweeps. With heavy usage you are likely to acquire around 5Tb per week.
2. **Large external RAID array** may be the best option for single user or lab. The RAID can be archived when full.
  - For the more common "core type" usage or more secure storage at regulated institutions you will need to sweep this internal storage onto user or institutional networked storage.
3. **Sweeping:**
  - a. This can be done in the cloud (RAID automatically backed up) or to an external HDD.
  - b. Never perform a "Move" files, instead, always "Copy." Then, review and check preferences to be sure the files are exactly the same down to the last byte. This will give you confidence that it is safe to delete the original data.
  - c. A more expensive option that significantly improves the speed of transferring files is a Solid State Drive (SSD).
  - d. The **ideal situation is every experiment and its images are saved on two hard drives, ("simple" RAID):**
    - i. **USB external HDD #1 = Archive hard drive**

Dedicate this HDD for archiving data. Either a zipped experiment with raw data only or the images folder copied over before processing analysis steps. These files will be smaller than analyzed files. When this HDD is combined with the one described below you can feel confident that your data is safe.
    - ii. **USB external HDD #2 = Working hard drive**

Dedicate this HDD for your processed data and for working on analysis. More space will be required to accommodate these bigger files. Assure you have enough space to complete all imaging and data processing. Connect this HDD to your workstation, if applicable, to run processing and analysis steps.

## 10 - Plate Layouts and Reports/Exports

---

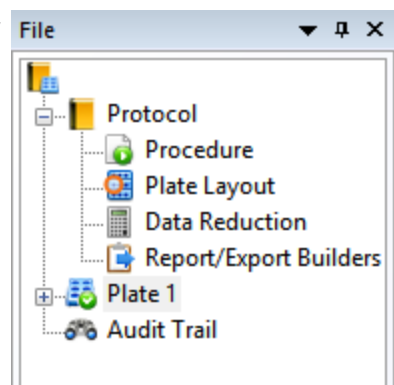
### *Customize Your Output*

|   |            |
|---|------------|
| <b>10.1 - Plate Layout</b> .....            | <b>205</b> |
| <b>10.2 - Reporting and Exporting</b> ..... | <b>207</b> |

### **Gen5 Layouts, Report/Export Builders**


In an experiment, Gen5 lets you create a customized plate layout by defining sample ID's, dilutions, concentrations, standards, controls, replicates and their placement/position on the plate.

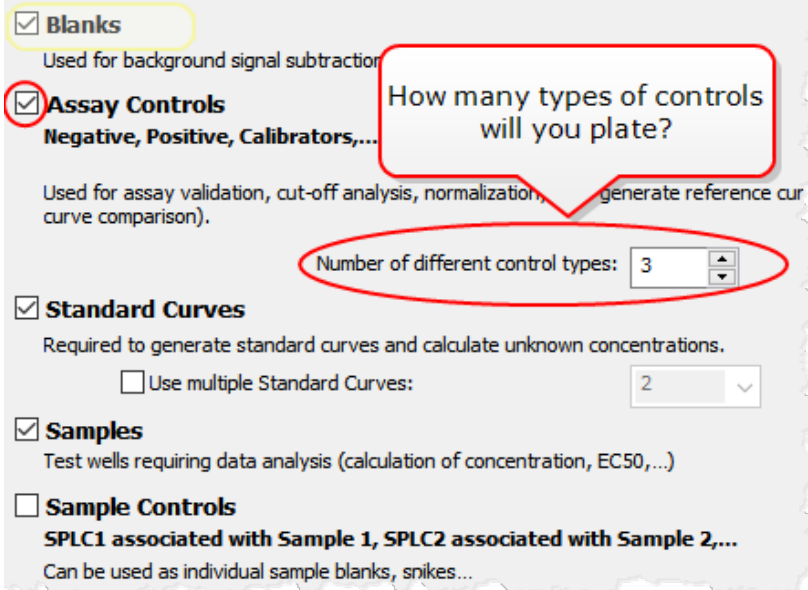
Along with data acquired in the experiment, this information can be exported to Excel or other formats and output in a Gen5's default report format.



### 10.1 - Plate Layout

It's easy to define the plate layout with Gen5's tools for identifying samples, standards, controls and blanks. Follow these steps:

1.  Select **Protocol>Plate Layout** or select it from the File tree.
2. In the Plate Layout Wizard that opens, select the well types used in your assay (check the boxes that apply).
3. Follow the wizard to define the **Concentrations** or **Dilutions**, if applicable, entering the values for each type. Use the Next and Back buttons as needed to add useful info for each type. Upon Finish, Gen5 opens a grid or plate map that represents your Plate Type.
4. Assign the well IDs to their corresponding locations in the plate map by clicking in the wells in the grid.



**Blanks**  
Used for background signal subtraction

**Assay Controls**  
**Negative, Positive, Calibrators,...**  
Used for assay validation, cut-off analysis, normalization, generate reference curve comparison).

Number of different control types: 3

**Standard Curves**  
Required to generate standard curves and calculate unknown concentrations.  
 Use multiple Standard Curves: 2

**Samples**  
Test wells requiring data analysis (calculation of concentration, EC50,...)

**Sample Controls**  
**SPLC1 associated with Sample 1, SPLC2 associated with Sample 2,...**  
Can be used as individual sample blanks, spikes...

## 10 - Plate Layouts and Reports/Exports

**Plate Layout**

Select a Well ID in the list on the left, then assign to the matrix.

Add... Delete

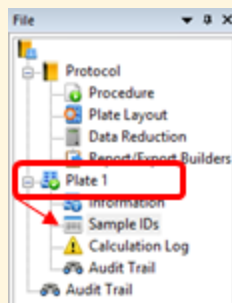
|   | 1   | 2 |
|---|-----|---|
| A | BLK |   |
| B | BLK |   |
| C |     |   |

<Empty>  
 BLK (x2)  
 Assay Control  
   NC  
   PC  
   CAL  
 Sample  
   SPL1

Serial Assignment  
 Replicates: 2

- In the left pane, select an item to assign to the plate. (The cursor looks like a pipette.)
- Click **Add** to create blanks, controls, standards, etc.
- Select **<Empty>** in the pane to clear previously assigned wells.
- Likewise, click **Undo** to correct errors and begin again.
- Experiment with the **Serial Assignment** tools to see if they're helpful. Disable "Auto Select" if not.

- When you select a corresponding starting number of Replicates in the Serial Assignment box, the IDs change accordingly.
- Keep **Next Dilution** box checked and click to assign replicates in either the row or column orientations.



- You can also assign sample IDs using the Plate icon in the File tree.

Serial Assignment

Replicates: 1

Next Dil.

Auto Select Next ID

The Serial Assignment dialog box shows a grid with 1 replicate highlighted in a red box, indicating row orientation. The 'Next Dil.' checkbox is checked, and the 'Auto Select Next ID' checkbox is unchecked.

Serial Assignment

Replicates: 1

Next Dil.

Auto Select Next ID

The Serial Assignment dialog box shows a grid with 1 replicate highlighted in a red box, indicating column orientation. The 'Next Dil.' checkbox is checked, and the 'Auto Select Next ID' checkbox is unchecked.

Set row or column orientation.



### 10.2 - Reporting and Exporting

#### Gen5 Quick Export

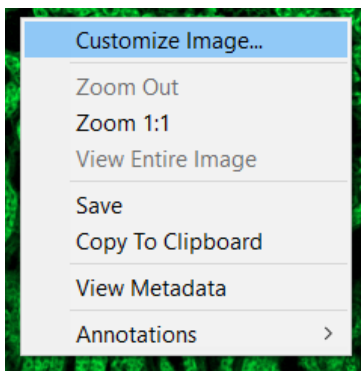


Quick export to Excel is offered in several views. Just click the Quick Export image or data buttons to instantly export to Excel. Only the currently displayed view will be exported.

#### Customize the Scale Bar

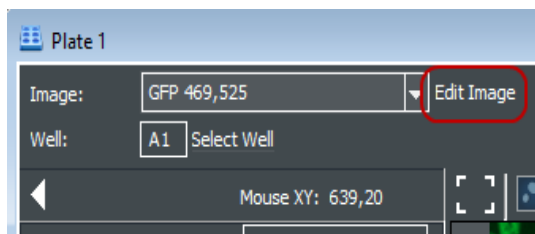
*Gen5 v.3.12 and higher*

Follow these steps to improve the appearance of the scale bar that Gen5 applies to image files.

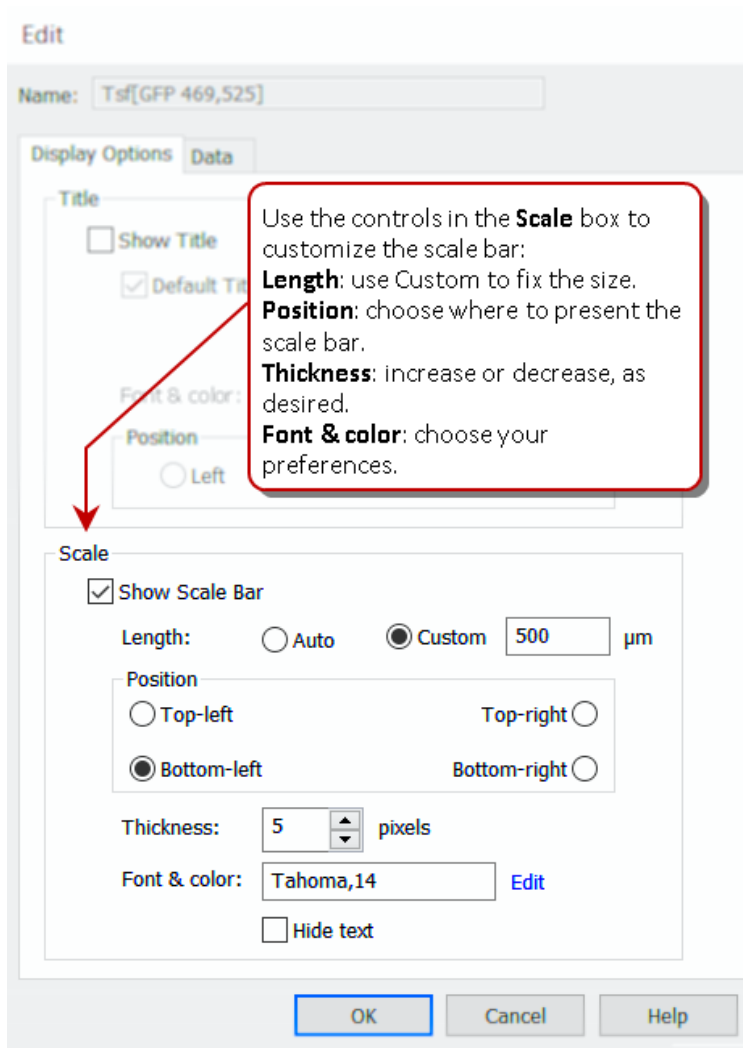


In Manual mode or a well zoom, **right-click** for a context menu and select **Customize Image**.

Or, in a well zoom, click **Edit Image**.



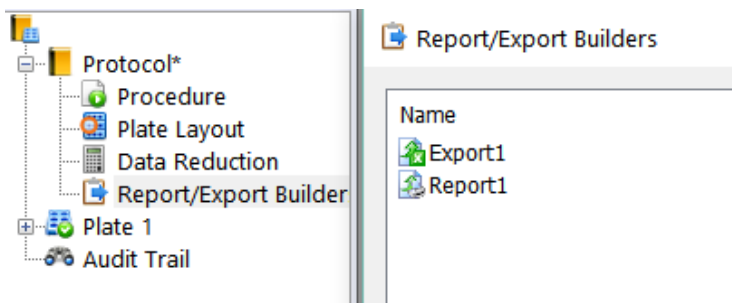
## 10 - Plate Layouts and Reports/Exports



Remember to customize the scale bar for the image set before adding it to a report or Power Export.

### Gen5 Report and Export Builders


In experiment mode, you can customize the reports to include only the most useful information.

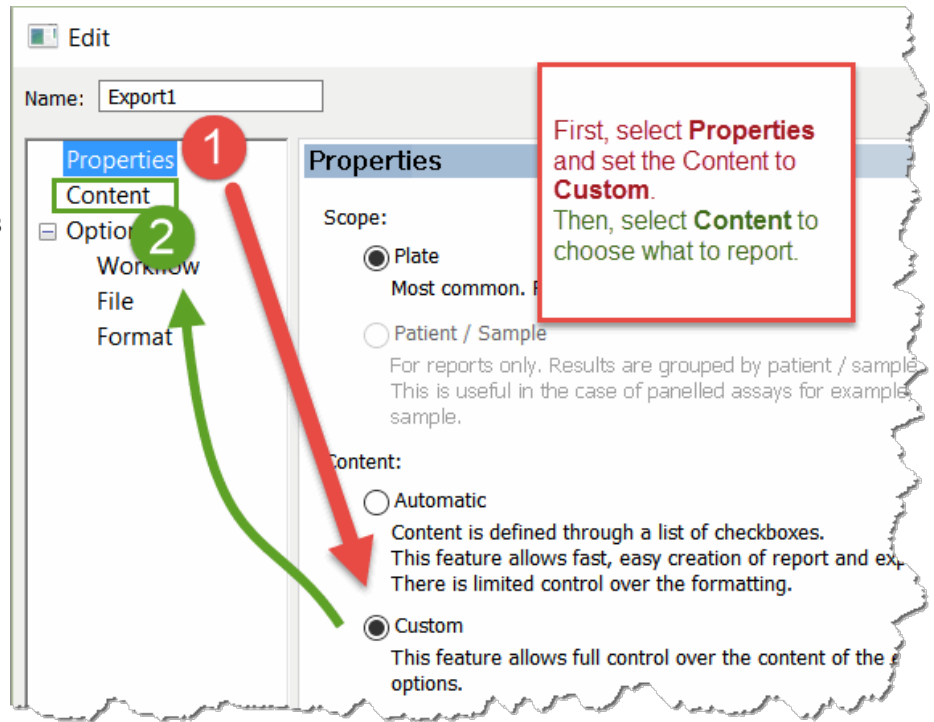




Explore the options available for including or excluding data and formatting the appearance of the report.

## 10 - Plate Layouts and Reports/Exports

To customize an export/report:

1. Click  (or select the builder from the File tree).
2. Respond to the Gen5's prompts. In the builder window, on the **Properties** page, keep the default "Automatic" selection under Content, or select **Custom**.
3. Then select **Content** in the left pane to specify the data to include in the report and to customize the layout.

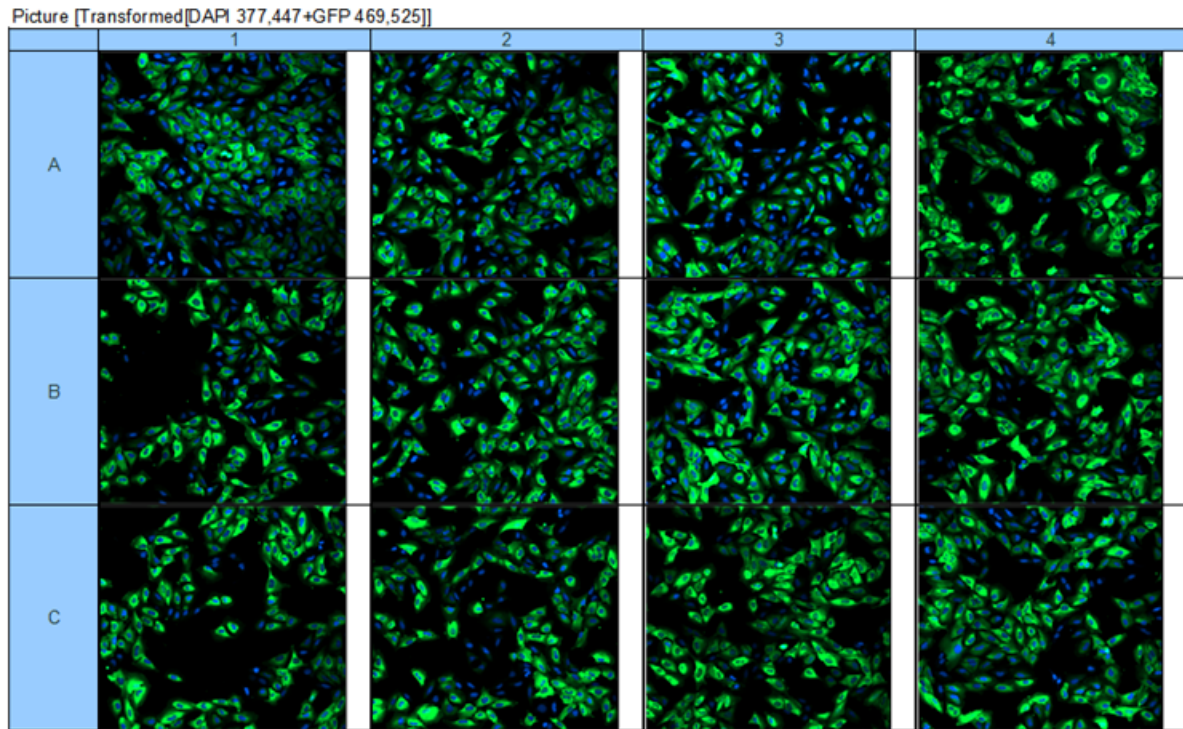


4. After defining or customizing a report/export, click:
  -  to export to Excel.
  -  to preview or print the report.

## 10 - Plate Layouts and Reports/Exports

### Increase Thumbnail Images in Power Exports

For imaging experiments, Gen5 gives you the ability to increase the size of thumbnail images in a matrix, statistics or analysis results table. The best results begin by preparing the Excel worksheet to accommodate the increased size of the images.



After defining the protocol, plate layout, and data reduction steps, define a Power Export:



Customize the **Power Export** template (change **Properties** to **Custom** as described earlier).

Edit Template

Then, select **Edit Template** to launch Excel to enlarge the height and width of cells.

## 10 - Plate Layouts and Reports/Exports

The screenshot shows the Microsoft Excel interface with the 'Format' menu open. The 'Cell Size' section is expanded, showing options for 'Row Height...', 'AutoFit Row Height', 'Column Width...', 'AutoFit Column Width', and 'Default Width...'. A red box highlights the 'Format' menu and the 'Cell Size' options. An orange arrow points from a text box to the 'Format' menu. The text box contains the following instructions:

Highlight the area the matrix will fill.  
Select the **Format** tool and increase **Row Height** and **Column Width**.

As usual, use the **Add-ins** toolbar to set up the report:

## 10 - Plate Layouts and Reports/Exports

The screenshot shows the Excel interface with the 'Edit' dialog box open. The 'Format' tab is selected, and the 'Image Export Format' section is highlighted. A callout box says 'Important: do not set to Plain Text'. A list of instructions is overlaid on the right side of the dialog.

1. On the worksheet, select the cell to place the matrix in and click **Matrix** on the **Add-ins** ribbon.
2. Select the data set with thumbnail images.
3. In the **Edit** window, review settings for Selection and Data, and revise as needed.
4. On the **Format** tab, choose a size increase option for the thumbnails.

Important: do not set to Plain Text

Increase image thumbnail resolution by: x 15

Note: Using x2 or greater impacts performances and increases the size of Excel workbooks.

Some trial and error may be necessary to properly size the images.

Note: this change does not apply to printed reports, QuickExport, nor CopyToClipboard.

## 10 - Plate Layouts and Reports/Exports

## 11 - Setup and Hardware

---

### *The Engineering Behind Your Imager*

This section provides detailed explanations of the hardware for each of our imaging instruments. Here you will find Laser Autofocus cube and LED/Filter cube installation and calibration information, and internal schematics of the imagers. You will also find Labware adapters for different vessels and their part numbers.

|   |            |
|---|------------|
| <b>11.1 - Instruments and Accessories</b> ..... | <b>215</b> |
| <b>11.2 - Objectives</b> .....                  | <b>221</b> |
| <b>11.3 - Imaging Cubes</b> .....               | <b>223</b> |
| <b>11.4 - Labware</b> .....                     | <b>233</b> |



### 11.1 - Instruments and Accessories

#### Instrument Components

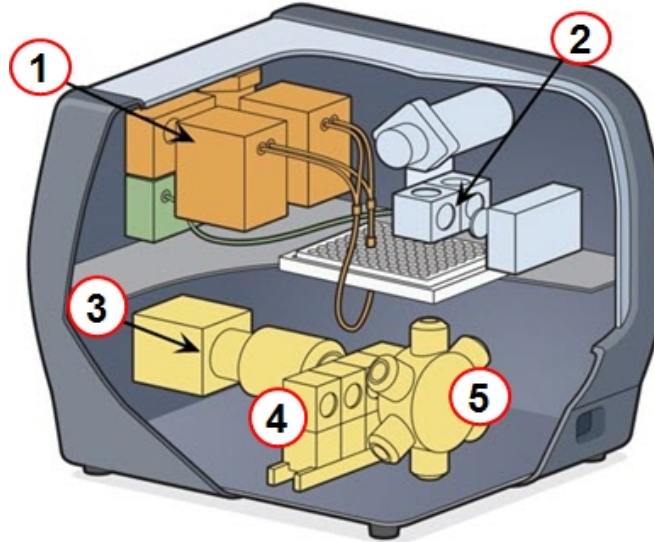
Shown in this Cytation cutaway illustration:

Multi-mode detection system components, top

1. Quadruple monochromator
2. Filter optical & Alpha laser components

Not shown but available in Cytation 5: Phase Contrast (which would replace the filter optical and Alpha laser components).

3. Imaging tube lens and camera
4. LED and Filter cubes
5. Objective turret



Fully equipped Cytation 5 with filter-based optics

## 11 - Setup and Hardware

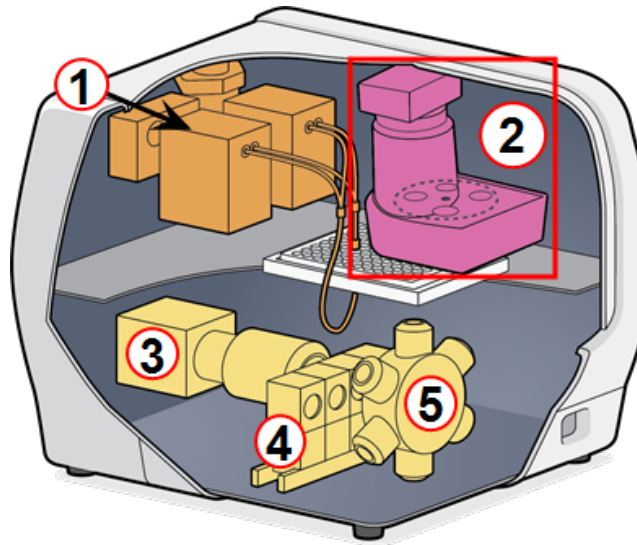
This Cytation 7 cutaway illustration shows some of the same components as above with the addition of an upright imager in the top:

1. Quadruple monochromator
2. Upright imager

All models have an upright imager with 4 fixed lenses and 2 brightfield light sources.

W-models also have an inverted imager:

3. Imaging tube lens and camera
4. LED and Filter cubes
5. Objective turret

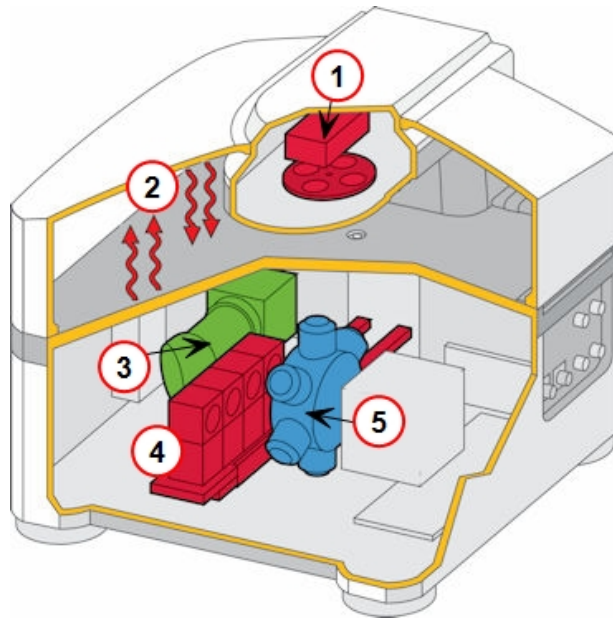


Fully equipped Cytation 7 with upright & inverted imagers

Shown in this Lionheart cutaway illustration:

Imaging system components:

1. Phase Contrast annuli,<sup>1</sup> Brightfield LED
2. Heaters in cover and base (Lionheart FX only)
3. Imaging tube lens and camera
4. LED and Filter cubes
5. Objective turret



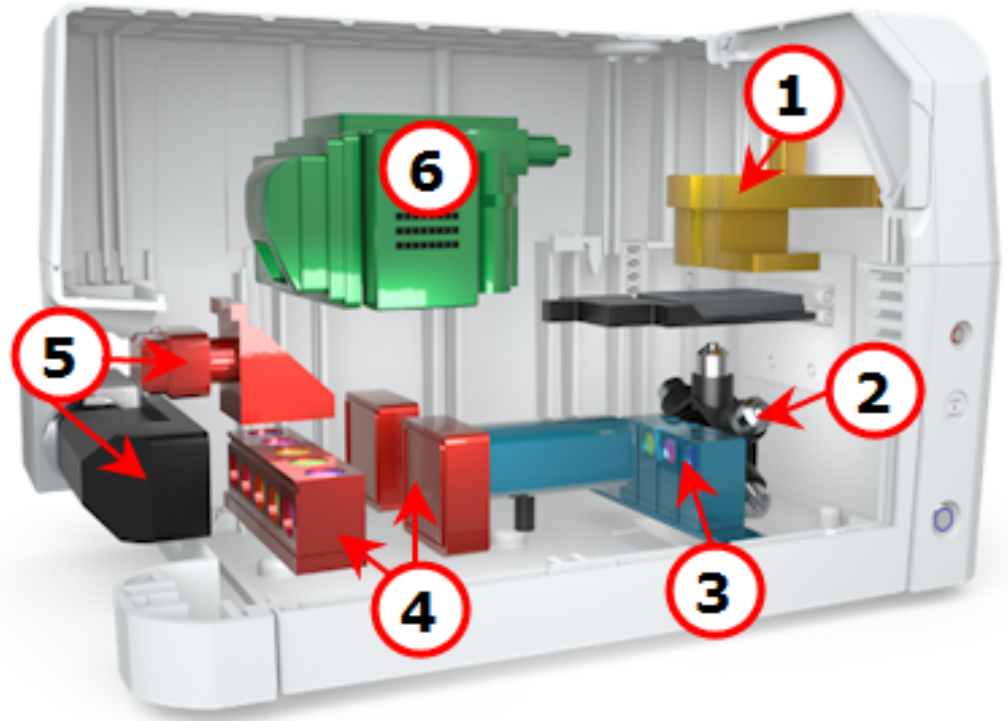
Lionheart FX

## 11 - Setup and Hardware

Shown in this Cytation C10 cutaway illustration:

Imaging system components:

1. Phase Contrast, Brightfield LED
2. Objective turret
3. Widefield LED and Filter cubes
4. Confocal filter cubes and disks
5. Imaging tube lens and camera
6. Monochromator for ABS, FL intensity, LUM



Cytation C10

1 — Not available for Lionheart LX

## 11 - Setup and Hardware

### Agilent Imager Feature Comparison

| Features                               | Lionheart FX | Lionheart LX | Cytation 5 | Cytation 1 | Cytation 7 | Cytation C10 |
|--|--------------|--------------|------------|------------|------------|--------------|
| Alpha detection                        |              |              | X*         |            |            |              |
| Variable bandwidth monochromators (FL) |              |              | X          |            | X          | X            |
| Phase contrast (4x-40x objectives)     | X            |              | X*         |            |            | X            |
| Incubation up to 65°C <sup>1</sup>     | X            |              | X          | X          | X          | X            |
| Color Brightfield (H&E stain)          | X            | X            | X          |            | X          | X            |
| Joystick positional control (optional) | X            | X            | X          |            | X          | X            |
| High capacity (6 objective) turret     | X            | X            | X          |            | X          | X            |
| Oil immersion objectives               | X            | X            |            |            |            |              |
| Extended dynamic range in FP, TRF, FI  |              |              | X          | X          |            |              |

<sup>1</sup>Temperature control varies: up to 40°C with Lionheart FX; 45°C with Cytation 1, Cytation 7, Cytation C10; and 65°C with Cytation 5.

## 11 - Setup and Hardware

### Dual Reagent Injectors



When applications require rapid inject-and-read capability, or when it's simply more convenient to dispense one or two reagents into a vessel inside the instrument, the optional syringe-drive Dual Reagent Injector Module is available. Installation is quick and tool-free. Gen5 software allows intuitive control over dispensing activities.

See **19 - Dual Reagent Injector**

*Not available for Lionheart LX*

### CO<sub>2</sub> / O<sub>2</sub> Gas Controller



Live cell assays require a controlled environment, including regulation of CO<sub>2</sub> and O<sub>2</sub> levels. The optional Gas Controller provides precise monitoring and control of both CO<sub>2</sub> (from 0 to 20%) and O<sub>2</sub> (from 1 to 19%) levels inside the instrument.

Lionheart FX offers an optional "Environmental Chamber" stage insert that supports a Gas Recharge feature to quickly restore gas levels after the cover has been opened. Learn more in the operator's manual.


See **20 - Gas Control**

*Not available for Lionheart LX*

## 11 - Setup and Hardware

### Joystick

Some users may find the optional joy stick makes it easier to find and focus on their samples, especially when using high-power objectives. The joy stick offers more precise and varied navigation than standard computer step controls. For example, the joystick permits movement in both X and Y axes simultaneously. Like Gen5's step controls, it offers precise focusing control. (PN 1320003)

 After connecting the joystick, restart the instrument to establish communication with it.

**Note:** Not available with Cytation 1 or 3.



## 11 - Setup and Hardware

### 11.2 - Objectives

Up to six objectives are supported on most imagers. Confocal imaging requires a 20X, 40X, or 60X objective.

|                       | Widefield      |              |              |                     |                       | Widefield & Confocal  |                       |                |
|-----------------------|----------------|--------------|--------------|---------------------|-----------------------|-----------------------|-----------------------|----------------|
| Magnification         | 1.25x          | 2.5x         | 2.5x         | 4x /<br>4x<br>Phase | 10x /<br>10x<br>Phase | 20x /<br>20x<br>Phase | 40x /<br>40x<br>Phase | 60x            |
| Numeric Aperture      | 0.04           | 0.12         | 0.07         | 0.13                | 0.30                  | 0.45                  | 0.60                  | 0.70           |
| Resolution (nm)*      | 5588           | 1863         | 3193         | 1719                | 745                   | 497                   | 373                   | 319            |
| Working Distance (mm) | 5              | 6.3          | 5.7          | 17                  | 10                    | 6.4-7.6               | 2.7-4.0               | 1.5-2.2        |
| Depth of Field (µm)   | 408            | 50.7         | 132.2        | 38.9                | 7.1                   | 2.9                   | 1.5                   | 1.1            |
| Field of View (mm)    | 10.4 x<br>10.4 | 4.7 x<br>4.7 | 5.8 x<br>5.8 | 3.2 x 3.2           | 1.3 x 1.3             | 0.65 x<br>0.65        | 0.32 x<br>0.32        | 0.22 x<br>0.22 |
| Manufacturer          | Olympus        | Zeiss        | Meiji Techno | Olympus             | Olympus               | Olympus               | Olympus               | Olympus        |

**Note:** Confocal imagers do not support Zeiss 2.5X objectives.

See Install & Calibrate Objectives, LED-Filter Cubes, Confocal Cubes on page 225.

#### Important information about Objectives

Objectives need to be calibrated after installation and before first use (except oil objectives, which are not calibrated). Calibration is performed via the Gen5 Imaging Configuration tab shown above. The goal of calibration is to:

- Locate the center of the objective's optical axis relative the plate carrier in the X-Y plane (horizontal),
- Find the focal point of each objective relative the plate carrier surface in the Z-axis (vertical),
- Reduce pixel shift for each of the filter cubes installed.

- **Important:** All "W" model imagers and non-W models with high-power objectives (40X - 60X air objectives) require an "objective setup plate" during Auto Calibration. Use the objective setup plate provided with your instrument.

## 11 - Setup and Hardware

- Cytation C10 and Lionheart "W" models require PN 1942519 objective setup plate.
  - Cytation "W" models require PN 1852501 (which supports FL Illumination Correction).
  - Other Cytation and Lionheart models use PN 1222531 to calibrate their high-power objectives.
- Correction collar: 20X, 40X, and 60X air objectives have a correction collar. Start with position .17 on the collar for cover slips and glass-bottom plates or .5 for standard plastic microplates. Readjust collar as needed to support different plate types, *(as described on page 1)*.

**Phase Contrast Objectives:** At the factory, Agilent installs and calibrates the required phase contrast components, a phase contrast objective and matching aperture. If adjustments are needed, contact Agilent Technical Service.



### 11.3 - Imaging Cubes

Widefield imaging uses LED cube-Filter cube assemblies. Confocal imaging uses confocal cubes, (*as described on page 230*).

#### LED Cube and Filter Cube Assemblies

For fluorescence widefield imaging, the imager can contain up to four LED cube and filter cube assemblies, one for each channel of image acquisition color. The cubes are mounted on a mechanical slide, and are easily removed and exchanged.

Numerous wavelengths are available to support most known applications of fluorescence microscopy. The cubes are joined to work together:

- bottom cube contains a high-powered LED and focusing lens,
- top cube contains the excitation filter, dichroic filter, and emission filter.

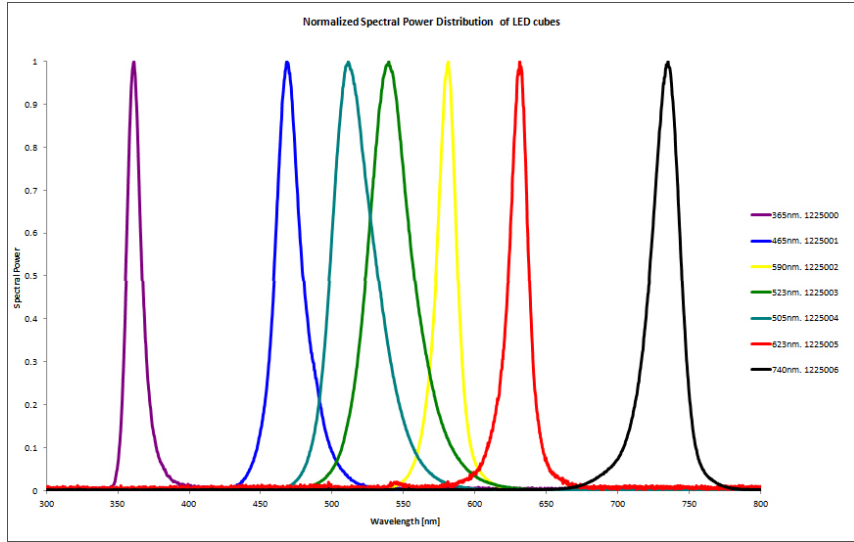
The wavelength of the LED is matched to the color requirements of the filters in the top cube.

- Gen5 helps ensure the LED cube is a proper match to the filter cube by limiting your configuration options to matching components.
- LEDs are calibrated for light intensity **every time** the instrument is powered up. LED calibration ensures a stable signal for the duration of an experiment.
- Different LEDs have different maximum allowed current. So, it is important to correctly identify the wavelength and position of the LED cube in Gen5's **Instrument Configuration** prior to powering up the instrument.



Easy access to slider for LED and filter cube assemblies (Cytation shown above)

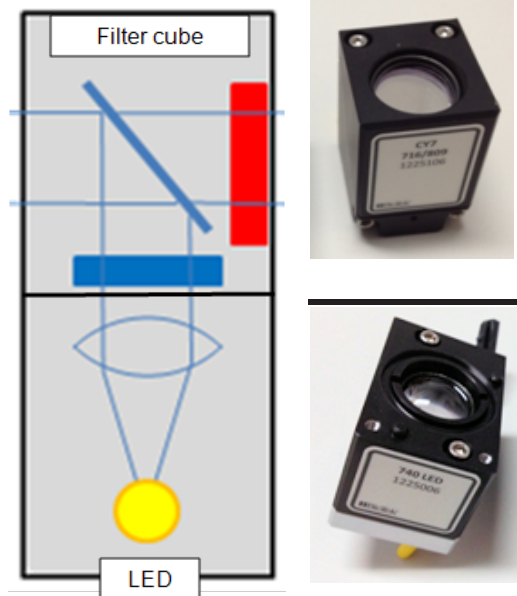
## 11 - Setup and Hardware



LED cube (bottom) and filter cube (top)

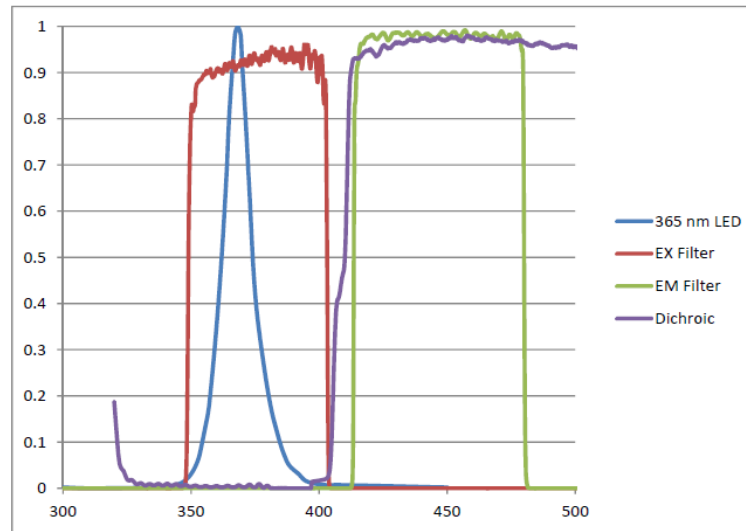
Typical LED Spectra

**Visit the Gen5 Document Library for comprehensive listings of LED and filter cubes, and objectives.**



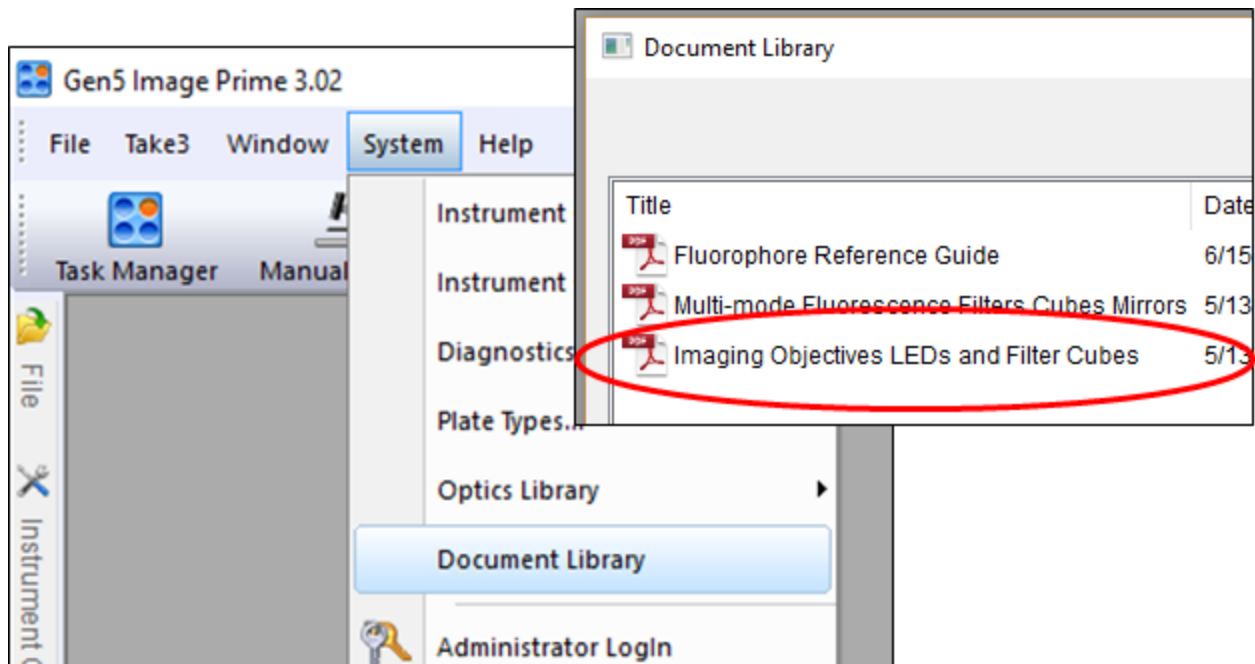
## 11 - Setup and Hardware

DAPI (p/n 1225100)



sample data

In Gen5, select **System > Document Library** for *Imaging Objectives, LEDs and Filter Cubes* or get the latest information on Agilent's website: [www.biotek.com](http://www.biotek.com).



### Install & Calibrate Objectives, LED-Filter Cubes, Confocal Cubes

**Important:** To prevent damage to the LED cubes, follow the onscreen instructions for installing the LED-Filter cubes. You must tell the imager which LED cubes are going to be installed in which location before you install them and run Auto Calibration.

## 11 - Setup and Hardware

Select **System>Instrument Configuration**. Double-click the imager item and click **Setup**.

Reader Setup - CytationC10 (Com4)



Imaging Configuration
Dispenser 1
Dispenser 2
Probe Size
Temperature Offset
Absorbance
Basecode

**Objective Configuration**

| Position | Objective            |        |
|----------|----------------------|--------|
| 1        | 1220519/4X PL FL     | Access |
| 2        | 1320516/10X PL FL Pt | Access |
| 3        | 1320517/20X PL FL Pt | Access |
| 4        | 1320518/40X PL FL Pt | Access |
| 5        | 1220545/60X PL FL    | Access |
| 6        | <none>               | Access |

Get Values

Send Values

Auto Calibration

Manual Calibration (Advanced)

Phase Ring Configuration

WFOV FL Illumination Correction

Quick Calibration  
 Autocal Without Pausing  

Only to be used if Auto Calibration fails.

**LED and Filter Cube Configuration**

| Position | Filter Cube          | LED Cube |
|----------|----------------------|----------|
| 1        | 1225010 / Laser Auto | 1225010  |
| 2        | 1225101 / GFP        | 1225001  |
| 3        | 1225100 / DAPI       | 1225000  |
| 4        | 1225102 / Texas Red  | 1225002  |

**LED and Filter Cube Change Procedure:**

1. Set new configuration.
2. Click 'Send Values' button.
3. Power off instrument.
4. Install new LED cube and filter cube.
5. Power on instrument.
6. Wait for self-test to complete.
7. Press carrier button to silence chirp.
8. Click 'Auto Calibration' button.

**Tell Gen5 and the instrument (Send Values) where the LED filter cubes and other imaging components will be installed.**

**Confocal Filter Cube Configuration**

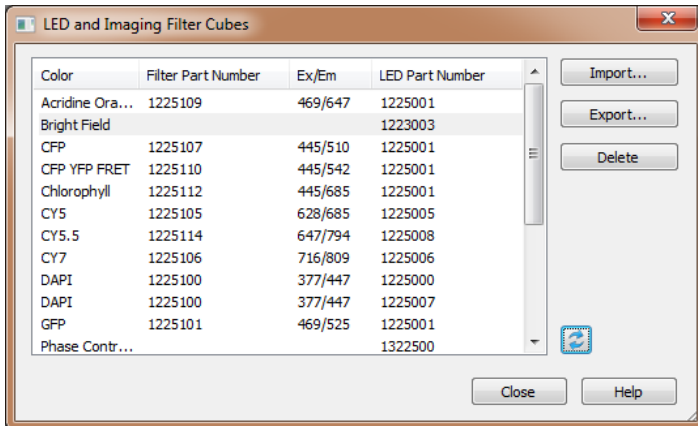
| Position | Filter Cube          | Laser Wavelength(nm) |
|----------|----------------------|----------------------|
| 1        | 1945103 / Confocal D | 405 nm               |
| 2        | 1945104 / Confocal G | 470 nm               |
| 3        | 1945105 / Confocal R | 520 nm               |
| 4        | 1945102 / Confocal C | 640 nm               |

**Important:** All “W” model imagers and non-W models with high-power objectives (40X - 60X air objectives) require an “objective setup plate” during Auto Calibration. Use the objective setup plate provided with your instrument.

- Cytation C10 and Lionheart “W” models require PN 1942519 objective setup plate.
- Cytation “W” models require PN 1852501 (which supports FL Illumination Correction).
- Other Cytation and Lionheart models use PN 1222531 to calibrate their high-power objectives.

## 11 - Setup and Hardware

**Make sure Gen5's 'Instrument Configuration' exactly matches the actual contents of the LED and Filter Cubes and Objectives.**

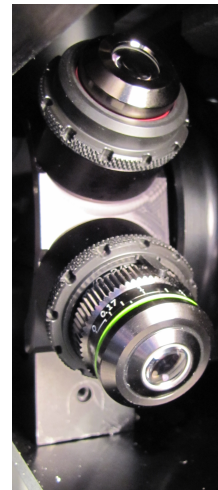
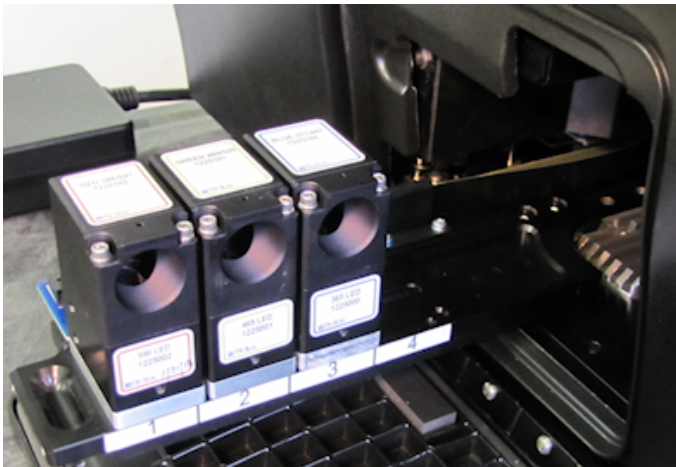


Select **System>Optics Library>LED and Filter Cubes**.

If any components are missing from the list, import them from Agilent's website: [www.biotek.com](http://www.biotek.com).


Swap the objectives and/or the imaging filter cubes when needed. To prevent over powering the LED cubes, you must first tell the imager which LED cube configurations are going to be installed.

**Important:** Wear gloves when changing components to avoid contaminating them.



### Step 1: Update Gen5 Settings

**First**, use Gen5 to update the imager's onboard settings:

1.  In Gen5, select **Reader Setup**.
2. On the **Imaging Configuration** tab, set the objective, LED cube and filter cube, and confocal cube configurations for your components.

**Recommendation:** Generally it is best to install your lowest power objective, beginning with 4X, in position #1, and higher power objectives in the subsequent positions. However, it is best to install objectives with power lower than 4X in the last positions. Sample sequence: 4X, 20X, 60X, 2.5X, 1.25X.

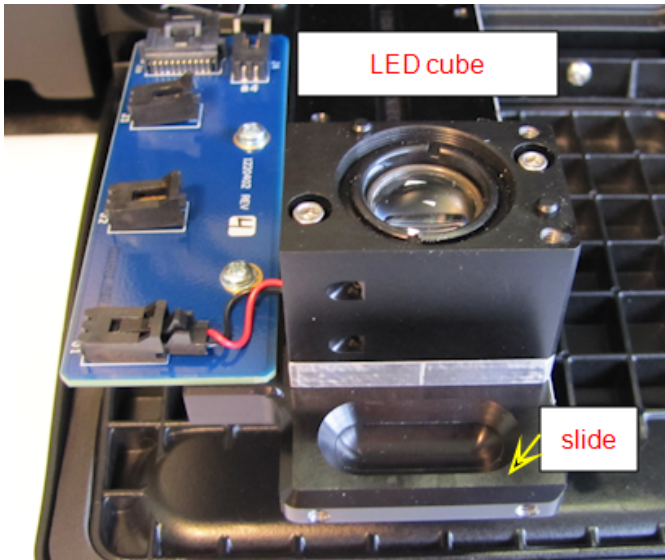
## 11 - Setup and Hardware

3. In the Objective Configuration area, click the **Access** button to move the turret to that position.
4. When defined, click **Send Values**. (Keep this Gen5 window open until the rest of the procedure is completed.)

### Step 2: Physically install components

Open the access door.

#### LED-Filter Cubes:



**Tools:** 3/32 hex/Allen wrench

1. Reach in and pull out the filter slide.
2. Place the new LED cube in the defined position (Position 1, 2, 3, or 4).
3. Use the hex wrench to screw the LED cube into the filter slide.
4. Plug the LED's wire connector into the socket on the circuit board.
5. Place the imaging filter cube on top of the LED cube and screw it to the LED cube.

#### Objectives:

- Screw each objective into its defined position.
  - Do NOT over tighten!
  - Screw securely: Especially with high-power objectives, make sure the objective's adapter threads properly into its place in the turret. Pay close attention when installing them. If you feel a stop or misalignment, remove the objective and try again. Verify proper installation by making sure there is no gap between the adapter and the turret.

#### Confocal cubes:

See Install the Confocal Imaging Filter Cubes on page 230.

### Step 3. Run Auto Calibration

1. Turn on the imager. After a couple minutes, expect to hear a chirping sound indicating that calibration is not completed. Press the plate carrier eject button to silence the alarm.
2. On Gen5's **Imaging Configuration** tab, click **Auto Calibration**.

This will take several minutes.

## 11 - Setup and Hardware

Quick Calibration

Autocal Without Pausing

Autocal Without Pausing lets you walk away from the imager during calibration, (i.e., user interaction is not needed to complete the process). When not all components have been changed, Quick Calibration saves time by only calibrating the new components.

### Install the Laser Autofocus Cube



#### Laser Autofocus - Optional Accessory

Install the Laser Autofocus cube (PN 1225010) in Position 1 on the slide (follow procedure for installing imaging LED and Filter cubes).

| Position | Filter Cube            | LED Cube |
|----------|------------------------|----------|
| 1        | 1225010 / Laser Auto I | 1225010  |

Use the 3/32 Hex wrench to tighten the cube's captive screws.  
Plug the cube's wire clip into the socket.

## 11 - Setup and Hardware

### Install the Confocal Imaging Filter Cubes

*Confocal imaging models only*

**Tools:** 3/32 Hex wrench

**Important:** Wear gloves when handling the confocal filter cubes. Be careful not to touch imaging components.

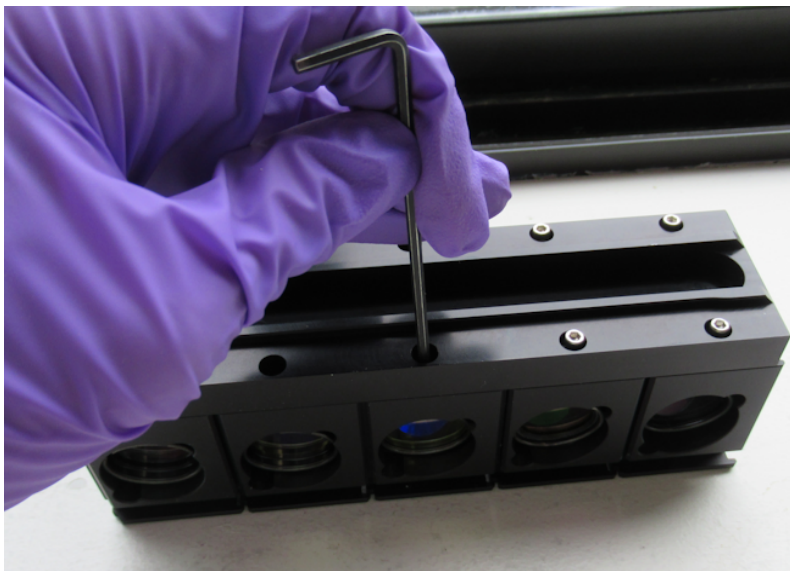


Position 5 is reserved for the "Path Correction" confocal imaging cube. Be sure to position the cube labeled "Path Correction" in Position 5 on the confocal cube slider.

This cube does not need to be configured in Gen5.



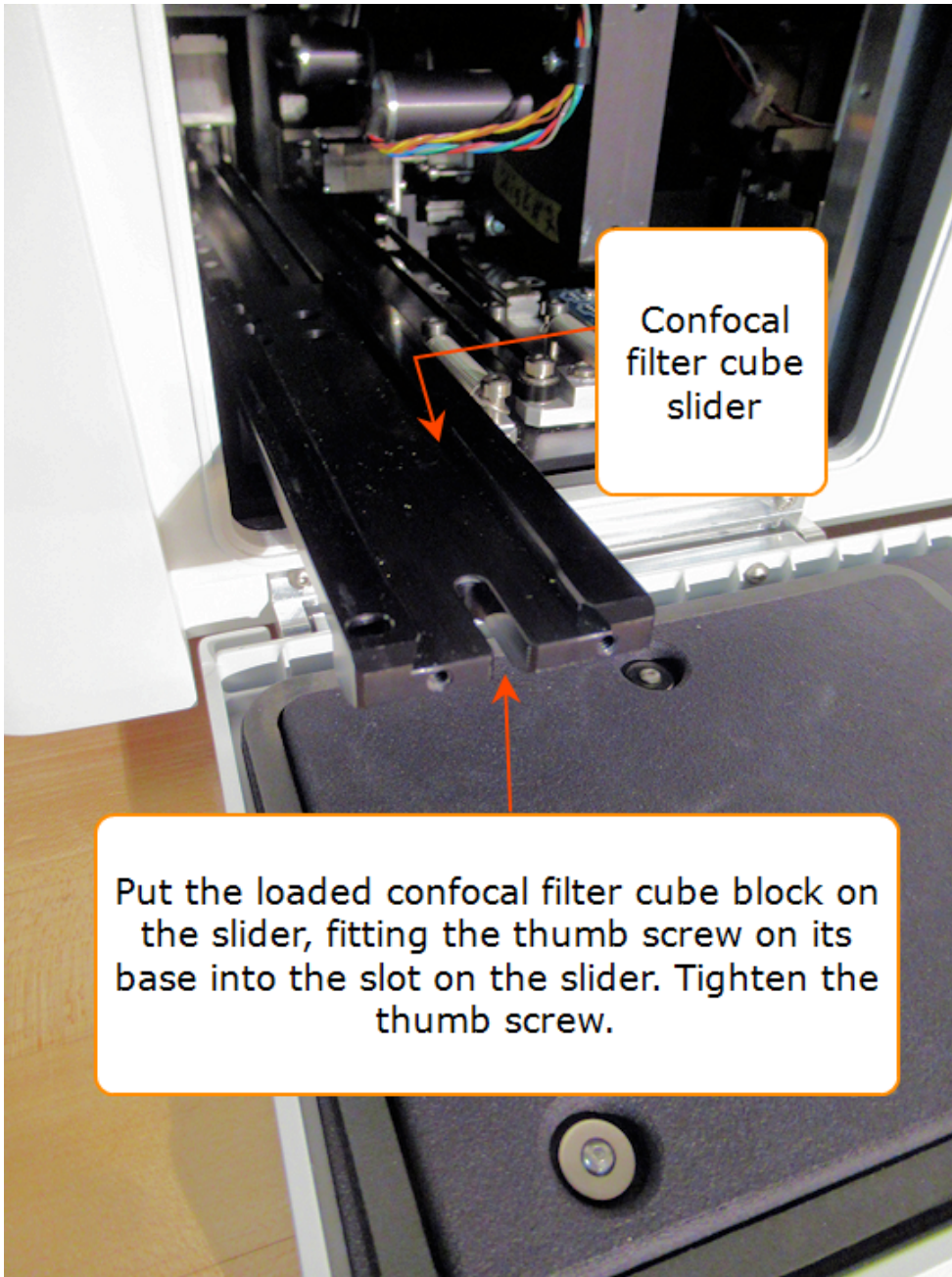
1. Line up the confocal filter cubes (upside down) in the order defined in Gen5. Make sure the Path Correction filter cube (shown above) is in Position 5.





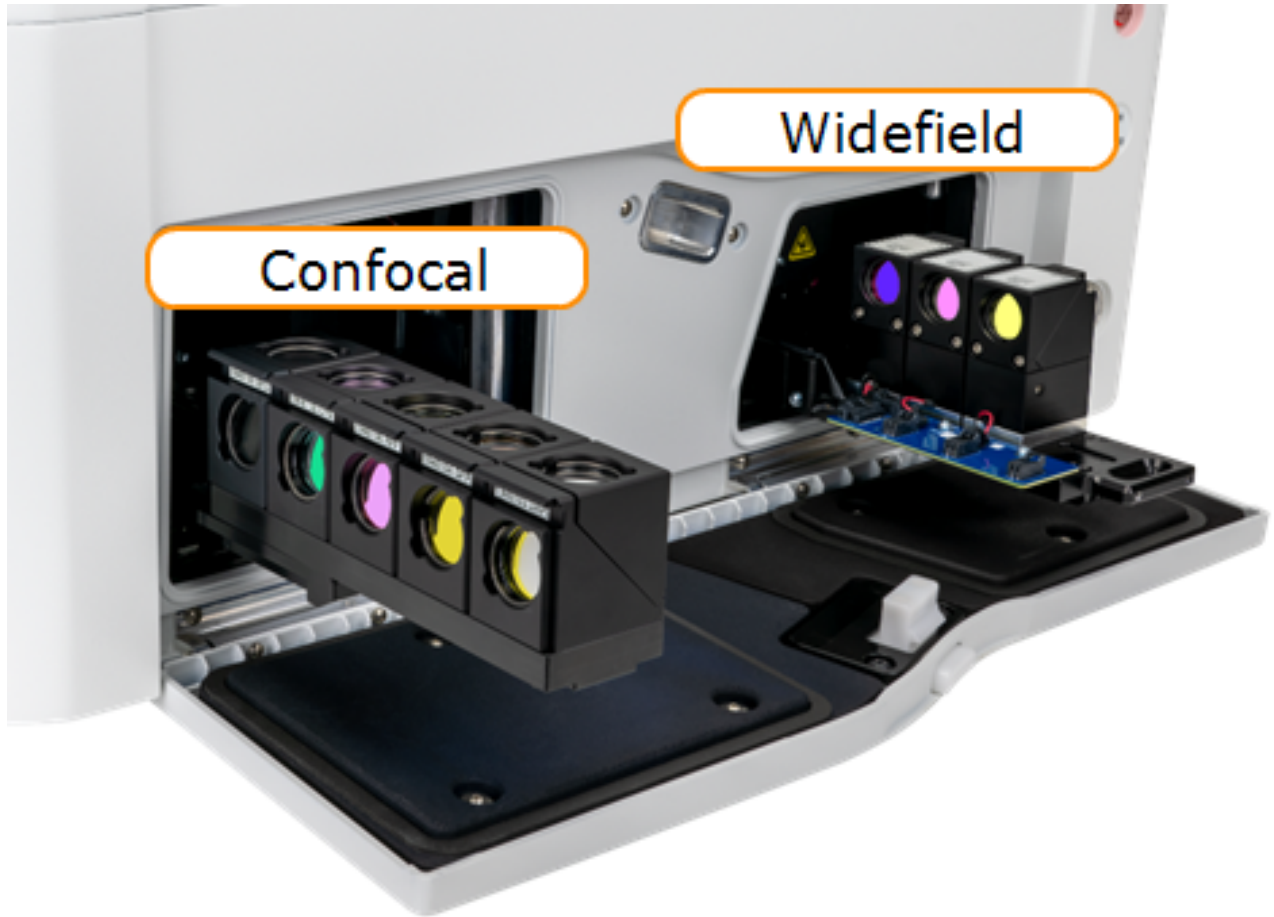
## 11 - Setup and Hardware

- Put the confocal cube slider on top of the cubes in the correct orientation to position the cubes correctly. (The thumb screw is at Position 1.) Use the hex wrench to tighten the captive screws holding each cube.



- Open the front access door. On the far left side, gently pull the confocal filter cube slider out.
- Put the loaded confocal filter cube block on the slider, fitting the thumb screw on the bottom into the slot on the slider. Tighten the thumb screw.

## 11 - Setup and Hardware



Confocal Filter Cubes and LED Cubes-Filter Cubes Installed

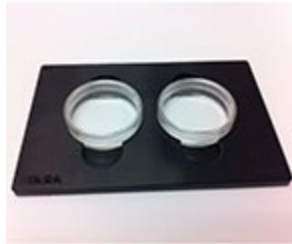
### 11.4 - Labware

#### Microplates and Other Sample Vessels

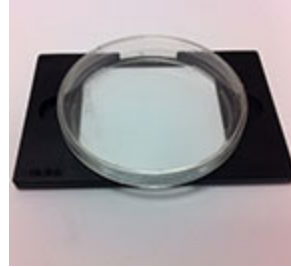
Imaging can be done in numerous sample vessel formats, including 6- to 384-well microplates, microscope slides, various size Petri dishes and Corning® 25 cm<sup>2</sup> rectangular, canted-neck cell culture flasks. And, custom vessels that match the basic microplate geometry can be defined in Gen5 for use in imaging.



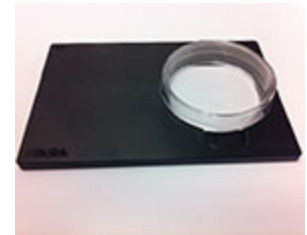
Slide holder  
PN 1220548



Petri dish holder  
PN 1222240 38.7 mm  
PN 1222246 35.5 mm



100 mm Petri dish holder  
PN 1222242



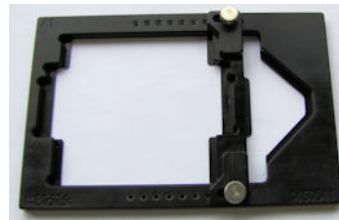
60 mm Petri dish holder  
PN 1222241



T25 Corning flask  
PN 1222210 (Cytation)  
PN 1452275 (Lionheart)



Cell counting chamber  
PN 1322131 & 1320016



Multi-vessel adapter  
PN 1450541



Nunc 4-well in adapter

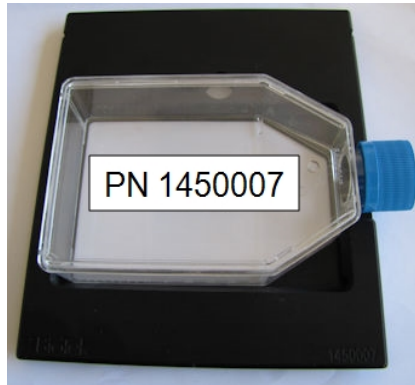
## 11 - Setup and Hardware

### Lionheart Stage Inserts

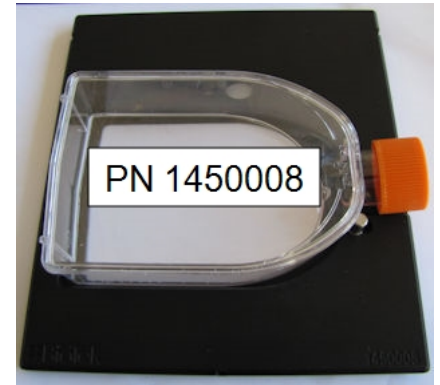
Optional stage inserts may fill your assay requirements:



Environmental chamber for lidded plates  
(For Lionheart FX only - LFX & LFXW)



T-75 square flask



T-75 U-shaped flask

| Dispense Tips     |
|-------------------|
| <none>            |
| 1450532 - Offset  |
| 1450533 - Aligned |

The screenshot shows a software interface for selecting a stage insert. A dropdown menu labeled "Stage Insert" is open, showing a list of options: "1450006 - Humidity", "1450007 - T75 Flask", "1450008 - T75 Flask U", "1450527 - Standard Microplate", and "1530500 - Onix Stage Insert". The "1450007 - T75 Flask" option is highlighted. To the right of the dropdown are two buttons: "Send Value" and "Refresh". A red box highlights the "Send Value" button, and a red arrow points from a text box to it. The text box contains the instruction: "Be sure to click **Send Value** after choosing the Stage Insert."

When changing stages, go to **System>Instrument Configuration>Setup** and change the stage setting to tell the Lionheart which stage is installed.

Lionheart FX only: When using the T-75 flask stages, set "Dispense Tips" to **<none>**, if they have been previously configured.

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## Notices

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## In This Book

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### Document Revision History

| Part Number | Revision | Date          | Changes         |
|-------------|----------|---------------|-----------------|
| 1321031N    | A        | February 2022 | Initial release |

Original Language - EN



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