

# Agilent Cytation C10

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## Confocal Imaging Reader - Cytation C10 -

The Agilent BioTek Cytation C10 confocal imaging reader combines automated confocal and widefield microscopy with conventional multimode microplate reading. It gives exquisite resolution and optical sectioning capabilities for many sample types.

Objectives (Olympus): 4x BF, 4x Ph, 20x Ph, 40x Ph, 63x

Widefield Fluorescence: DAPI, GFP, TRITC, CY5

Confocal: 6-line Laser, filter cubes for DAPI, GFP, TRITC, CY5

Transmitted light: Brightfield, High Contrast Brightfield (HCBF), Phase Contrast, Colour Brightfield

Camera: Sony IMX 264 CMOS

Imaging: 6- to 386-well plates, imaging up to 1536-well plates; slides, chamber well slides

Temperature control (18-45 °C) and CO<sub>2</sub> control;

Gen5 Image+ software (version 3.12) for image acquisition, procedure and experiment design, experiment runs, image pre- and postprocessing, image and data analysis

Some applications: Label-free cell counting, time-lapse live cell imaging, 3D cell culture, Slide scanning, cell viability/toxicity, cell migration, whole organism imaging, cell cycle analysis, confluence, transfection efficiency, stem cell differentiation, genotoxicity, ELISA, automatic ROI identification, Luciferase reporter assays, nucleic acid and protein quantification, cell growth, apoptosis.

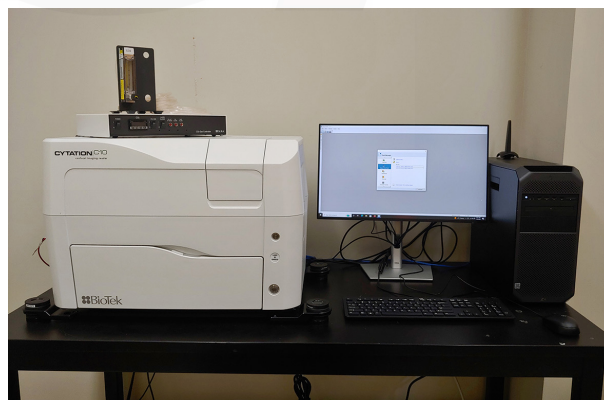
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## *User Guidelines*

1. When you begin to use these facilities please provide a Billing Authorization Sheet including the supervisor's signature and the Trust Fund Account number.
2. Before any unsupervised access is granted, users must enroll in supervised training sessions during which they will review with the confocal manager how to operate the equipment properly and safely. The time required for the training sessions will vary depending upon the user's demonstrated competency with the equipment. Billing will be at the "Training" fee rate.
3. The instruments are booked in Clustermarket. Booking can be done up to the moment the user wants to start but cancellation and editing of the booking have a cut-off limit of 2 hours before the start time. Any cancellations or changes after the cut-off limit need to be done by the confocal manager.
4. For any planned after hours use of the confocal system, please make arrangements with the confocal manager for access to the hallways.
5. Users are expected to bring all their own supplies including pipets, slides, coverslips, computer disks, etc. However, the facility will provide immersion oil and lens paper.
6. Files saved to computer hard drives must be removed as soon as possible. All computer hard drives will be cleared on a regular basis - it is the users' responsibility to manage their own image files. USB keys are not allowed on any of the instrument computers! The facility offers a variety of other options for data transfer.
7. Please notify the confocal manager immediately of any problems that you encounter with the equipment - it is essential that we work together in taking care of the facility. Improper care of the equipment will result in rejection of access to the facility.

## Cytation C10 Confocal Image Reader: General Information

### Objectives

Objective	Immersion	Working Distance (mm)	Coverslip (mm)	Confocal	NA	Depth of field ( $\mu\text{m}$ )
4x	air	17	0.17	No	0.13	39
4x Ph	air	17	0.17	No	0.13	39
20x Ph	air	6.6-7.8	0.0-2.0	Yes	0.45	2.9
40x Ph	air	2.7-4.0	0.0-2.0	Yes	0.60	1.5
60x Ph	air	1.5-2.2	0.0-2.0	Yes	0.70	1.1

### Correction Collar (20x, 40x, 60x)

With the 20-60x objectives you can not only image through a glass coverslip but also through up to 2 mm thick plastic (e.g., specific well plates). To achieve this, these objectives have a correction collar that needs to be adjusted for your sample. The bottom thickness of your plate/slide is specified by the manufacturer and needs to be defined when you setup the plate type.

### High Power Objectives

#### 40x and 60x Objectives: Limitation

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 not obvious when defining the Plate Layout but it is visible when selecting the wells to read in a read step (click the 'Full Plate' button). Gen5 identifies this limitation in the plate map:

'X' - no part of the well can be imaged

'O' - only a portion of the well can be imaged.

### High Contrast Bright Field (HCBF) Aperture

Significant improvements to cell counting have been seen when using a custom aperture in bright field imaging. This modification yields high-contrast images of adherent cells that can be easily counted by Gen5.

# Cytation C10 Startup and Sample Setup

## System Startup

- 1) Start the laser engine and turn the key to 'On' (Fig. 1A, circle and arrow)
- 2) Turn on the Plate Reader (Fig. 1B, arrow)
- 3) Turn on the computer and log into Windows
- 4) Start Gen5 software

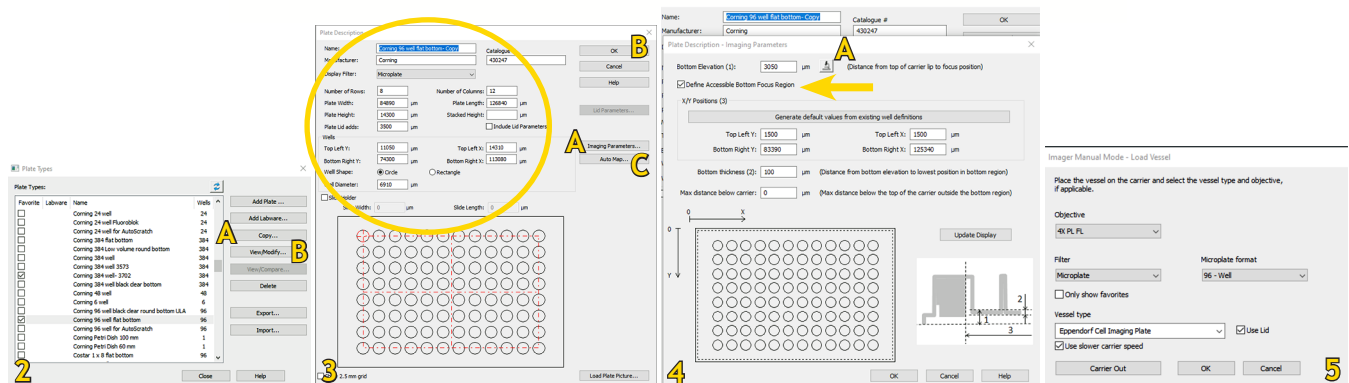


## Plate Definition and Setup

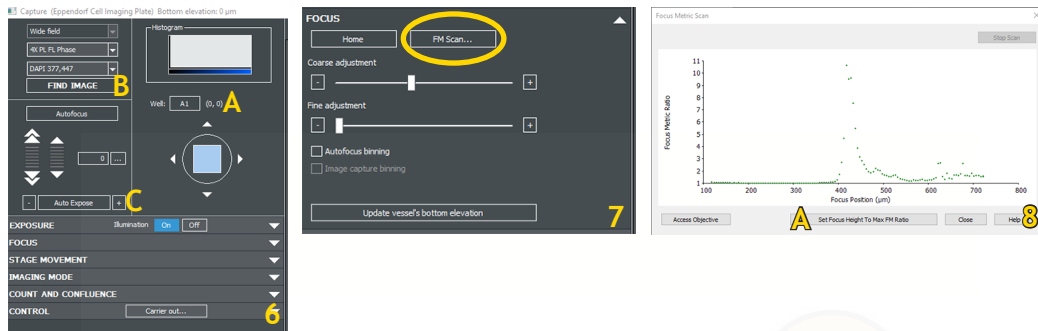
The choice of cell culture vessel significantly affects image quality and assay success, especially with higher power objectives. Best choice are glass or optical-bottom plates.

The proper plate definition for your vessel is crucial for the experiment setup. 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.

1. In the menu, select 'System' - 'Plate Types'.
2. Find the Plate Type you are using (or one most similar to it), click 'Copy' (Fig. 2A) and give it a unique name. Select 'View/Modify' (Fig. 2B) and adjust the measurements of the well plate (Fig. 3, circle).
3. Select 'Imaging Parameters' (Fig. 3A).
4. In the 'Plate Description - Imaging Parameters' window (Fig. 4): make sure that 'Define Accessible Bottom Focus Region' is checked (Fig. 4, arrow) and click the microscope icon (Fig. 4A).
5. Imager Manual Mode (IMM) will open with the 'Load Vessel' window (Fig. 5). Select 'Use lid' - 'Carrier out' - place the vessel on the imager's carrier or stage and click 'OK' - 'Yes' to the warning.



6. In IMM, select objective and filter cube. Use the strongest channel for your sample (Fig. 6).
7. Accept the default well selected, A1, or select 'Well' to take measurements at various parts of the plate (Fig. 6A).
8. Run 'Find Image' (Fig. 6B) or select 'Auto Expose' (Fig. 6C) and then 'FMScan' in the 'Focus' tab (Fig. 7), if a fluorescent channel is selected). When you get a defined peak: click 'Set Focus Height to Max FMRatio' (Fig 8A).
9. Click 'Save Settings' in IMM. Gen5 will update the Bottom Elevation value automatically and close IMM. Click 'OK' in the Image Parameter window (Fig. 3B).



10. Finally, select 'Auto Map' (Fig. 3C). In the pop-up window (Fig. 9A), the read method should be 'Image' - select 'OK' - the Read Step window will pop up (Fig. 10) - Select objective and channel (Fig. 10A, B) - click 'OK' and the window will close. In the Automap image select 'Run' (Fig 9, arrow). At the end of the run you will see the images of the two selected wells - adjust the offset either with the arrows below the image or by dragging the white circle to match the imaged well and select 'OK' (Fig. 11). 'Auto map' closes automatically.
11. If you haven't renamed your plate type yet, do this now and click 'OK'. The new plate is now added to the list.



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. If you are seeing an image focusing problem across your plate, take 2-4 more measurements in the corners and the center.

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.

## Imager Manual Mode

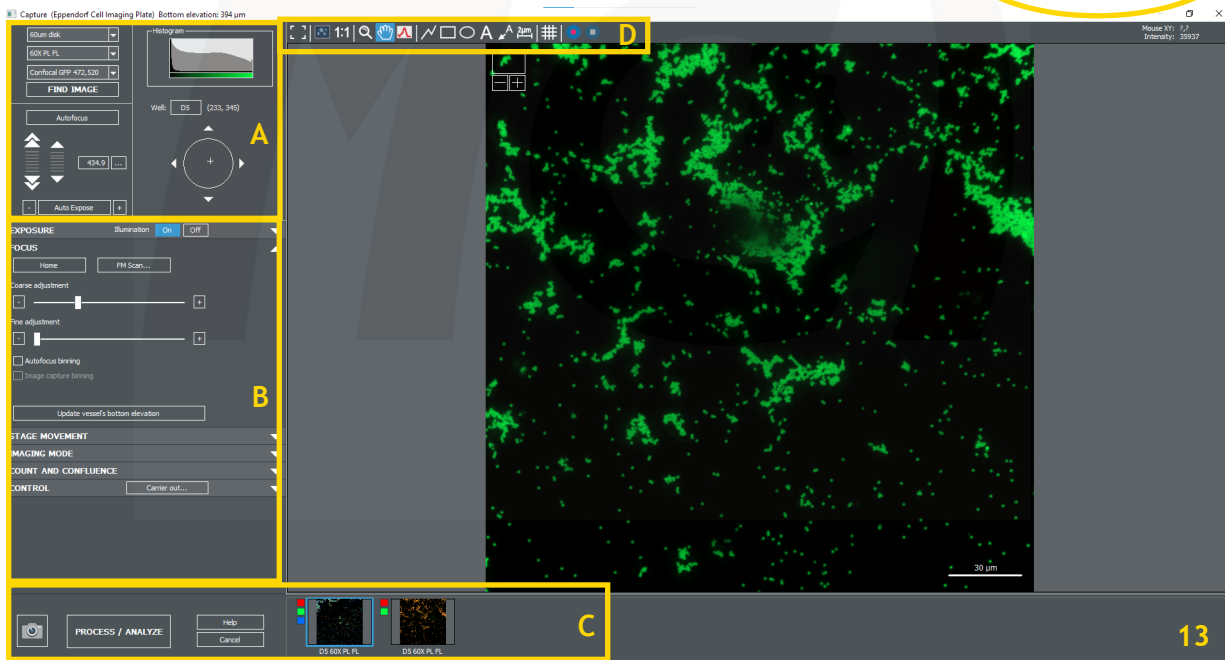
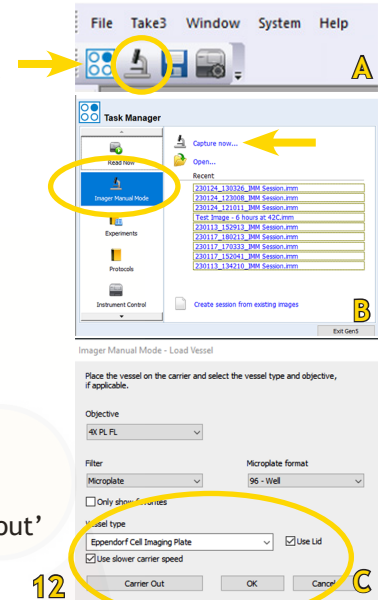
Manual Mode allows you to acquire a single field at a time in up to 6 colours. It is a good starting point to fine-tune focusing and determine the best parameters to apply in an experiment.

IMM sessions are saved with the file extension .imm and can be opened again at a later time from the Task Manager: select 'Imager Manual Mode' and your file (Fig. 12B).

### Start Imager Manual Mode

Click the 'Microscope' icon on the main menu toolbar (Fig. 12A) or open the Task Manager (Figs. 12A arrow, 12B):

1. Select 'Imager Manual Mode' (Fig. 12B).
2. Select 'Capture now' (Fig. 12B, arrow). The 'Load Vessel' dialog will open (Fig. 12C).
3. Select your plate type, check 'Use lid' (if applicable) and select 'Carrier out'
4. Put your plate on the carrier/stage and click 'OK'.



### User Interface

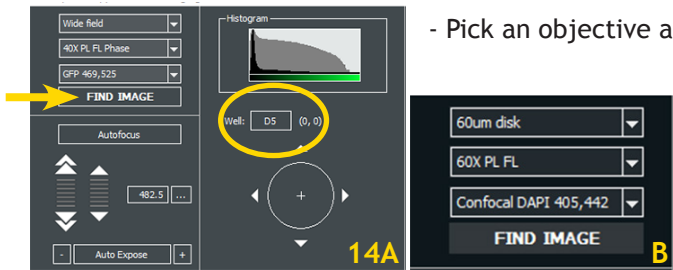
The Manual Mode window will open with a live view of your first well (by default, in well plates, this is A1 or, at higher magnification, B2 or C3).

The Manual Mode window comprises 4 primary sections plus the live image (Fig. 13):

Navigation (A) - Imaging Parameter Controls (B) - Acquisition & Processing (C) - Annotation Tools (D)

## Basic Image Acquisition

### Navigation (Figs. 13A, 14)



- Pick an objective and channel (Fig. 14A)

If you use the confocal imager (Fig. 14B):

1. Set magnification to 20X, 40X or 60X.
2. Choose the 60  $\mu\text{m}$  disk, (not Wide Field)
3. Select the confocal filter cube.

- 'Find Image' (Fig. 14A): Gen5 iteratively applies autofocus and exposure settings when you select this button. **Important:** Select 'Find Image' in widefield imaging only - it is not recommended for confocal.

- 'Well' (Fig. 14A): Click the button to open a plate map and to navigate within your carrier. Click on a well to move to it. You can navigate within the well using the arrow keys or by single mouse-click directly in the well area.

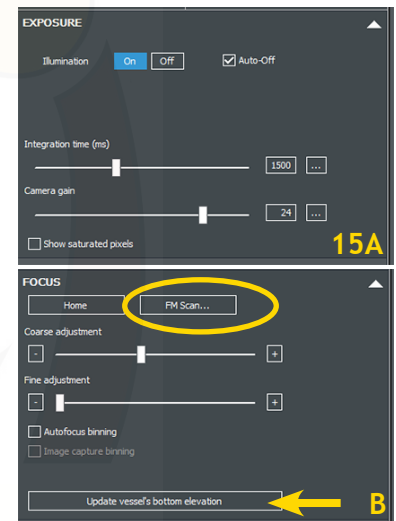
### Exposure (Figs. 14A, 15A)

You can use the plus/minus buttons on the 'Auto Exposure' button (Fig. 14A) or expand the Exposure panel to access user-controlled sliders (Fig. 15A).

### Focus (Figs. 14A, 15B)

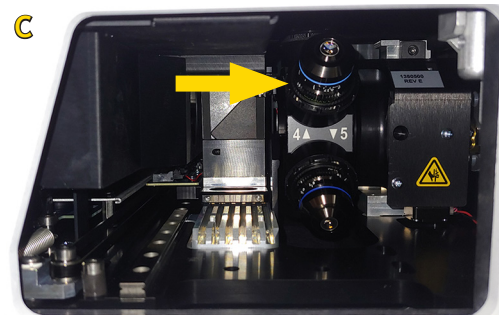
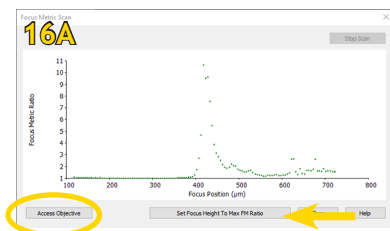
If images are not perfectly in focus, try increasing the Exposure settings, and expand the Focus panel to run 'FM Scan' (Fig. 15B, 16A). When you find a good peak, select 'Set Focus Height to Max FM Ratio' (Fig. 16A, arrow).

If necessary, slightly adjust the 'Auto Expose' and 'Autofocus' controls until you are satisfied with the quality of the image, going back and forth between expose and focus. You can also update the bottom elevation of your plate from this panel (Fig. 15B, arrow).



If you need to adjust the correction collar of the 20, 40, or 60x objective:

In the 'Focus' panel, select 'FMScan' - 'Access Objective' (Fig. 16A), then open the front bottom door (Fig. 1B); in the right compartment, the chosen objective will be facing upwards towards you (Fig. 16C, arrow). Adjust the collar (do not touch the lens!!), close the door, click 'OK' and close the window in the software.



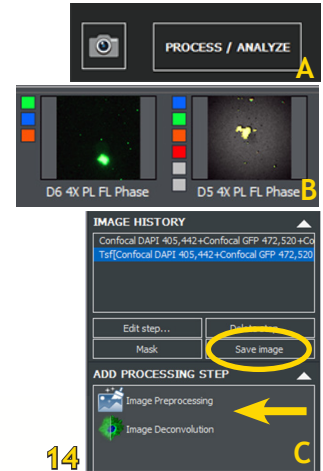


Acquisition and processing (Figs. 13C, 14)

The camera button captures the image with the current settings (Fig 14A). Gen5 creates a thumbnail of the image, then overlays up to 6 channels for that image (Fig. 14B). Adding more than 6 channels or moving in X/Y/Z generates a new image. Colour squares beside the thumbnail image indicate the channels acquired (Fig. 14B).

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

Select 'Process/Analyze' (Fig. 14A) and your desired processing step (Fig. 14C). Gen5 will save the collection of thumbnails and present them for processing and analysis as you proceed. For more details, see pages 20 and onward. When you're in 'Process', you can also save the images acquired in IMM (Fig. 14C).



## Experiment Mode

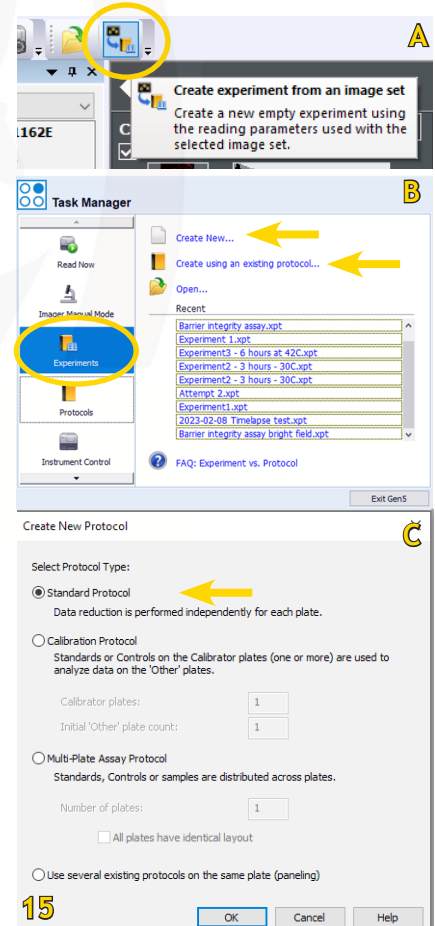
### Creating an Imaging Experiment

There are 3 ways to create an imaging experiment:

#### From a Manual Mode Session

Once you've acquired an image with optimal settings and applied processing and analysis steps as necessary, you can convert these settings to Experiment Mode (file extension .xpt), which automates acquisition of multiple samples.

1. In Manual Mode, after saving your images, select the image of interest.
2. In the main menu, click the 'Create experiment from an image set' icon (Fig. 15A). The 'Procedure' window will open and you can edit your experiment parameters (see page 10).



#### From Scratch

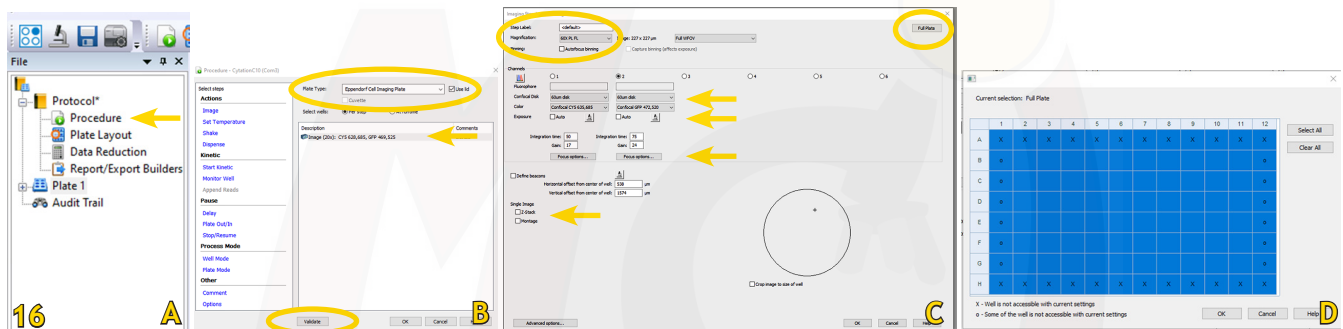
Create a new experiment from scratch from the Task Manager: select 'Experiments' - 'Create New' and opt for a Standard protocol (Fig. 15B, C).

#### From a Saved Protocol

Create a new experiment based on a saved protocol from the Task Manager ('Create using an existing protocol', Fig. 15B).

After creating a new experiment from scratch or based on a saved procedure, you can edit it.

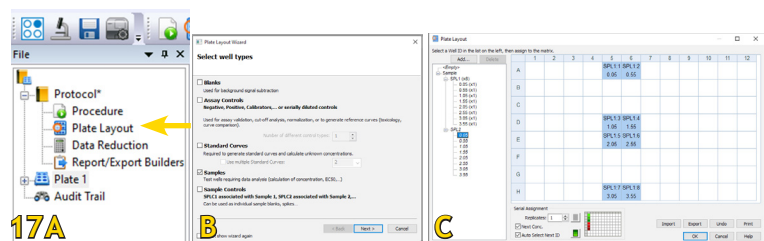
- Select 'Procedure' (Fig.16A)
- Plate Type: Pick your plate definition from the Plate Type drop down list. Check "Use Lid" if applicable. (Fig. 16B).
- To edit or add a step, double-click 'Image' or select one of the options in the left panel (i.e., Kinetic step) (Fig 16B). The 'Imaging Read Step' Window will open (Fig 16C).
- Select the Objective to use (Fig. 16C).
- Choose the light source and channels (Fig. 16C).
- Add Z-Stack or Montage if desired (Fig. 16C)
- Exposure: To use Auto Exposure, check 'Auto' and click on the microscope icon to open IMM. Select both positive and negative wells (highest and lowest intensity) for best auto exposure results. Select 'Save Settings' and close IMM.
- Select 'Focus options' to define the focus strategy (Fig. 16C).
- Select 'Full Plate' to see your well-plate and define the wells to be imaged. (Figs. 16C, D).
- Click 'OK' to close the 'Read' step.
- Select 'Validate' to check your setup (Fig. 16B).
- Click the 'Read' button to start the experiment.



## Plate Layout

It's easy to define the plate layout with Gen5's tools for identifying samples, standards, controls and blanks.

1. Select 'Protocol' - 'Plate Layout' (Fig. 17A).
2. In the Plate Layout Wizard that opens, select the well types used in your assay (Fig. 17B, 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 information 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 (Fig 17C).



# Advanced Acquisition Setup and Experiment Design

## Image Capture for Cell Counting

### Defocusing for Better Contrast

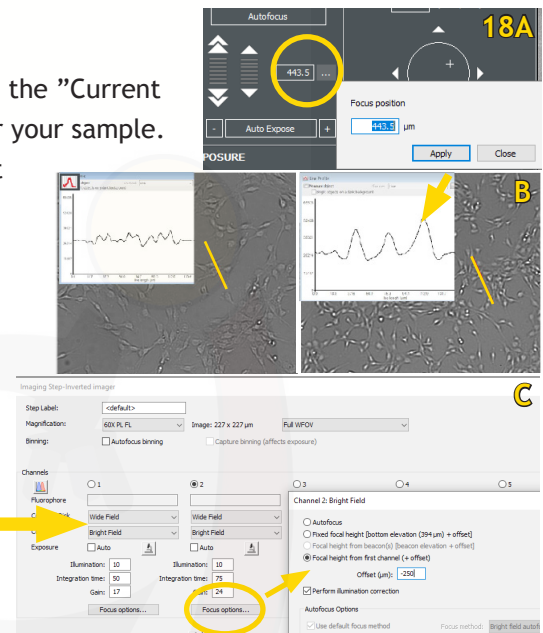
Slightly defocusing the bright field image is recommended to improve cell counting. Usually, this is an iterative process of trial and error to determine how to achieve the highest contrast while retaining acceptable focus.

### In Manual Mode

1. Find the optimal focus settings.
2. Defocus the image by clicking on the 3 dots (Fig. 18A) and reduce the "Current focus position" by  $-250\ \mu\text{m}$ . This is a good starting point but may differ for your sample.
3. Use the Line Tool after defocusing to evaluate the setting's effect on separating background and foreground signals. The goal is to find well-defined intensity peaks for defocused cells (Fig. 18B).

### In Experiment Mode

1. Find the optimal focus settings.
2. Perform two Brightfield reads (= 2 channels), one "in focus" for the best images and one "defocused" for the best cell counting. For the second channel, select Focus options. Select 'Focal height from first channel (+offset)', and enter a defocusing offset (Fig. 18C).
3. Use the Line Tool after defocusing as described above.



## Image Montage (Stitched Image)

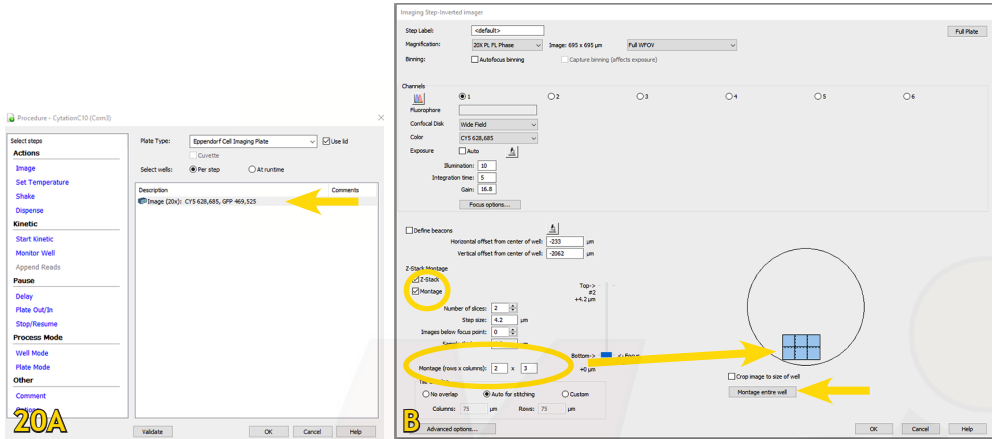
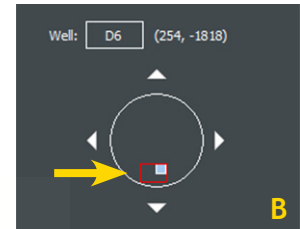
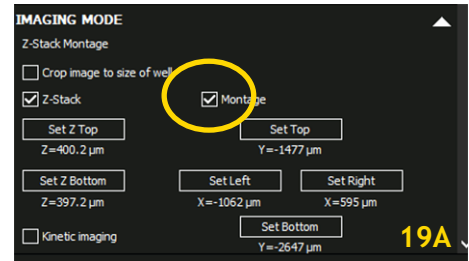
Montaging (=tiling, stitching) allows you to capture a larger field-of-view while maintaining the resolution of your desired objective. Montage is a good choice when: many cells are required for analysis, or large objects contain information beyond a single field-of-view.

### Montage in Manual Mode

1. Expand the 'Imaging Mode' panel (Fig. 19A).
2. Enable Montage. Navigate the field-of-view with the software controls, and set the boundaries with 'Set Top', 'Set Bottom', 'Set Left' and 'Set Right'.
3. Red lines will appear on the well map as you set boundaries (Fig. 19B). When finished, you will see a red box indicating the area to be covered by the montage.
4. After defining the area you want to capture, click the camera button. The default 'Auto for stitching' setting gives 10% overlap between tiles.

## Montage in Experiment Mode

1. In the Procedure, double-click 'Image' and select 'Montage' (Figs. 20A, B).
2. Adjust the number of rows and columns to define the capture area, which will be shown in the well map (Fig. 20B). If the entire well is desired, you can click on 'Montage entire well'.
3. You can also define offsets for the montage center or use beacons (see pages 14-17).

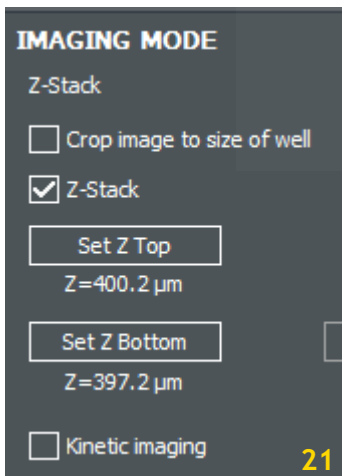


## Acquisition of a Z-stack

Z-stacking captures multiple images (up to 200 slices) in multiple focal planes.

Use Z-stacking when analyzing: thick samples, hanging-drop assays, whole organisms, assays run in matrigel.

**Best practice:** 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).



## Manual Mode Z-Stack

1. Expand the Imaging Mode panel and enable Z-Stack (Fig. 21). Gen5 always shows the current z-position as you move in the z-axis.
2. Use the Autofocus control '+/-' buttons to find the z-axis position that best defines the start of the sample area you want to capture.
3. Click the 'Set' buttons for both top and bottom position when you have found the top and bottom in-focus images of the sample, respectively.
4. Click the camera button to capture the Z-stack.

Based on capture height and objective depth-of-field (=step size), Gen5 determines the sample thickness, step size, and the number of optical slices.

## Experiment Mode Z-Stack

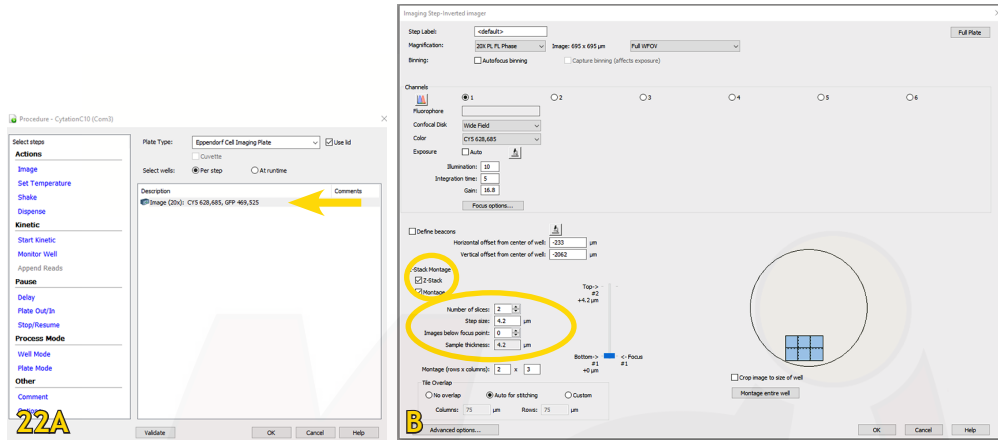
To define a Z-stack directly in experiment mode,

1. In the Procedure, double-click 'Image' and select 'Z-Stack' (Figs. 22A, B).
2. Set the parameters

Stepsize: defaults to the depth-of-field of the objective, it can be changed if needed;

Number of slices: defines the Sample thickness.

Focuspoint: by default, the Bottom position is the focus point. You can use the slider or define a number of images below focus point to create an alternative capture range.



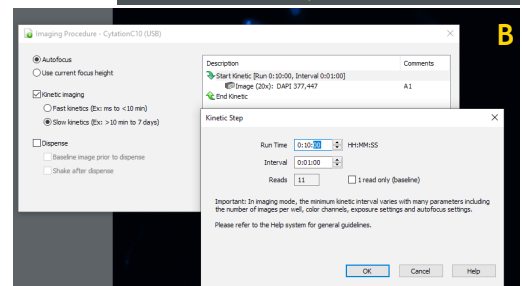
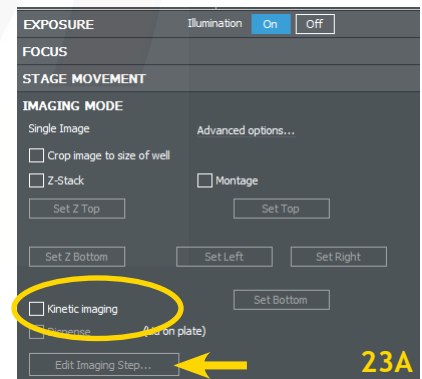
## Kinetic Image Capture (Time Lapse)

Time-lapse image capture is available in both Manual and Experiment Modes. Several factors affect the interval duration, including: Number of detection channels - Integration (exposure) time - Autofocus method - Binning.

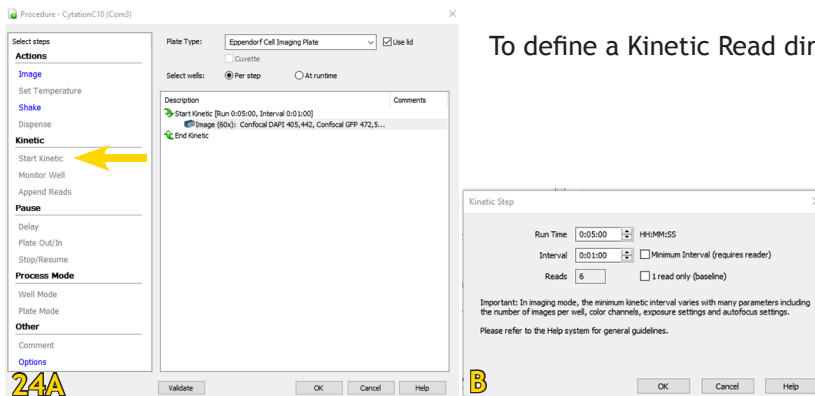
Integration times to achieve 10 frames per second (fps) must be set to 50 milliseconds (ms) or lower; integration times to achieve 20 fps must be 20 ms or lower (with binning turned on). Fast kinetics must be performed in 'Well Mode'.

## Manual Mode Kinetic Acquisition

1. Expand the 'Imaging Mode' panel, (Z-Stack and Montage can also be included in a Kinetic loop) and check 'Kinetic imaging' (Fig. 23A).
2. Click 'Edit Imaging Step' (Fig. 23A).
3. In the pop-up window, double-click 'Start Kinetic' to define the interval (the time between imaging steps), and total run time (Fig. 23B).
4. Click on the camera icon to execute the run.



## Experiment Mode Kinetic Acquisition

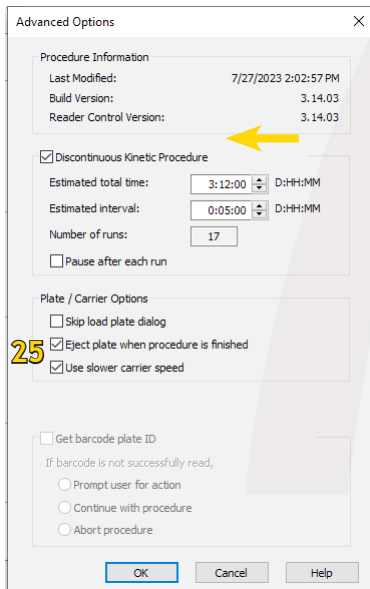


To define a Kinetic Read directly in experiment mode:

1. In 'Procedure', select 'Start Kinetic' in the left panel (Fig. 24A).
2. Define run time and interval (Fig. 24B).
3. Click 'Validate' and 'OK'.

## Discontinuous Kinetics

Set Up a Discontinuous Kinetic Experiment:



1. From the Task Manager, select 'Protocols' - 'Create New'
  2. When defining the procedure, select 'Options', and select 'Discontinuous Kinetic Procedure' (Figs. 24A, 25).
  3. Enter 'Estimated total time' and 'Estimated interval' in Days:Hours:Minutes. Gen5 uses your estimated timelines to setup the data views and formulas with place holders 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 Readstep(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.
  7. Remove the plate and process (e.g., add reagent, incubate, etc.) until it is time for the next reading.
  8. For the next reading time, open your 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.

## ROIs and Beacons

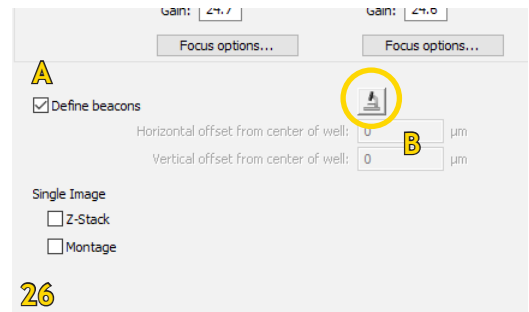
Two methods are available for identifying and imaging Regions of Interest (ROIs) in an experiment:

- A. Add Beacons manually to mark ROIs: Place a beacon on each ROI in every well individually when creating the experiment, or assign a pattern of beacons to multiple wells simultaneously.
- B. ROI Identification Experiments: Discover ROIs using low magnification, image ROIs using high magnification.

## Add Beacons to mark ROIs

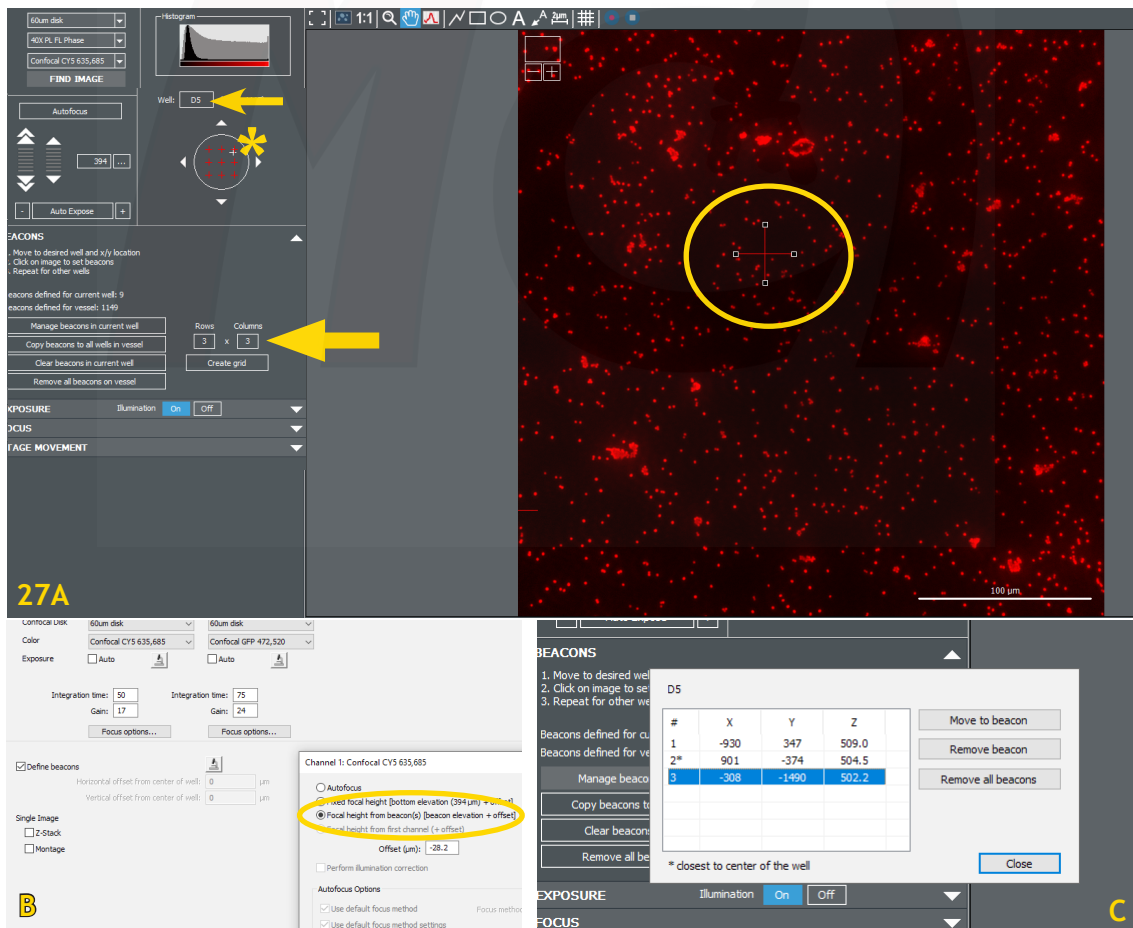
By default Gen5 captures images at the center of the well. Two ways to change the X/Y position of the capture area in an experiment are:

- A) Beacons (Fig. 26A): to identify regions of interest manually, when the ROIs vary from well to well (e.g, sparse or patchy cell population or spheroids).
- B) Horizontal and vertical offsets (Fig. 26B): When the region of interest is not in the well center but occurs in the same area (X/Y coordinates) of every well (e.g., biofilm accumulates around edges of wells).



### Defining beacons in experiment mode

1. In the 'Imaging Read Step' dialog, set the Objective and Colour and select 'Define beacons' (Fig. 26A), then click the microscope icon to define the beacons. Gen5 launches a special IMM session to let you define beacons (Fig. 27A).
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.



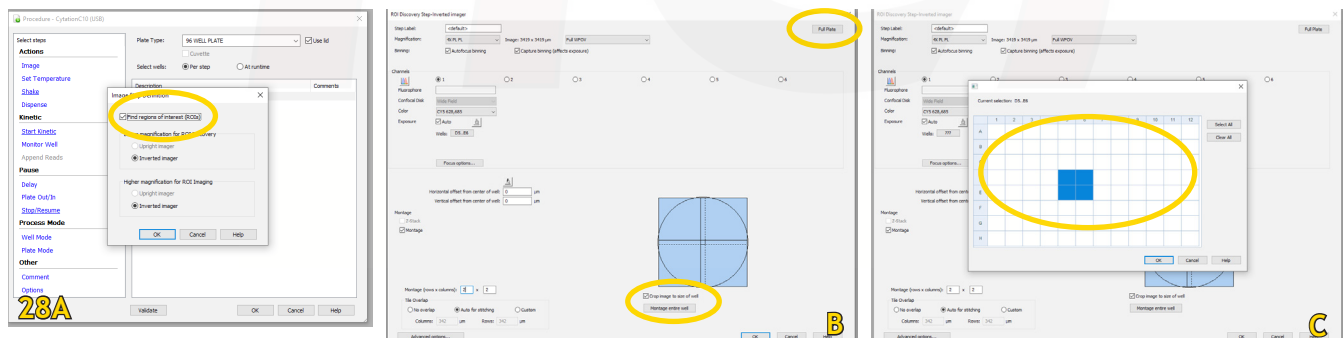
- Set focus and exposure, change the focus option in the Image Read Step from 'Autofocus' to 'Best focus of Beacon' if necessary (Fig. 27B).
  - Click in the centre of the image, this adds the xyz coordinates. A red cross in the image itself and a red cross in the well map mark the spot of the beacon (Fig. 27A).
  - To define beacons in another well, repeat steps 2 through 5.
  - You can also use a grid to define multiple beacons in a well (Fig. 27A, asterisk). Beacons are evenly spaced in the well when a grid is applied. To apply a grid to a well, enter the number of Rows and Columns and click 'Create grid' (Fig. 27A).
- Using a grid in combination with "Copy beacons to all wells in vessel" is a fast way to apply beacons to multiple wells. You can add, move, delete, and manage the beacons. To change the grid dimensions, first clear beacons from either the current well or the entire plate.
- Note: Gen5 prompts you to delete any existing beacons before executing the copy function.
- Gen5 activates the multi-beacon controls once several beacons are defined (Fig. 27C). Use the top control 'Manage beacons in current well' to evaluate and modify beacon selection.
  - When you are finished defining beacons on that plate, click 'Save settings'.
  - In the read step, click the plate button to see where beacons are defined. Hover the mouse over the well to see its offsets.

### Defining ROIs in experiment mode

This automates the identification of ROIs in your well plate: Wells are scanned at low magnification in a 'Discovery Step' with 1 channel only; Gen5 identifies ROIs automatically, then switches to the defined higher magnification and additional/other channels, stacks or montages, and images all identified ROIs in the 'Imaging Step'.

### Step by Step ROI Imaging

- Create a new experiment, select 'Image' and check 'Find regions of interest (ROIs)' (Fig.28A). Click 'OK'



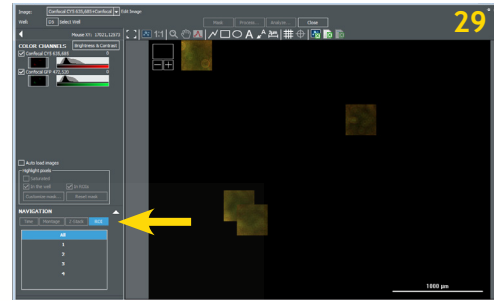
- In the first discovery step at lower magnification, define the entire potential field for the ROIs:
  - Use the 'Montage entire well' shortcut to cover the entire well, if useful (Fig. 28B).
  - Click 'Full Plate' (Fig. 28B) and then limit the wells to be imaged, if applicable (Fig. 28C). This setting will be fixed for ROI imaging also, except that only wells with one or more defined ROIs are imaged.
- Check the correction collar setting (20x-60x objectives) with the FM Scan.
- Do an 'Autofocus' routine to see if it works for your sample - otherwise, 'Beacons' are a better option.



5. Retain the default settings in the ROI Settings dialog (Fig. 28B), at least initially. You may want to adjust the ROI margin after gaining some experience with the process.
6. Define the image acquisition step with the desired magnification and colour channels plus additional options as in any experiment (this will be different from the discovery step!). Click ‘Validate’ to make sure the procedure can be performed as defined.
7. Add processing steps if desired/needed.
8. Read the plate.

## Viewing ROIs

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.



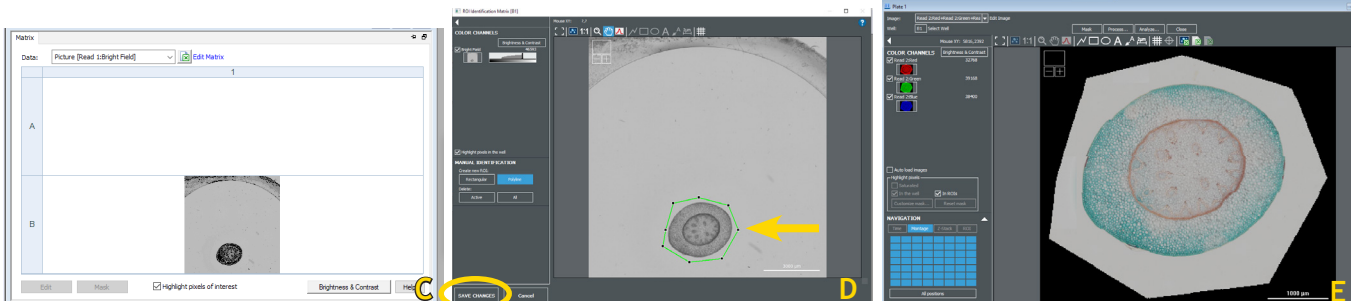
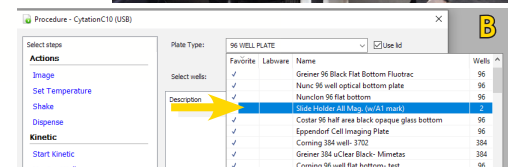
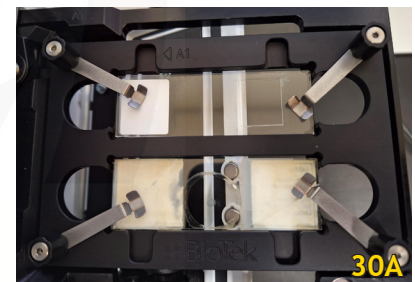
## Slide Imaging

The slide scanning workflow is very similar to ROI imaging described above. You will need the slide insert for this (Fig. 30A) and the equipment needs to be calibrated (true???).

Colour brightfield imaging: The colour option offers no exposure control, due to its nature of filter imaging. Brightness and contrast can be adjusted in B&C after image acquisition, if needed.

## Set up Slide Scanning in Experiment Mode:

1. Select ‘Experiment’ - ‘Procedure’
2. Select ‘Slide Holder All Mag’ as plate type (Fig. 30B)
3. Double-click on ‘Image’
4. Select ‘Find ROI’ and define discovery and imaging steps as described above (Fig. 28). The discovery scan will not be saved; it uses binning and displays the montage image without stitching (Fig. 30C). The discovery scan can also be performed in brightfield gray scale (saves time).
5. In the preview, define the ROI. Select ‘Save changes’ (Fig. 30D).
6. Select ‘Run’ and the system will perform the imaging step and display the final montage or image (Fig. 30E).



## Image Review and Processing

### Image Review

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

You can view each channel separately by selecting/deselecting the checkboxes under 'Color Channel'.

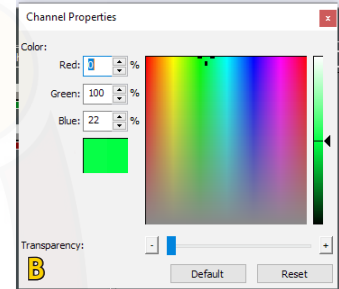
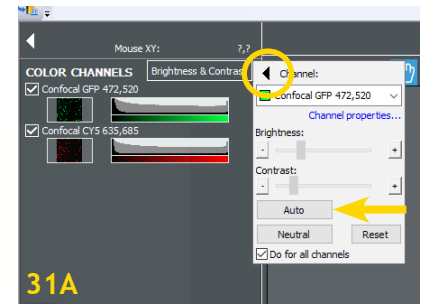
After acquisition in Manual or Experiment Mode, the Brightness and Contrast (B&C) Control window and the annotation toolbar are immediately available.

### 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 (Fig. 31A). B&C modifications do not alter your raw data in any way, they merely improve the display on the monitor.

Choose 'Auto' (Fig. 31A, arrow) to let Gen5 make the adjustments.

'Channel properties': Select this to apply transparencies and/or adjust color gradients to improve the image (Fig. 31B).



### Annotation Tools

Common software annotation tools are offered after image capture. They can also be used in graphs and other elements of the Gen5 dataset (Fig. 32). Whenever the toolbar is present, you can add notes to draw attention to areas of interest in the image or chart.



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 tools to draw polylines, rectangles, and circles, apply text, 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

To use various annotation tools:

- Click and drag across the image.
- Grab the element's nodes to resize it.
- Hover over a positioning tool, to move it.
- Double-click an item to change its properties.
- Right-click for a context-sensitive menu.

Select 'Properties' to change the colour and other elements of the item.

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

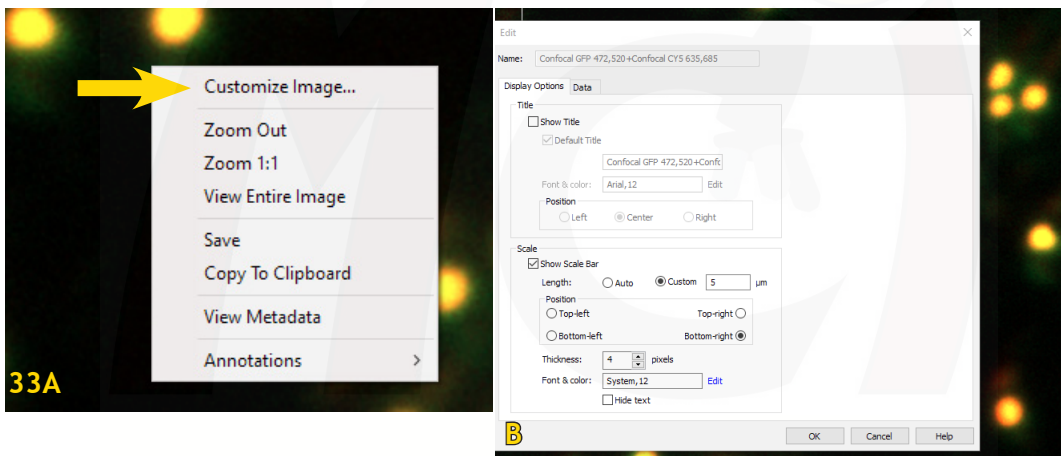
After using a tool to add an item to the image, right-click on the element and select 'Properties' to change its appearance.

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

## Customize the Scale Bar

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' (Fig. 33A). Or, in a well zoom, click 'Edit Image'. Adjust the values (Fig. 33B).



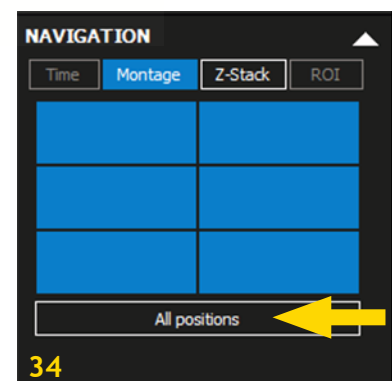
## Viewing Montage and Stitched Images

After capturing the montage image, Gen5 presents a special Control box to let you view the whole raw image (unstitched) or an individual tile (Fig. 34):

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.

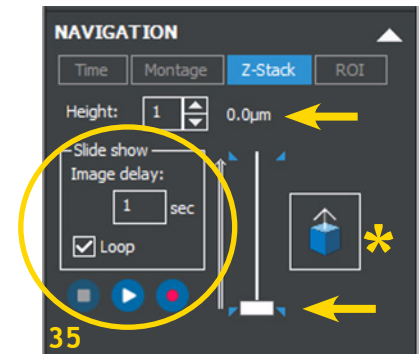


## Viewing a Z-Stack

After capture, Gen5 presents a Z-Stack Control (Fig. 35). You can use:

- The slider or 'Height' scroll buttons to display one slice at a time in sequence
- Z-Stack Slide Show: to view the slices like a slideshow
- Z-Stack Movie: click the record button to make a movie
- 3D Viewer: click to render the z-stack in 3D (Fig. 35, asterisk).

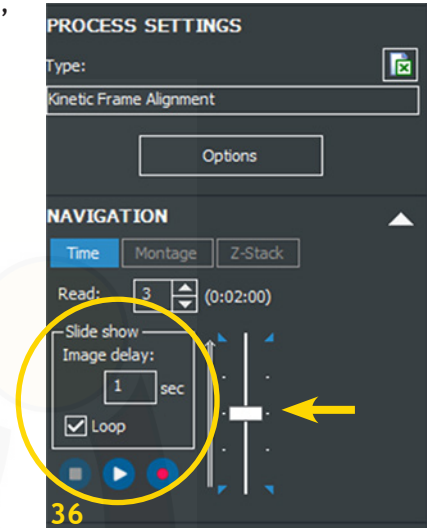
In experiment mode, set the plate's Matrix Data to 'Picture' without a 'Z Proj' label to open the Z-Stack Control and view the individual slices.



## Viewing a Kinetic Sequence

After capture, Gen5 presents a Time Control (Fig. 36). You can use:

- The slider to display the time points one by one in sequence,
- Time Slide Show: to view like a slideshow and define the image delay
- Time-Lapse Movie: click the record button to create a movie.



## Image Processing

The images presented immediately after acquisition are truly raw data. Image processing refers to the mathematical modification of images to improve their quality.

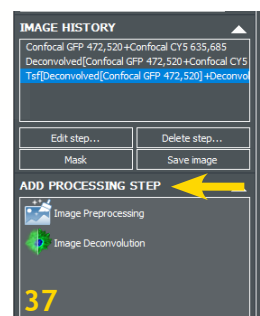
Gen5 creates a new data set for each processed image. You can compare the data sets by selecting them in the 'Image History' box (Fig.37). Double-click the processed data to modify its parameters.

Gen5 prepopulates the processing steps, depending on the parameters of the acquired images.

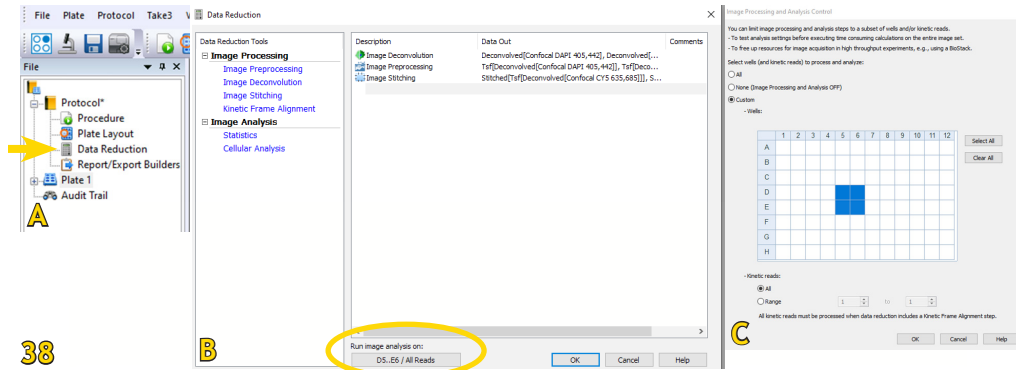
These methods include:

- Image Preprocessing (Background Flattening and Smoothing)
- Digital Phase Contrast
- Deconvolution
- Image Stitching
- Z-Projection
- Kinetic Frame Alignment

In IMM, select the process step under 'Add Processing Step' (Fig. 37).



In experiment mode, select ‘Data Reduction’ (Fig. 38A) and select from the available processing steps (Fig. 38B). As general rule, always start with ‘Deconvolution’ if needed, any other processing step should be performed afterwards. Just like in image acquisition, you can select which wells you want to process/analyze: Select ‘Data Reduction’ - ‘Run image analysis on:’ to see your well-plate and define the wells to be processed/analyzed (Figs. 38B,C).

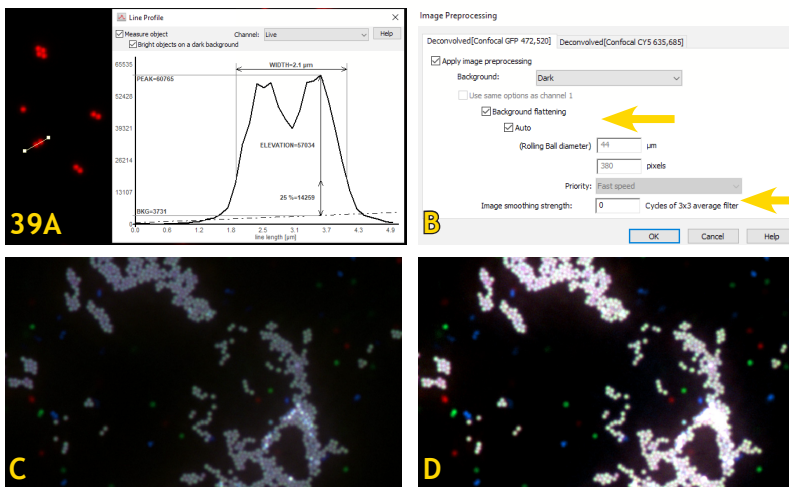


## Image Pre-Processing

Gen5’s image preprocessing step eliminates weak background signal and smoothes objects’ edges to create a cleaner, sharper image. Unlike ‘Brightness and Contrast’ changes, Image Pre-processing alters your data!

### Background flattening

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. Use the Line Profile tool to estimate size and show comparative pixel intensities (Fig. 39A). It is useful for determining the optimal rolling ball diameter: with a poorly chosen rolling ball size, legitimate signal can 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 (Fig. 39B).



### Important:

- The preprocessing step takes longer, the smaller the selected diameter of the rolling ball.
- If deconvolution is performed after the rolling ball preprocessing, it can introduce artefacts like a black square in the signal area (meaning the rolling ball diameter was set too small).

## Image smoothing

A method of removing noise at the perimeter of an object, can also be added in this step (Fig. 39B). 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.

### Important:

Every time you choose 'Image preprocessing', it increases image brightness (Figs. 39C,D) - select B&C and select 'Neutral' to restore the brightness to its original state. 'Reset' reverts to the previous state (Fig. 31A).

## Digital Phase Contrast



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 in high-density plates and corrects the uneven illumination and halo effect that is otherwise observed.

Gen5 performs three steps to apply digital phase contrast to acquired images (Figs. 40A,B):

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 Processing Step' in the Image Processing area (Fig. 40A).

In Experiment Mode, select 'Data Reduction' and click 'Digital Phase Contrast' (Fig. 40C).

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

Set the 'Structuring element size' (Fig. 40D) to match the size of the objects of interest in the image.

Too large = geometric artifacts will distort the image.

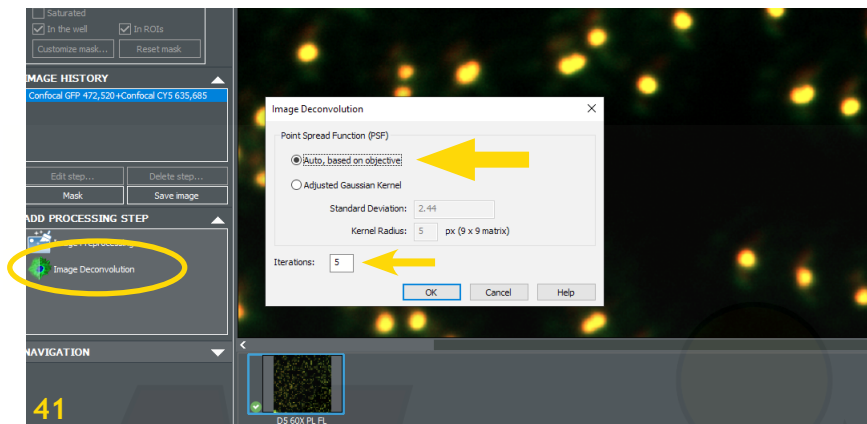
Too small = true signal in the sample will be lost.

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

## Deconvolution

Deconvolution (Richardson-Lucy) removes the “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.

Select the ‘Image Deconvolution’ Processing step in IMM or in experiment mode - Select “Auto, based on objective” - Define the number of iterations - Click ‘OK’ (Fig. 41).



## 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 (Fig. 42).

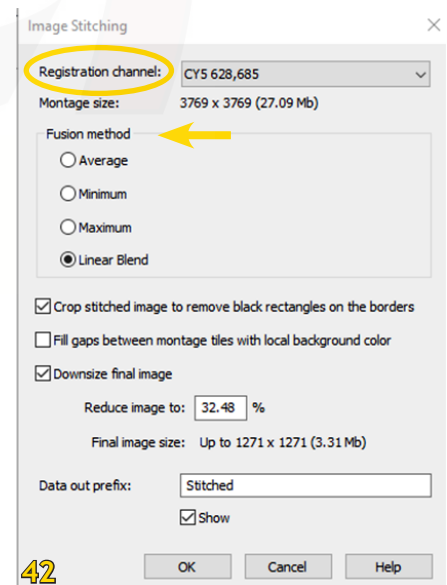
‘Registration channel’ is the channel that will be used to determine the tile alignment. Choose the channel with the most signal and coverage, often this is your nuclear stain.

‘Fusion Method’ 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 montage 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.

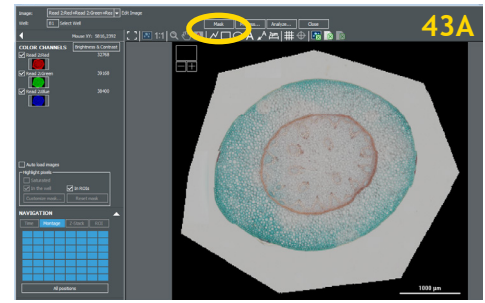
‘Downsize final image’ reduces the final image size. A full-resolution image will be (2.2 MB [minimum size of each tile] X number tiles in the montage). Downsizing improves computer performance and storage space, but also reduces the resolution of the resulting montage.



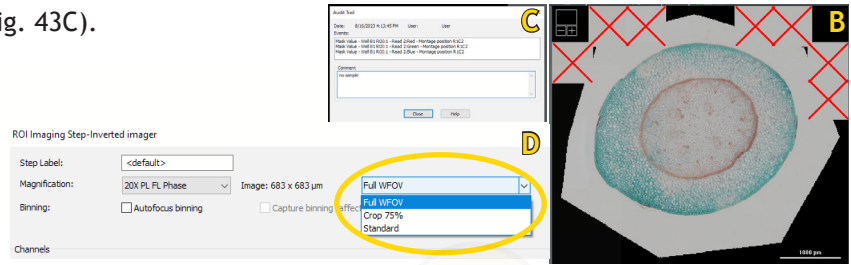
### Exclude Unwanted Tiles

‘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 (Fig. 43A).
2. Select the tile you want to mask by clicking in the image or using the Navigation tools.
3. Click ‘Mask’ (Fig. 43A). Gen5 marks the tile with a red X (Fig. 43B).
4. Gen5 prompts for an audit-trail comment when a mask is applied. Keep notes on the reasons for the mask (Fig. 43C).



Sometimes, channels are misaligned at the edge of wells if they are in liquid media. This is due to the meniscus artefact. Use ‘Crop 75’ (Fig. 43D) to remove the outer edges of your stitched image.



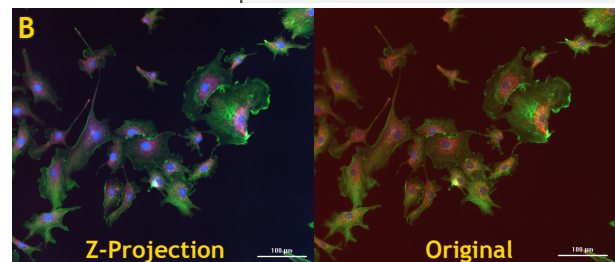
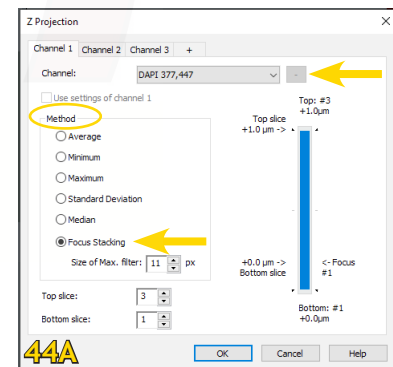
### Z-Projection

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.
- In Experiment Mode, define a Data Reduction step.

#### Specify method and slices for Z-projection (Fig. 44)

1. In the Z Projection window, select a colour channel. For a multi-channel z-stack, click the ‘+’ tab for each additional colour. By default, additional channels 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. However, ‘Focus Stacking’ doesn’t work well for thick objects like organoids or confocal images. For those images, a regular Z-Projection is better. Mostly the ‘Maximum’ algorithm works best. If the sample shows large variation in intensities across the Z slices, try the ‘Standard Deviation’ algorithm.
3. Use the Top and Bottom slice scroll buttons or drag the slider to limit the projection to a subset of slices.





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 (Fig. 35, asterisk and Fig. 45A).

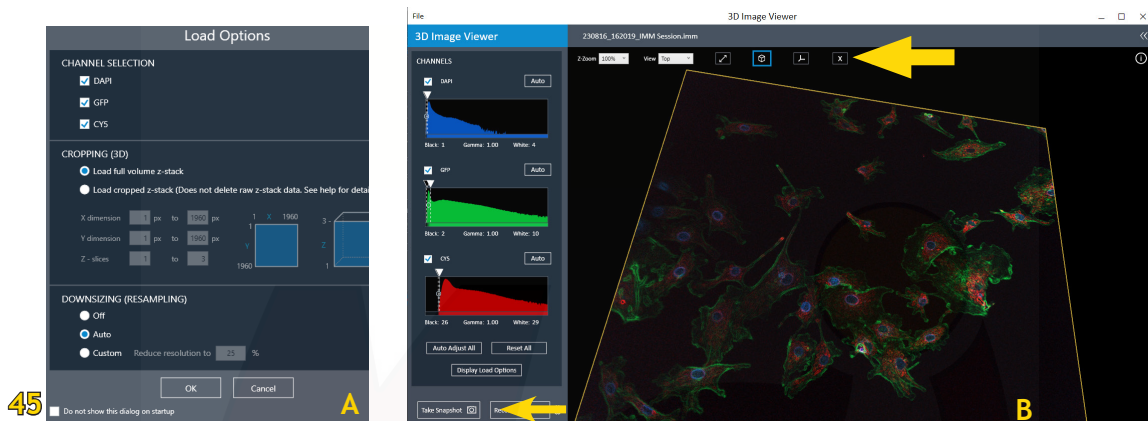
Interactive Controls (Fig. 45B): Several controls for manipulating the 3D projection are provided. Experiment with them to determine the best rendering of your sample. Begin by applying the bounding box to enhance the 3D dimensions. Toggle the X, Y, and Z planes to add these views to the projection, 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 gray scale at various positions in the stack. Right-click and drag on the plane to change its brightness and contrast.



Use Histograms to improve projections (Fig. 45B): Histograms for each colour 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. 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.

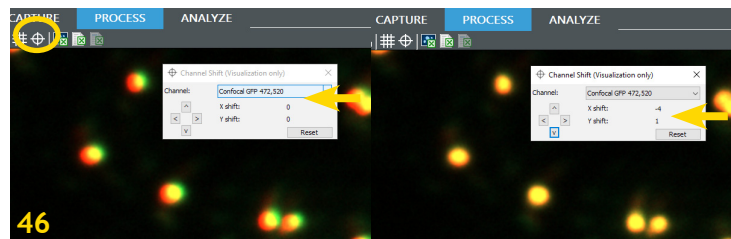
3D Snapshots and Movies (Fig. 45B): Use the controls at the bottom of the 3D Viewer to capture snapshots and movies of the current 3D projection. Snapshots can be saved as TIF or JPEG. Click the 'Options' button to customize the movie settings. This is especially useful for reducing the file size of the movie.

## Channel Shift

Fluorescent channels can be shifted to each other. This systemic error is constant throughout the image and can be corrected by using fluorescent beads as control (Fig. 46):

Select the 'Channel Shift' icon

Select the fluorescent channel to align  
Define X and Y shift by moving the target image pixel by pixel in the correct position.

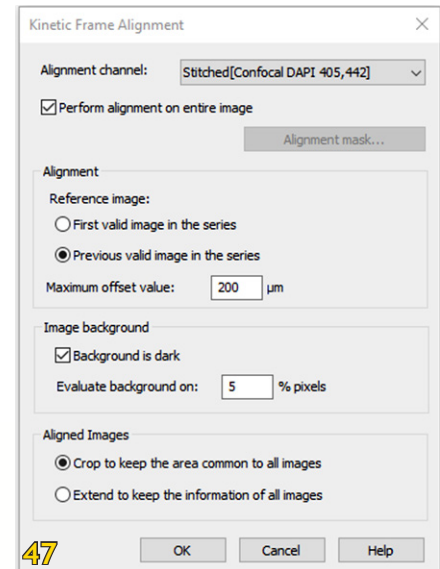


## Kinetic Frame Alignment

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 (Fig. 47).

In Manual Mode, click Z-Projection in the 'Image Processing' area.  
In Experiment Mode, define a Data Reduction step.

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.



## Kinetic Z-Stack Montage

You can combine the advanced acquisition methods (Time-lapse - Z-stack - Montage)

Such a complex image compilation will produce significantly larger data files depending on the number of colour 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 harddrive? 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 4096MB. Stitched images (after downsizing) must be smaller than 1GB (1024MB).

# Data Management

## Saving Files

### File Management System

Experiments and Protocols are saved on the C: drive in the 'Public' folder. Imager Manual Mode (IMM) files and images are saved on the E: drive (external hard drive) in your lab folder (E:\Cytation C10 User Data\Images\your lab folder").

**Important:** 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 subfolder under the experiment folder when moved so that the experiment and image folders can automatically relink.

## ***Saving Images***

When a Gen5 session is saved, all acquired images are automatically saved in your Image Library.

Raw images are 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 reanalysis 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 Image' function. 'Save Image Raw Data' - saves the image in 16 bit TIFF format. Multi-channel images can be saved as separate files per image or as stacked images in a single TIFF file.

'Save Picture for Presentation' - saves the image or current display in colour, 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.

### 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"

### Recording and saving time-lapse movies

Gen5 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.

### Saving movie files

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

- wmvis - Windows default video file format
- mp4 - industry standard video file format

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.

## ***Reporting and Exporting Experiment Data***

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.

## Shutdown

### 2-HOUR-RULE:

**Don't switch off anything** if someone is signed up within 2 hours after you finish!  
Only close the software and log off your Windows account.

- 1) Save your data and export them to OneDrive
- 2) Close Gen5
- 3) Log off your account
- 4) **Shut down computer**
- 5) **Switch off Plate Reader**
- 6) **Turn Laser key to 'Off'**
- 7) **Switch off laser engine**

