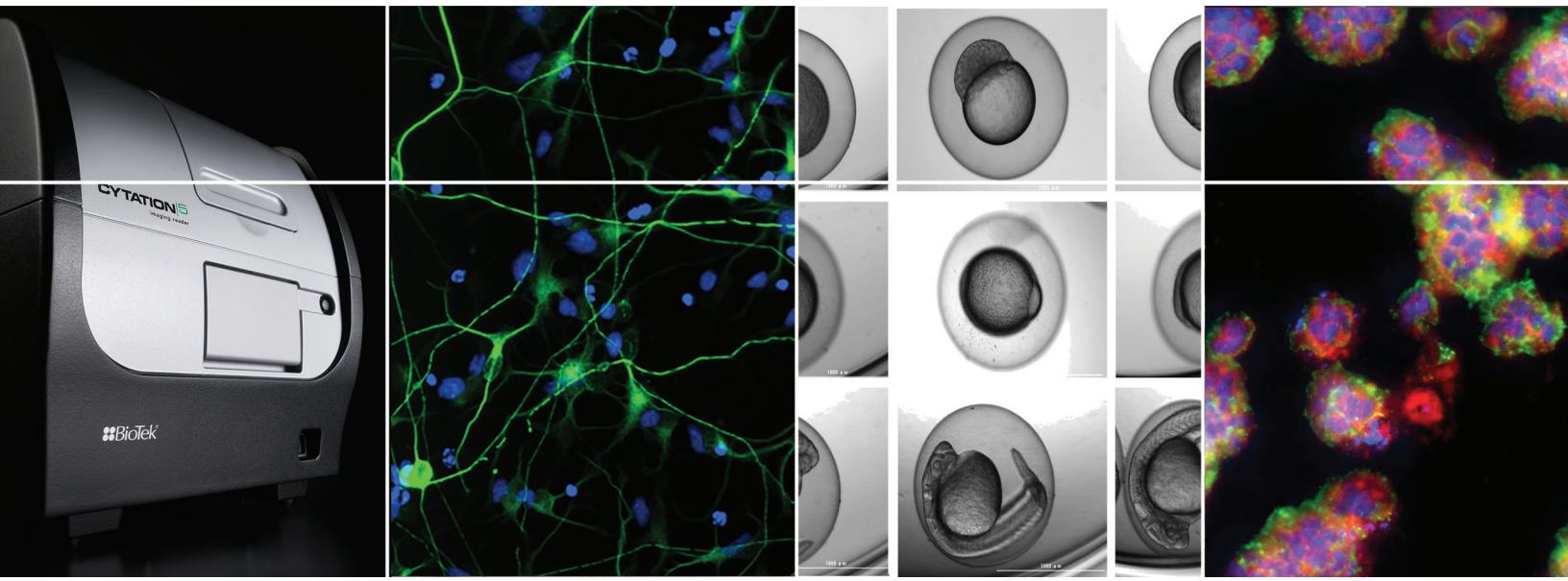
Cytation™ Cell Imaging Multi-Mode Reader

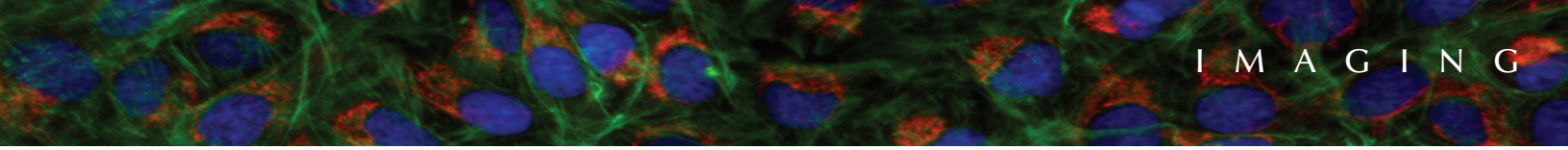
An Imaging Primer



Think Possible

Fisher Scientific

BioTek®



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1. Introduction

Why Image?

The old adage "a picture is worth a thousand words" holds true, even in cell biology. Viewing cells, the most basic unit of life, has been going on for 400 years, since the advent of the microscopy instruments of Hooke and van Leeuwenhoek. The biggest benefit to life sciences is fluorescence microscopy and an ever increasing array of fluorescent probes that light up molecules involved in cellular signal transduction.

German organic chemists in the late 19th and early 20th century developed the first useful probes, albeit for completely different applications at the time – paint! Hoechst 33342, for example is a widely popular nuclear stain used today. The discovery of antibodies and Albert Coons' demonstration of their fluorescent labeling in the mid-20th century opened the door for the use of a virtually unlimited array of fluorescent probes of unique specificity to proteins of interest in the cell. Yet perhaps the most significant addition to the arsenal of fluorescent probes is the work of Roger Tsien in developing green fluorescent protein (GFP), and a subsequent family of photoproteins as fusion proteins used to visualize cellular protein trafficking in real time. These probes and the advances in microscopy instrumentation offering spatial resolution that defies the diffraction limit of light have led to the adoption of imaging techniques as the primary tool in every cell biologist's toolbox.

Why Microplates?

Most imaging is done on microscope slides: one slide per experiment. Microplates offer the ability to perform multiple experiments in a convenient, inexpensive vessel that minimizes reagent use (experiment cost!). Microplate vendors provide myriad protocols for seeding almost any cell into microplates, whether immortalized, primary, or stem cells. These protocols allow for a broad range of applications to be conducted rapidly and easily including qualitative cell staining of fixed cells, live cell imaging, and both quantitative live cell and end-point assays.

Microplate imaging of cells is currently largely reserved to expensive instrumentation in the High Content Screening (HCS) market. Although powerful in their ability to image cells in microplates and reduce reagent expenditure on a per experiment basis, these instruments are beyond the budgets of most university laboratories. Their high cost is driven by the development of software algorithms to enable specific imaging assays (e.g., neurite outgrowth), data storage needs, and imaging rapidity to enable screening of thousands of compounds in a reasonable time frame.

Why Imaging in a Microplate Reader?

The microplate reader is a universal laboratory instrument. It is used in myriad applications such as DNA and total protein quantification, ELISAs, enzyme kinetics, cytotoxicity and many other assays. Many readers offer multiple detection technologies including the most common: absorbance, fluorescence, and luminescence, and also more esoteric modes such as FRET, TR-FRET, and fluorescence polarization. The microplate reader is typically a workhorse in the common laboratory for quantitative measurements. Adding an imaging mode to its detection capabilities provides an even more indispensible instrument for the laboratory.

And, the combination of imaging and microplate detection modes is greater than its parts. The two detection modalities can be used together to provide unique capabilities. Quantitative measurements using the microplate reader can be used to trigger the imaging of wells based on whether a certain threshold intensity is achieved. This serves to reduce significantly the number of wells requiring imaging, reducing not only total analysis time, but also image data storage requirements – two well-known bottlenecks in HCS applications.

2. Cytation Hardware

Overview

Three separate and distinct optical systems reside in a fully equipped Cytation instrument. The multi-mode microplate detection system includes a quadruple monochromator-based optical path and a filter-based optical path. An Alpha Laser module can be added to the filter optics. The imaging section contains a CCD camera with tube lens, filter cubes with matched LED light source and up to six objectives. Additionally, a Phase Contrast imaging device is offered, but it cannot be combined with the filter-based optical system.

Shown in this cutaway illustration:

Multi-mode detection system components, top:

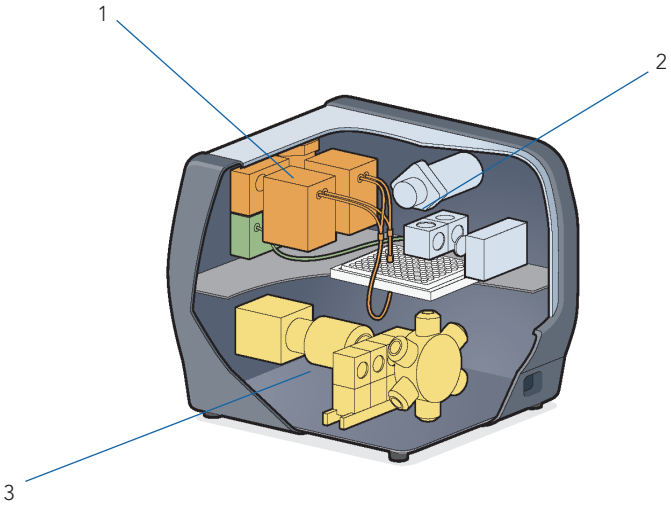
1 Quadruple monochromator and Alpha laser optics

2 Filter optical components

Not shown but available: Phase Contrast optical components (which would replace the filter optical components)

Imaging system components, bottom:

3 Imaging system components

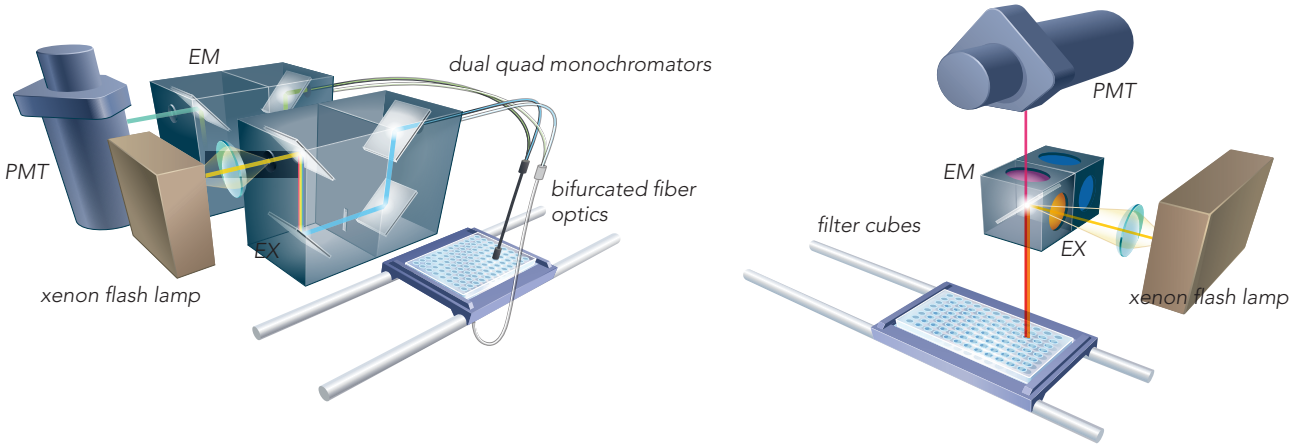


Multi-Mode Detection System

The multi-mode microplate detection system in Cytation is a patented design. This Hybrid Technology™ optical system contains flexible monochromator-based optics that becomes a high-performance patented hybrid system with the addition of a filter-based optical module.

The monochromator optics uses a 4th-generation quadruple grating design that provides a broad excitation or emission wavelength range, selectable in 1 nm steps. This system supports top and bottom fluorescence intensity, UV-Vis absorbance and high performance luminescence detection. It is the ideal system for all the standard microplate applications found in life science research laboratories.

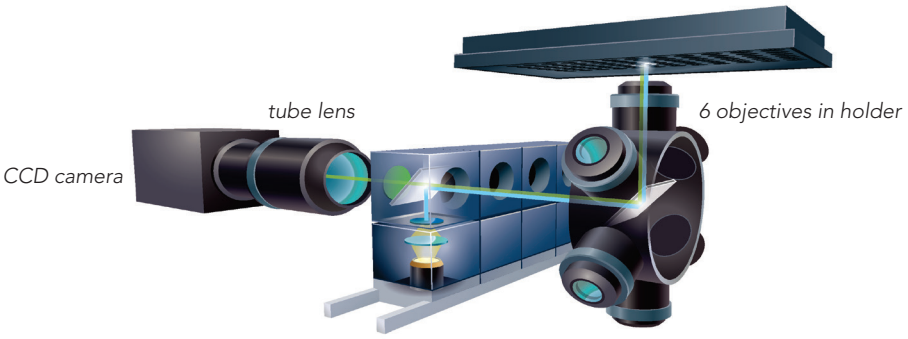
The filter module is a completely independent optical system that includes its own light source and a high-performance dichroic-based wavelength selection system. With its very high optical efficiency, the filter module supports advanced detection modes such as fluorescence polarization, time-resolved fluorescence & TR-FRET and filtered luminescence (e.g., BRET). An optional alpha laser module supports AlphaScreen®, AlphaLISA®, and SureFire® assays.



The quadruple monochromator (left) and filter- based (right) optical components and light paths of the multi-mode detection system. Note that each has its own light source, wavelength selection and PMT detectors.

Imaging System

Cytation's automated digital microscopy and camera optical system is completely contained within the base of the instrument (except in Phase Contrast models, some optical components replace the filter optics in the upper section of the reader). Optical components fit in a compact arrangement for efficiency, performance and usability.



The major components of Cytation's digital microscopy system are shown above.

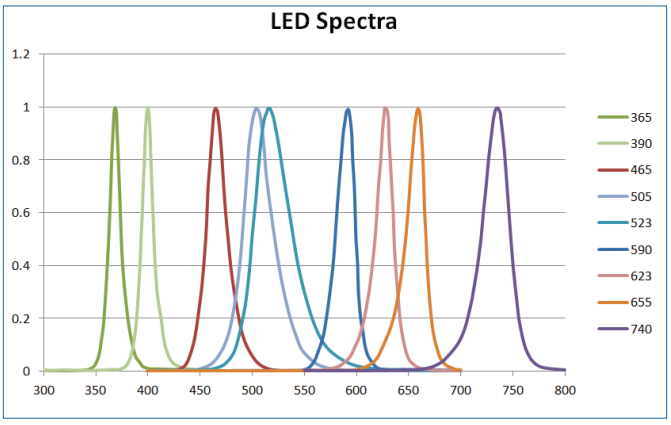
LED Cubes & Filter Cube Assemblies

Cytation can contain up to four two-piece LED and filter cube assemblies, one for each channel of image acquisition color. The imaging cubes are mounted on a mechanical slide, and are easily removed and exchanged.
Numerous colors are available, in addition to the standard red, green, and blue (RGB) measurements, to support most known applications of fluorescence microscopy. The two 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 LED base is matched to the color requirements of the filters in the top cube.

Easy access to slider for LED and filter cubes assemblies.



Normalized spectral power distribution of LED cubes.

LED cube (bottom) and filter cube (top).

Objectives

Essential to Cytation's digital microscopy function are the objectives. The imaging system supports (up to) six objectives. High-quality objectives, including "phase" models, from Olympus, Meiji, and Zeiss are available in numerous magnifications, and are easily user-exchanged as the application requires. The objectives are mounted on a software controlled turret that supports the up and down adjustments critical to an autofocus capability.

Cytation objectives are easily user-exchanged.

Camera

Cytation uses a grayscale CCD camera with a Sony chip for image collection. An incorporated tube lens captures the images directly through the selected objective. Excitation (EX) and emission (EM) filters in the LED-filter cube assemblies are used in fluorescence processing. For live cell and unstained assays, color image processing, called Color Brightfield, is performed using red, green, and blue LEDs to produce a high-resolution full-color image. The CCD sensor is 1384 x 1036 pixels, with a 1280 x 960 image size. The 16-bit camera records over 65,500 different intensity values per pixel in the image.

Additional Hardware Options & Accessories

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 microplate inside the reading chamber, the optional syringe-drive Dual Reagent Injector Module is available. Installation is quick and tool-free – the module sits compactly atop the Cytation. Gen5 software allows intuitive control over dispensing activities.

Cytation 5 with optional Dual Reagent Injector installed.

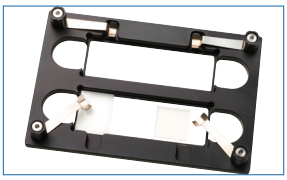
CO_2 / O_2 Gas Controller

Live cell assays require control of the environment, including regulation of CO_2 and O_2 levels. The optional Gas Controller for Cytation provides precise monitoring and control of both CO_2 (from 0 to 20%) and O_2 (from 1 to 19%) levels inside the instrument. Along with the dual reagent injectors, temperature control to 65 ^{0}C and orbital and linear shaking, Cytation provides an ideal platform for live cell applications.

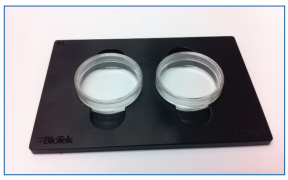
Cytation with dual reagent injectors and CO_2/O_2 controller is a compact workstation for live cell imaging or quantification applications.

Microplates and Other Sample Vessels

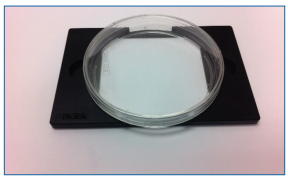
The Cytation supports imaging in numerous sample vessel formats, including 6- to 384-well microplates, microscope slides, cell counting chambers, cell culture and Petri dishes in various sizes and Corning® 25 cm^2 rectangular, canted-neck cell culture flasks. Other vessels that match the basic microplate geometry can be defined in Gen5 for use in imaging also.



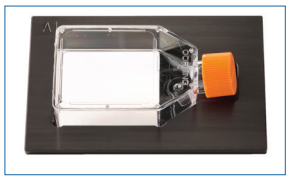
Slide holder



35 mm Petri dish holder



100 mm Petri dish holder



T25 Corning flask

Cell counting chamber

Joystick Control

Some users may find the optional joystick makes it easier to find and focus on their samples. The joystick 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.

3. Gen5™ Image Capture and Storage

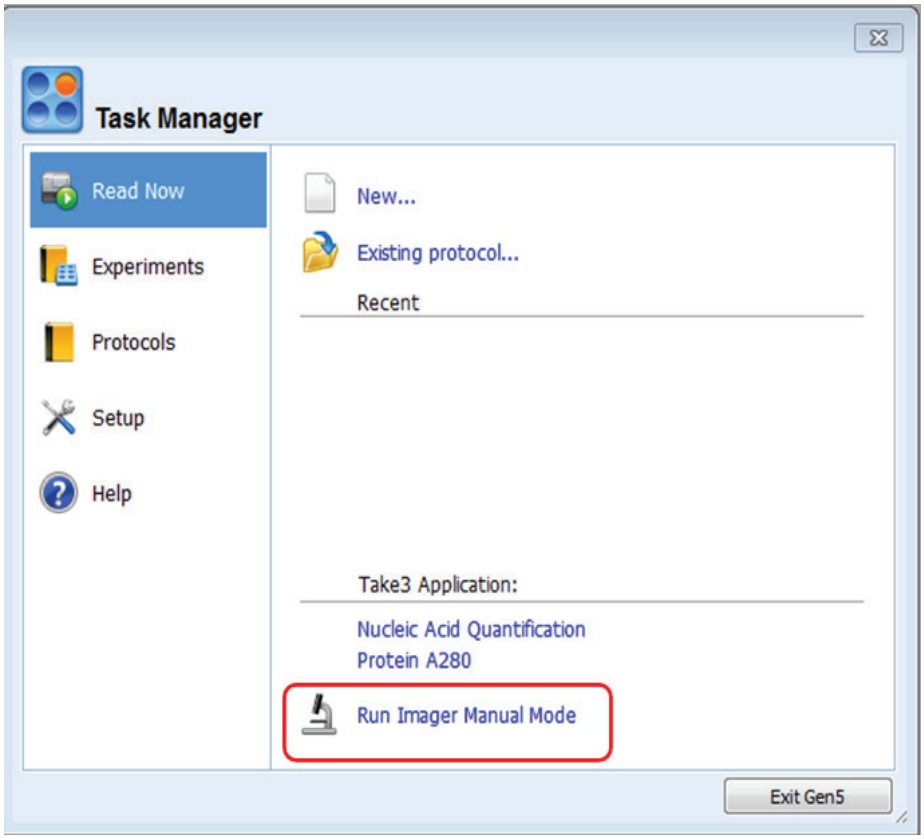
Image Capture

Cytation offers two modes for image collection: manual image capture (Manual Mode) and automated image capture (Experiment Mode). Manual image capture allows a user to interact with their sample in real time, similar to working with a standard microscope, giving the user complete control over navigating around the sample, adjusting focus, adjusting camera exposure settings, selecting objective type, and taking single or multichannel images. Manual mode is ideal for capturing images on a low number of samples, quickly checking the condition of cells or samples, confirming samples are properly stained prior to starting an automated run, and for capturing images to be analyzed with an external software application.

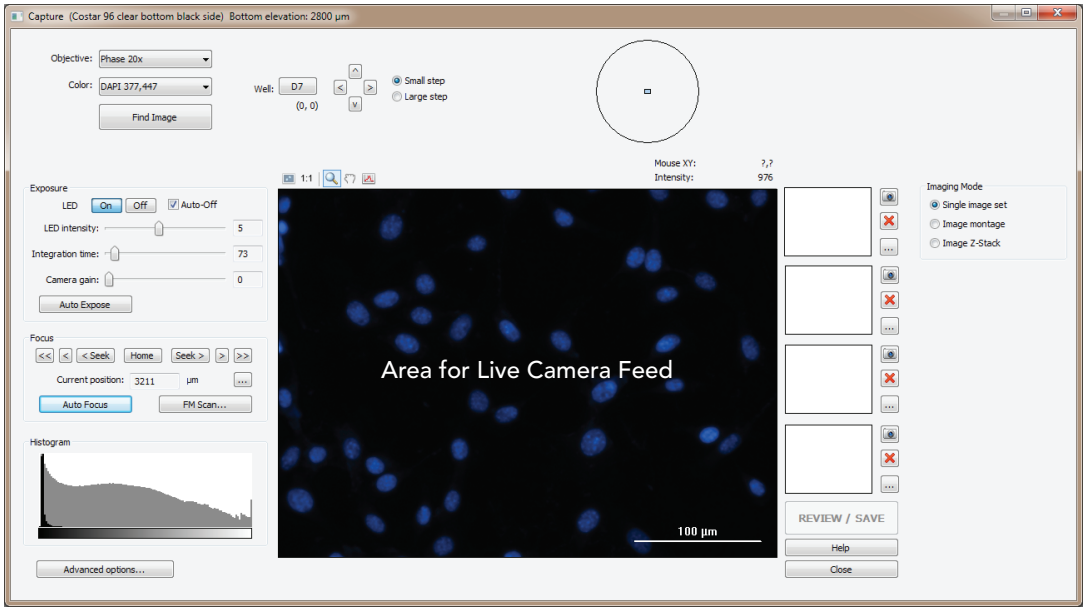
Experiment mode takes advantage of the Cytation's automated stage and Gen5's automated processing to collect images from multiple wells or samples. Experiment mode can capture and analyze images from many samples, as in a 96- or 384-well microplate. It is also useful in combination with Cytation's multi-mode microplate reader: experiments can be defined to capture images only in wells whose fluorescence intensity values, measured by the plate reader, fall above a user-defined threshold, for example. This allows hit picking wells for imaging based on specific criteria, decreasing the amount of data collected and stored, and reducing the amount of image QC performed in downstream steps.

Manual Mode: Manual Image Capture

The *Run Imager Manual Mode* selection is easily accessed through the Gen5 Task Manager:



Gen5's manual mode Capture window presents all the controls for the imaging function in Cytation. In manual mode, the live camera feed is dynamically displayed to the user on the screen (as shown in the Gen5 screenshot below). Gen5 only displays controls that apply to the selected imaging mode. For example, exposure is done automatically in Color Brightfield mode so exposure controls are hidden when this mode is selected. As adjustments such as exposure and focus are made, the displayed image is updated accordingly.

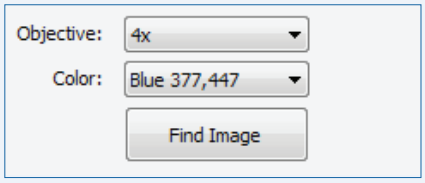


Each fluorescent dye/marker in the sample requires its own image; selecting the *Color* pairs the excitation/emission filter with the color of your fluorescent marker. An additional brightfield option allows for viewing samples with transmitted light. A color brightfield option overlays transmitted-light images with red, green, and blue (RGB). The phase contrast optics module significantly improves brightfield imaging. Up to 4 image channels can be acquired from a field-of-view of the sample.

Manual Mode Controls

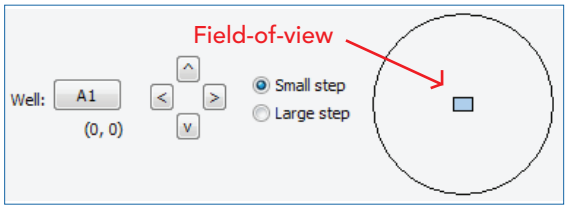
Best Practice Tip:

The *Find Image* button is ideal for a new user who is unfamiliar with exposure and focus settings. After selecting a well to image, the *Find Image* function will go through iterations of exposure and focal plane adjustment until it displays a properly focused and exposed image to the user, eliminating the need for manual adjustments.



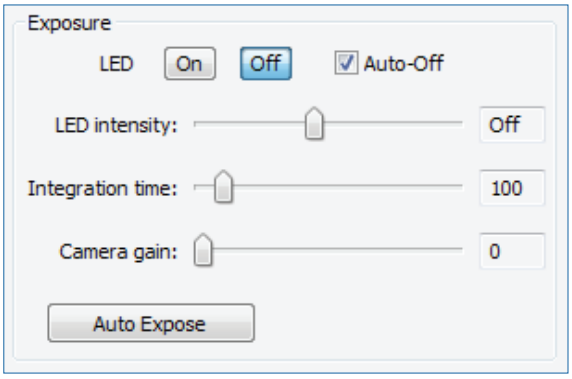
Navigation controls to select focus area

The *Well:* button is used to select the well and the region of the well (or slide) to image, while the Small step and Large step adjustments allow navigation around the well or slide. Orientation of the field of view relative to the well/slide is provided, and the movement of the field of view is tracked as small and large steps are taken around the sample.



Exposure

LED intensity, *Integration time*, and *Camera gain* controls are provided to override auto-exposure (when necessary) to optimally expose the image. Because fluorescent samples quickly degrade under constant illumination, the LED can be powered on or off. For fluorescent imaging, the *Auto-off* selection guards against accidental photobleaching of a sample by turning off the LED when the Cytation is idle. The LED must be ON when imaging a sample.



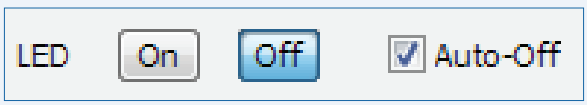
Best Practice Tip:

When setting exposure, be aware of any saturated image pixels, which indicate too high an exposure setting. Over-exposed images lose detail and can impede image analysis. Use the *histogram* to help ensure the sample isn't overexposed.

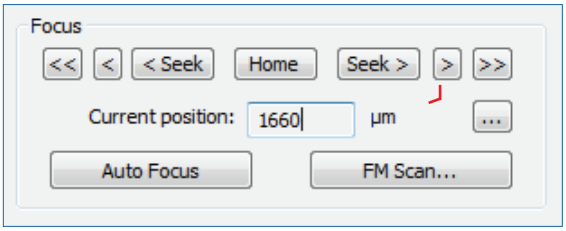
The 3 variables affecting exposure can be manually adjusted or auto exposed. Manually increasing *LED intensity*, *integration time* and *camera gain* individually will increase the brightness of the live and acquired image. LED intensity can be controlled up to 100% intensity output. Integration time is set in milliseconds and is comparable to a camera shutter speed, (i.e., the higher the integration time, the longer the sample will be exposed to the camera sensor creating a brighter image). Integration Time can be adjusted to a maximum of 1000 milliseconds (1 second). Camera gain controls the sensitivity of the camera's CCD sensor. Low camera gain settings have less inherent "noise" in the image than higher camera gain settings. The *Auto Expose* button will iteratively adjust these 3 variables and set values accordingly to get a properly exposed image for the Objective and Color selected.

Best Practice Tip:

Long exposures of LED light on fluorescent dyes will begin to bleach or degrade their fluorescent intensity, causing poorer image quality and are also detrimental to live cells, decreasing their survival time. Keep the LED *Auto-off* selection checked when working with fluorescent dyes.

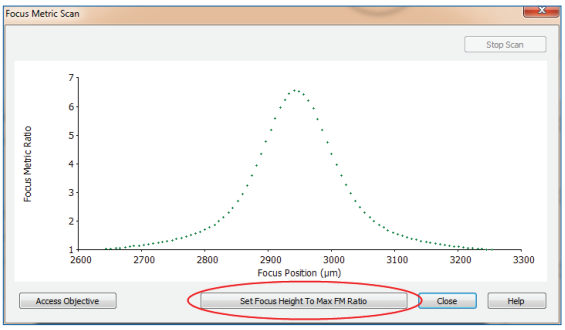


Focus



Focus parameters are available for both fine and coarse focus adjustments, similar to a standard microscope. *Seek* begins progressive movements in the direction chosen (objective moves either up or down) until the user stops the movement. *Auto Focus* runs a software autofocus algorithm to search for the plane of focus.

Performing an *FM Scan* displays a curve showing where the focal plane contrast is the greatest based on a Focus Metric Ratio. The peak Focus Metric Ratio is the plane of optimal sample focus. Note that *Auto Focus* is based on the dimensions of the plate type selected by the user; be certain to select the proper plate type when starting the imaging session.

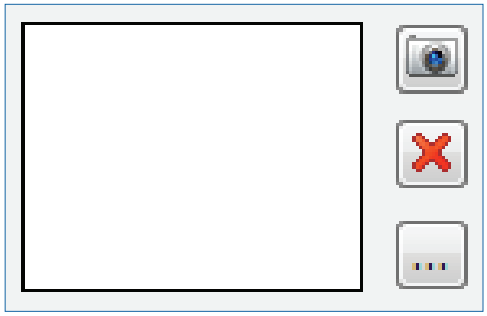


... Click the 3-dot button in the focus controls to specify a focus position and to move the objective to it.

Capture

The camera icons next to the image thumbnail holders make it easy to acquire a single-channel image (1 color) or multiple color channels. Except in Color Brightfield, it's possible to acquire up to 4 separate images of the same field-of-view for each unique fluorophore or brightfield channel of the sample. Clicking the camera icon takes an image with the current exposure and focus settings.

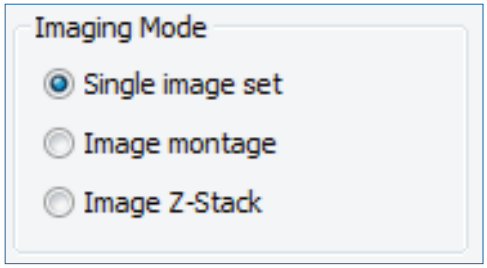
Note: *When acquiring a multi-channel image set of the same field-of-view with 2.5x - 20x magnifications, typically the optimal focal plane found for channel 1 can be applied to channels 2-4 without readjusting the focus settings.*



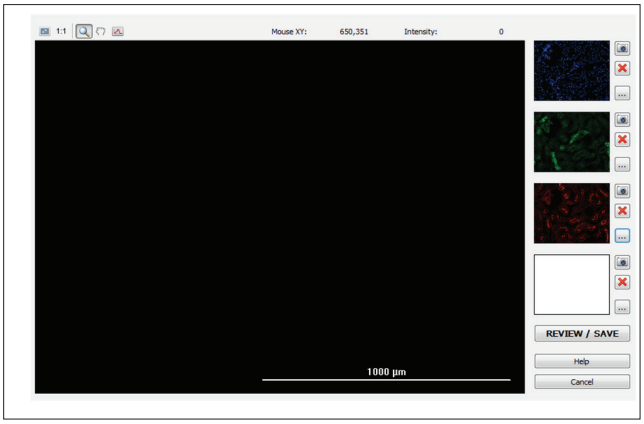
- Deletes the selected image.
- ... Open or import a previously acquired TIF file image for viewing or analysis.

Imaging Mode

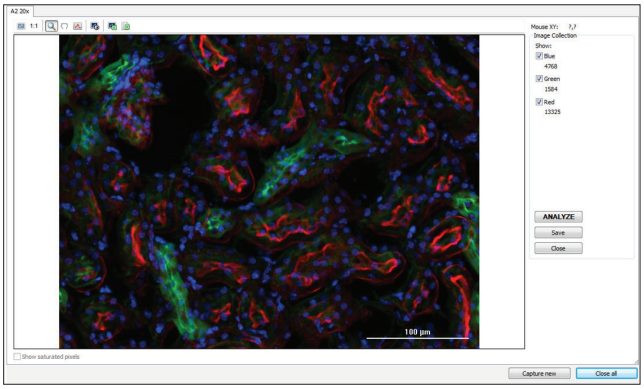
In both manual mode and experiment mode, Gen5 offers Image montage and Image Z-stack methods. Montage captures multiple images in the same focal plane that can be stitched together to make one image of the whole. Z-stack captures multiple images in multiple Z-axis planes, and presents them singly or in a slide-show. After acquiring the images, Gen5 combines user-selected images in the Z-stack to produce a 3D Projection or Z-Projection. In both modes, images can be analyzed individually or combined, in individual channels or all channels.



In the screenshot below, images in 3 channels, one for each of 3 fluorescent dyes of blue, green and red are captured.



When a user is satisfied with the captured image, and all three images are acquired, selecting *Review/Save* proceeds to the Image Review section where additional contrast/brightness adjustments can be made to the 3-color overlaid image and the image Saved or Analyzed.

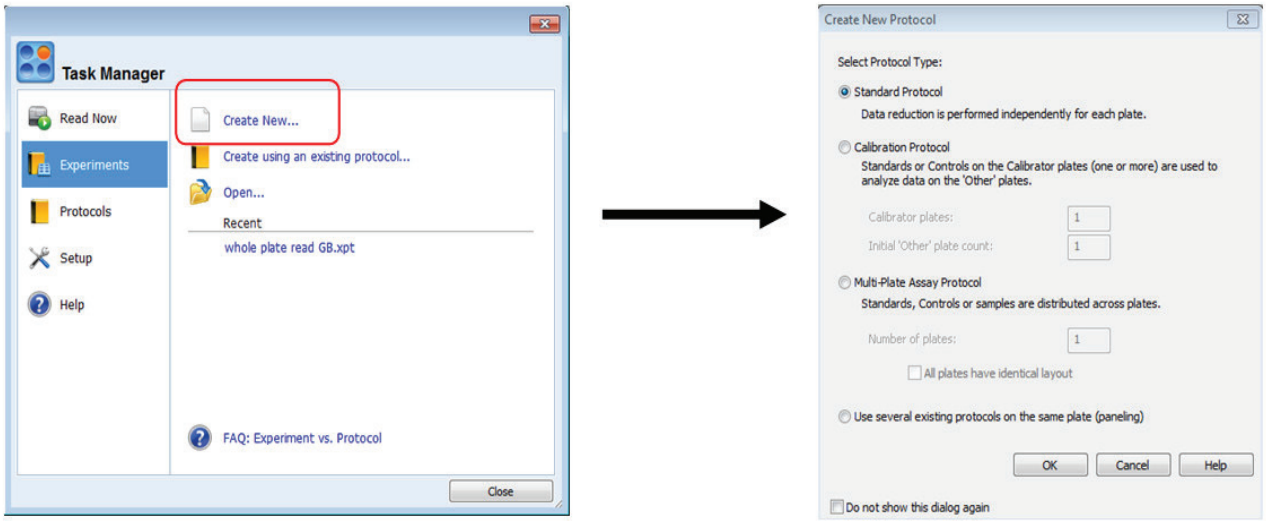


In review mode, the user can view and/or save each color channel individually, along with the option to *Analyze* the image set. *Capture New* allows additional images to be captured without closing the already captured image set.

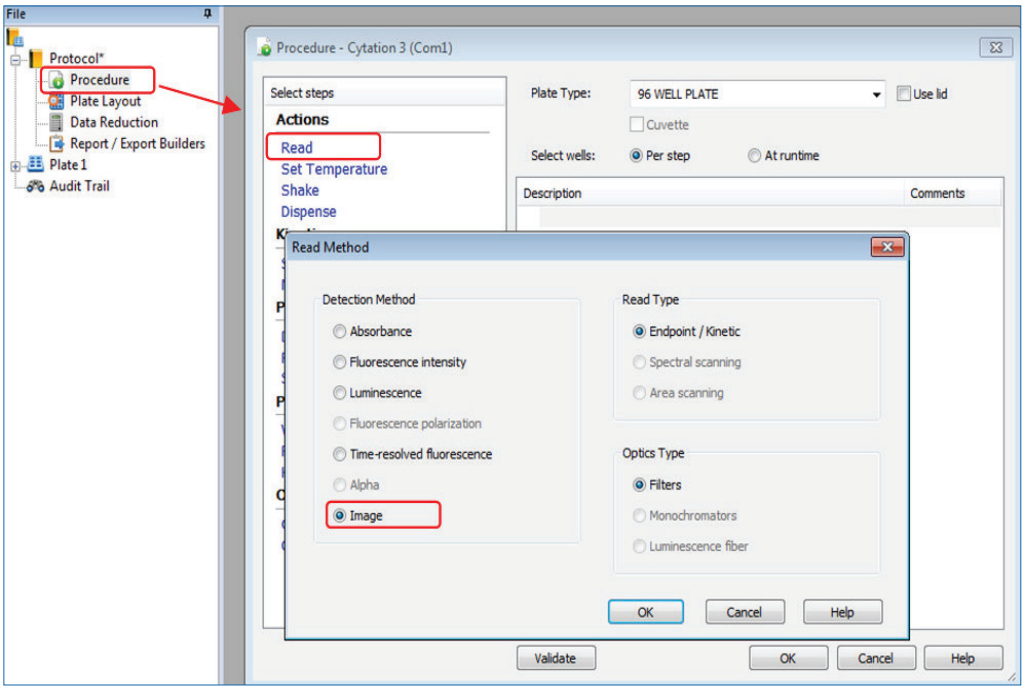
Experiment Mode: Automated Capture using Gen5

Gen5 software controls the Cytation for automatic capture of images in a microplate, a slide, or other vessel; in all wells or a subset of wells in a microplate, or in a defined area in a vessel; and in a montage or Z-stack of images. Automated image capture parameters can be used alone or in combination with the Cytation's monochromator or filter-based optics.

For automated image capture, a new Gen5 Experiment is created via the Task Manager, typically using a standard protocol.



Gen5's procedure step allows the user to select **Image** as the detection method, with end point/kinetic and filters selected by default (as shown in this Gen5 screenshot):



Cytation supports multiple imaging read modes: end point, time-lapse, montage, and Z-stacking.

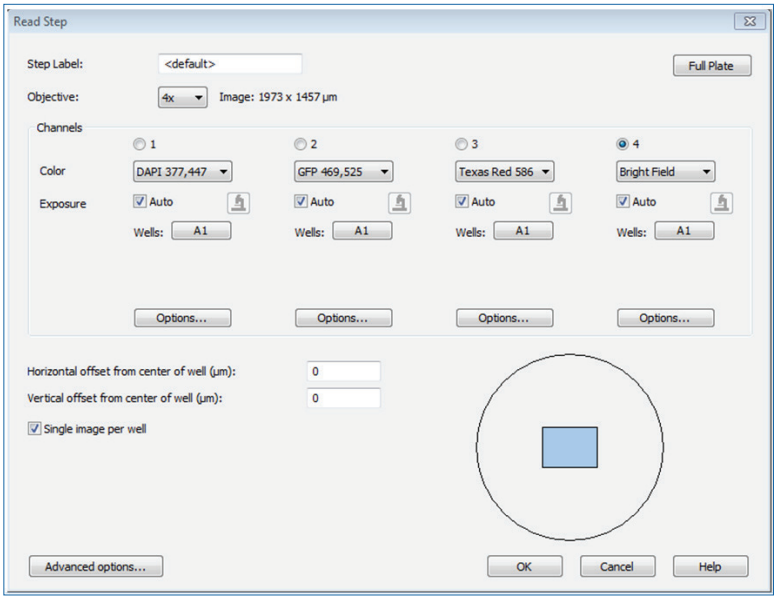
- **End point** measures each well or slide once, using one to four color channels. Measurement is done by default in the center of the well or slide, but offsets can be applied, when necessary, to take off-center measurements.

- **Time-lapse** (called kinetic in Gen5) enables repeated measurements of all samples over a defined time period. Two process modes are offered: well mode and plate mode. In well mode, the entire time lapse measurement is done on one sample before going to the next. Intervals between images can be as low as 0.5 second. In plate mode, the entire microplate is read at each time point and the interval between two data points is at least 6 minutes for a 96-well plate (more if multiple color channels are used).

- **Montage** measurements allow reading an array of images per sample location (well). All images are presented together (with or without image stitching) at the end of the read process for an overview of the entire sample. Each individual image file is saved separately. Montage is compatible with both end-point and kinetic (time lapse) read modes.

- **Z-stack** mode captures images on multiple planes in the z-axis. Gen5 processes the images, based on user-specified selections, into a Z-Projection or compiled 3D image. Image and cellular analysis can be performed on individual slices (images) or on the Z-Projection. Z-stacking is compatible with both end-point and kinetic (time lapse) read modes.

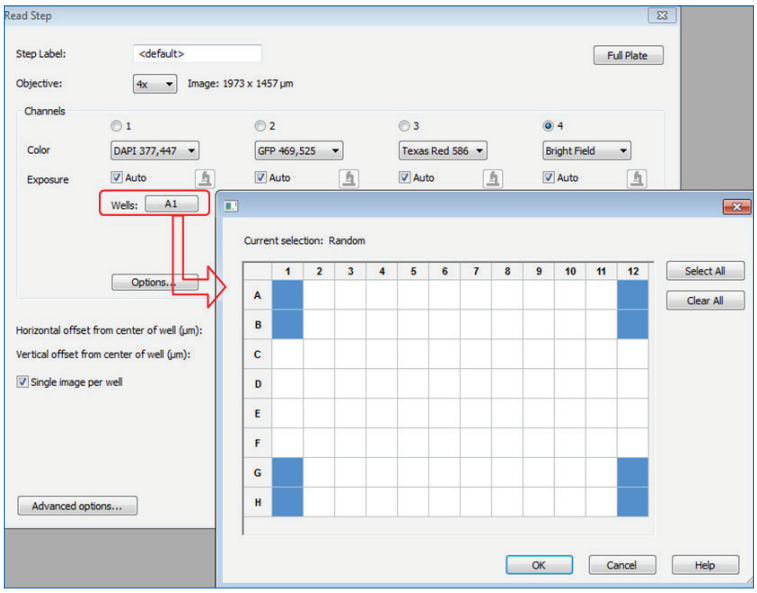
The objectives and channels for imaging are selected in the Read Step. In this example, blue, green, red, and brightfield images will be acquired in the 4 channels using the 4x objective. Exposure for each of the 4 channels can be set per channel automatically or manually. By default, the entire plate is selected for imaging; the *Full Plate* button allows the user to select a subset of wells in the microplate for imaging.



A single image per well is acquired by default and the image can be offset from the center of the well by adjusting the horizontal and vertical offset from the center of well. A montage or Z-stack of images can be acquired in each well by deselecting *Single Image Set* and defining the target area (screenshot not shown). Upon clicking *OK*, the settings are saved and the step added to the procedure.

Best Practice Tip:

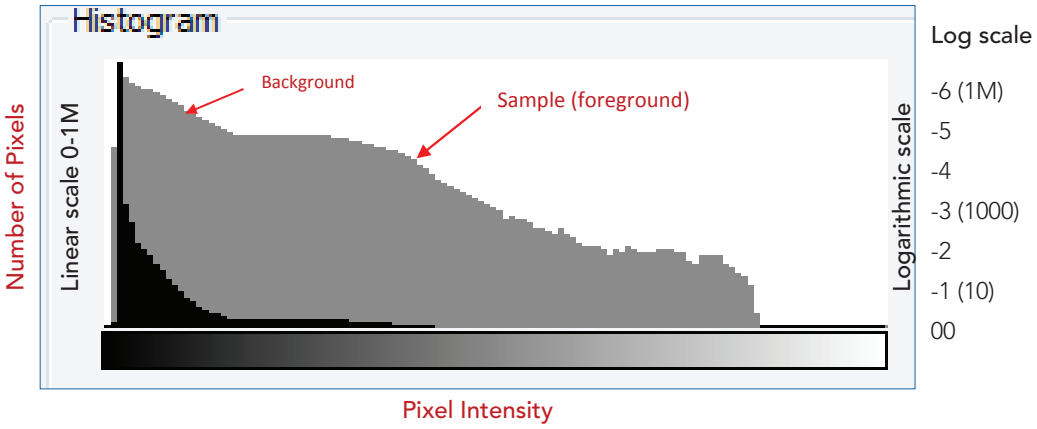
When using Auto Exposure, select wells that represent the dynamic intensity range of the assay: pick a minimum of two wells that represent the lowest signal (Min wells) and two that represent the highest signal (Max wells). Auto exposure takes the average exposure settings for these wells and applies the values to the entire plate. When Exposure is set to *Auto*, Min and Max wells can be identified by clicking the *Wells* button (circled in red in this screenshot) and selecting the appropriate wells.



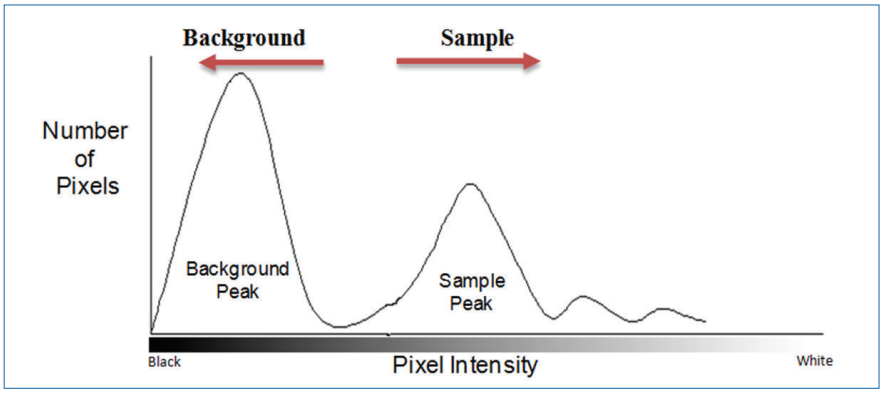
After the imaging read step has been added to the *Protocol Procedure*, you begin image acquisition by saving the Experiment and selecting in the Toolbar. If additional Plate Layout, Data Reduction and Report/Export Builder steps are required, complete the additional steps, save the Experiment and then select in the Toolbar.

Histogram

The image histogram concept is fundamental in imaging and is useful for a variety of applications. Every image captured by the Cytation has a corresponding image histogram. The histogram is especially useful in Gen5's Manual Mode to help fine tune image capture settings. It provides information on 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.



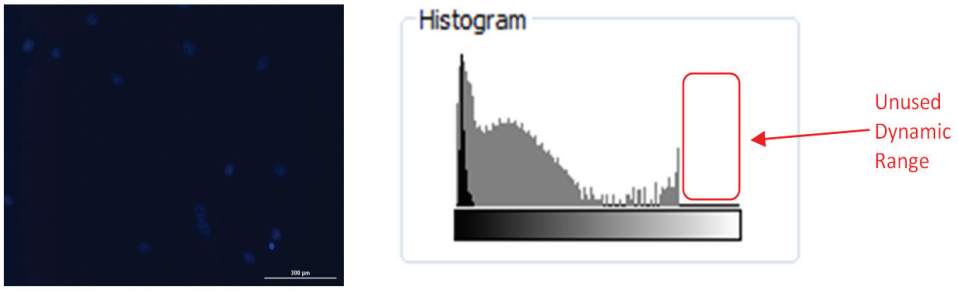
Each channel in a captured image has its own separate histogram. With fluorescent images, the histogram typically has a large peak of dark pixels showing the black background and a smaller bright pixel peak showing the foreground signal of interest.



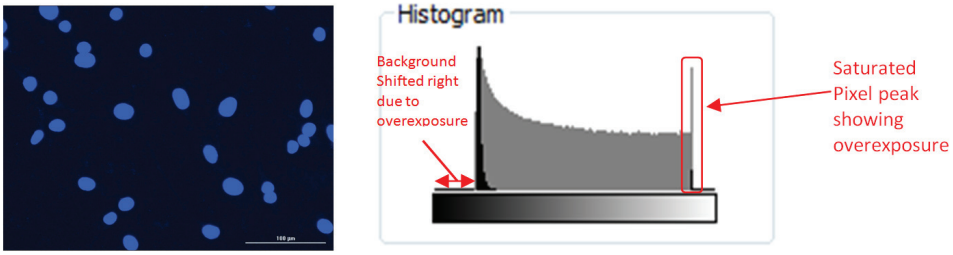
Improving Image Capture: Image Histogram and Optimal Exposure

After capturing an image, the image histogram can verify that the camera's dynamic range is being used effectively without causing over or underexposure. 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.

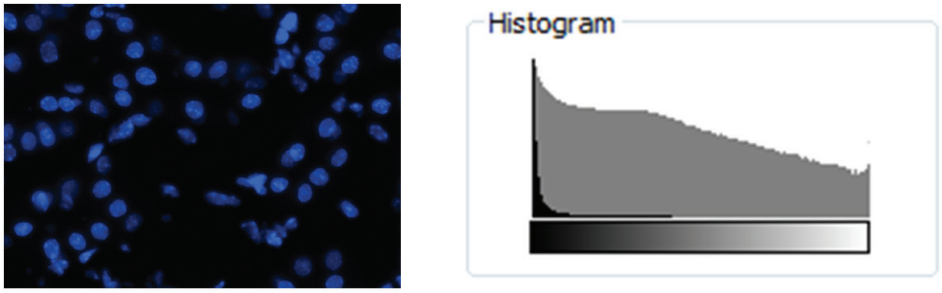
Examples of underexposed, overexposed and properly exposed images and histograms are provided below.



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



Overexposed image and histogram of DAPI stained cell nuclei. Note the loss of texture detail in the saturated nuclei in the image. The histogram also reflects overexposure; a histogram peak at its maximum intensity shows an accumulation of pixels that have saturated the camera's dynamic range and the background peak has shifted to the right, showing an increase in background intensity.



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

Reviewing an Image: Image Histogram and Brightness and Contrast Adjustment

When reviewing an image, Gen5 provides tools for adjusting brightness and contrast per channel. Adjusting brightness and contrast (B&C) in an image does not affect image analysis; it allows Gen5 to appropriately display images on a computer monitor. Adjustments are primarily for:

- Visualization on the computer monitor (to address a mismatch between dynamic ranges of Cytation images and the computer monitor display).
- Optimizing image quality prior to saving the image for other purposes

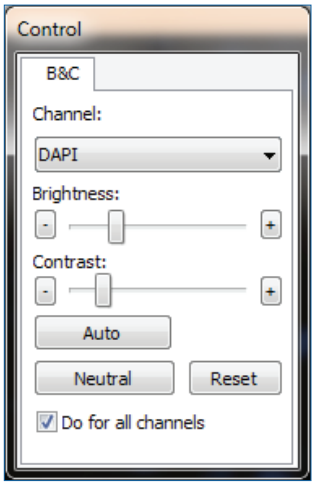
As adjustments to brightness and contrast are made, image histograms update dynamically to reflect the changes. Image brightness and contrast adjustments are subjective and vary image to image, channel to channel, and monitor to monitor, so it is performed on a case by case basis.

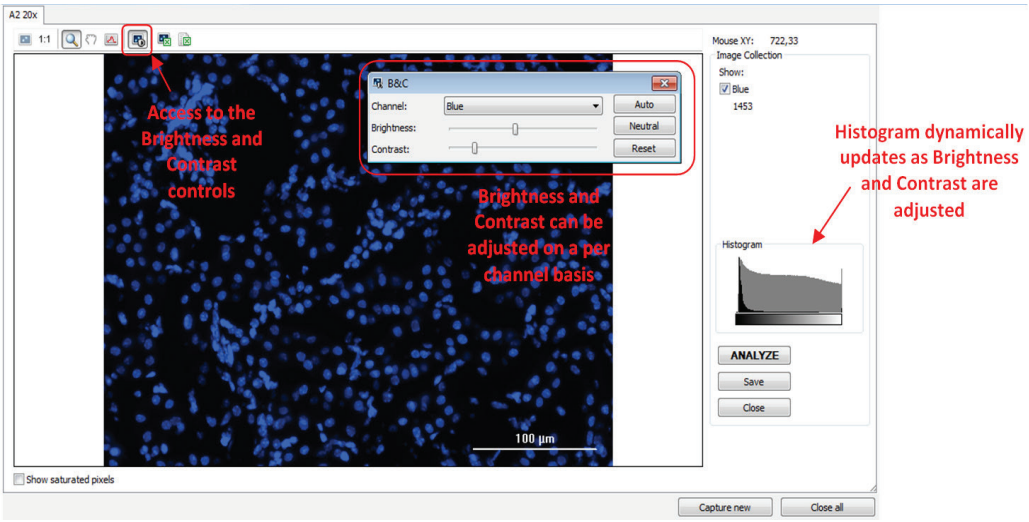
Where appropriate, the Gen5 interface provides a button  to open and close the B&C (brightness and contrast) control. In combination with visual affirmation, the image histogram provides instant feedback on how the image is affected by the adjustments. All images in a set or in an experiment will have the same brightness and contrast settings applied. This allows for legitimate cross comparison between all images acquired at the same exposure settings.

Gen5's brightness and contrast controls:

 The B&C button toggles the brightness and contrast adjustment control. You can select one channel or all channels to apply the adjustment to.

Note: *Adjusting the brightness and contrast does not affect analysis, but it improves image viewing.*





Best Practice Tip:

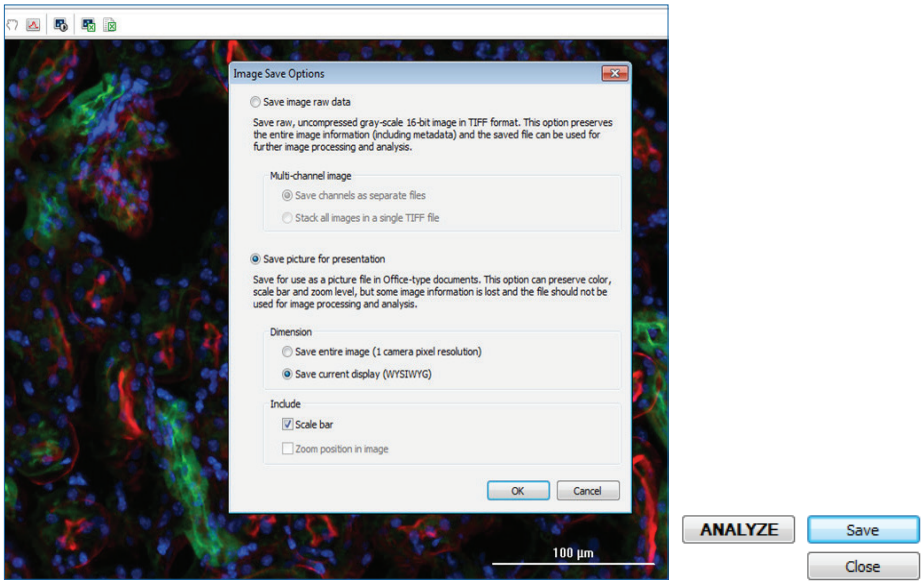
Let Gen5 adjust the brightness and contrast "for all channels" simultaneously. For fluorescent imaging, Gen5 keeps the image background as black as possible. If necessary, you can then view one channel at a time to make final small adjustments. Avoid over applying brightness or contrast adjustments, as details of the color can be lost.

Image Storage & File Formats

Image storage and file format options vary based on the image acquisition mode: *Manual Mode* or *Experiment Mode* (automated).

Manual Mode: Image Storage and File Format Options

After an image or image set is acquired with Cytation, the user advances to the *Review/Save* window, where the Save button accesses the Image Save Options screen (shown below).



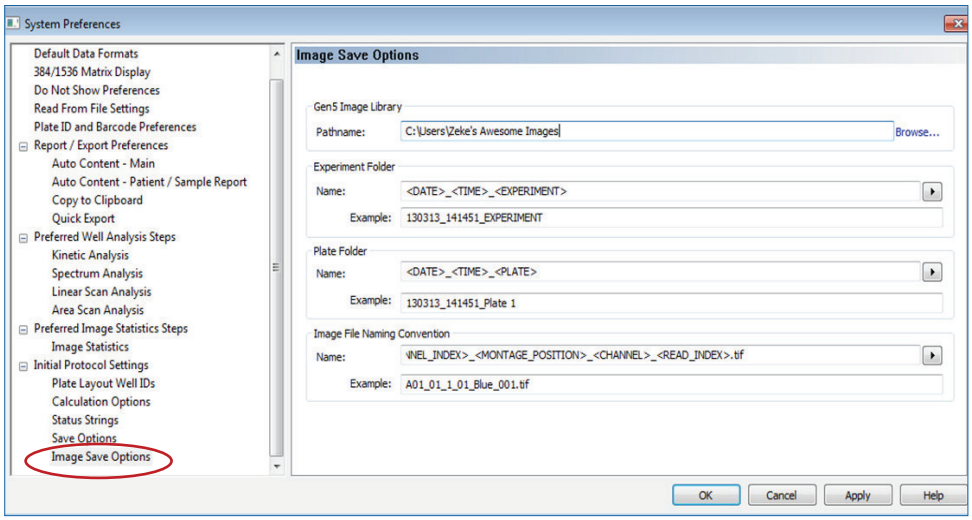
Save Image Raw Data saves the image in TIF format. This option is best for saving the highest quality image in the native grayscale format for use in other image analysis software packages and for re-analysis with Gen5. Multi-channel images can be saved as separate files per image or as stacked images 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 these commonly used formats: TIF, JPEG, PNG, GIF, BMP and EMF.

Note: *The multiple image formats available allows them to be used not only in Gen5 software, but also in 3rd party imaging and image analysis software. Image formats ideal for presentations or documents include JPEG, PNG and GIF. Image formats ideal for publication purposes include TIF and BMP.*

Experiment Mode: Image Storage and File Format Options

Gen5 provides tools for customizing automated storage location and file-folder naming for Experiments and Plates, and an *Image File Naming Convention* for images captured automatically in an experiment. Images acquired with a Gen5 experiment are saved in TIF format by default. TIF format allows for re-analysis at a later point in Gen5 or analysis with third party image analysis software. The *Image Save Options* are shown below:



From the Gen5 main menu, select **System>Preferences**

In either manual or experiment mode, images can be saved to the local hard drive, to an external drive, or to a network drive. This helps reduce the bioinformatics burden of maintaining an in-house database.

Best Practice Tip:

Each raw image is 2 MB and a 96-well plate with one image per well requires 200 MB of storage space. A local hard drive could easily be filled, so it's recommended to consult with your IT group for guidelines on an appropriate image storage location.

4. Image Processing and Analysis

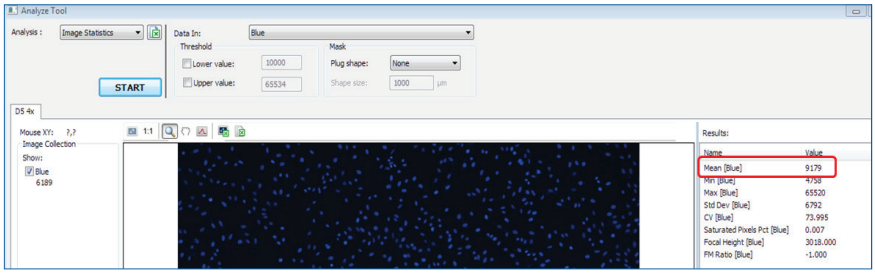
Image Statistics Calculation Tool

Image Statistics calculates general statistics on the image's pixel values, such as average pixel intensity, standard deviation of pixel values, or percentage of saturated pixels. Image Statistics can be generated in manual mode or in experiment mode as a *Data Reduction* step in a Protocol or Experiment. Three main use cases are described below with relevant application examples.

Scenario 1: No **Threshold** and no **Plug** are defined. This method provides data similar to plate reader data, but also provides an image for additional information for QC or other purposes. The intensity in the entire image is measured. Example applications that are ideal for this method include:

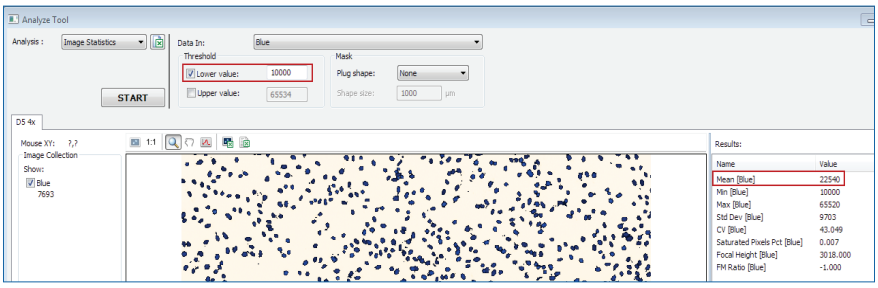
1. Assay Development: When transitioning a cell-based assay from microplate reader format to imaging format, this method can compare mean intensity values measured on the image with mean intensity values acquired on the plate reader to show correlation between detection methods.

2. Running a cell-based assay where all that is needed is a well mean, but the user requires an image for QC or hit confirmation purposes.



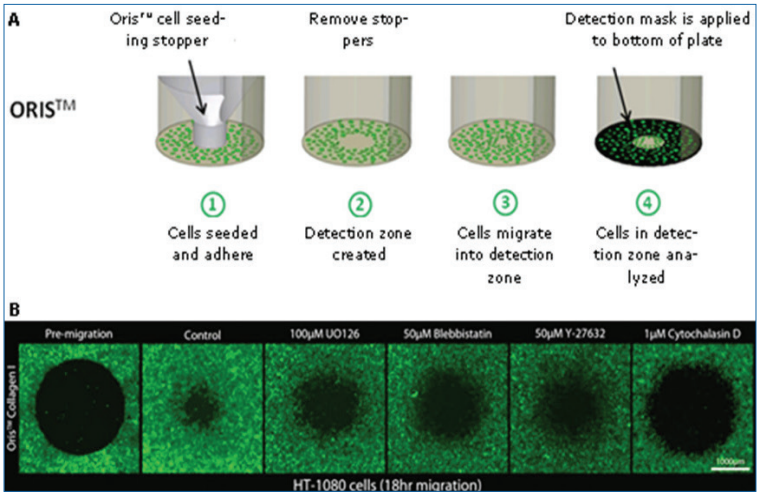
Scenario 2: A **Threshold** is used but no **Plug** is defined: This method increases detection sensitivity by measuring only the signal in the image above or below a specified intensity threshold value. Intensity values in Cytation images range from 0 to 65,534, so thresholds can be set anywhere in this range. Setting a lower threshold limits measurements to objects with intensities above that threshold, and setting an upper threshold only counts pixels in the image below the threshold. Assay sensitivity is increased because well-level measurements only return statistics on the signal of interest in the well without diluting measurements by the dark background in the image. An application that benefits from this method is:

1. Dose Response in a fluorescence intensity cytotoxicity assay: Setting a lower threshold (10,000 in the below example) to evaluate only DNA stained nuclei, the well-level Mean Blue value only measures actual stained cells. The Mean Blue will show a dose response as cell numbers decrease due to toxicity. This choice increases sensitivity (compared to not using a threshold) because the Mean Blue is not affected by the dark background in the image. In the below screenshot, threshold outliers are highlighted in white. Note the increase in the Mean Blue value compared to Scenario 1, which is affected by the dark background.



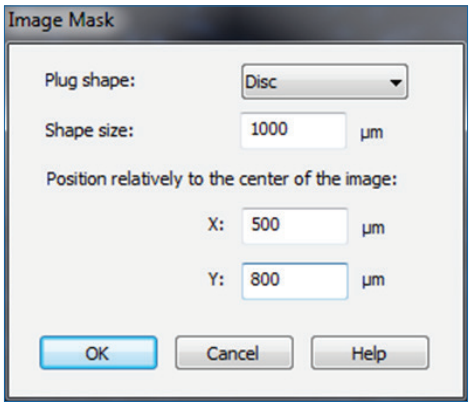
Scenario 3: No Threshold is used but a **Plug** is defined: This method defines a plug that narrows the image statistics to a particular region in the center of the image. The plug size and shape (disc or square) are user-specified. An example application employing this method is:

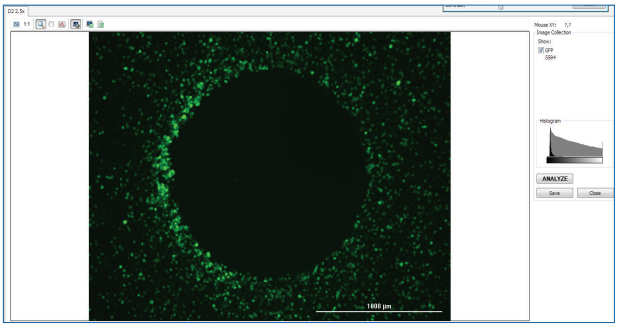
1. Migration assay like the Platypus ORIS™ Cell Migration Assay: In this assay, a plug is positioned in the center of a microplate well. The plug blocks any cell attachment or growth. Cells are seeded around the plug in the well. Once cells attach and are treated, the plug is removed and the amount of cell migration into the vacant area left by the plug is measured.

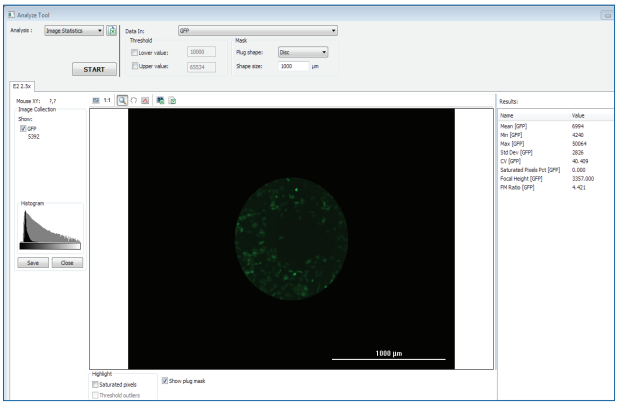


Schematic of the Platypus ORIS cell migration assay, indicating position of the stopper (or plug) in the center of the well.

Fluorescence intensity measured in the "detection zone" defined by the plug is a measure of cell migration. By defining a plug in image statistics, measurements are taken only in the area of interest where cells are expected to migrate, increasing the sensitivity of the assay versus analyzing the entire image.

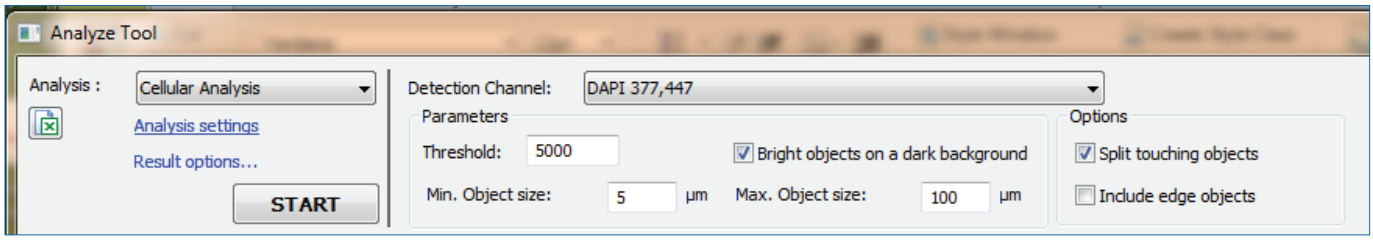




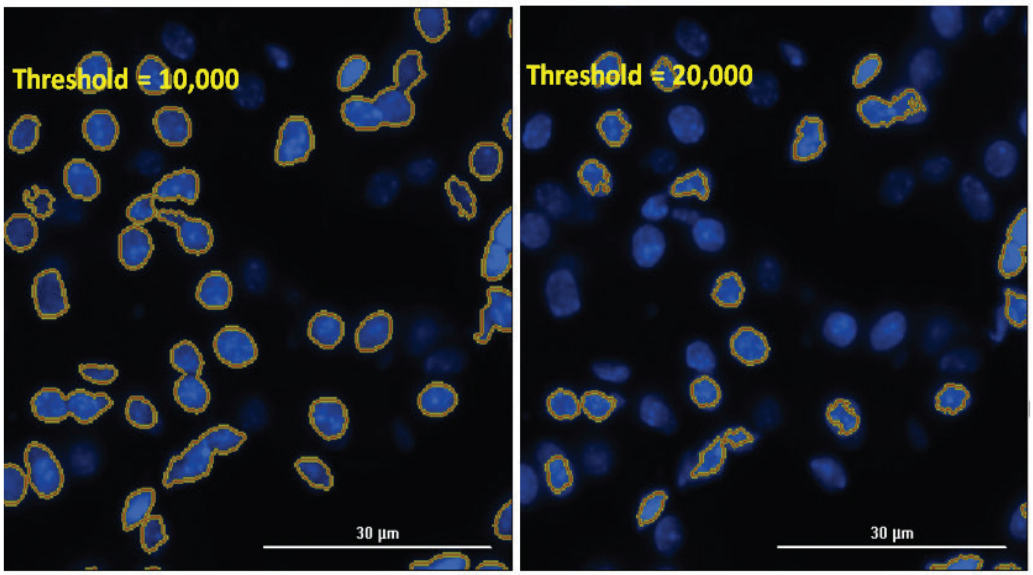


Scenario 4: Using a **Threshold** and a **Plug**: This method extends the analysis capabilities of the Oris assay described above. Defining an intensity threshold in addition to a plug can generate values for %Confluence, Total Intensity, and Total Area, all great indicators of whether or not cell migration is occurring and how rapidly.

Cellular Analysis Calculation Tool



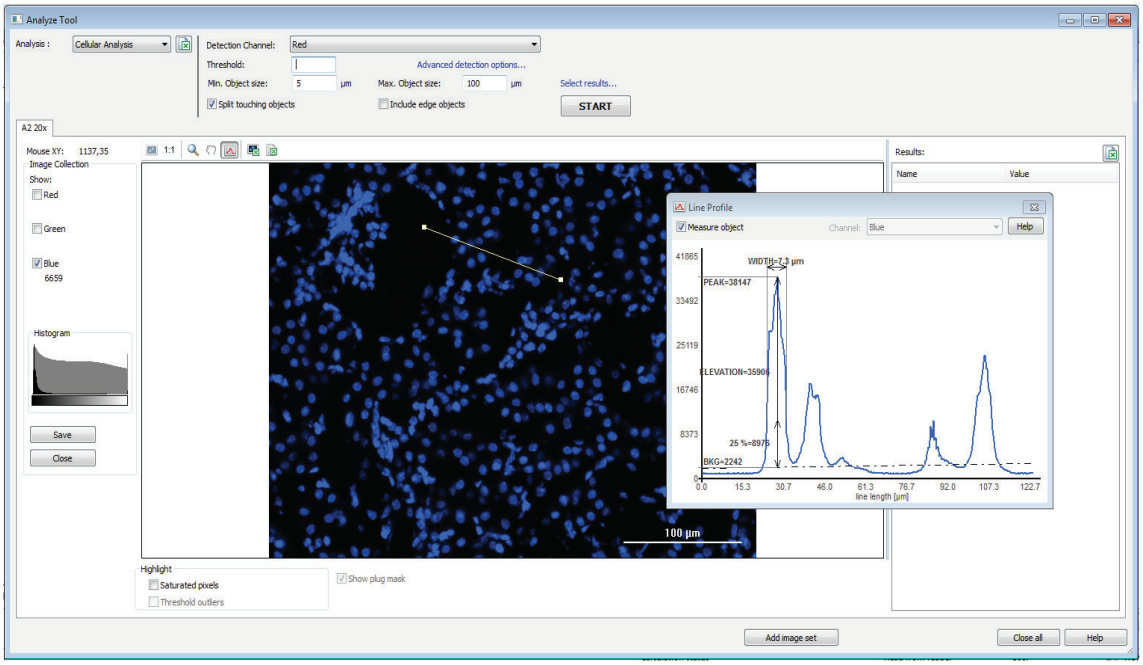
In *Cellular Analysis*, images can be analyzed for cell level details, like object size, intensity, and circularity measurements. A total count is returned for all identified objects in the image which is useful for applications such as cytotoxicity and proliferation studies. The most critical parameter when the Analyze Tool is set for Cellular Analysis is *Threshold*. A threshold sets the minimum pixel intensity value for the signal of interest; it 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 65,535 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 20,000-65,534 are included. The images below illustrate the masking behavior at two thresholds:



Any fluorescently labeled image can be set as the Detection Channel, allowing any RGB fluorescent label to be 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 *View 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 images that represent the maximum intensity offers the best likelihood of analyzing all images optimally.



The Line Profile viewing window in Gen5™ is shown above. The selected line is depicted in the image display.

Best Practice Tips:

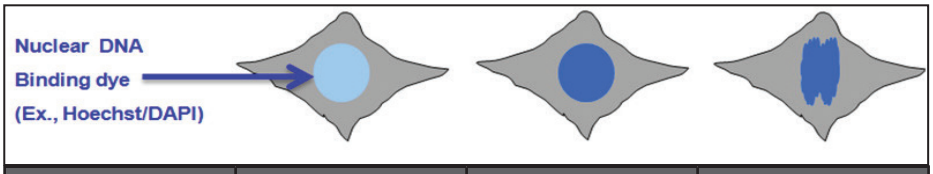
- The *Split Touching Objects* feature is recommended in applications where object number is important, such as proliferation, cytotoxicity, or migration studies to separate two or more objects whose masks are touching. The Split Touching Objects feature may not be useful or recommended in applications 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.

The measurements that are returned for each object per channel are provided below. By default, *Count* and *Area* measurements are limited to the Channel 1 mask, however all channels return intensity measurements. To add *Count* or other statistics to a secondary channel, refer to "subpopulation analysis" below.

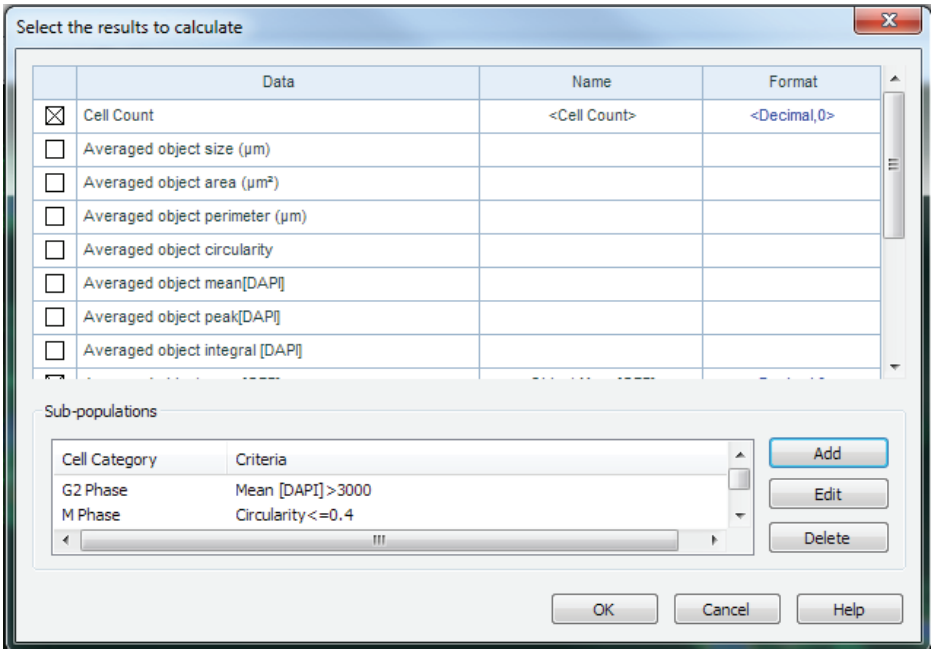
Channel 1	Channel 2	Channel 3	Channel 4
Object #	Mean (Ch2)	Mean (Ch3)	Mean (CH4)
Object size	Peak (Ch2)	Peak (Ch3)	Peak (Ch4)
Object circularity	Integral (Ch2)	Integral (Ch3)	Integral (Ch4)
Object area	STD DEV (Ch2)	STD DEV (Ch3)	STD DEV (Ch4)
Object perimeter			
Mean (Ch1)			
Peak (Ch1)			
Integral (Ch1)			
STDEV (Ch1)			

New users often find it challenging to apply the available measurements in Cellular Analysis to actual applications. An example application is provided below, illustrating 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. 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 stages of the Cell Cycle.

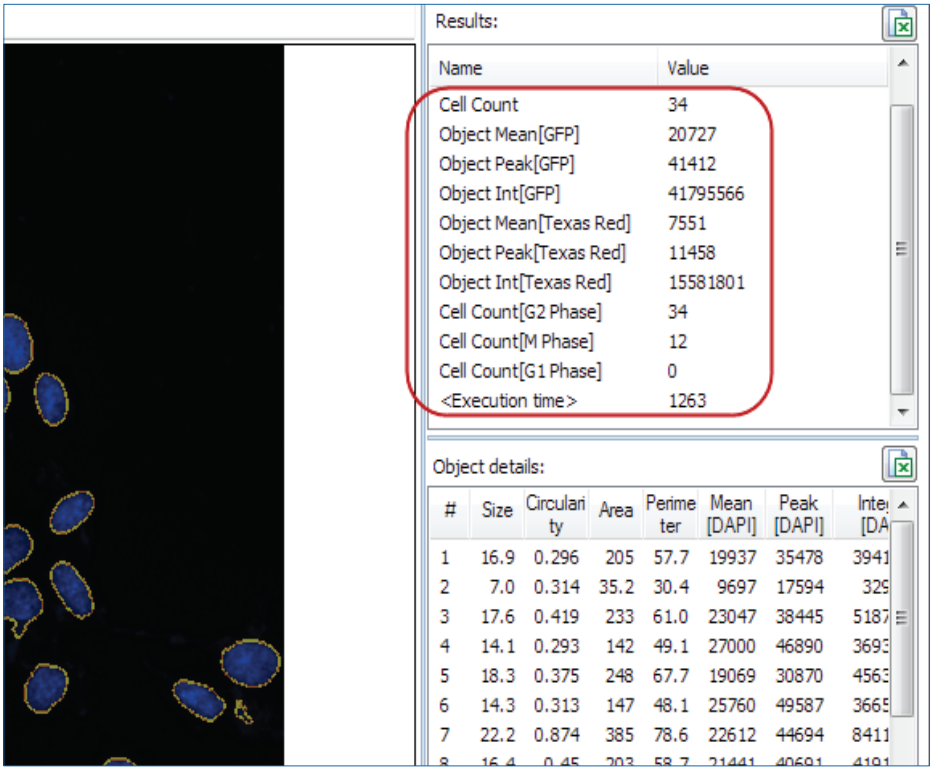


Measure	$G_{0/10/1}$	G2	M
Object #	Stable	Stable	Stable
Object size	Normal	Normal	Skewed aspect ratio
Object circularity	Normal	Normal	Skewed aspect ratio
Object area	Normal	Normal	Unclear
Object perimeter	Normal	Normal	Skewed aspect ration
Mean Intensity	Normal	Brighter	Brighter
Peak Intensity	Normal	Brighter	Brighter
Integral Intensity	Normal	Brighter	Brighter

Sub-population analysis is defined in Gen5 using the "Results Options…" link of the *Cellular Analysis* tool. In this example, Gen5 is set to report on the total population (Total Cell Count), and to report a cell count on 3 sub-populations (G1, G2, and M phases) based on specific intensity or shape criteria.



The results are reported after analysis, as shown here:



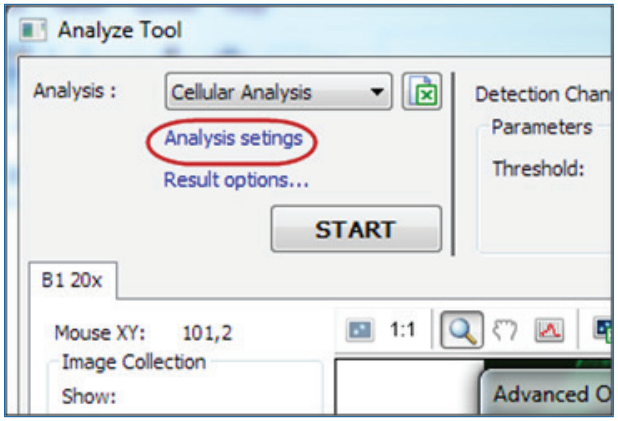
All images, image statistics, and cellular analysis results can be instantly exported to Excel.

Additional examples of biological applications of each measurement are:

Measurement	Biological Examples
Cell count	Cell counting: Proliferation assay; Toxicity assay; Replacement assays for MTT, BRDU or tritiated thymidine Object counting: Colony counting, count of lysosomal spots using a dye such as lysotracker
Object size	Cellular hypertrophy; cytoskeletal changes; formation of syncytia, colonies, clusters or tubes
Object area	Cellular hypertrophy; toxicity; formation of syncytia, colonies, clusters or tubes
Object sum area	Assays looking for total area change, like cell growth, migration, colonies
Object perimeter	Cytoskeletal or phenotypic cellular changes to drug treatment
Object circularity	Cytoskeletal or phenotypic cellular changes to drug treatment
Mean (Ch1,2,3,4)	Any Intensity change assay where protein of interest has increase/decrease of intensity; translocation of protein to nucleus; Cell Viability assays; Colocalization of signal if the Means of two Channels are used for analysis
Object STDEV	Assays looking for texture variations. Example: nuclear health
Peak (Ch1,2,3,4)	Apoptosis or Mitosis where nuclear intensity increases the Peak intensity may also increase
Integral (Ch1,2,3,4)	Useful when Area is critical to take into account, but intensity is relatively unaffected. Examples include colony counting and neurite outgrowth using a cell membrane stain of cells trated with and without NGF.
Object sum integral	Assays looking for total intensity change, taking area into account: cell growth, migration, colonies

Image Processing

For the best possible analysis of cellular images acquired with Cytation, Gen5 performs several pre-processing steps to improve the efficacy of image analysis. These options are available via the *Analysis Settings* link in the Cellular Analysis window in manual mode and in the *Advanced detection options* link of a Cellular Analysis Data Reduction step in experiment mode. Three attributes of the image can be optimized: image flatness, image noise, and elevated background levels.



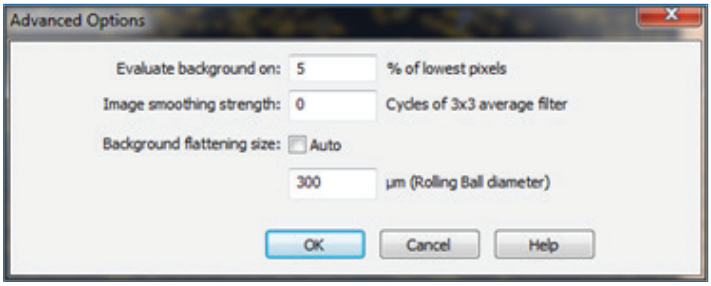
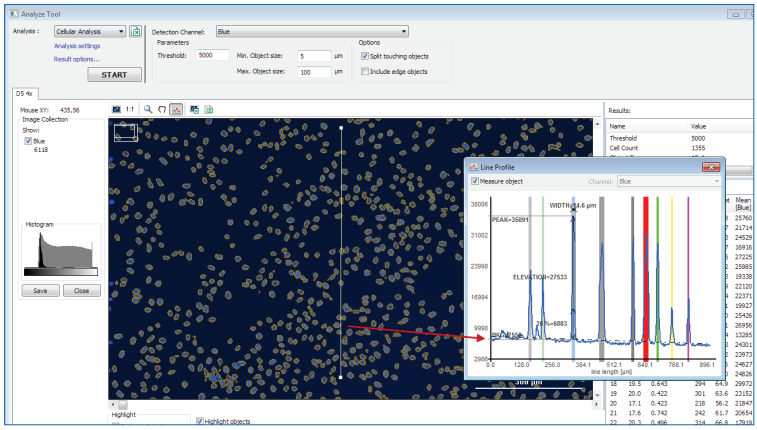


Image Flatness

The first pre-analysis step corrects the image for flatness. *Background flattening size* looks at a portion of the image around each pixel, specified in the Rolling Ball diameter box. The local background is evaluated in this region, and then subtracted from the pixel. It then moves to the next pixel, evaluates the local background within the set diameter around that pixel and repeats the background measurement and subtraction. This corrects for uneven background signal across the image, as is seen in the screenshot below, where the line tool has been drawn from the image border to border to show variation of intensities. The red arrow indicates the background level, which slopes downward from the top to the bottom of the image (left to right in the Line Profile chart). Uneven background makes object identification less accurate. When background flattening is used:

- If initial background is higher, more background is subtracted
- If initial background is lower, less background is subtracted



Correcting for local background variations makes the background across the image much more uniform, improving the accuracy of subsequent steps in cellular analysis. If the rolling ball diameter is set too small, then background removal may be too aggressive. If the rolling ball diameter is too big, distinguishing the background from the cells or objects may be impossible.

Best Practice Tip:

Background Flattening pre-analysis is highly recommended. Gen5's *Auto* selection for automatic background flattening is recommended for new users (and Gen5 performs this way by default). When manually adjusting the Rolling Ball Diameter, always enter a value at least two times the largest object diameter in the image or image set. Otherwise, the formula to subtract local background may inadvertently remove true signal of interest, making cellular analysis less accurate.

Image Noise

The screenshot above also illustrates small intensity variations in the background intensity levels which are referred to as image noise. Image noise can make image analysis more difficult because it can create small specks of noise that are mistakenly measured, and it can cause jagged object borders which makes cellular measurements such as Circularity, Perimeter, and Area less accurate. To prevent this, *Image smoothing* is routinely used in image analysis to obtain a smooth digital picture. Image smoothing decreases the noise, as seen in the line profile, so the background is less variable, providing more accurate object border identification and better spatial measurements.

***Note**: Over-smoothing may distort the image resulting in erroneous object splitting, object counting, and spatial measurements. Lower smoothing numbers cause less smoothing than higher numbers.*

Elevated Background

A third phenomenon noted in the above line profile is an elevated background, above a 0 intensity level. Ideally, the background would be perfectly black, represented by an intensity value of 0. But in reality, small amounts of residual fluorescent stain and other factors typically cause an elevated background level. Background Flattening (Rolling Ball) removes some of the increased background when correcting for image flatness, but the background still remains elevated. "*Evaluate Background on % of Lowest Pixels*" helps address this. For example, if 5% is specified, Gen5 will increase the threshold value defined for cellular analysis by the intensity value at the fifth percentile of image intensities. This essentially removes the impact of the lowest 5% of background intensities in the image by raising the threshold value an equal amount to compensate for it. This improves the ability to accurately identify objects of interest during cellular analysis.

5. Key Applications

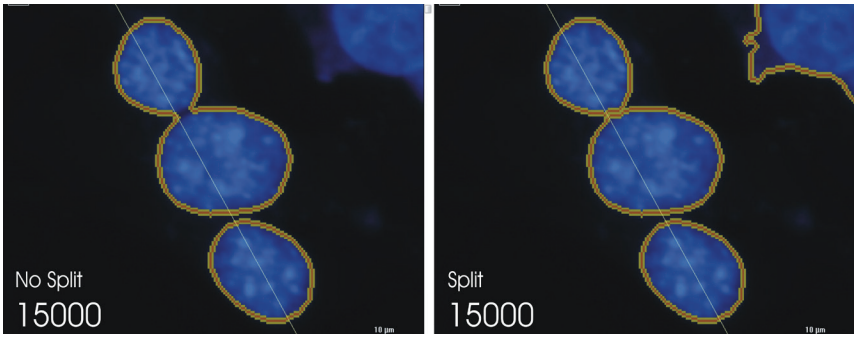
Overview

Incorporating true automated microscopy into a multi-mode microplate detection system provides the means to streamline imaging processes and make assay workflows much more efficient. Data collection and visualization of cellular processes are performed in parallel, providing information-rich phenotypic and meaningful quantitative data. Many publications describing novel applications performed in Cytation Cell Imaging Multi-Mode Reader are available online at: www.biotek.com/techresources and on www.cellimager.com. Some key application examples include:

Analysis of Nuclear Stained Cells

Using the Cytation 3 Cell Imaging Multi-Mode Microplate Reader with DAPI-stained Cells

The imaging and analysis of fluorescently stained cells has traditionally been accomplished using manual microscopic methods with low numbers of samples, while the analysis of higher sample numbers requires the use of very expensive dedicated microscopes. Here we demonstrate digital fluorescence microscopy using the Cytation 3, a novel microplate reader capable of both whole well intensity measurements using a PMT and digital inverted microscopy with a CCD. Gen5 software provides cell segmentation and counting in microplate wells. One-color staining of cells is performed in 96-well microplates and imaged using 4x and 20x microscope objectives.

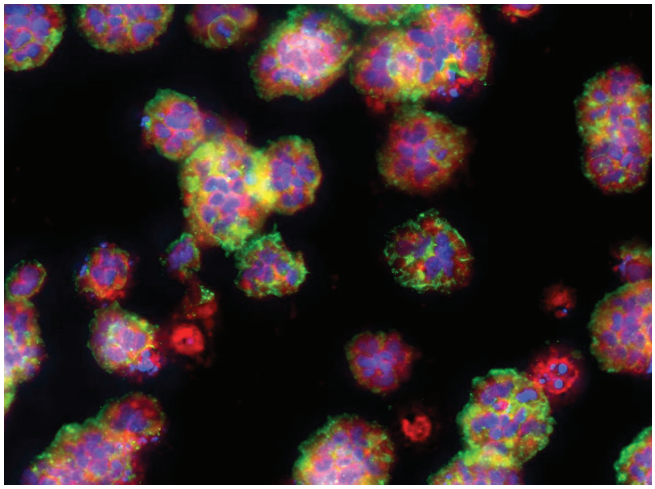


Use of Gen5 software's "Split touching objects" on cell counting. Scale bar indicates 10 µm.

Z-Stacking of Single Plane Digital Widefield Fluorescent Images

Incorporation of the Cytation ™ 3 Cell Imaging Multi-Mode Reader and combineZP Software to Create Deconvoluted, Stacked Images of 3D in vitro Cell Models

Three-dimensional (3D) cellular models have the potential to become a fundamental research tool in cell biology. This is because cell culture performed in this manner reestablishes cell-cell and cell-extracellular matrix interactions that mirror what's seen within real tissues. Histology slides, containing tissue samples mounted onto microscope slides, also continue to be an important research and diagnostic method in the clinic in laboratory. The multi-dimensional cell structures present complications for optical microscopy due to their thickness in the z-axis. Here we present a method to capture and project multiple single z-plane images using digital widefield microscopy.

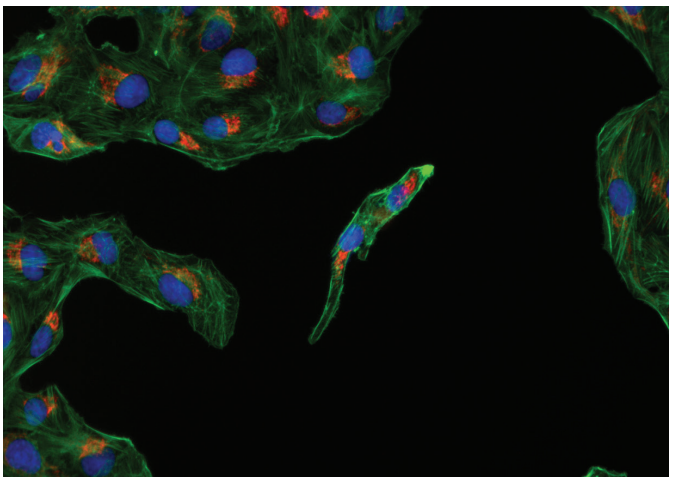


HCT116 cell z-stack image at 20x.

Automated Tissue Culture Cell Fixation and Staining in Microplates

Using the EL406™ Combination Washer Dispenser to Prepare Samples for Imaging with the Cytation™ 3 Cell Imaging Multi-Mode Microplate Reader

Fluorescence microscopy has traditionally been performed on microscope slides, but there is a growing trend towards the use of 96- and 384-well microplates as this allows a greater number of samples to be easily processed and automated. This is certainly true in the field of High Content Screening. Here we describe the use of the EL406 Combination Washer Dispenser to automate the fixation and staining processes typically used prior to fluorescence imaging.

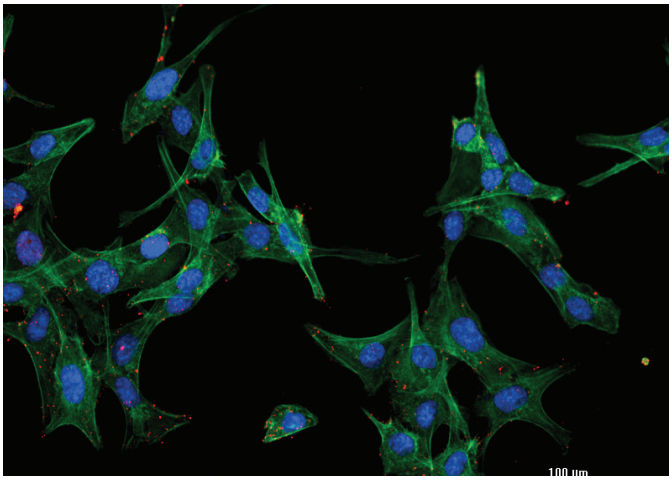


U-2 OS cells stained for mitochondria, F-actin and nucleus. Scale bar indicates 80 µm.

Multiplexed Assay for IL-6 Secretion and Cell Viability Using an Epithelial Ovarian Cancer Cell Line

Using the Cytation™3 Cell Imaging Multi-Mode Microplate Reader to Monitor IL-6 Secretion using HTRF detection and Cell Viability Using Digital Widefield Fluorescence Microscopy

Epidermal growth factor receptor (EGFR) is expressed in up to 70% of epithelial ovarian cancers (EOCs). High levels of IL-6 have also been found in the ascites of EOC patients. EGFR activates multiple signaling cascades including the activation of NFkB, which is known to activate the transcription of inflammation-related proteins such as IL-6. In this work we will demonstrate a multiplexed assay for inhibitors at the level of EGFR and NFkB to monitor IL-6 secretion and cell viability. The multiplexed assay work flow uses an assay plate and HTRF detection plate. Following treatment of the cells, supernatant is transferred to the HTRF detection plate where IL-6 concentrations are determined; then cell viability is assessed in the assay plate using fluorescent probes and imaging. All assays were conducted on the Cytation™ 3 Cell Imaging Multi-Mode Reader.



SKOV-3 cells; blue is DAPI-stained nuclei, green is Phalloidin actin staining.

6. Best Practices

Plating, Incubating & Handling Cell Plates

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 paying close attention to the details in sample preparation will significantly benefit the assay results.

Here are some best practice tips to improve sample preparation:

- Cell Plating:
  - After plating cells, allow them to sit on the lab bench 15 to 20 minutes prior to placing in incubator. This gives cells time to settle more evenly, eliminating much of the uneven distribution within wells and the edge effect that is often noted.

- Cell Growth:
  - Do not stack plates in an incubator. This causes uneven heat and humidity distribution in the plate 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 is a common solvent used for compound or drug addition to cells. Cells can typically handle a final concentration between 0.25-1% DMSO (cell-type dependent). DMSO has a higher density than water so it is important to appropriately 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.

- Imaging Microplates:
  - Clean the bottom of the microplate wells or slide surface prior to imaging the sample.
  - When imaging a previously stained plate that is stored at 4°, allow the plate to warm to room temperature, otherwise the bottom of plate collects condensation that causes blurred images
  - Check that the correct microplate is selected in Gen5 Data Analysis Software; selecting the incorrect microplate causes autofocus failures due to incorrect microplate dimensions.

Dye Compatibility Chart for RGB Channels

Common Blue, Green and Red Dye / Fluorophores								
BLUE	excitation	emission	GREEN	excitation	emission	RED	exitation	emission
Hoechst 33342	352	449	Fluorescein	490	515	Texas Red	589	615
DAPI	358	461	GFP	488	509	Alexa Fluor	591	618
Hoechst 33258	352	449	FITC	485	520			
Other Blue, Green and Red Dye / Fluorophores								
BFP	382	448	AlexaFluor 488	495	519	DyLight	593	604
Whole Cell Stain Blue	350	450	Whole Cell Stain Green	493	518	AlexaFluor 568	579	604
Coumarins	350-360	430-450	YOYO-1	491	509	BODIPY-TRX	589	617
AlexaFluor 350	347	442	YO-PRO-1	491	509	SpectrumRed	587	612
CPM	385	471	SpectrumGreen	497	524	Calcium Crimson	590	615
Calcein Blue	375	420	Fluo-4	494	516	X-Rhodamine (XRITC)	580	605
Cascade Blue	400	420	Calcein	494	517	mCherry	587	610
						Live/Dead Fixable Red		
DyLight 350	353	432	Acridine Yellow	470	550	dead cell stain	595	613
Acridine	362	462	Bodipy FL	502	540			
AlexaFluor 405	401	421	Cell Tracker Green	490	540			
Calcein Violet	401	451	LysoSensor Green	448	503			
LysoSensor Blue	374	424						
LysoTracker Blue	373	422						
Pacific Blue	405	455						
Quinine Sulfate	349	461						
Y66H (fluorescent prot	360	442						
Live/Dead Fixable Blue stain	344	442						

***Note**: The Cytation supports many more dyes/fluorophores than listed here with over 15 filter sets available.*

Objective Specifications

All Cytation objectives are long-working distance objectives (required for measurement in microplates).

Magnification	1.25x	2.5x	2.5x	4x	10x	20x	40x	60x
PN standard phase	1220562 N/A	1220539 N/A	1220549 N/A	1220519 1320515	1220518 1320516	1220517 1320517	1220544 1320518	1220545 N/A
PN adapter phase	1220560	None	1220560	1222062	1222062	1222236	1222236	1222236
Numeric aperture	.04	.12	.07	.13	.30	.45	.60	.70
Working distance	5 mm	8.7 mm	5.7 mm	17 mm	10 mm	6.4-7.8 mm	2.7-4 mm	1.5-2.2 mm
Depth of field	328 µm	33.6 µm	88.2 µm	24.4 µm	4.22 µm	1.81 µm	1.01 µm	0.74 µm
Field of view (mm)	7.14 x 5.35	3.57 x 2.67	3.57 x 2.67	2.23 x 1.67	.89 x .67	.45 x 33	.22 x .17	.15 x .11
Manufacturer	Olympus	Zeiss	Meiji Techno	Olympus	Olympus	Olympus	Olympus	Olympus
Ring color	Black	N/A	Brown	Red	Yellow	Green	Light blue	Cobalt blue
								

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