SARTORIUS

Simplifying Progress

Incucyte® SX5 Training

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Agenda

Introduction and Applications

Incucyte® Hardware and Best Practices

Software Overview

Acquire

Visualize

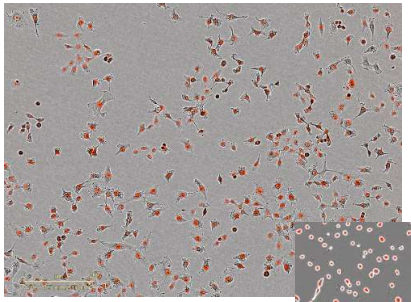
Process

Data Export

Conclusion

Incucyte® Live Cell Monitoring and Analysis

Acquire



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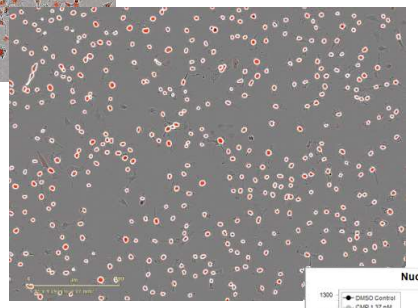

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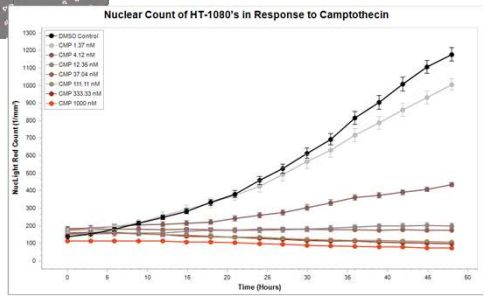
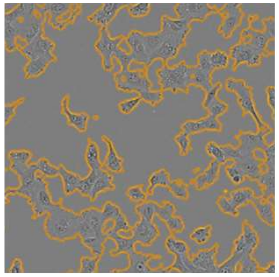
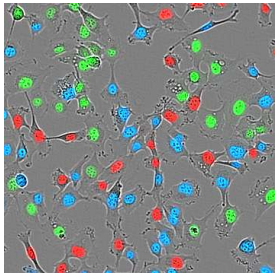
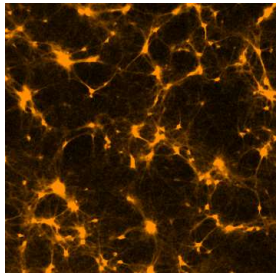
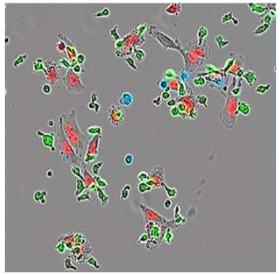
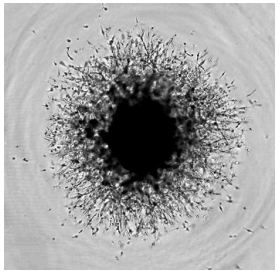
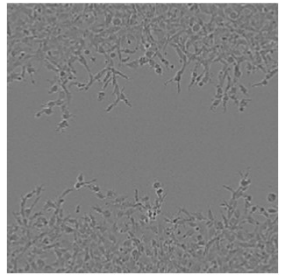


Incucyte® Key Applications

Cell Health & Proliferation	Cell Function	Cell Movement & Morphology
▪ Cell Counting	▪ Immune Cell Killing	▪ Scratch Wound Migration
▪ Viability	▪ Immune Cell Activation	▪ Scratch Wound Invasion
▪ Apoptosis	▪ Antibody Internalization	▪ Chemotaxis
▪ Cytotoxicity	▪ Phagocytosis	▪ Neurite Outgrowth
▪ Tumor Spheroid	▪ NETosis	▪ Spheroid Invasion
▪ Cell Cycle	▪ Neuronal Activity	▪ Dilution Cloning
▪ ATP Metabolism	▪ Live-Cell Immunocytochemistry	
▪ Mitochondrial Membrane Potential		
▪ Organoid Culture QC		

Continuously monitor & analyze cells inside your incubator using simple mix-and-read protocols!

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Incucyte® SX5 Overview

3 Optical Module Options

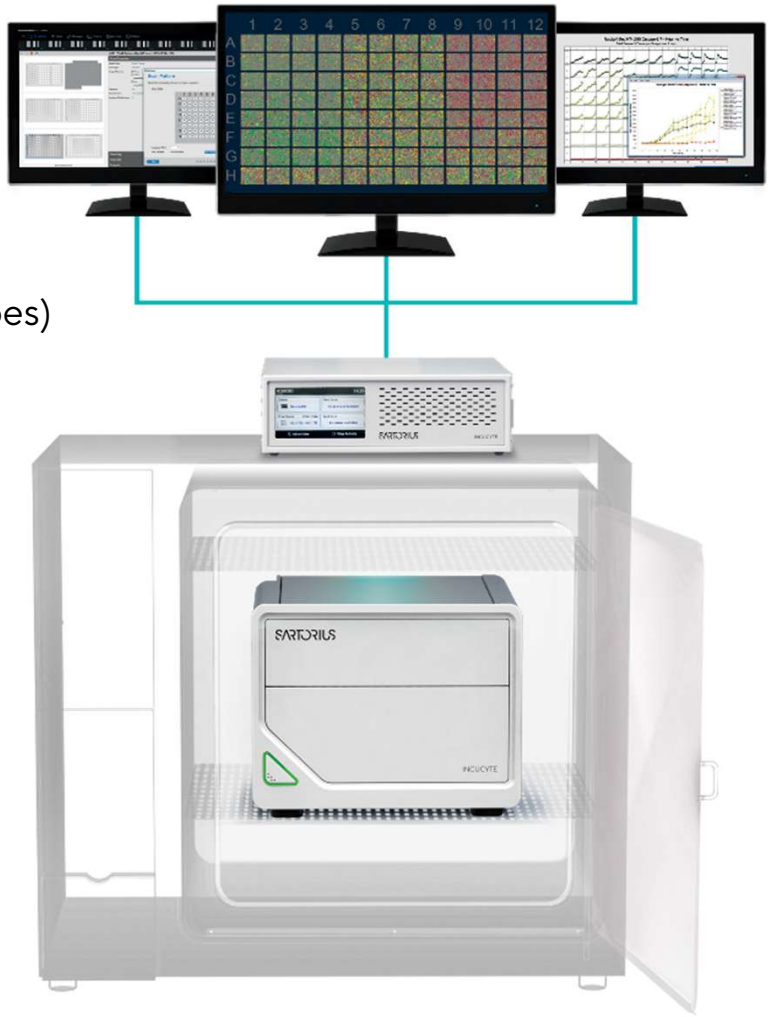
- All modules have HD Phase (brightfield with Spheroid and Organoid scan types)

- Green/Orange/NIR 

Channel	Excitation	Emission
Green	453 – 485 nm	494 – 533 nm
Orange	546 – 568 nm	576 – 639 nm
NIR	648 – 674 nm	685 – 756 nm

- Green/Red 

Channel	Excitation	Emission
Green	441 – 481 nm	503 – 544 nm
Red	567 – 607 nm	622 – 704 nm

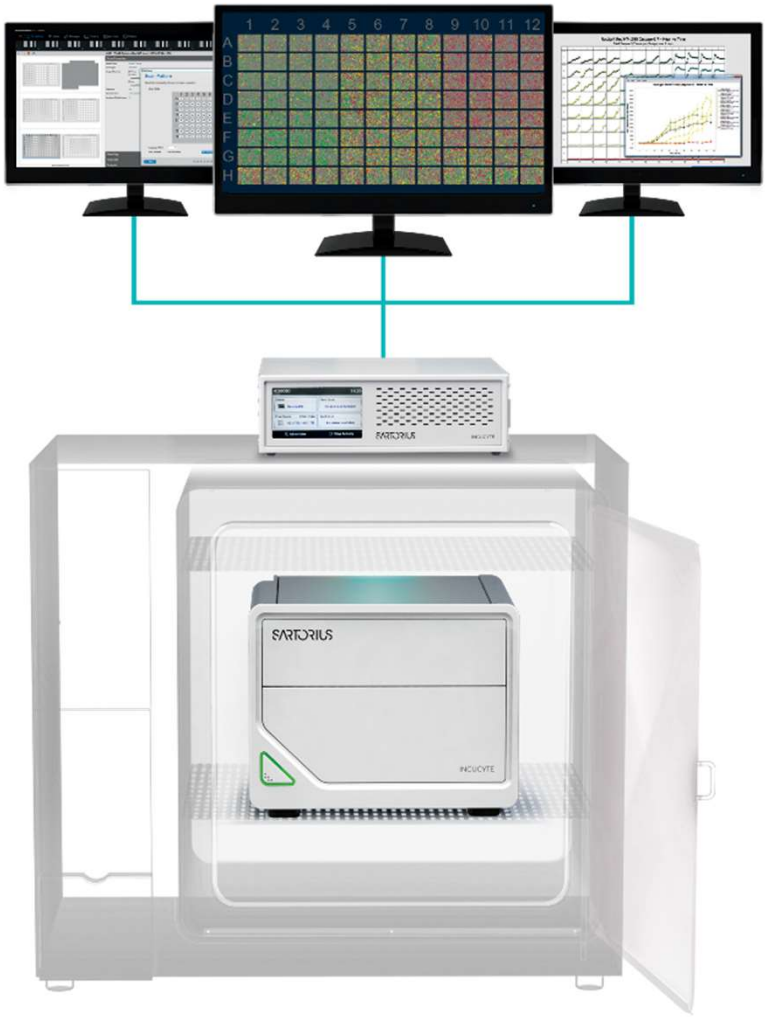


Incucyte® SX5 Overview

3 Optical Modules

- Metabolism 
- Dual Excitation, Single Emission for ATP Analysis

Channel	Excitation	Emission
Green	473 - 498 nm	NA
Orange	524 – 550 nm	565 – 591 nm
NIR	648 – 674 nm	685 – 756 nm



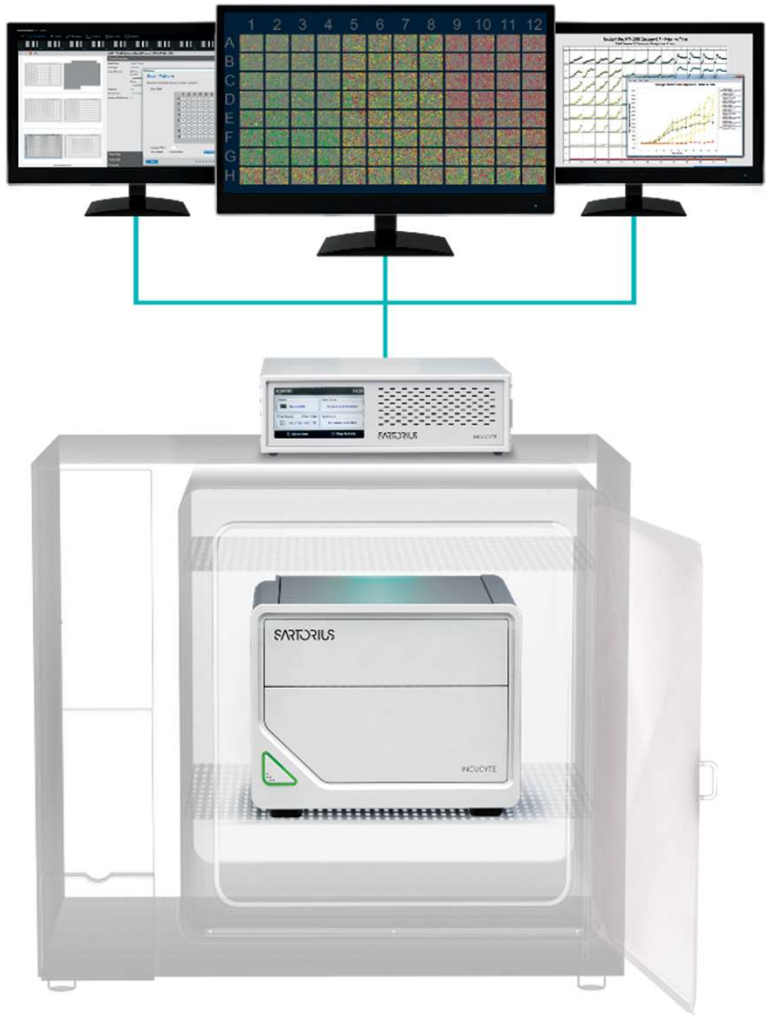
Incucyte® SX5 Overview

Objectives

- 4x, 10x, 20x on an automated turret

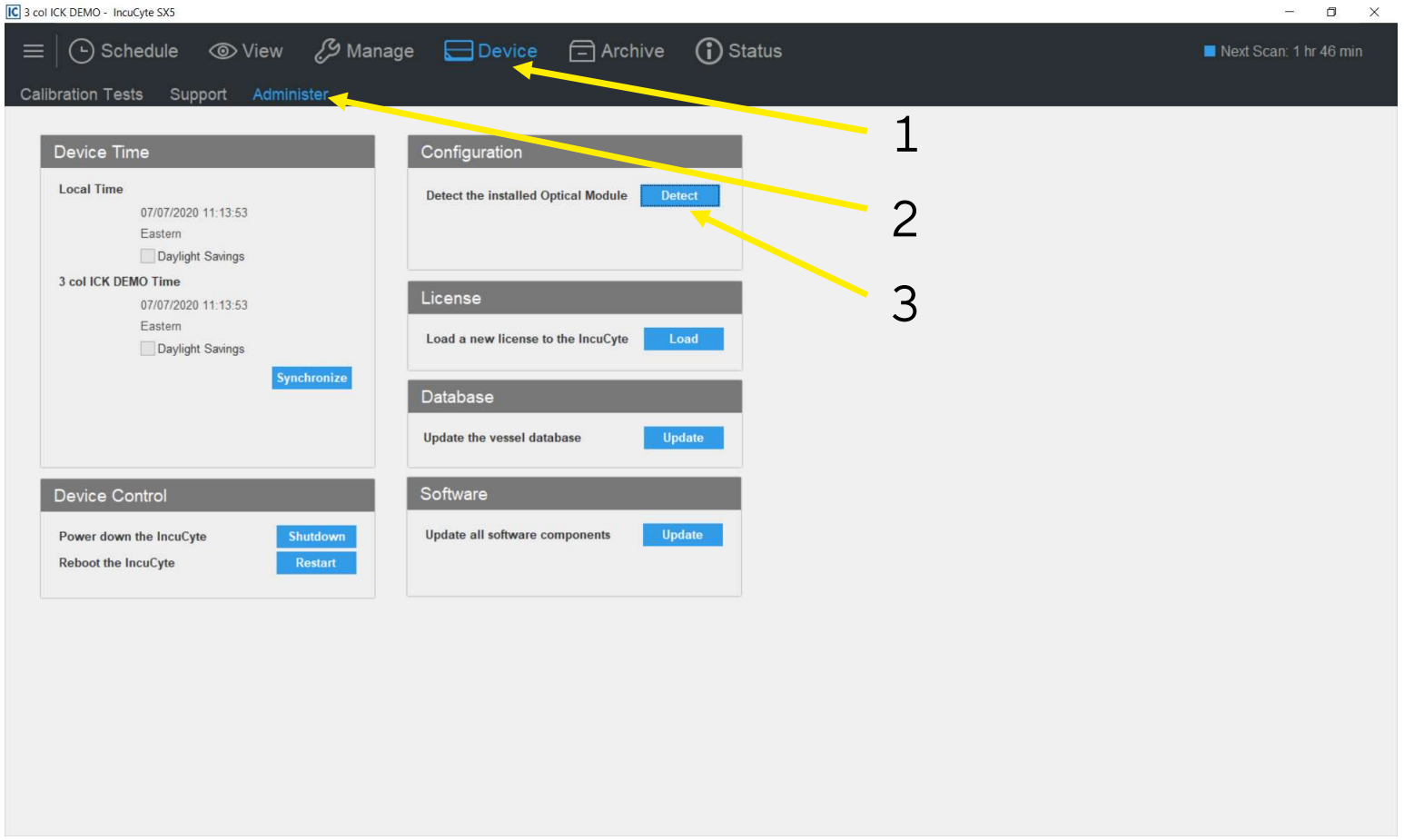
- Compatible with most tissue culture vessels :
- Multiwell plates, dishes, flasks and slides
- >600 vessels from >30 manufacturers

- **Software**
- Remote access
- Guided interface
- Unlimited user licenses



Swapping the Optical Modules

- Detect the installed optical module in the Device menu
- Physically swap the module and then detect again to update to the new Optical Module
- Swapping modules will cancel upcoming scans



Remove the Front Cover of the Incucyte® SX5 Gantry

- Make sure that the Incucyte® SX5 is powered off
- Locate the two Cover Tabs on the bottom of the Front Cover and push outward to disengage Cover from Incucyte® SX5
- Pull Cover down and away from the Incucyte® SX5 to remove
- Place Cover to the side and in a safe location

Remove the Incucyte® SX5 Optical Module Assembly

- Identify the Optical Module and Tab on the front of the Optical Module
- Flip Tab up and turn 90 degrees counterclockwise until the detent feature disengages
- Pull the Optical Module away and out of Incucyte® SX5
- Place Optical Module in a secure location or back into the proper packaging. The Pelican™ case which contains the optional Optical Modules is fitted to hold any Optical Modules designed for the Incucyte® SX5

Remove the Incucyte® SX5 Phase Lamp

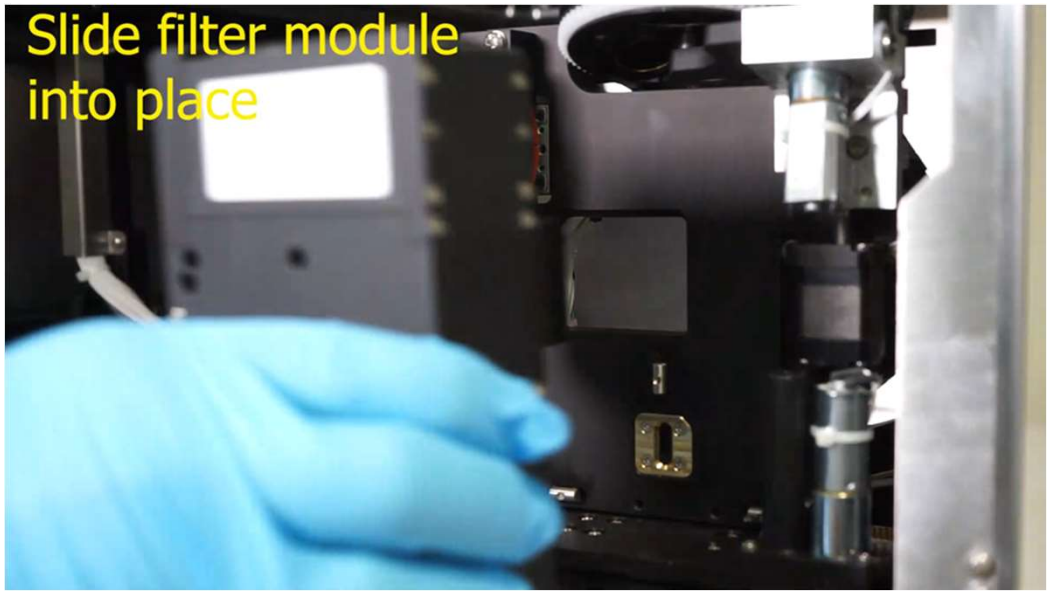
- Identify the Phase Lamp by looking for the top-right cut-out in the Front Support, where the Label on the Phase Lamp will be in-view
- Manually pull out the Drawer to the furthest extent and remove any Trays or Vessels
- Reach up and into the top of the Incucyte® SX5 towards the Label and grasp the Phase Lamp, front and back
- Pull the Phase Lamp to the right side of the Incucyte® SX5 – in the direction of the arrow
- Carefully, remove the Phase Lamp from the Incucyte® SX5
- Place Phase Lamp in a secure location or back into the proper packaging

Install the Incucyte® SX5 Phase Lamp

- Remove the new Phase Lamp from box or packaging
- With the Drawer still in the pulled-out position, guide the Phase Lamp into position
- Gently guide and push the Phase Lamp towards the left side of the Incucyte® SX5 until an audible and tactile click is noted
- The Phase Lamp will not seat into the proper position if not properly aligned

Install the Incucyte® SX5 Optical Module Assembly

- Remove new Optical Module from box or packaging
- Flip Tab up and turn 90 degrees clockwise or counterclockwise so that the Tab is vertically aligned
- Guide the Optical Module into the same position within the Incucyte® SX5 that the prior Optical Module was removed
- While gently holding the Optical Module in position, flip up and turn the tab 90 degrees clockwise until detent engages
- Flip Tab down and into the proper recess in the Optical Module
- Replace any Trays or Vessels that were removed earlier and manually push the Drawer back into the Incucyte® SX5



Replace the Front Cover of the Incucyte® SX5 Gantry

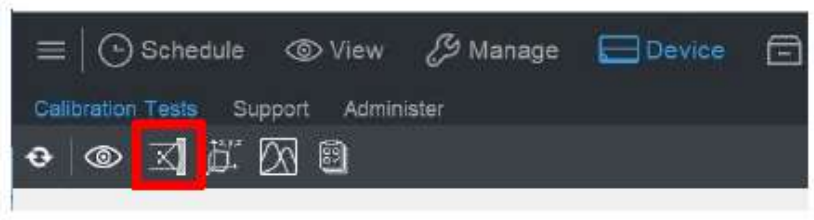
- Guide the Front Cover back into position on the Incucyte® SX5 by leading with the top and guiding upward, using the Guide Hooks for proper placement and orientation
- Once the Cover is in place and flush with the rest of the Incucyte® SX5, push the two Cover Tabs inward to engage Front Cover
- Power Controller on and allow the Incucyte® SX5 to run the warmup sequence before beginning any scanning

Incucyte® SX5 Function and Best Practices

- Incucyte® SX5 is NOT an incubator and will not regulate the environment
- Fans draw air into the gantry so conditions inside the Incucyte® are the same as the incubator but there is no active environmental control
- Operating Environmental Conditions
  - 0°C to 42°C
  - 5% to 95% Relative Humidity, Non-Condensing
- Best Practices
  - Set Incubator Temp 0.5°C below desired temp (e.g. if 37°C is desired, then set incubator to 36.5°C)
  - Check water pan and humidity settings 2-3 times per week
  - Always wear gloves and practice sterile technique

Incucyte® SX5 Maintenance

- The exterior of the Incucyte® gantry can be cleaned with 70% alcohol during routine incubator cleaning
- Vaporized multi-purpose disinfectants (e.g., Virkon) or acidic solutions can be corrosive to machinery and should NOT be used to clean the incubator
- Follow the manufacturer's recommendations for routine incubator maintenance but do NOT heat sterilize, if this is needed, remove the Incucyte® gantry from the incubator and move the controller if it is on top of the incubator
- The Incucyte® can be sterilized with vaporized hydrogen peroxide treatment
- Calibration is recommended every 6 months



Automated Image Acquisition and Auto-Focus

- Image based auto-focus
- Performs a series of wide (coarse and fine) sweeps to assess several image planes
- Finds plane of highest contrast and collects phase, green, and red image at the same plane
- Auto-focuses on every well, every plate, and every time point
- Seeding negative control wells to the upper left corner of a microwell plate can be beneficial

Live Cell Experimental Optimization

Accurately weigh and concentrate samples

Ensure water is free of impurities

Filter media and cell culture of unwanted debris

Pipette with accuracy and comfort

Prevent contamination with routine tests and aseptic technique

Cell Culture Best Practices

Pipetting Rules to Remember

- Hold the pipette vertically, when aspirating
- Immerse the tip only 2-3 mm
- Pre-rinse 3-5 times before pipetting (forward)
- Pause consistently after aspiration
- Dispense at a 30-45° angle
- Pipette against the inside wall of the receiving vessel
- Operate with smooth and consistent thumb action
- Reverse pipetting significantly reduces bubble formation

Accurately Pipette Cells and Samples: Technique is Key for Accurate Results

Tacta Mechanical Pipettes

Picus Electronic Pipettes

- Pipette with accuracy and comfort to ensure:
- Cells are evenly distributed
- Cell densities are accurate
- Volumes of treatments are accurate for dose response analysis
- Set-up high throughput assays rapidly and efficiently

Cultures should be Free of Contaminants: Technique is Key for Accurate Results

Microsart® AMP Mycoplasma Kit

MD8 Air Samplers

MYCAP™

- Check cell culture routinely for mycoplasma contamination
- Ensure cell culture environment has the lowest concentration of airborne contaminants
- Aseptically remove samples and close containers without contaminating contents

Weigh, Filter, & Concentrate: Technique is Key for Accurate Results

Sartoclear Dynamics® Lab Kits

Vivaspin® Turbo

Vivaflow®

MA37 Infrared Moisture Analyzer

Cubis® MSA

Quintix® & Secura®

- Filter cell cultures and media to ensure samples are free of unwanted debris
- Concentrate proteins, antibodies, and conditioned media from mammalian cell culture and test effects on cellular function
- Accurately weigh samples to ensure downstream assay set-up and analysis are optimal

Ultrapure Water for Multiple Applications: Technique is Key for Accurate Results

arium® pro

arium® mini

- Adapt an ultrapure water system for different applications
- Ensure water for cell culture applications is free of impurities

Live Cell Experimental Optimization

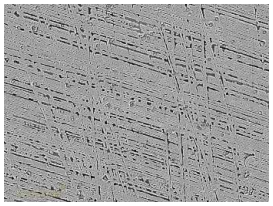
Live Cell Experimental Optimization

Condition	Things to consider
Cell Seeding	• Density may need to be optimized (always seed cells at least 10% confluence) • Allow cells to settle at ambient temperature for **20 minutes** before placing into the incubator for optimal cell distribution • Non-adherent cells may require a coating for optimal cell distribution
Media	• Ensure sufficient medium per well (especially 96 and 384-well plates) • Schedule media changes if longer than 3 days • No need to use phenol red-free medium • High riboflavin (e.g. Fischer's) can give green autofluorescence
Reagents	• Follow protocol recommendations for optimization • Make up stock solution containing reagents to dilute treatments
Treatments	• Always run a positive and negative control • Run at least 3 replicates for most applications
Plate Type	• Choose plates optimised for the experiment, black walled are not necessary • Good optical clarity assists focusing and makes analysis simpler
Imaging	• Always verify data with images • Minimize scanning frequency and fluorescent channel exposure for sensitive cell types

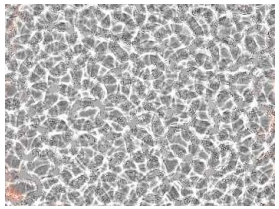
Best Practices for Image Acquisition

Image Artefacts to Avoid	How to Get the Best Image
Empty Wells (No Cells)	Edit the scan pattern to exclude wells that do not contain cells. Ensuring >10% cell confluency in all wells is recommended (unless scanning in dilution cloning mode)
Media Droplets on the Lid	Carefully wipe away
Fingerprints/Scratches/Debris	Avoid touching tops/bottoms of vessels (even with gloved hands) and using vessels with scratches/debris
Writing on the Vessel	Label vessels on the sides or in areas where imaging will not occur
Condensation	Allow the plate to warm-up for ~20min in the incubator/ Incucyte before scanning or use a pre-warmed lid
Vessels Not Being Positioned Correctly	Take trays out of the Incucyte and place on a flat surface. Lock microplates into place with well A1 in top left corner
Bubbles	Remove bubbles with ethanol vapor (De-Bubbler / Bubble Buster). Remove the inner straw from a wash bottle, add 70% ethanol and gently squeeze vapor over the wells to clear bubbles

Scratches



Condensation



The De-Bubbler / Bubble Buster

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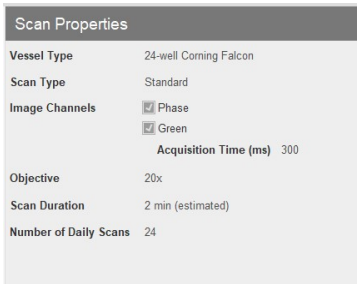
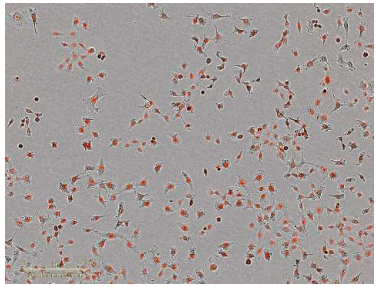
Live Cell Analysis Software Training

4 Steps to Image Analysis

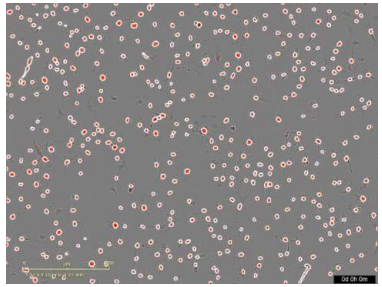
Acquire Images



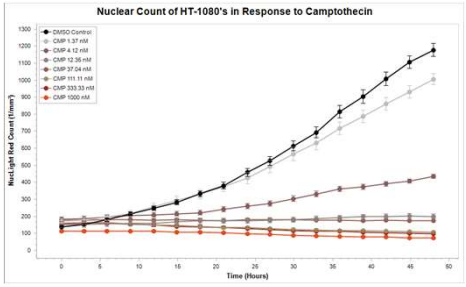
View Images



Process Images

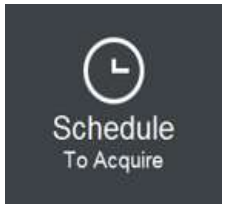


Export Data



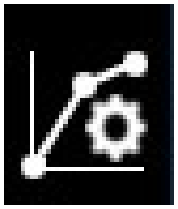
Incucyte® : A Guided Interface

Schedule Scans

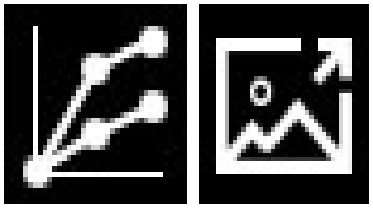


Vessel View

Analysis Definition



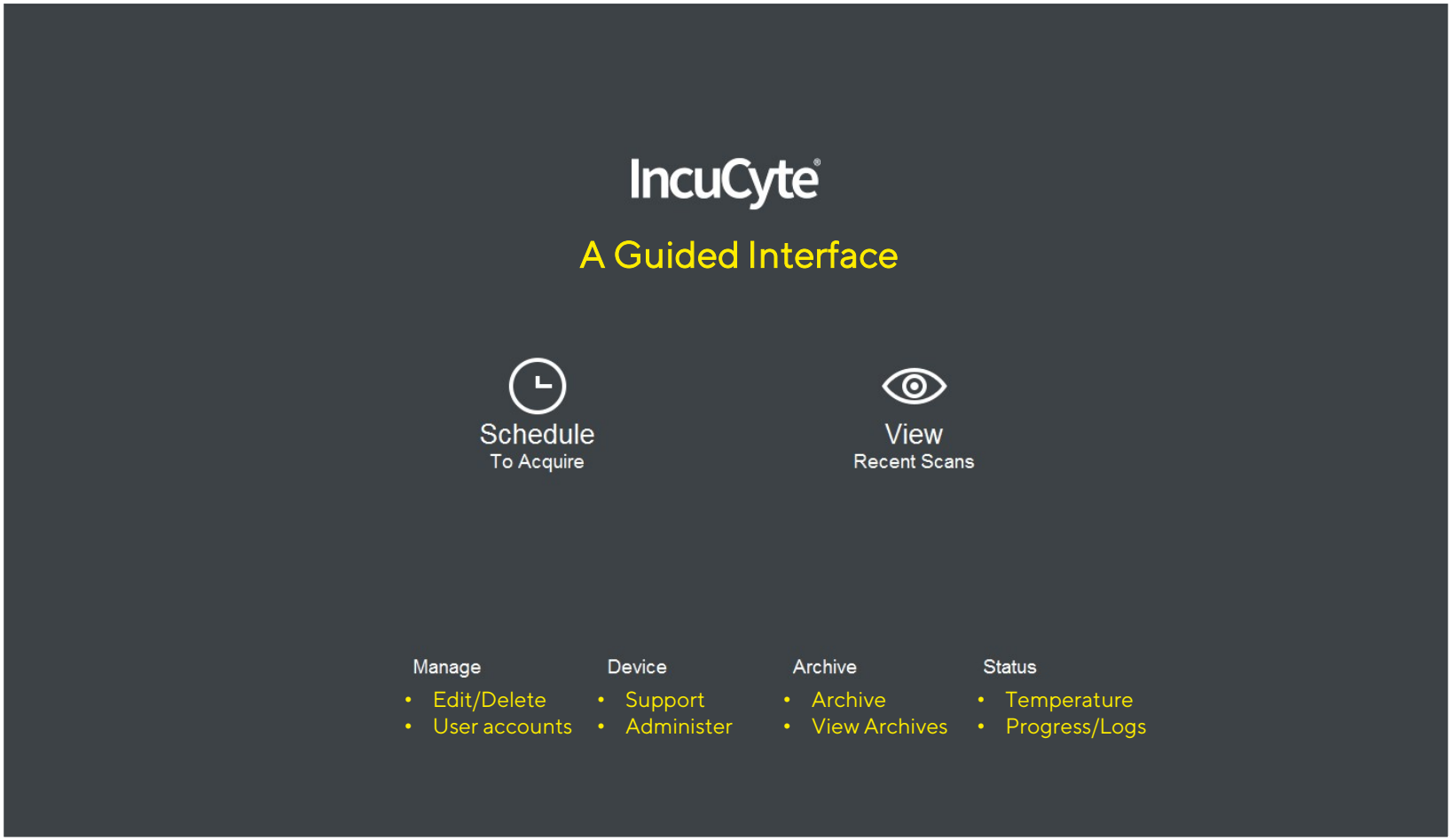
Graphing and Exporting



Logging into the Incucyte® SX5

- Incucyte®: Instrument identifier assigned by your FSE at installation (e.g. IC70014 or an IP address, 192.168.128.130 for a direct connection)
- User Name and Passwords can be set up by the Admin
- Main Window opens up to guide you through the interface

Navigating the Incucyte® Software



Data Management and Archiving

- The Incucyte® SX5 has 27.3TB of storage capacity across 4 hard drives
- Data is stored in a RAID format so is duplicated across different hard drives
- However, routine data backup is encouraged (Incucyte Manual page 151)
- Incucyte® data can be archived via the network or a directly connected hard drive
- Once archived, data can be retained on the Incucyte® SX5 or deleted
- Deleted data cannot be restored to the Incucyte® SX5 , however, reanalysis of the archived data set can be performed on the users own PC through the Incucyte® GUI
- Routine database management and removal of older experiments is recommended periodically

User Accounts

Function	Admin	Standard	Limited	Guest
Add/Remove/Edit Scheduled Scans	Any	Any	No	No
View Vessels	Any	Any	Any	Any
Create Analysis Definitions	Any	Any	Any	No
View Analysis Definitions	Any	Any	Any	Any
Edit/Delete Analysis Definitions	Any	User Owned	User Owned	No
Edit Vessel Documentation	Any	Any	Any	No
Delete Vessels	Any	User Owned	No	No
Archive Vessels	Any	Any	Any	No

Individual user accounts are recommended

Admin users can also perform software updates, load licence updates and run calibrations

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Getting Started – Inserting a Vessel

Device Status	LED Colour	LED State
Idle	Green	Pulsing
Active	Red	Solid
Pre-Scan (15 Seconds Before a Scan)	Yellow	Flashing
Drawer Open	Yellow	Flashing
Front Panel Removed	Red	Pulsing
Device Error	Red	Flashing

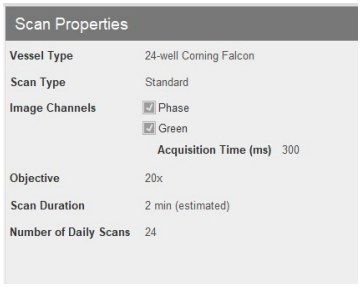
Opening the gantry unit and inserting a vessel

- Check (Software, touch display and LED-state) before opening
- Opening whilst scanning will lose that timepoint, but not the whole experiment

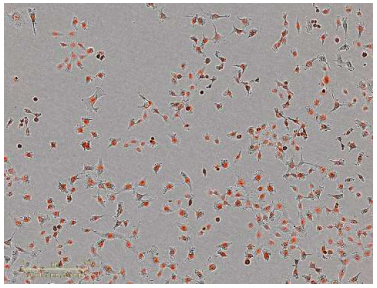
Scheduling Scans

4 Steps to Image Analysis

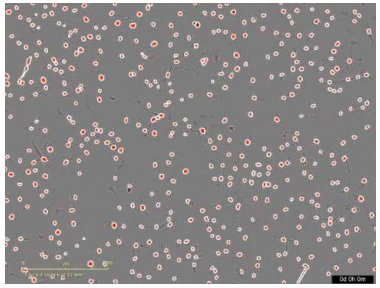
Acquire Images



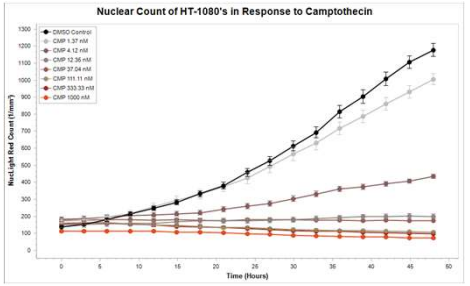
View Images



Process Images

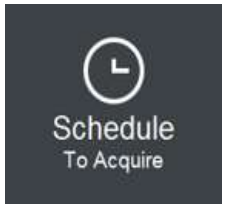


Export Data



Incucyte®: A Guided Interface

Schedule Scans



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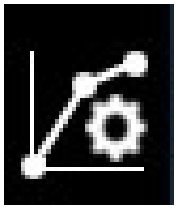
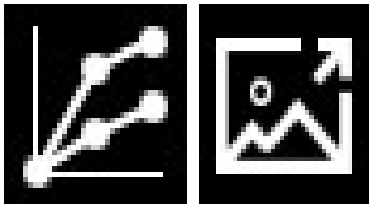
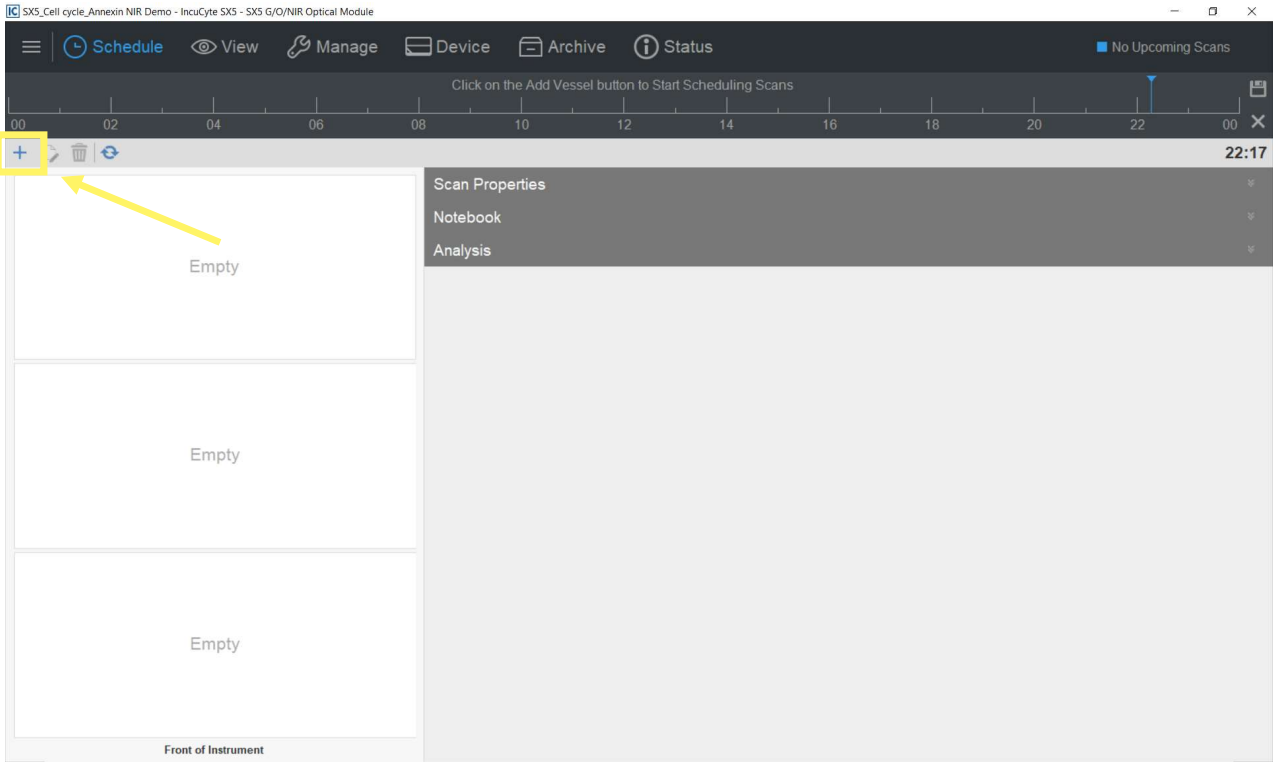


Graphing and Exporting



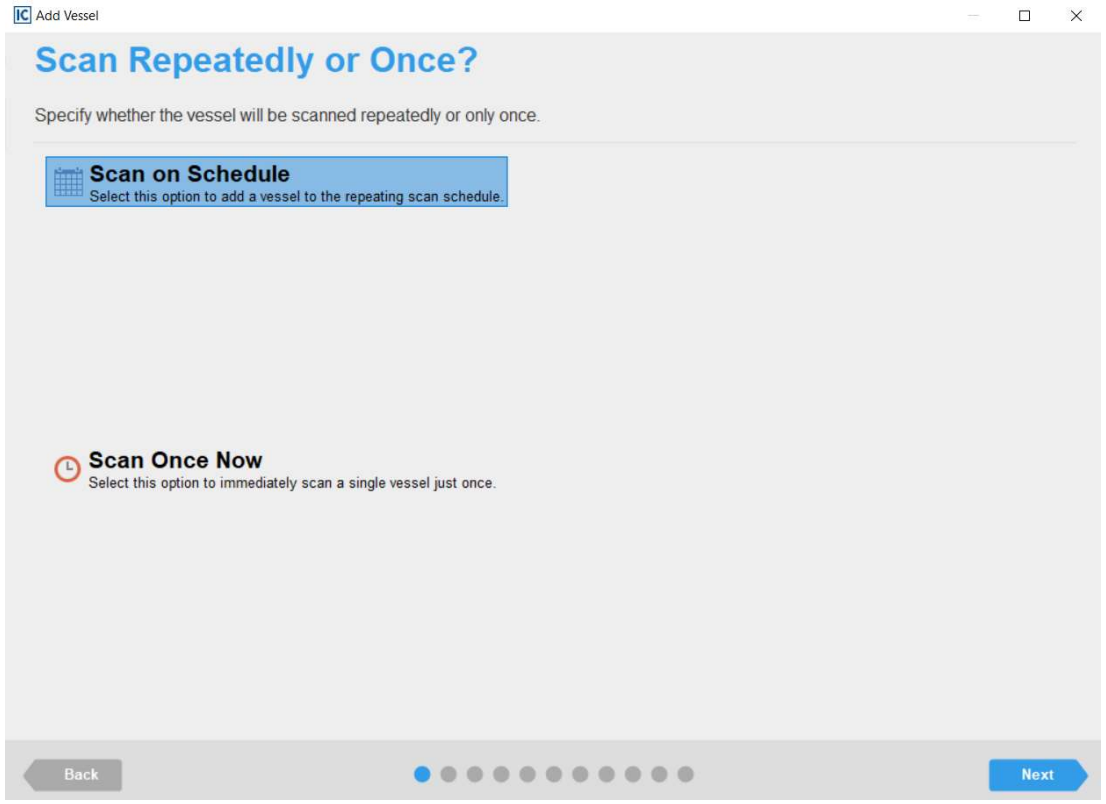
Scheduling Scans: Launch the Guided Interface



Launch the Guided Interface

- Click the blue + icon
- Will open a new window

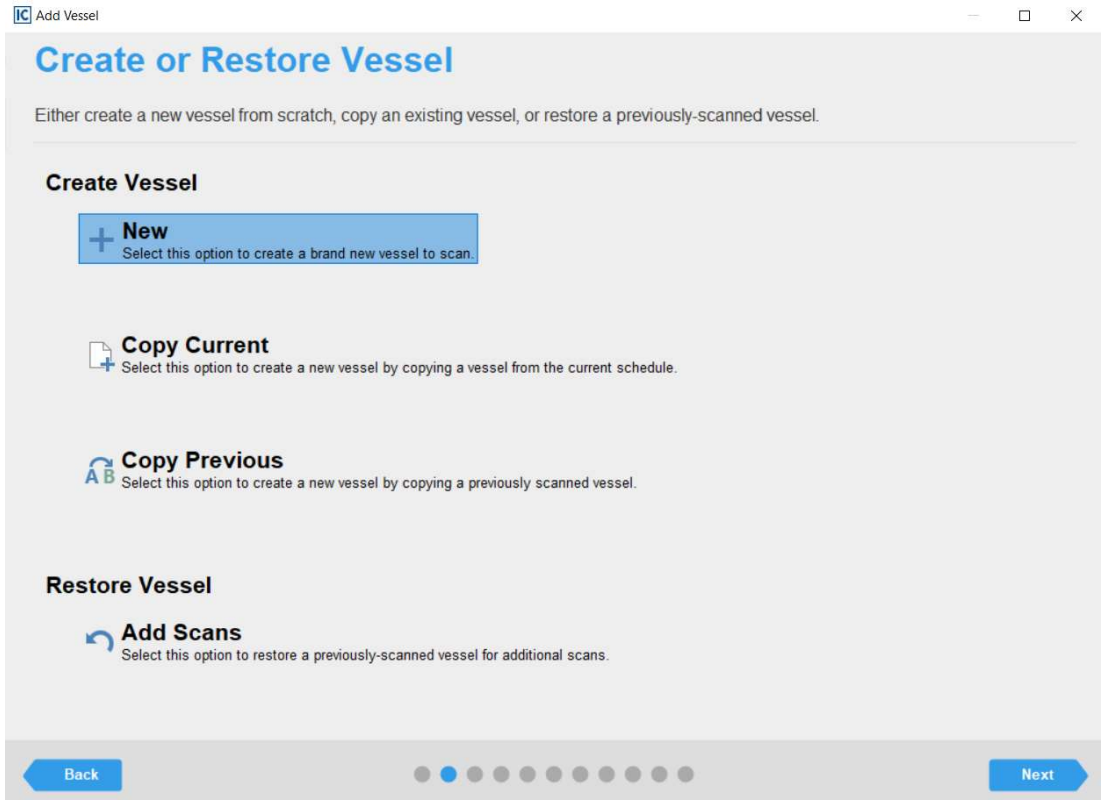
Scheduling Scans: Repeating or One Off?



Scan Repeatedly or Once?

- Most scans will be on a repeating schedule
- Scan Once Now is useful for a quick look at a vessel, e.g., for assessing confluence

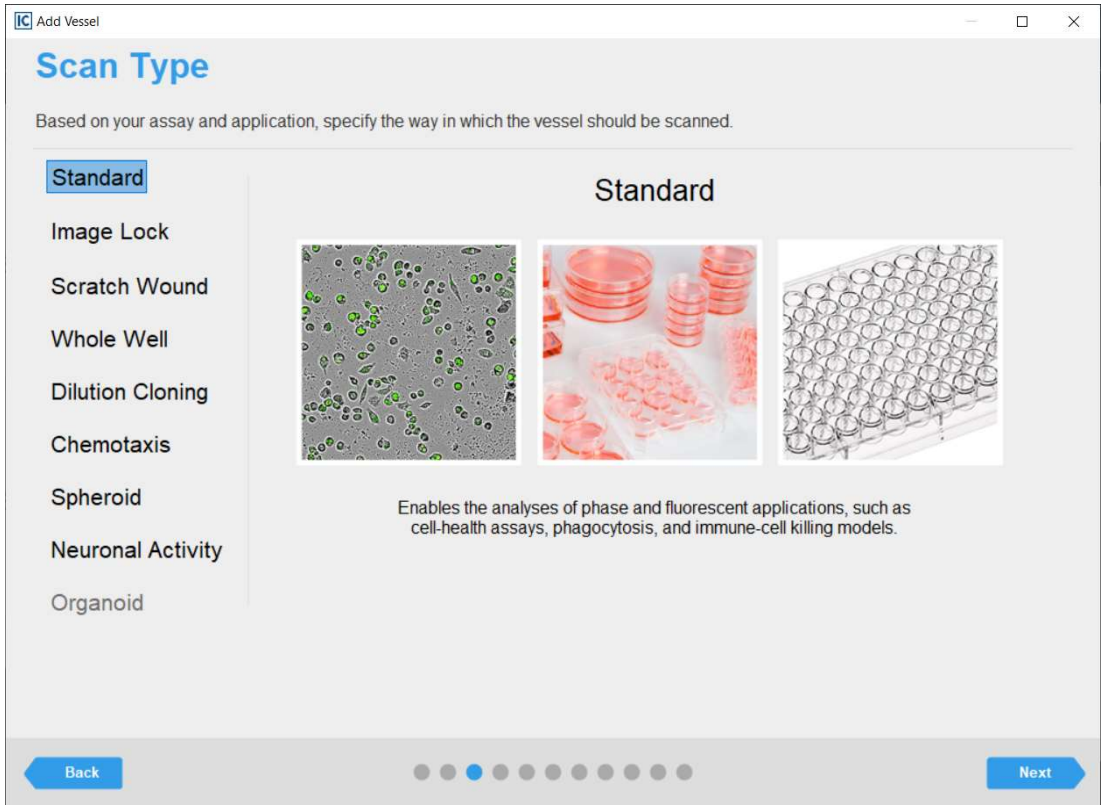
Scheduling Scans: New or Copied Vessel?



Create or Restore Vessel

- Create a new vessel from scratch
- Copy a vessel currently in the Incucyte or one scanned previously, e.g. a replicate plate
- Restore a previously scanned vessel and add scans to that same experiment

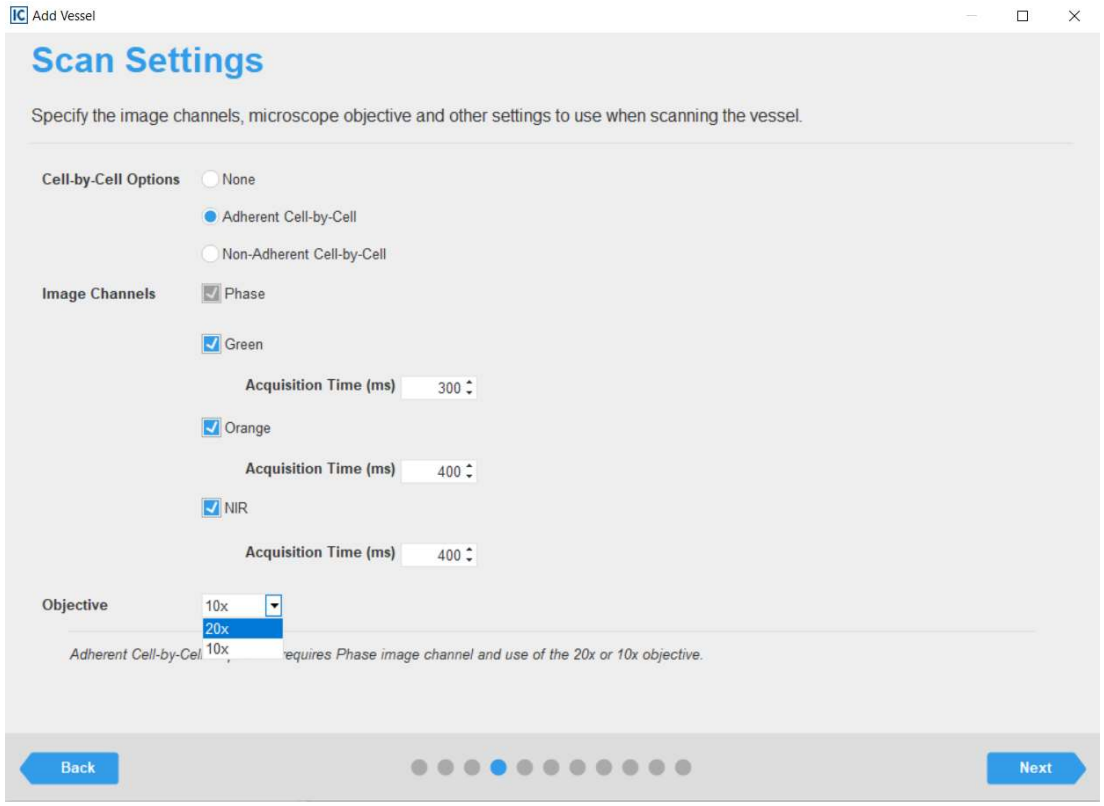
Scheduling Scans: Types of Scan



Scan Type

- Standard scanning is used for a large number of assays including cell health assays and to select Cell-by-Cell scanning
- Application specific scan types will automatically pre-select appropriate vessels / channels / objectives

Scheduling Scans: Channels and Objectives

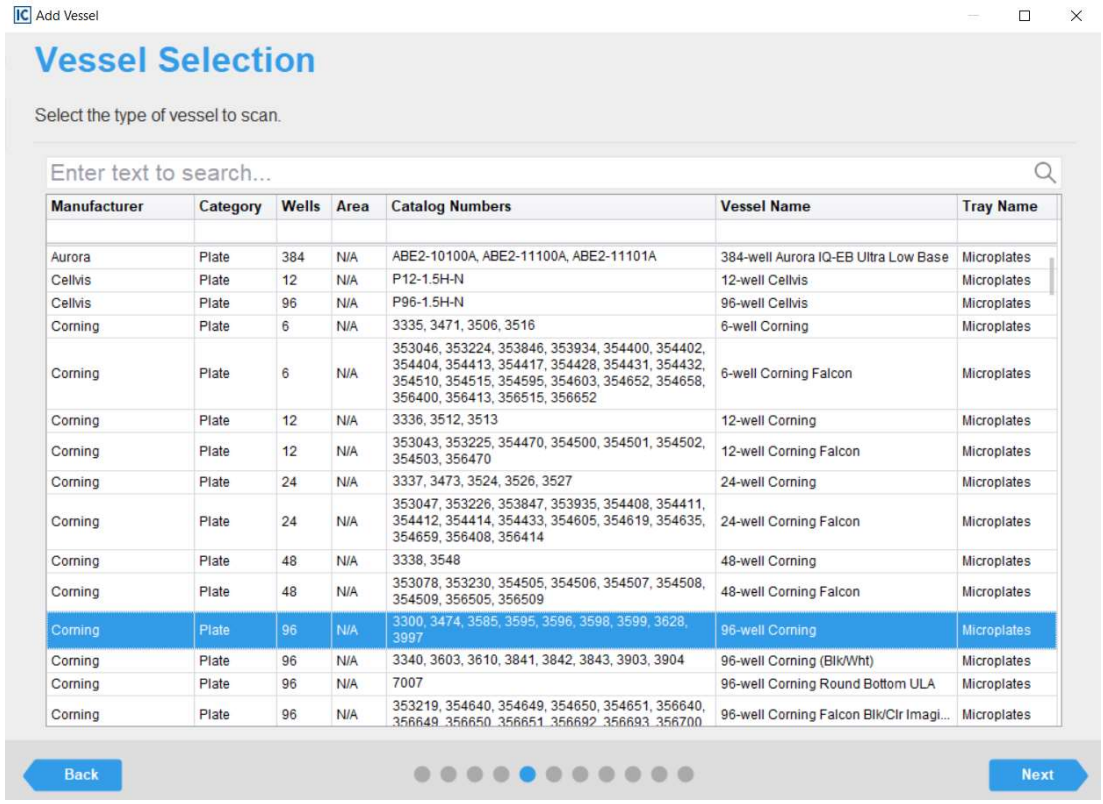


Scan Settings

- Cell-by-Cell scanning, if available, can be enabled at this point
- Each vessel can be scanned using a different selection of channels
- Default acquisition times will work for most applications
- Choice of objective is also vessel specific (some imaging modes require specific objectives)

Acquire

Scheduling Scans: Choose a Vessel

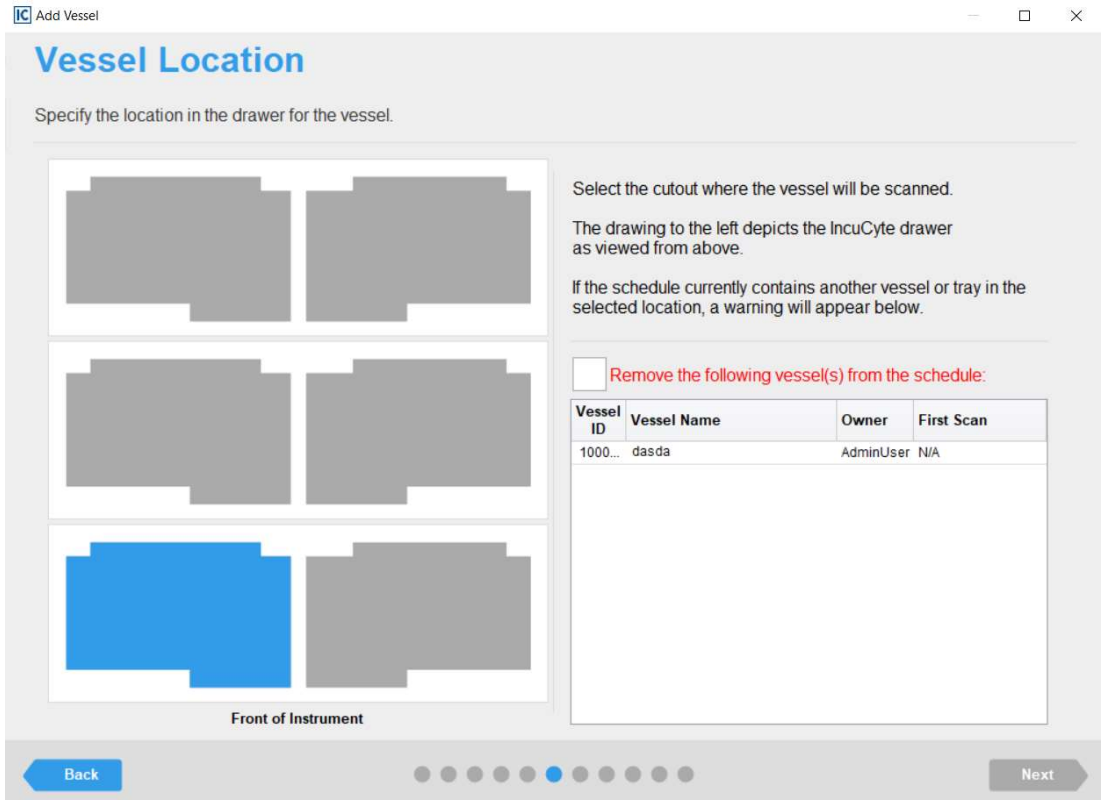


Manufacturer	Category	Wells	Area	Catalog Numbers	Vessel Name	Tray Name
Aurora	Plate	384	N/A	ABE2-10100A, ABE2-11100A, ABE2-11101A	384-well Aurora IQ-EB Ultra Low Base	Microplates
Cellvis	Plate	12	N/A	P12-1.5H-N	12-well Cellvis	Microplates
Cellvis	Plate	96	N/A	P96-1.5H-N	96-well Cellvis	Microplates
Corning	Plate	6	N/A	3335, 3471, 3506, 3516	6-well Corning	Microplates
Corning	Plate	6	N/A	353046, 353224, 353846, 353934, 354400, 354402, 354404, 354413, 354417, 354428, 354431, 354432, 354510, 354515, 354595, 354603, 354652, 354658, 356400, 356413, 356515, 356652	6-well Corning Falcon	Microplates
Corning	Plate	12	N/A	3336, 3512, 3513	12-well Corning	Microplates
Corning	Plate	12	N/A	353043, 353225, 354470, 354500, 354501, 354502, 354503, 356470	12-well Corning Falcon	Microplates
Corning	Plate	24	N/A	3337, 3473, 3524, 3526, 3527	24-well Corning	Microplates
Corning	Plate	24	N/A	353047, 353226, 353847, 353935, 354408, 354411, 354412, 354414, 354433, 354605, 354619, 354635, 354659, 356408, 356414	24-well Corning Falcon	Microplates
Corning	Plate	48	N/A	3338, 3548	48-well Corning	Microplates
Corning	Plate	48	N/A	353078, 353230, 354505, 354506, 354507, 354508, 354509, 356505, 356509	48-well Corning Falcon	Microplates
Corning	Plate	96	N/A	3300, 3474, 3585, 3595, 3596, 3598, 3599, 3628, 3997	96-well Corning	Microplates
Corning	Plate	96	N/A	3340, 3603, 3610, 3841, 3842, 3843, 3903, 3904	96-well Corning (Blk/Wht)	Microplates
Corning	Plate	96	N/A	7007	96-well Corning Round Bottom ULA	Microplates
Corning	Plate	96	N/A	353219, 354640, 354649, 354650, 354651, 356640, 356649, 356650, 356651, 356692, 356693, 356700	96-well Corning Falcon Blk/Clr Imagi...	Microplates

Vessel Selection

- Choose from a wide range of different tissue culture vessels
- Use the search tool to find your vessel of choice
- Some applications require specific vessels

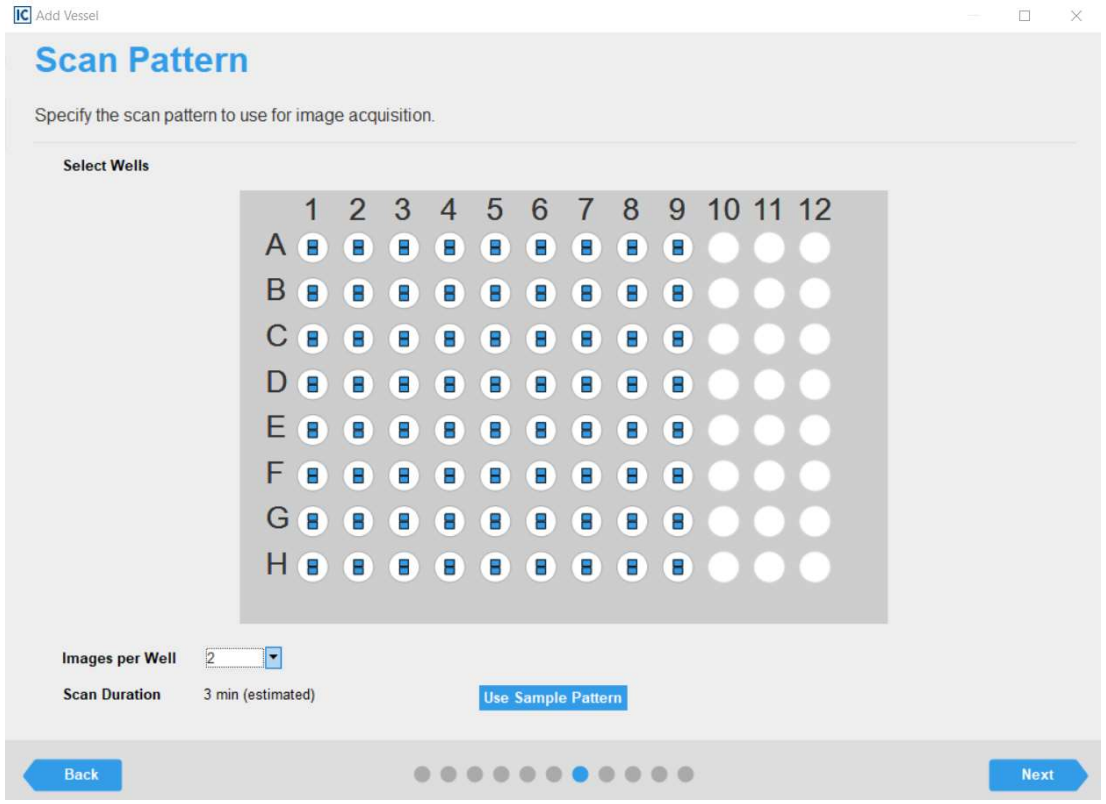
Scheduling Scans: Choose the Location



Vessel Location

- Physically load a vessel into the instrument, then schedule
- A warning will appear if trying to add a vessel to an occupied location

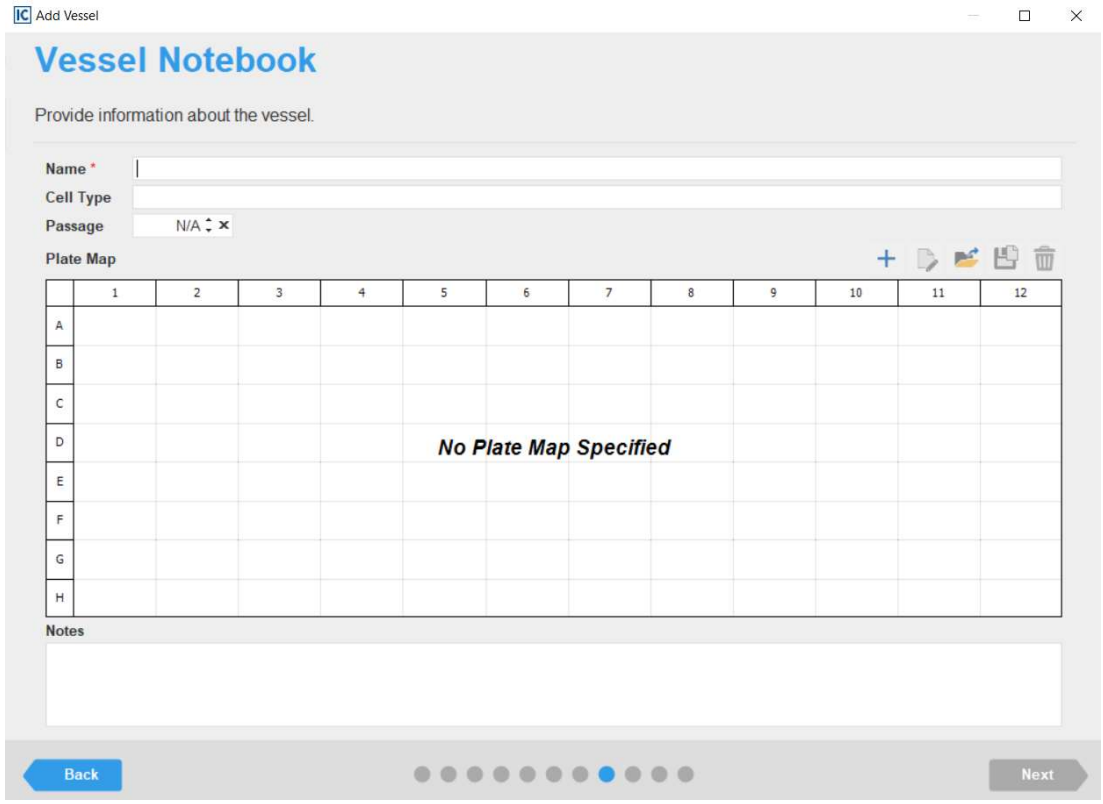
Scheduling Scans: Where to Scan



Scan Pattern

- Select wells containing cells, do not scan empty wells
- In a 96 well plate with the 10x objective, capture 2 images per well for adherent / homogeneous cultures
- Capture 4 images per well for non-adherent, non-homogeneous cells or with Cell-by-Cell imaging

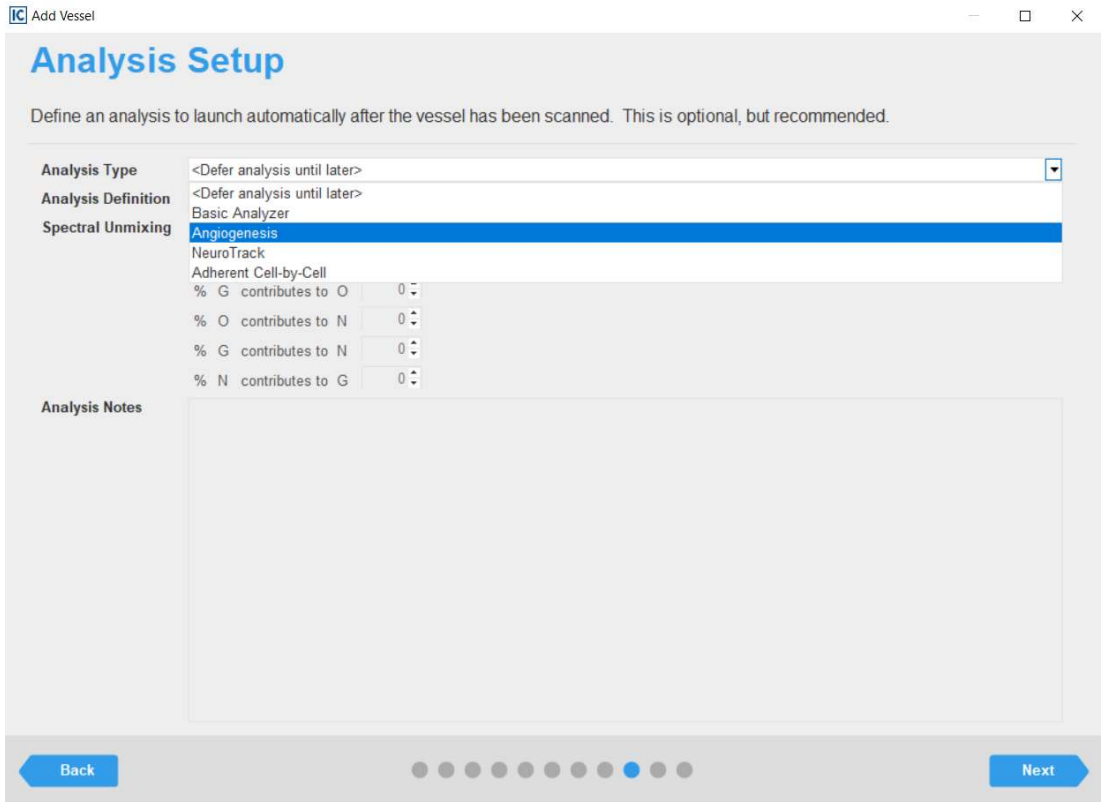
Scheduling Scans: Platemap & Notebook



Vessel Notebook

- Label every experiment clearly (make the name searchable)
- Add a Plate Map to record experimental design and automatically group replicates

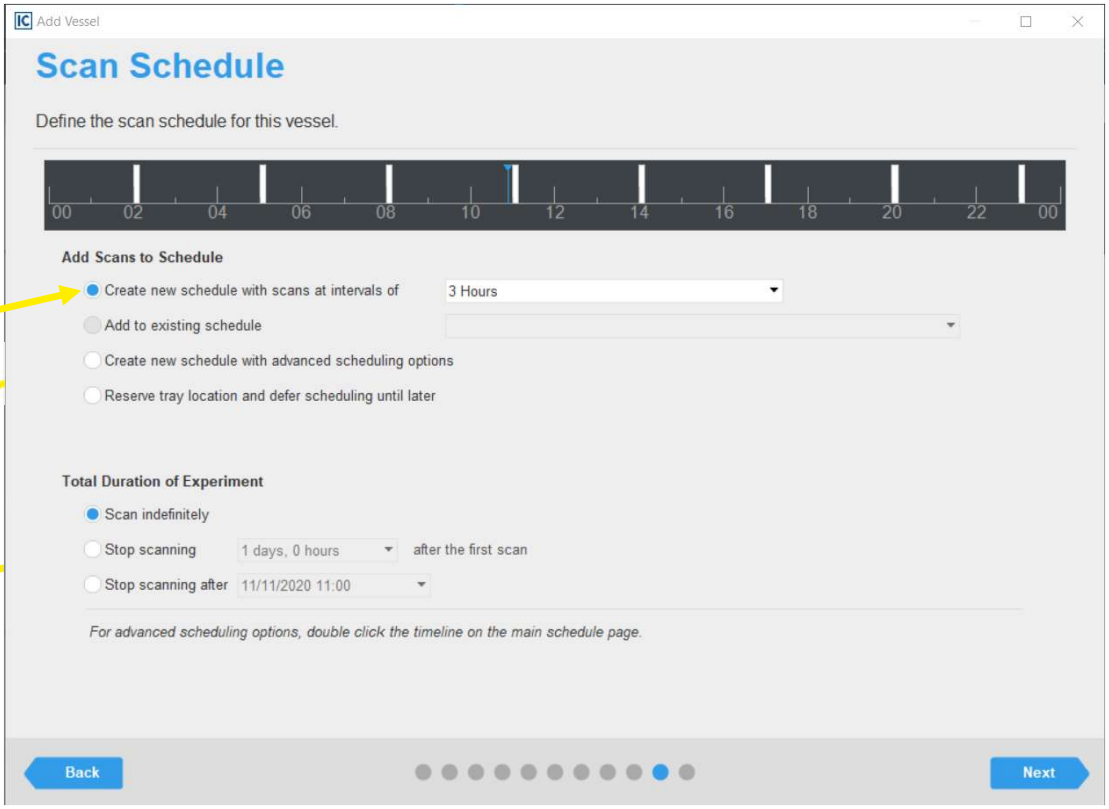
Scheduling Scans: Analysis with Acquisition



Analysis Setup

- Add analysis in parallel with image acquisition
- Only when an Analysis Definition as already been established
- Fluorescent images will need to be spectrally unmixed with previously determined values

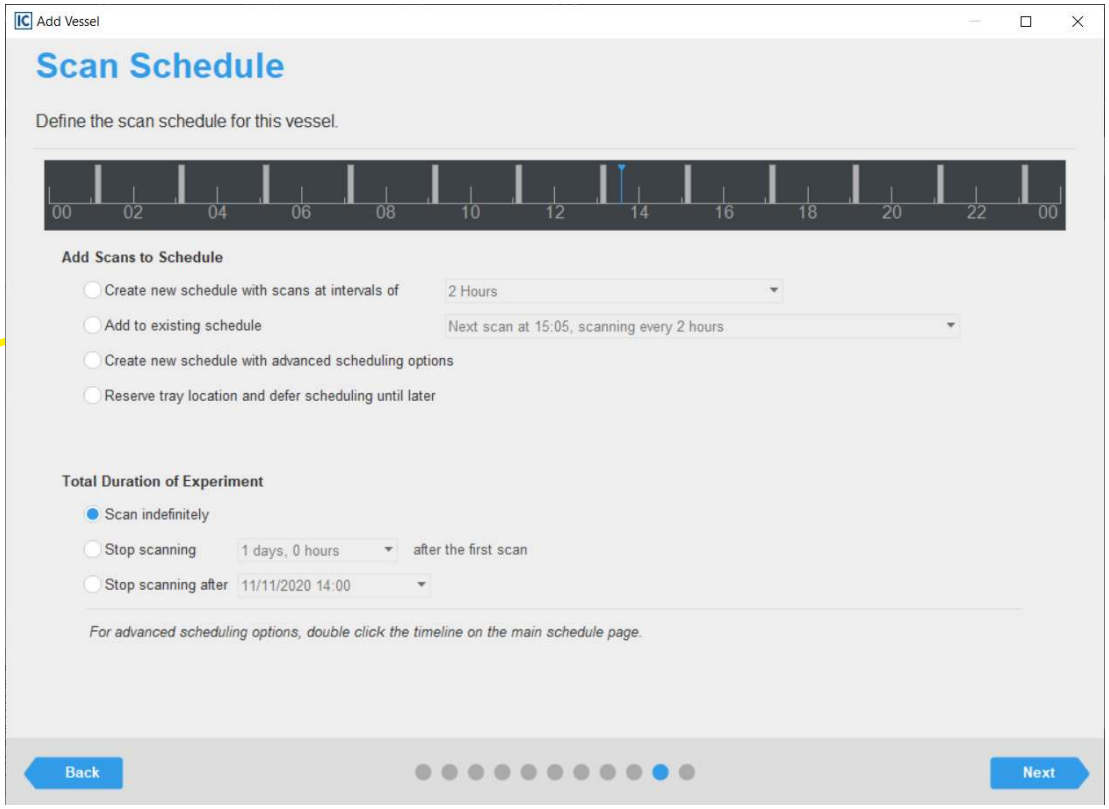
Scheduling Scans: Set the Scan Frequency



Schedule Scans

- Create new schedule, use advanced scheduling options or defer scanning
- Choose indefinite scanning or select an end time
- Fine adjustments can be made by click and drag

Scheduling Scans: Set the Scan Frequency – Second Vessel



Schedule Scans

- Create an independent second schedule if desired
- Add to an existing schedule to scan vessels sequentially at the same frequency
- Fine adjustments can be made by click and drag

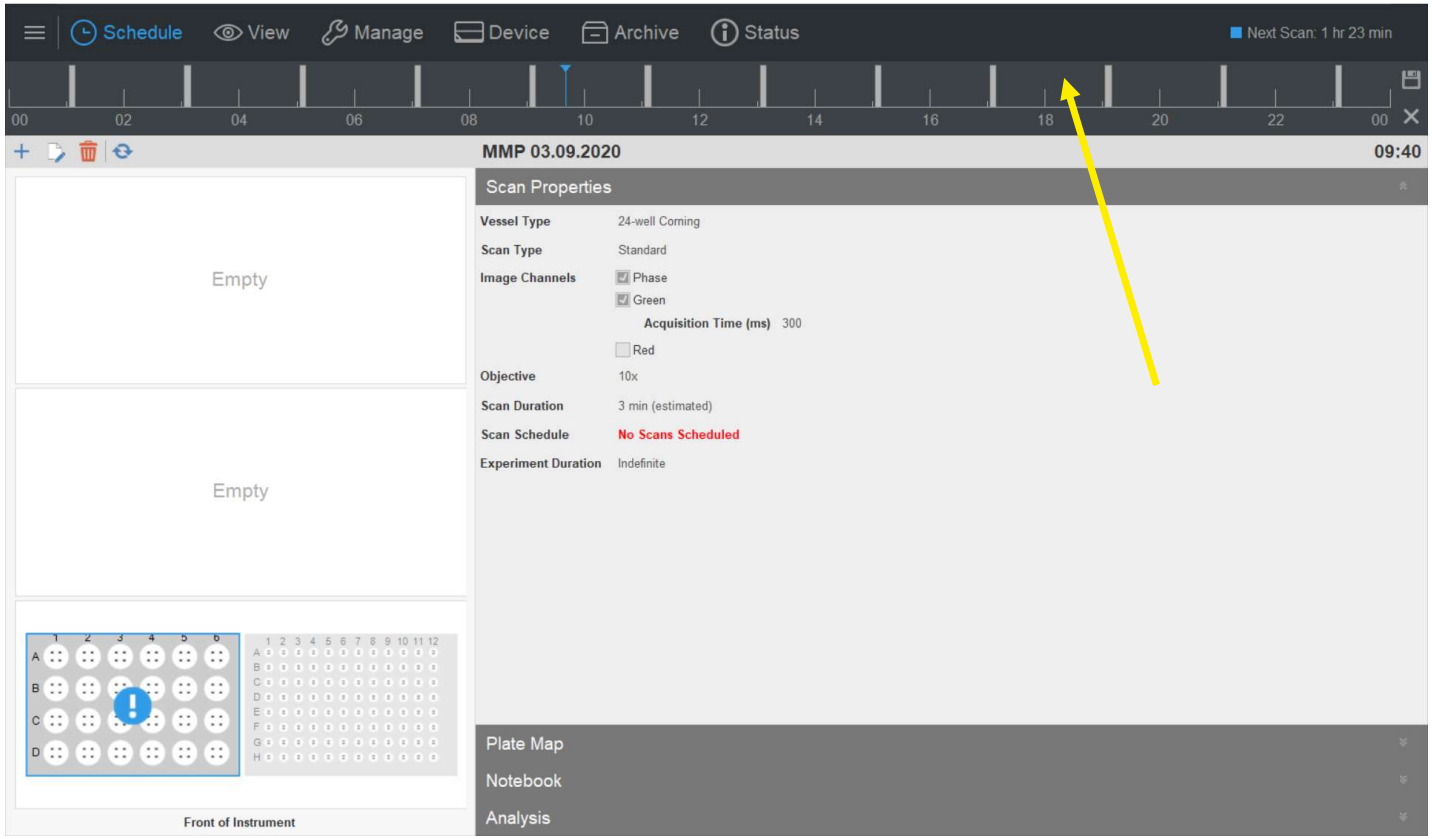
Scheduling Scans: Sampling Interval & Schedule

Sampling Interval : Do not over-sample (how fast is the process you want to quantify?)

Suggested Frequencies:

- 15-30 minutes interval for Phagocytosis or Immune Cell Killing
- 0.5-2 hours for Scratch Wound and Chemotaxis
- 2-4 hours for Proliferation and Cell Health
- 4-6 hours for Colony Formation and Spheroids

Scheduling Scans: Advanced Scheduling



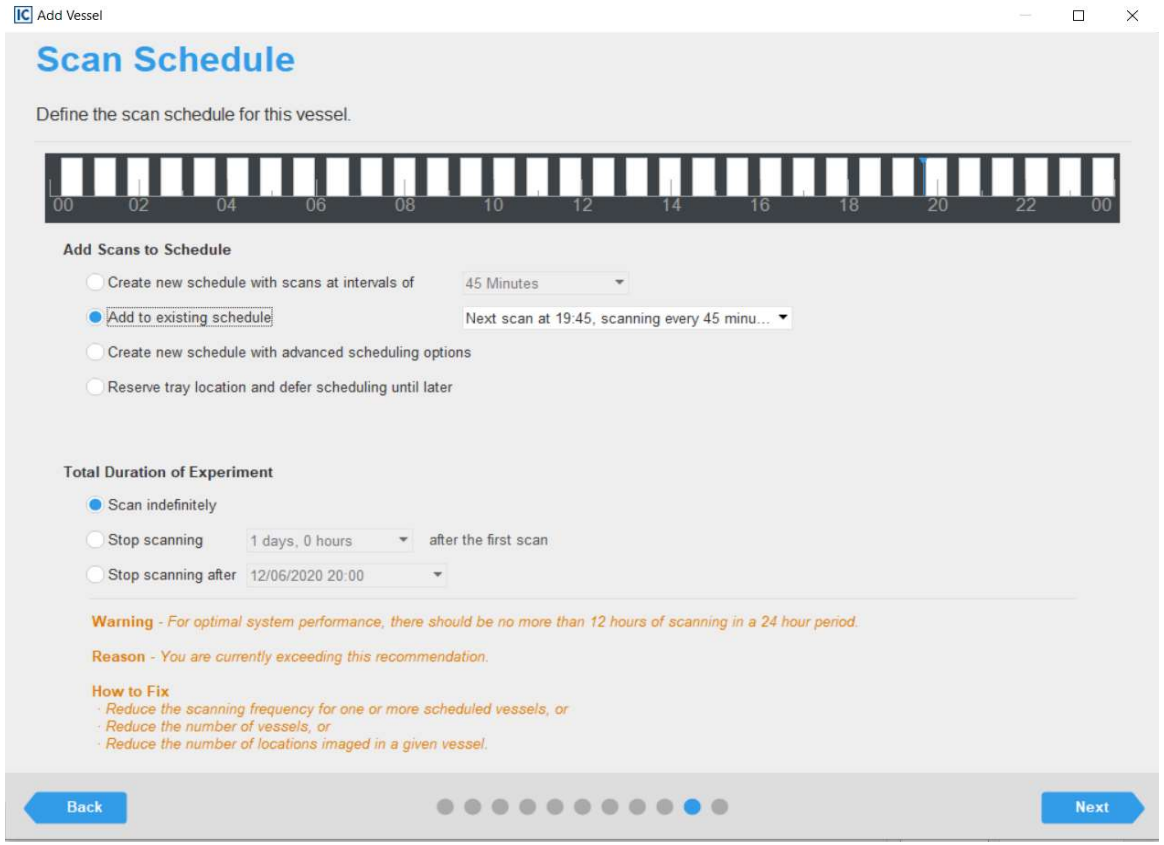
Advanced Scheduling

- Double clicking the timeline opens up advanced scheduling
- Allows manipulation of schedules

Acquire

Scheduling Scans: 2 Heat Threshold Warnings

Maintain Cell Health with Optimal Temperature Conditions

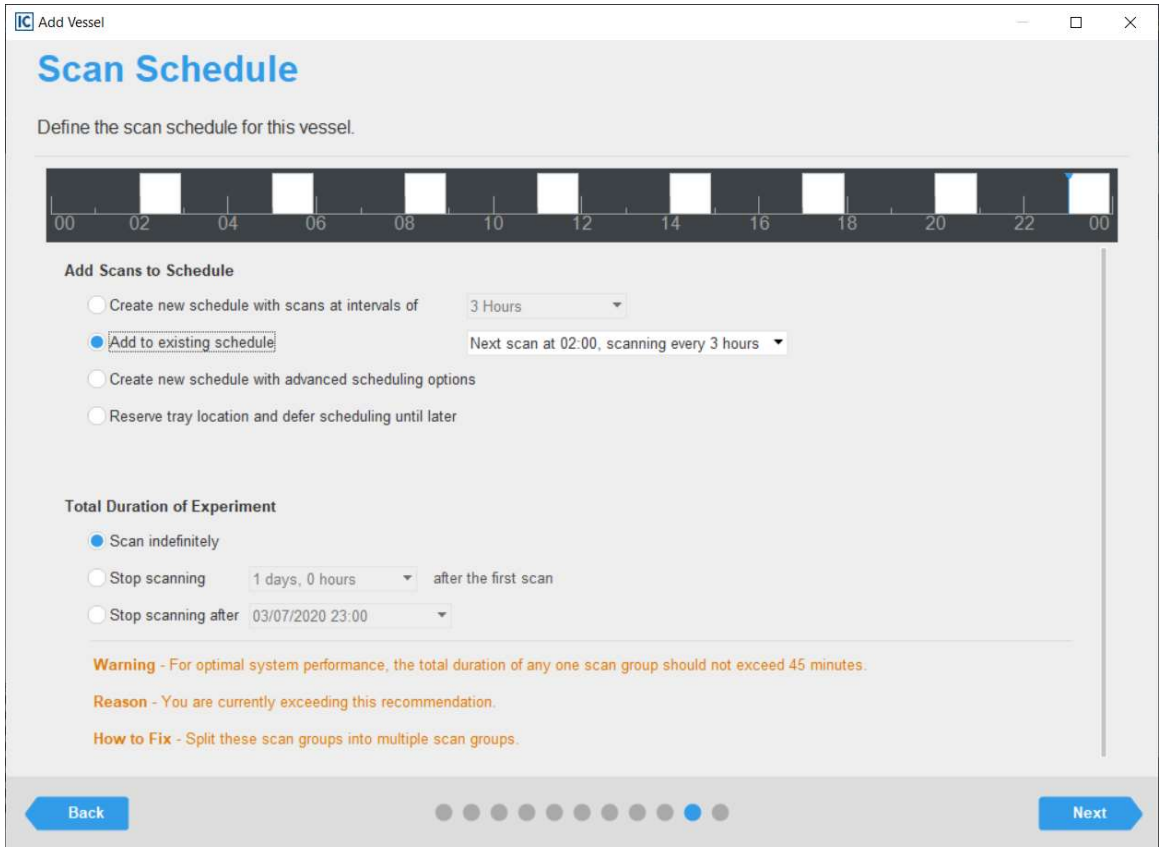


- For every 1 minute of scanning, there should be at least 1 minute of non-scanning
- 12 hours of scanning per day
- Reduce scan frequency if scanning in excess of guidelines

Recommendations in Orange : **Do NOT Ignore**

Scheduling Scans: 2 Heat Threshold Warnings

Maintain Cell Health with Optimal Temperature Conditions

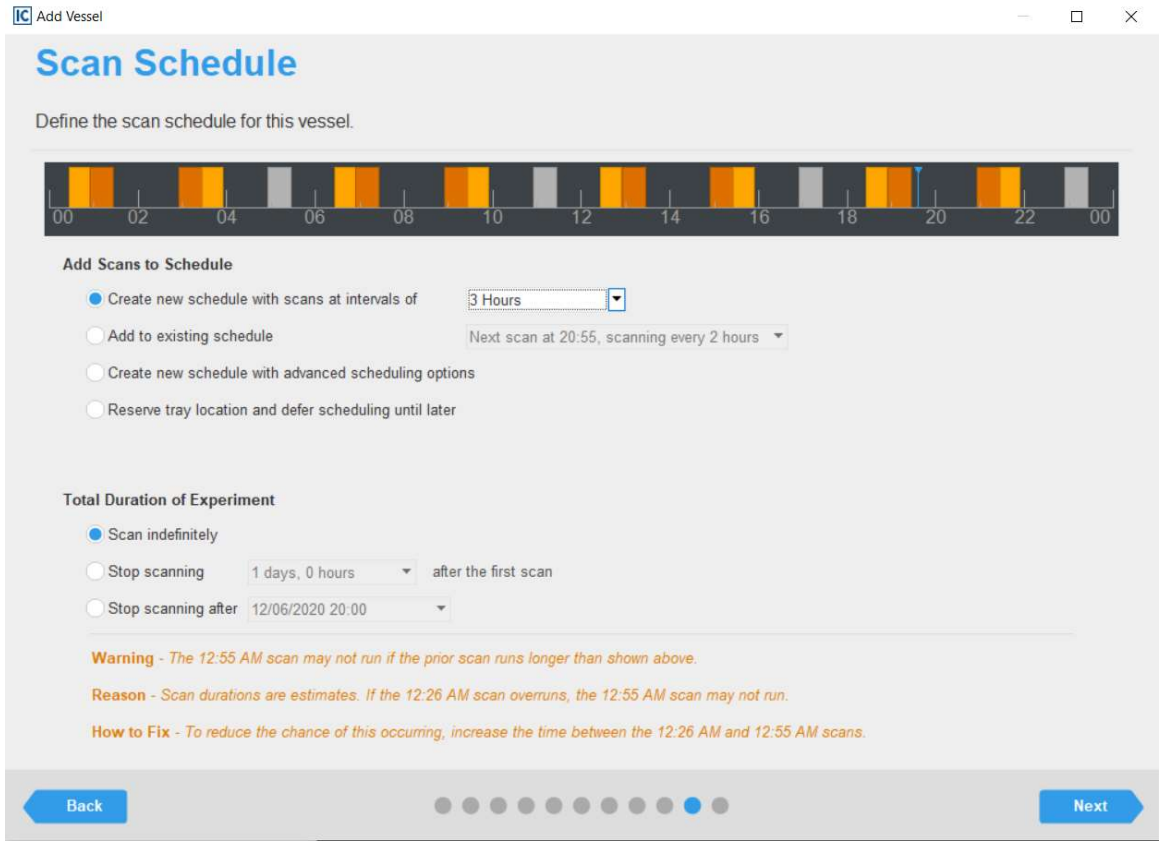


- Scan groups should not exceed 45 minutes
- Split long scan groups into two groups

Recommendations in Orange : **Do NOT Ignore**

Scheduling Scans: 2 Scan Overlap/Duration Warnings

Avoid Conflicts in Scheduling

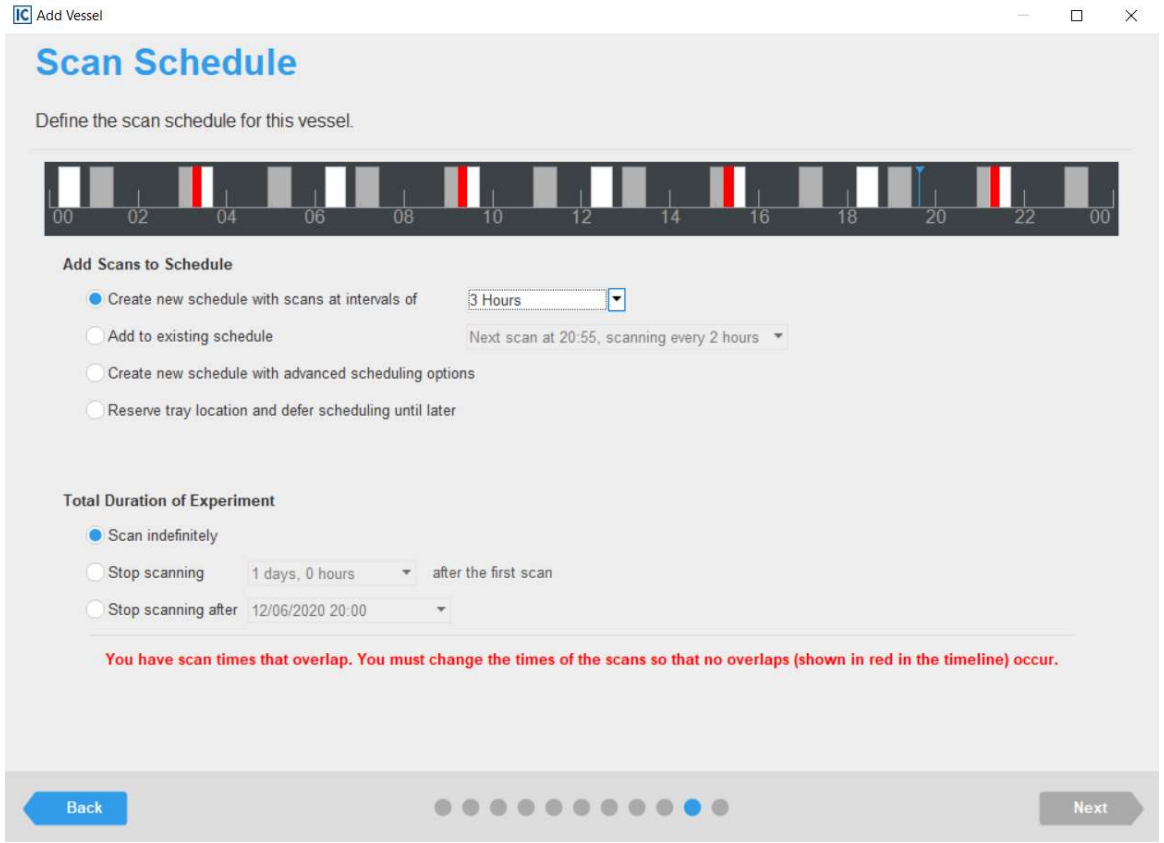


- Scan durations are estimates, if a vessel overruns, the subsequent group may be skipped
- Placing scan groups too close together will lead to the Incucyte showing the scans as orange bars
- Drag orange bars to add more time in between scans

Recommendations in Orange : **Do NOT Ignore**

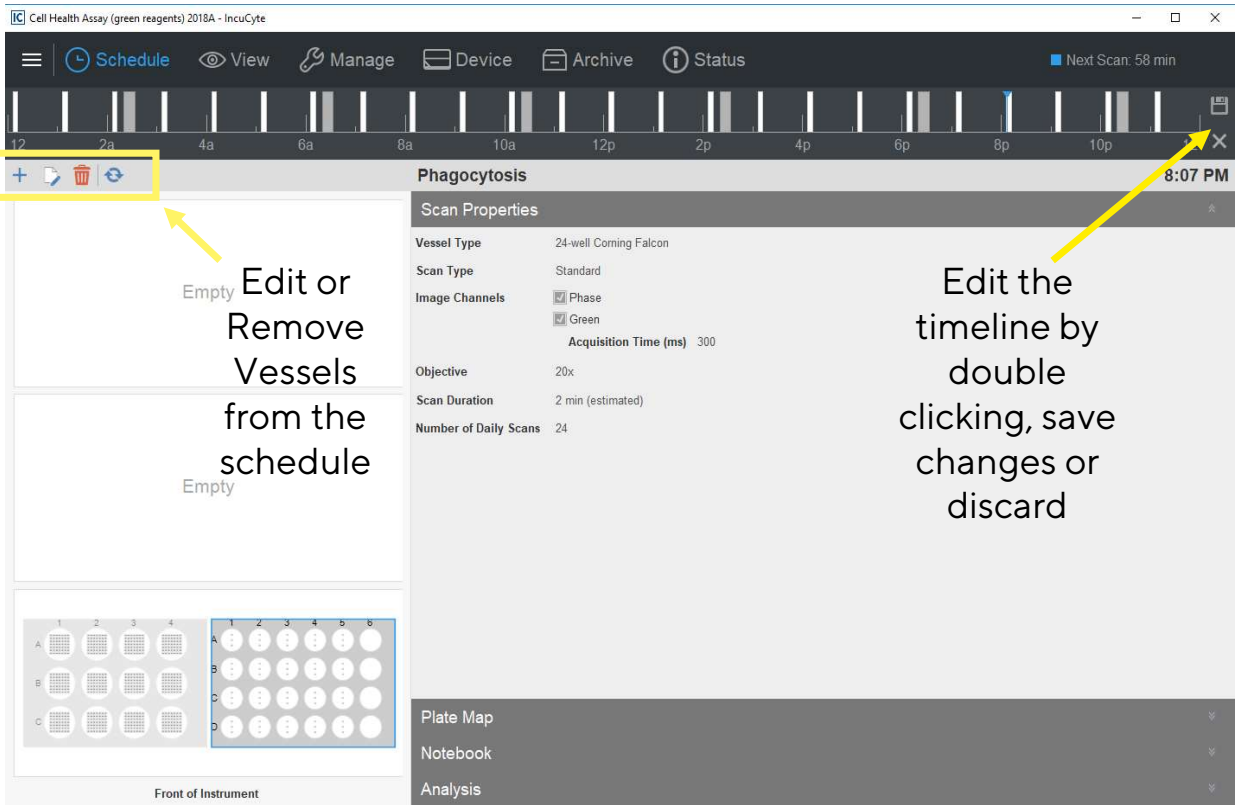
Scheduling Scans: 2 Scan Overlap/Duration Warnings

Avoid Conflicts in Scheduling



- Scans cannot be scheduled to occur at the same time
- The Incucyte will show overlapping scans as red bars
- Drag red bars to add more time in between scans
- Will not let you ignore

Scheduling Scans: Drawer Layout Summary



- Vessel selected in ‘blue’
- View Scan Properties/Notebook/Analysis
- Schedule is white for this vessel, other vessels are greyed out
- Add, Remove and Edit vessels using the icons above the tray layout

Agenda

Introduction and Applications

Incucyte® Hardware and Best Practices

Software Overview

Acquire

Visualize

Process

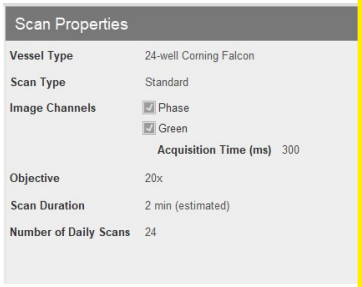
Data Export

Conclusion

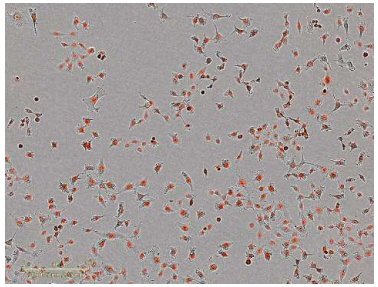
View Images

4 Steps to Image Analysis

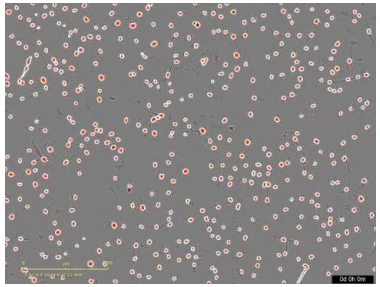
Acquire Images



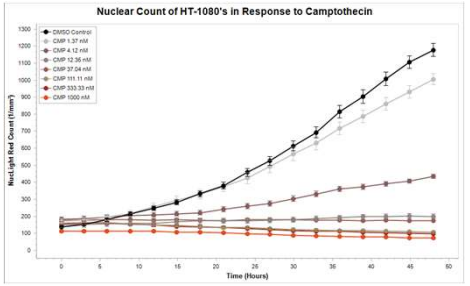
View Images



Process Images

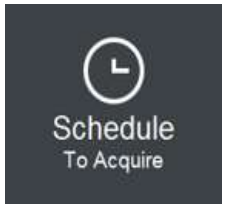


Export Data



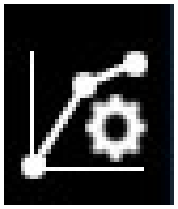
Incucyte® : A Guided Interface

Schedule Scans

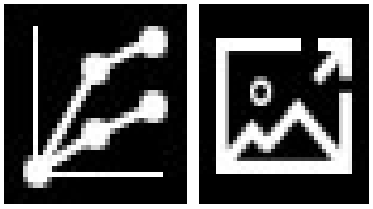


Vessel View

Analysis Definition

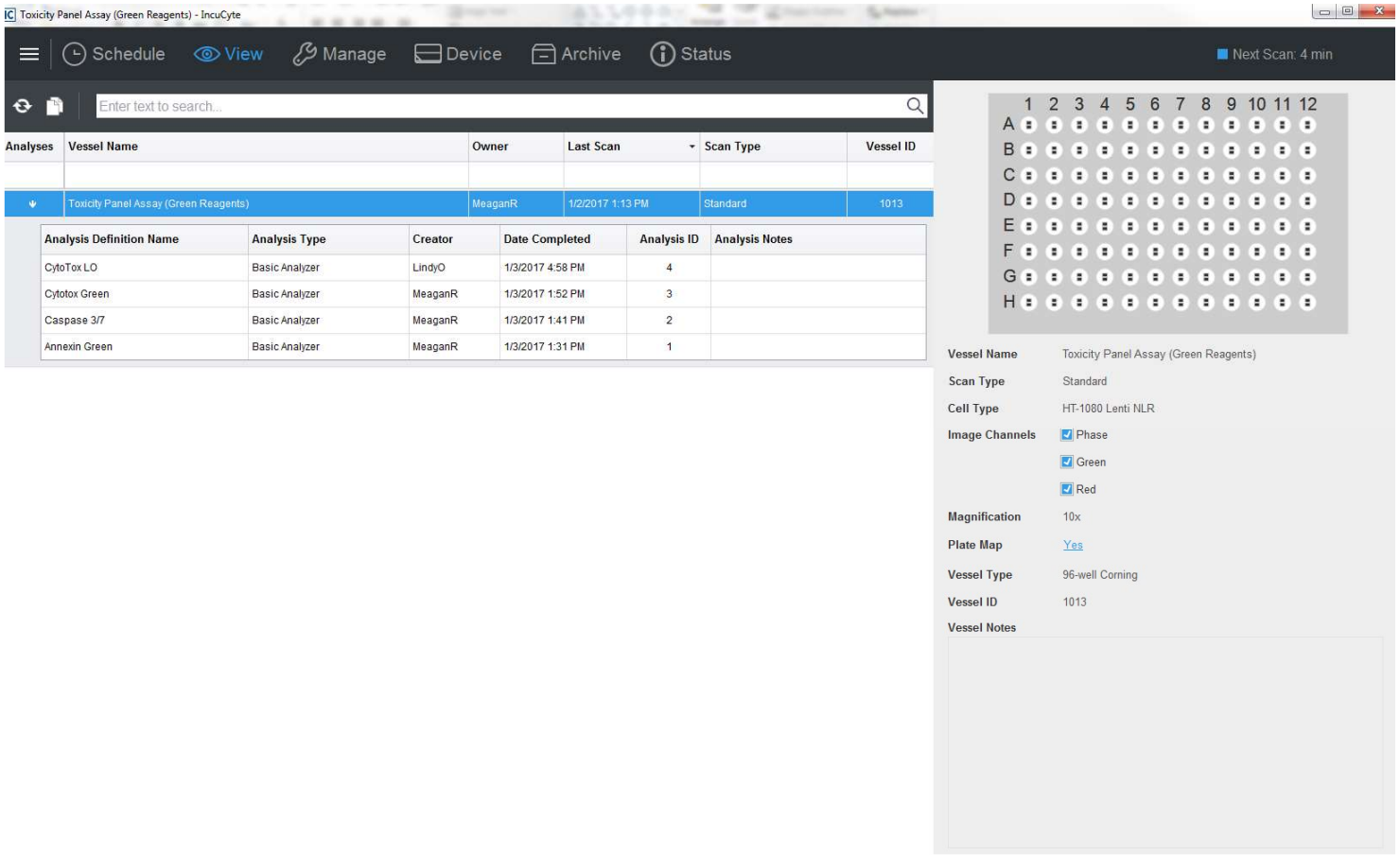


Graphing and Exporting



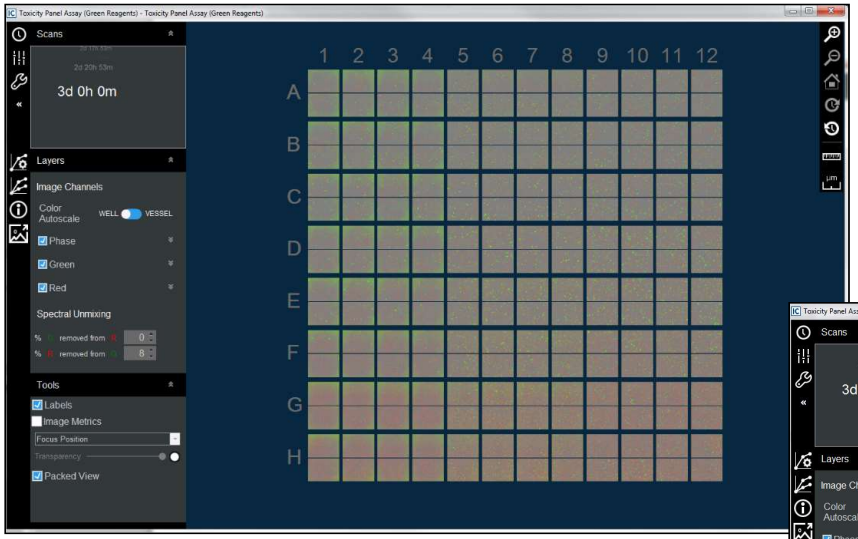
Visualize

Search for Experiments by Label/user/etc.



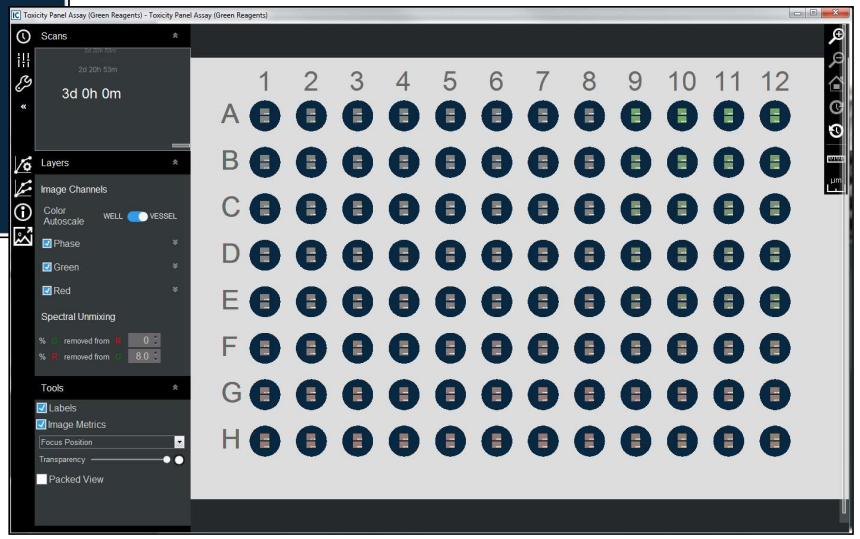
Vessel (Image) Display

Packed View



Quickly assess trends in data in default ‘Packed View’

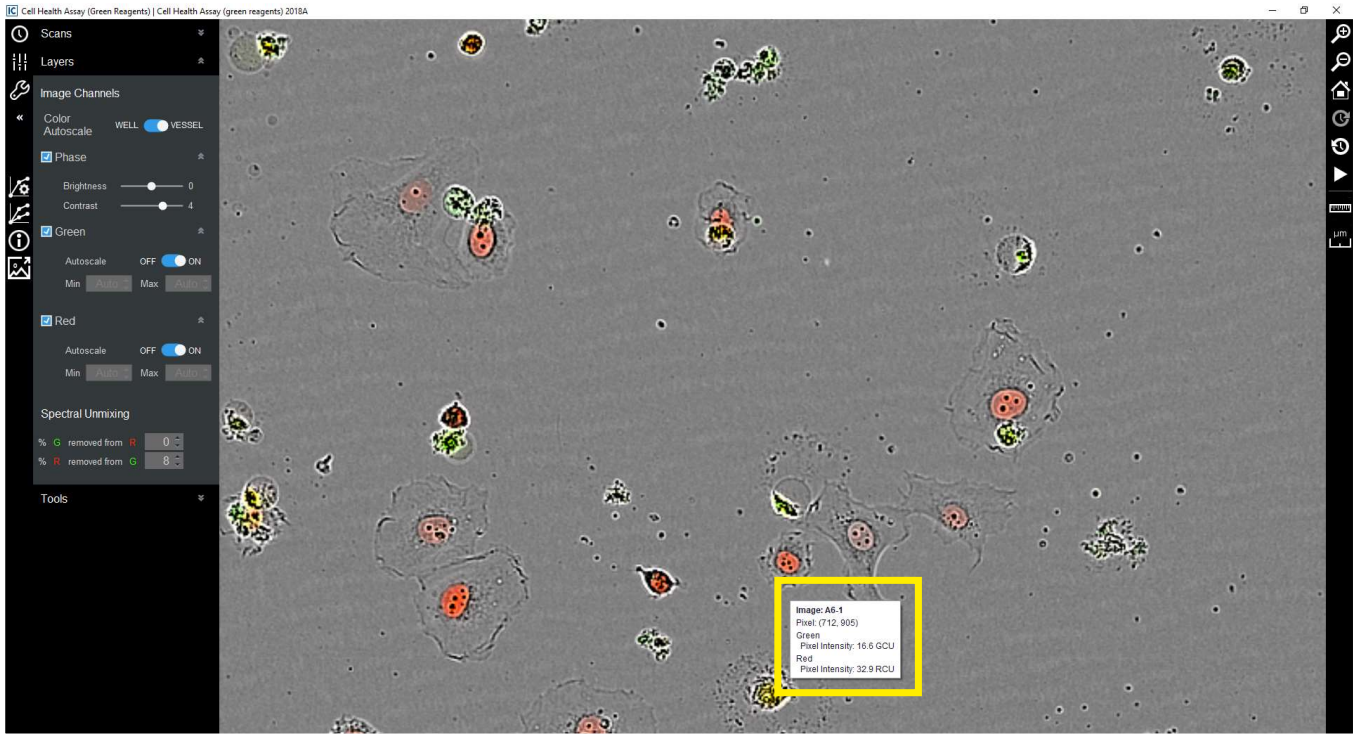
Unpacked View



Visualize location of images in the well

Visualize

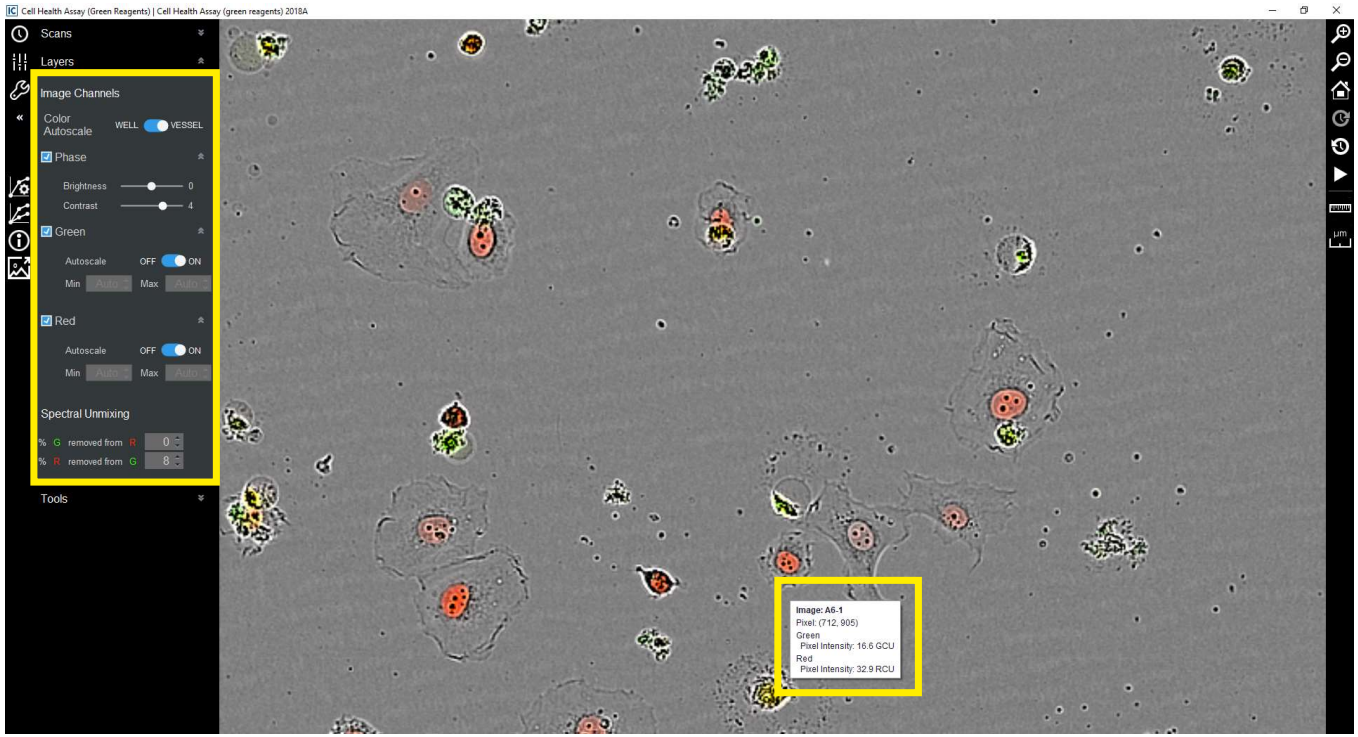
View and Interact with Images



Calibrated Images

- Compare images over time and between instruments
- Images made up of pixels with values used to define analysis
- Fluorescent pixel intensities are reported in calibrated units, GCU (Green), OCU (Orange) etc. Hover over any pixel to find these values.

View and Interact with Images



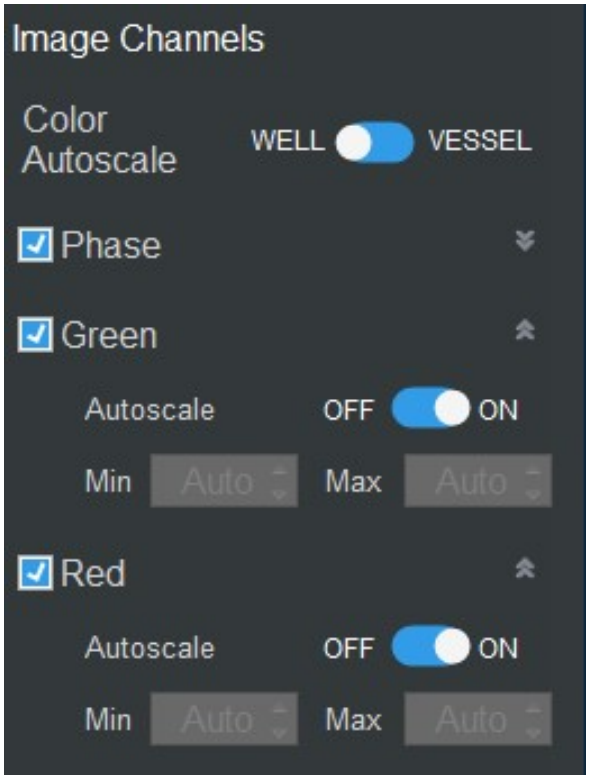
Visualize images

- Adjust Brightness/Contrast for phase-contrast
- Adjust min/max intensity settings for fluorescence
- Adjusting these values will not change the pixel values of the image

Autoscale

What is it?

- Sets a range of min/max intensities to account for changes in biological samples across different samples and time
- A ‘halo’ or ‘cross’ artifact might appear when imaging samples with dim signal

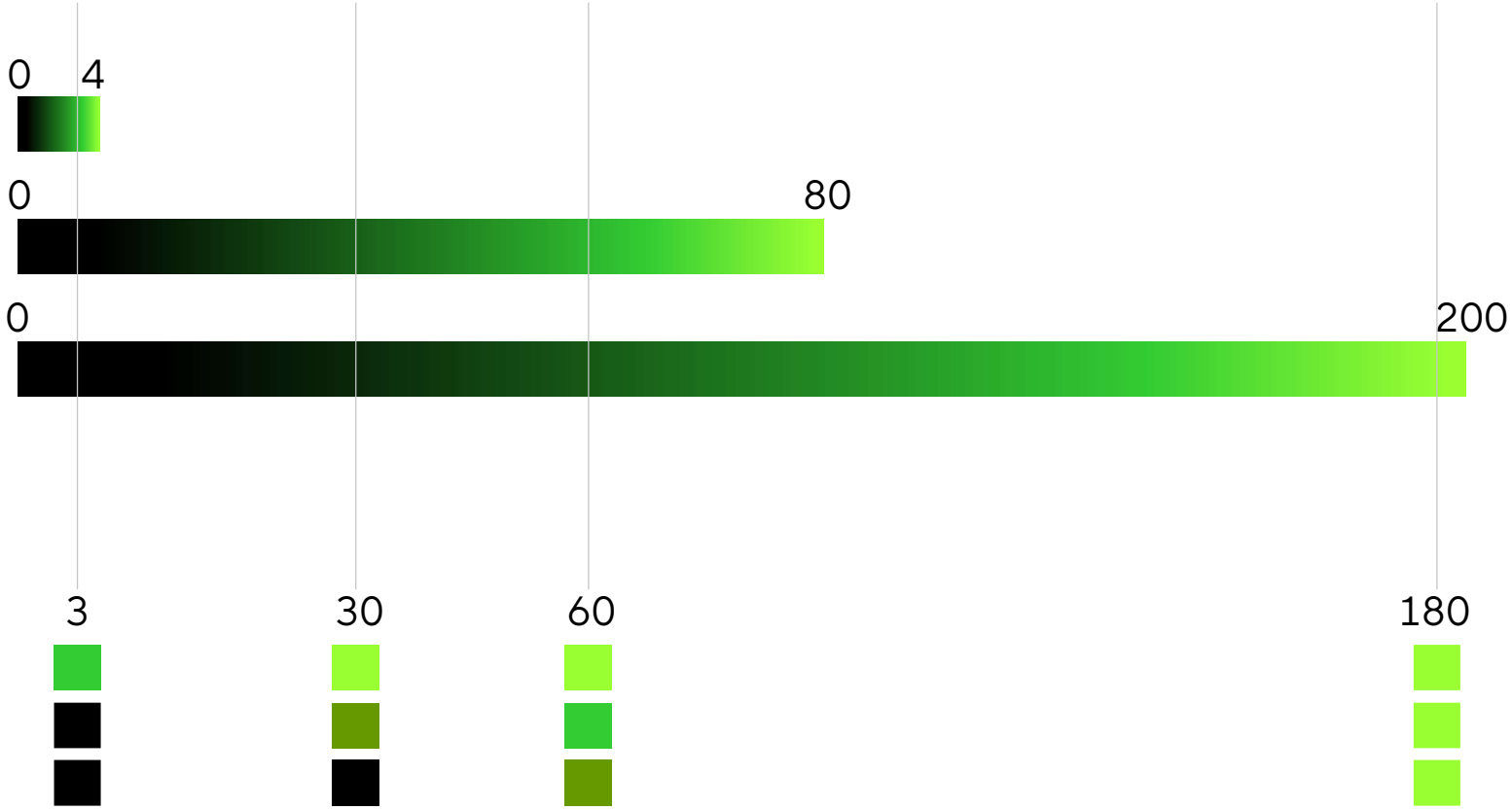


Minimum Green intensity in the Well/Vessel = Black

Maximum Green intensity in the Well/Vessel

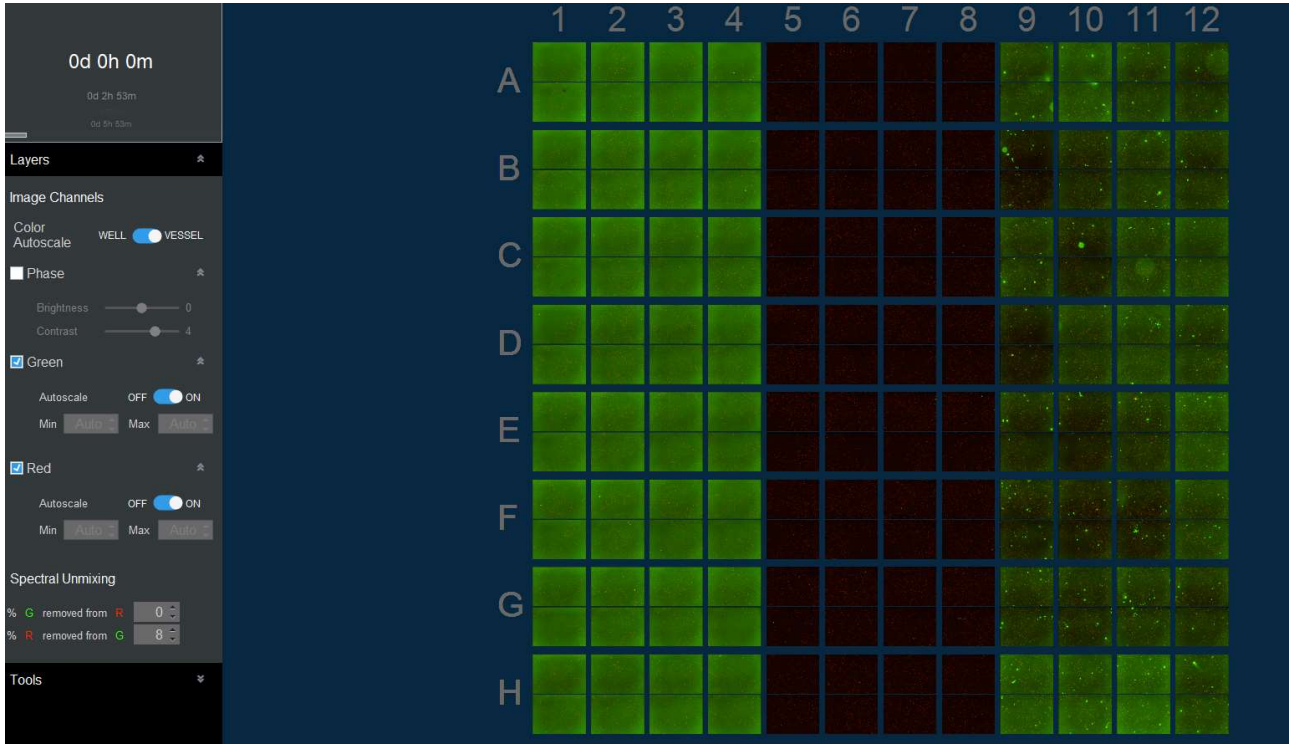
Visualize

Autoscale



Vessel – Autoscale

Applies an autoscale across entire vessel based on min/max brightness across entire plate



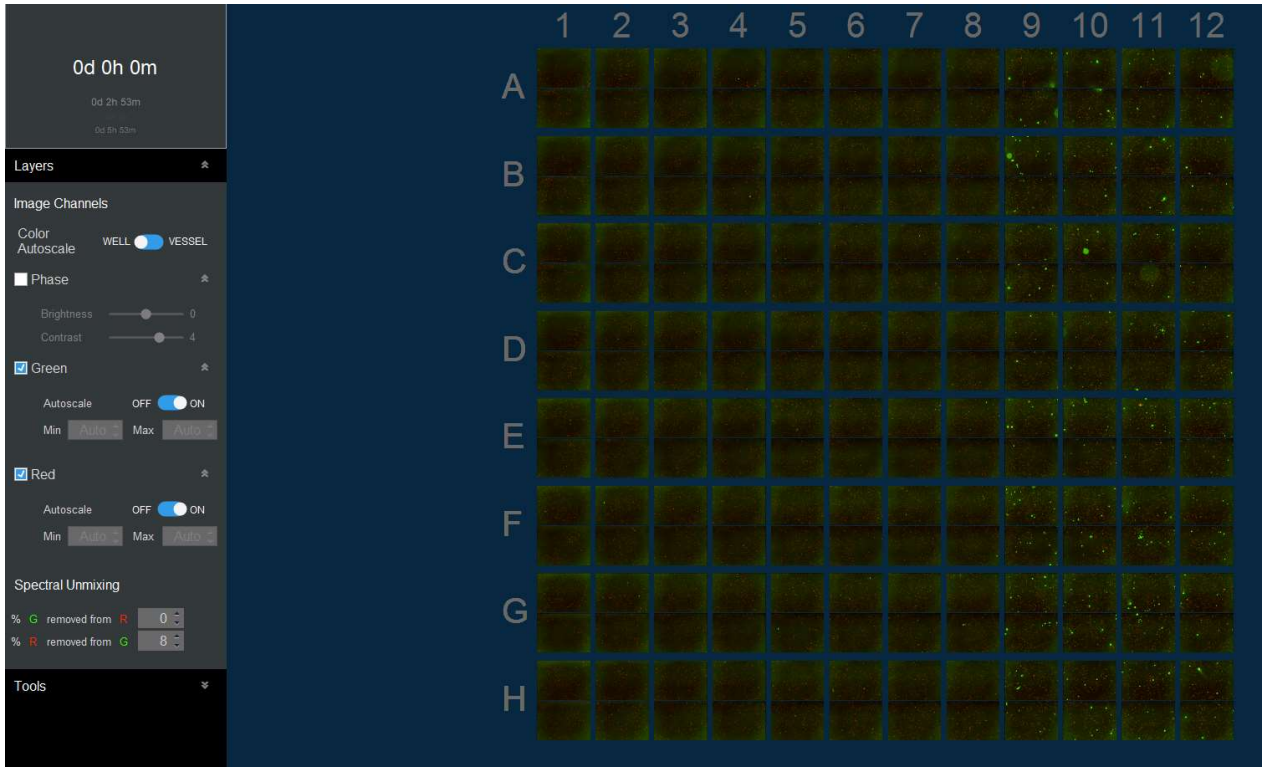
Ideal for experiments with the same reagent.

In this experiment, 3 sections of the plate were treated with different reagents of different fluorescence intensity; therefore, vessel autoscale is not recommended

Visualize

Well – Autoscale

Applies an autoscale for each individual well based on min/max brightness in each individual well



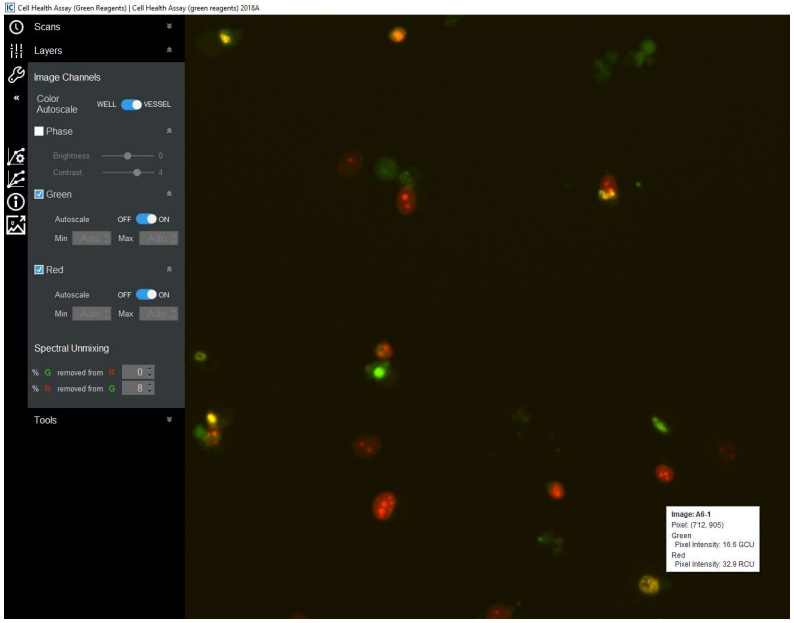
Ideal for experiments with different reagents and to determine the baseline intensities of individual wells.

In this example, well autoscale is recommended

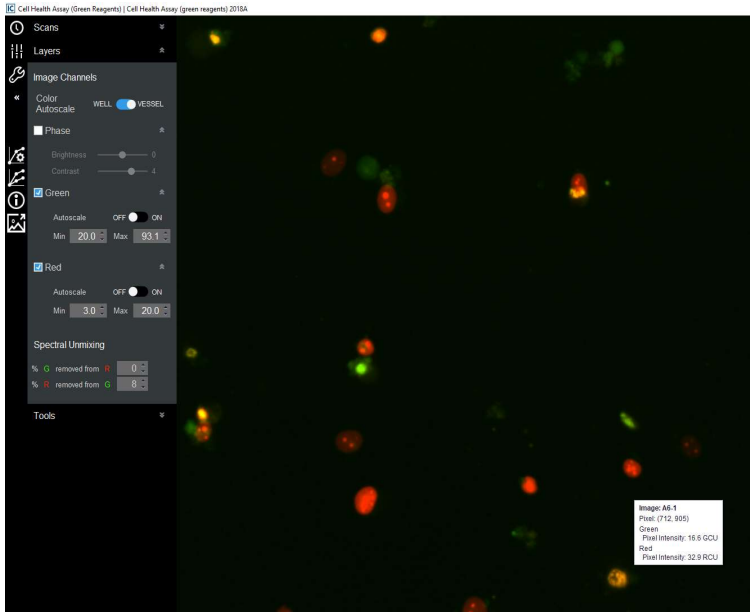
Turn off Autoscale (recommended for movie making)

Use the default autoscale settings to find a base line by turning off autoscale. Adjust min and max intensity values for optimal visualization (does not alter pixel intensity)

Auto-scale applied



Manually adjusted min/max settings

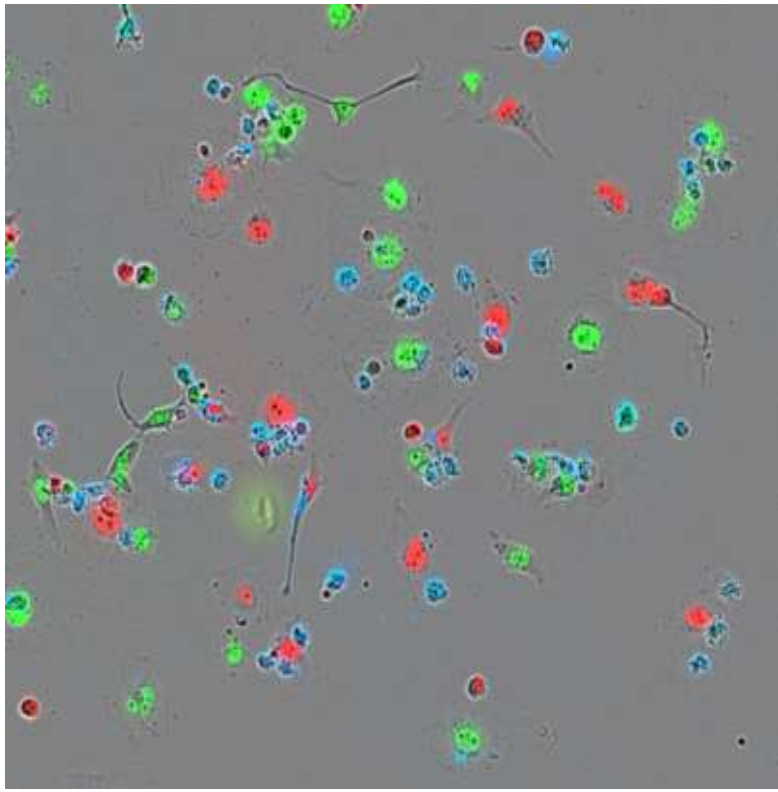


Example of same image (1 with auto-scale, 1 with manual adjustment). Pixel intensity remains the same despite changing the visualization range.

Analogy: Think of playing the same song at different volumes.

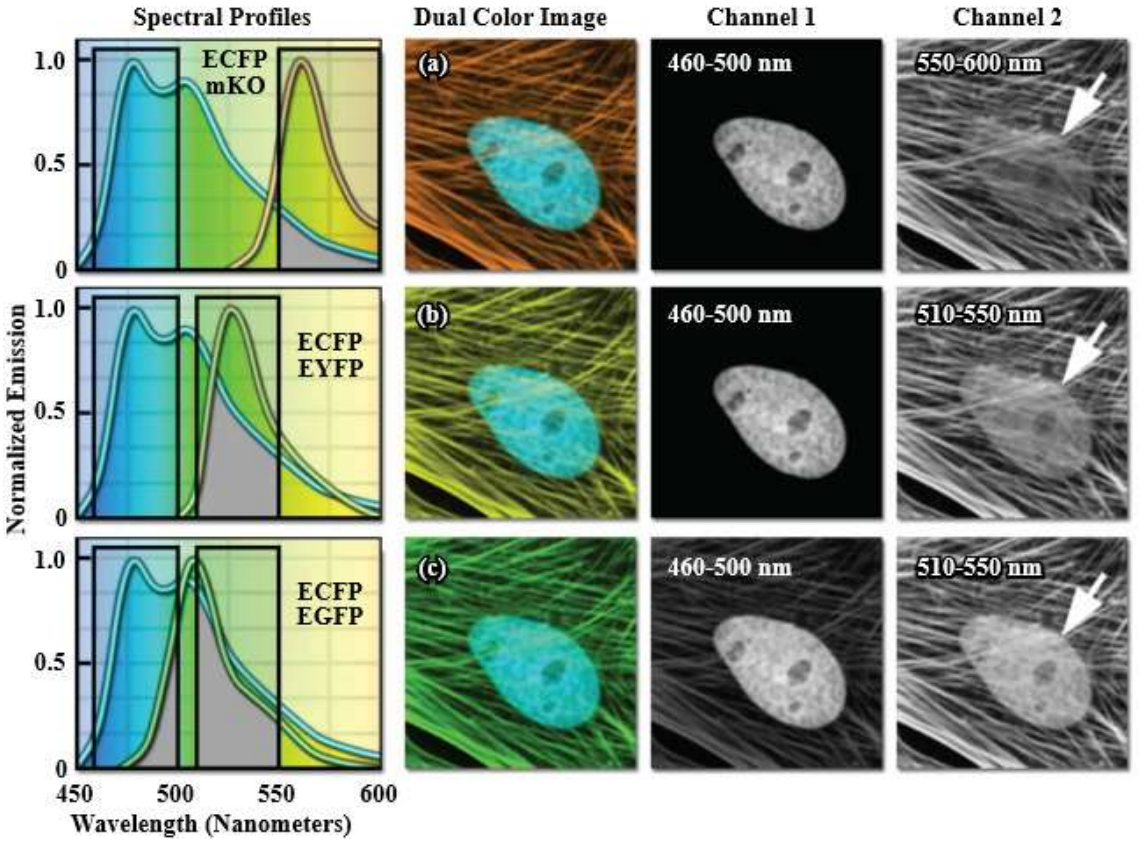
Why is the Near-IR Channel Image Blue?

- The NIR emission wavelength for the Incucyte® SX5 is a deep red which does not provide visual contrast with the emission wavelength of the orange channel
- These are spectrally separated optically but the perceived colors are very close visually
- We use a blue pseudocolor to represent NIR to enable easy differentiation between channels, particularly when displayed as a multichannel overlay image



Spectral Unmixing

- Most fluorophores have broad and uneven excitation and emission spectra
- Bleedthrough is the consequence of the excitation of one fluorophore by more than one excitation channel, leading to an emission readout in multiple channels
- We use Spectral Unmixing to separate and remove the contribution of undesirable emission signal between fluorophores, providing an accurate representation of the contribution of each fluorophore, imaged in the same sample

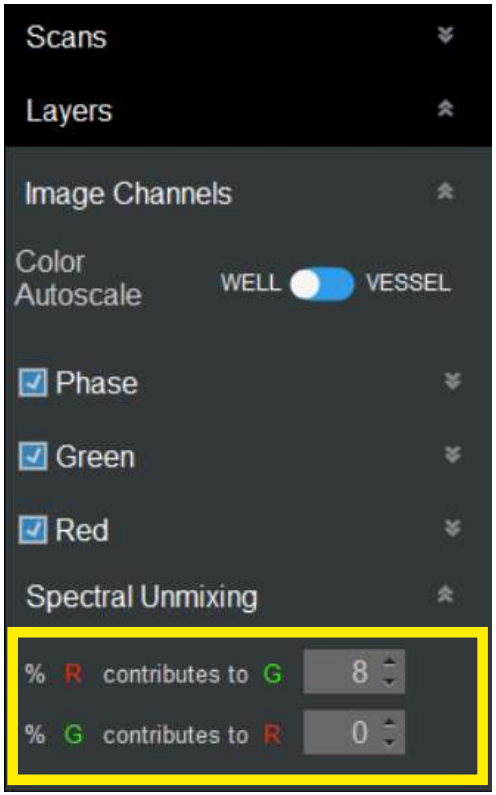


https://www.microscopyu.com/techniques/confocal/spectral-imaging-and-linear-unmixing

Spectral Unmixing

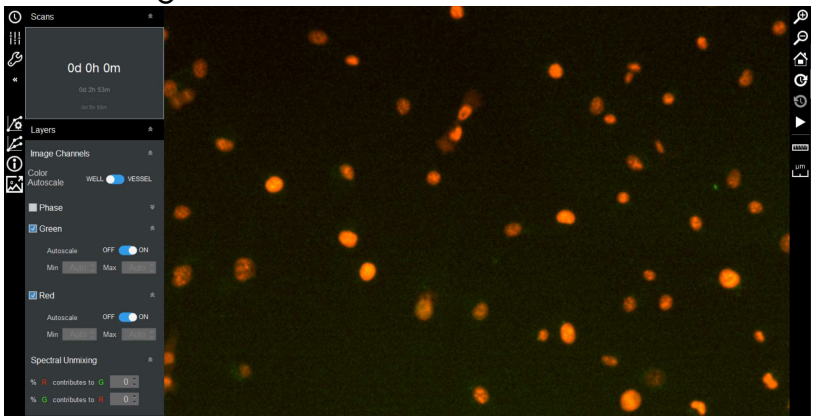
- Fluorescent Dye Optimization Experiment
  - To test the extent of spectral overlap of a fluorophore
    - Prepare wells with individual reagents only (Red or Green)
    - Image all wells with both Red and Green channels
    - Check for signal in both channels, if present in the opposite channel, Spectral Unmixing is required
    - For example, for a Red fluorophore with signal appearing in the Green channel
    - Increase the %R contributes to G until no signal is seen in the Green channel

Incucyte® Spectral Unmixing Tool

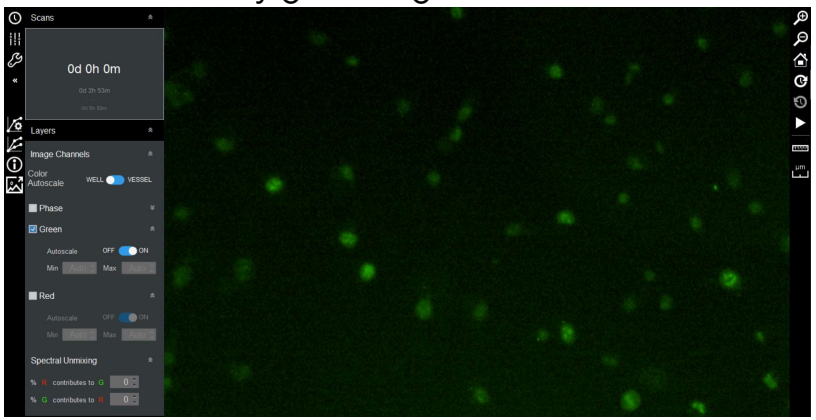


Spectral Unmixing Example

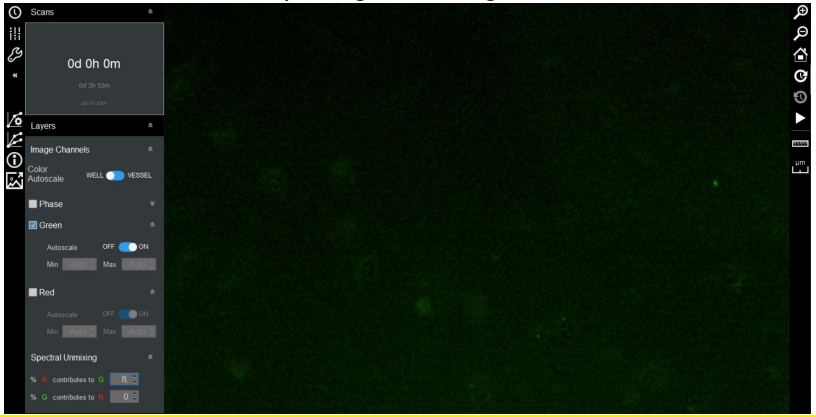
1. Red only fluorophore – imaged in both red and green channel



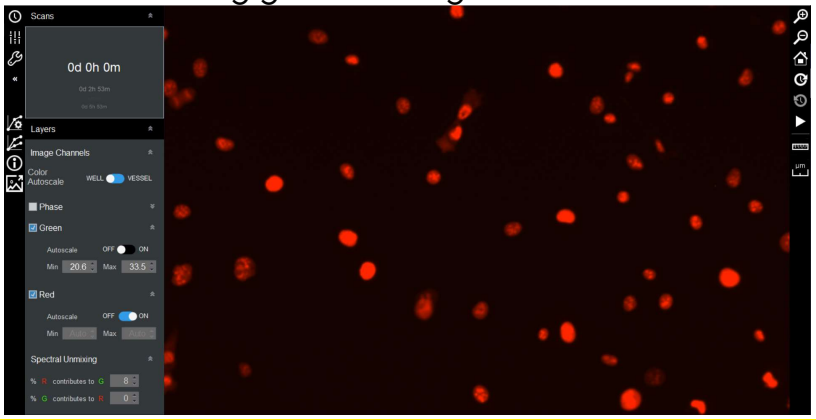
2. Turn off the Red channel and determine if there is any green signal



3. Increase the % R contributes to G until there is little/no green signal left



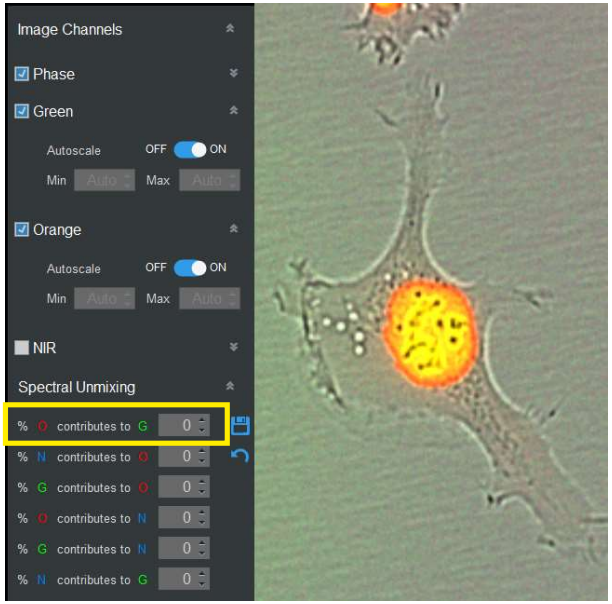
4. Adjust the min/max intensities to remove remaining green background



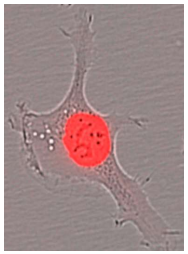
Nuclight Orange Spectral Unmixing with the Incucyte® SX5

No Spectral Unmixing

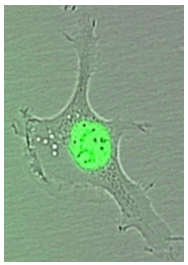
Green and Orange Channel



Orange channel



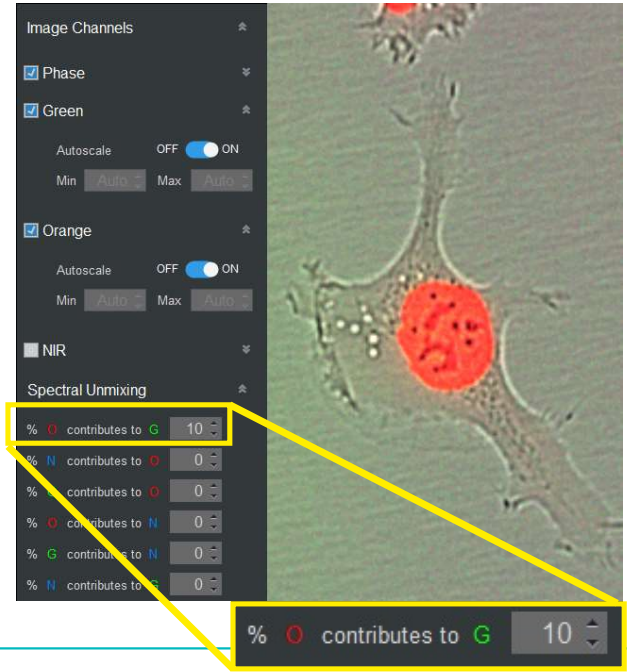
Green channel



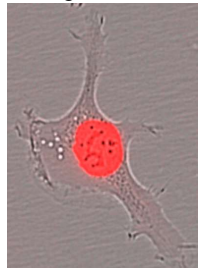
- Orange and Green overlap makes the color appear yellow
- There is no green fluorescent protein expressed in this cell
- Green signal is coming from the Orange bleeding into Green

Spectral Unmixing applied

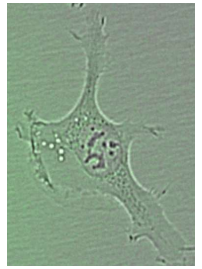
Green and Orange Channel



Orange channel



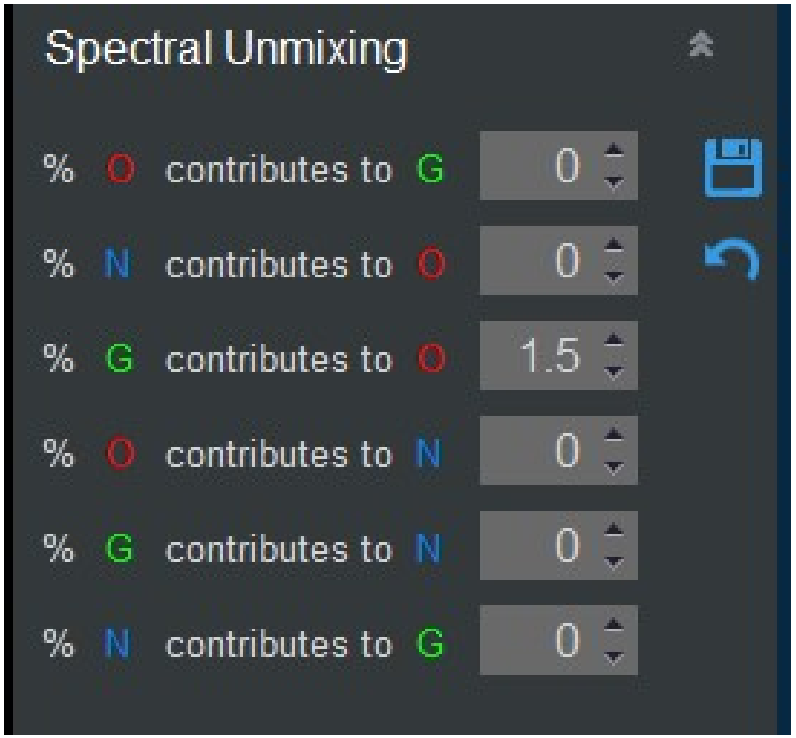
Green channel



- After Spectral Unmixing is applied, the bleed through Green signal is removed and the overlapping image appears as its true Orange color

Spectral Unmixing Using the Green/Orange/NIR Optical Module

- Using 3 fluorescent channels gives 6 potential Spectral Unmixing values to enter - the software lists combinations from most likely to least likely to occur
  - Bleed through from Orange reagents into the Green channel and NIR reagents into the Orange channel are most common
  - Green bleeding into Orange, or Orange bleeding into NIR is not as common but does happen
  - Example: Caspase 3/7 Green slightly bleeds into the Orange channel and requires a value of 1.5% G contributes to O as shown
  - Due to increased spectral separation, bleed through between NIR and Green channels is unusual, but still possible



Spectral Unmixing – Recommended Ranges for Incucyte® Reagents

Channel	Reagent	% O to G	% N to O	% G to O	% O to N	% G to N	% N to G
Green	Angiogenesis Prime Kit			-		-	
	Annexin V Green			-		-	
	Caspase 3/7 Green			1-2%		-	
	CytoLight Lenti			-		-	
	CytoLight Rapid Green			-		-	
	Cytotox Green			-		-	
	FabFluor IgG1, IgG2a, IgG2b			-		-	
	NucLight Green (BacMam?/lenti)			-		-	
	pHrodo e.coli, sa, zym Green			NR*		-	
Orange	Annexin V Orange	5-7%			-		
	NeuroBurst	-			-		
	NeuroLight	10-12%			-		
	NucLight Orange	10-12%			-		
	pHrodo cell labeling Orange	NR*			-		
	pHrodo e.coli, sa, zym Orange	NR*			-		
	MMP reagent	5-7%			-		
NIR	Annexin V NIR		1-2%				-
	NucLight NIR		1-2%				-
	NucLight Rapid NIR		1-2%				-
Green and Orange	FUCCI G/O	12-14%		-			

*Not Recommended for multiplexing

Agenda

Introduction and Applications

Incucyte® Hardware and Best Practices

Software Overview

Acquire

Visualize

Process

Data Export

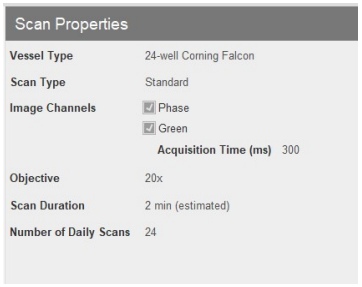
Conclusion

Process

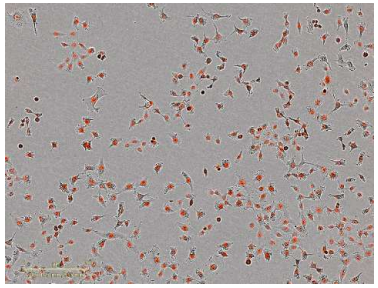
Create an Analysis Definition

4 Steps to Image Analysis

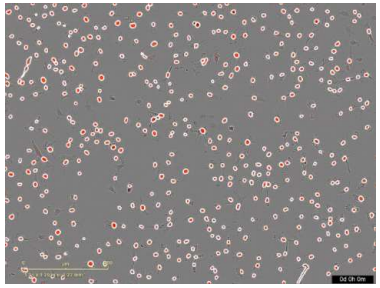
Acquire Images



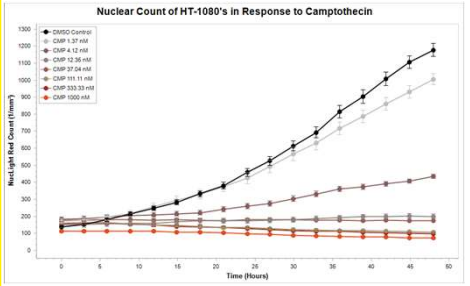
View Images



Process Images

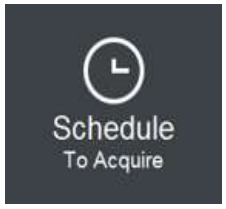


Export Data



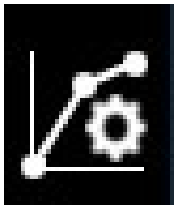
Incucyte® : A Guided Interface

Schedule Scans

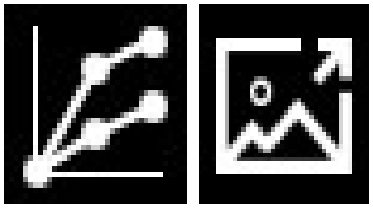


Vessel View

Analysis Definition



Graphing and Exporting



SARTORIUS

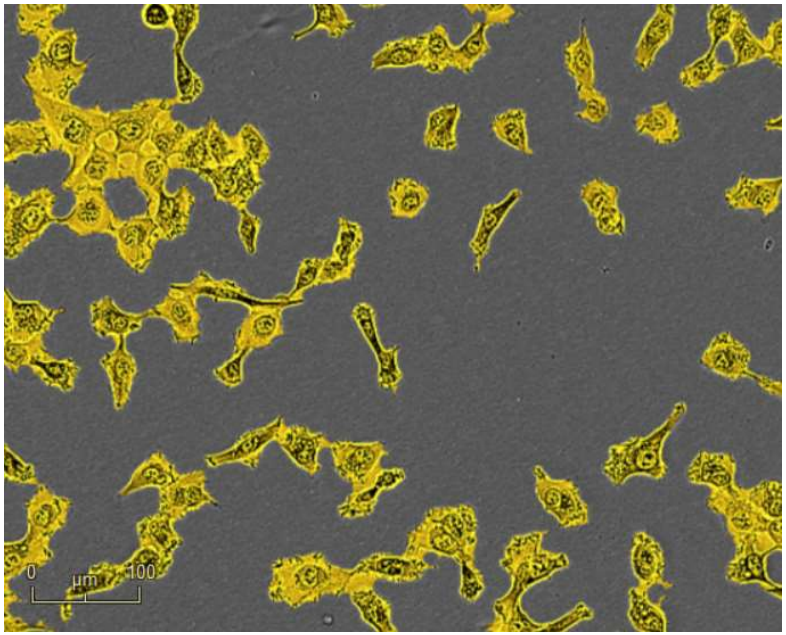
Defining Analysis Masks

What is a mask?

- An area of interest (cells, nuclei, etc.) within an image
- Define by eye which pixels in an image correspond to the desired object by adjusting analysis parameters
- The same analysis is applied to all selected images

When do I make a new mask?

- When changing:
  - Cell type
  - Fluorophore
  - Magnification



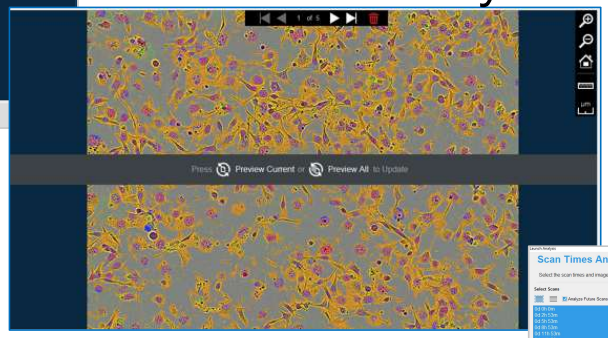
The pseudo yellow color in this image indicates which phase areas of the image are of interest (cells) and should be quantified in the data analysis

Image Processing: 3 Steps to Image Analysis on the Incucyte® SX5

1. Select Representative Images

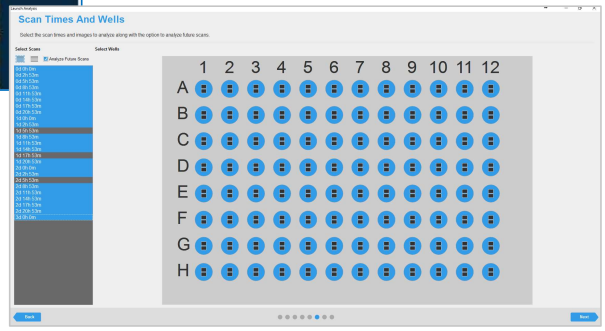
Include images from beginning/end, untreated/treated, dim/bright

2. Preview Analysis Definition



Adjust parameters to create mask

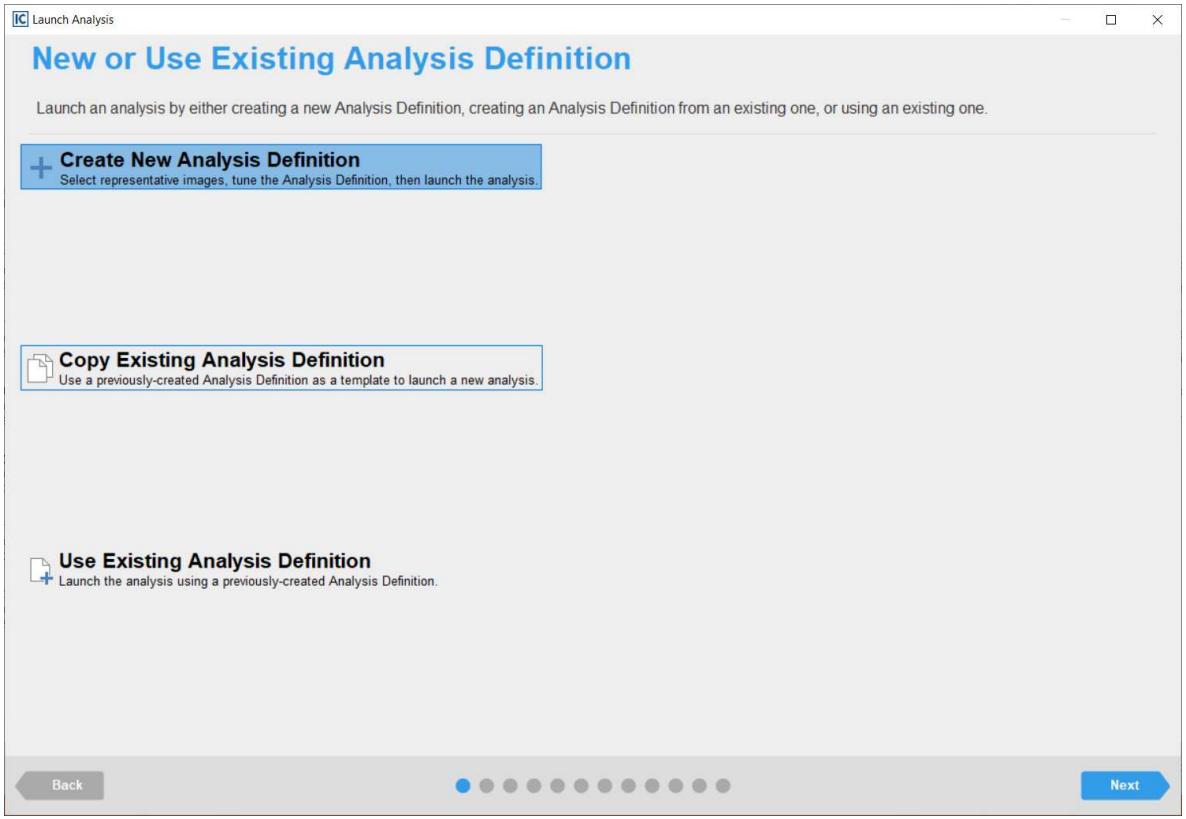
3. Select Time Points/Wells



Run analysis definition on all images/time points of choice

SARTORIUS

Image Processing: New or Existing Analysis

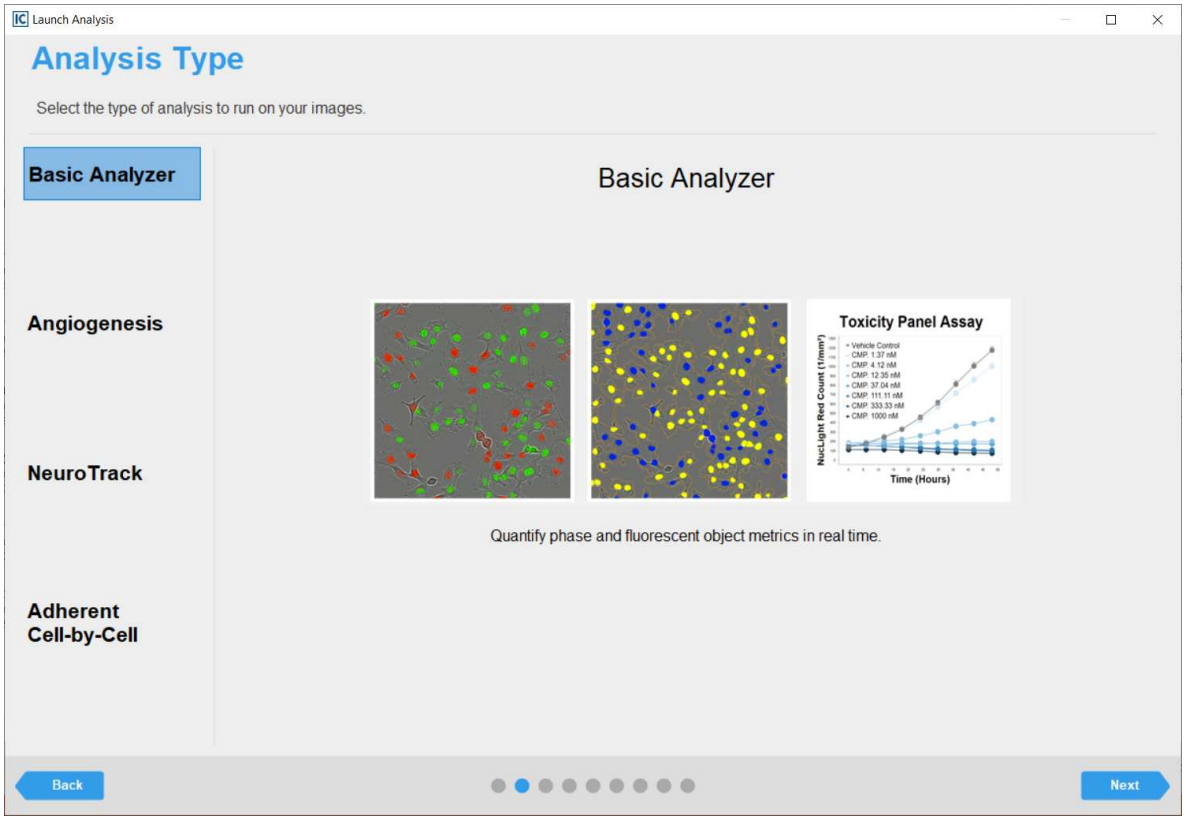


Analysis

- For a new experiment choose a New Analysis Definition
- If a repeat of a previous experiment use or copy an Existing Definition

Process

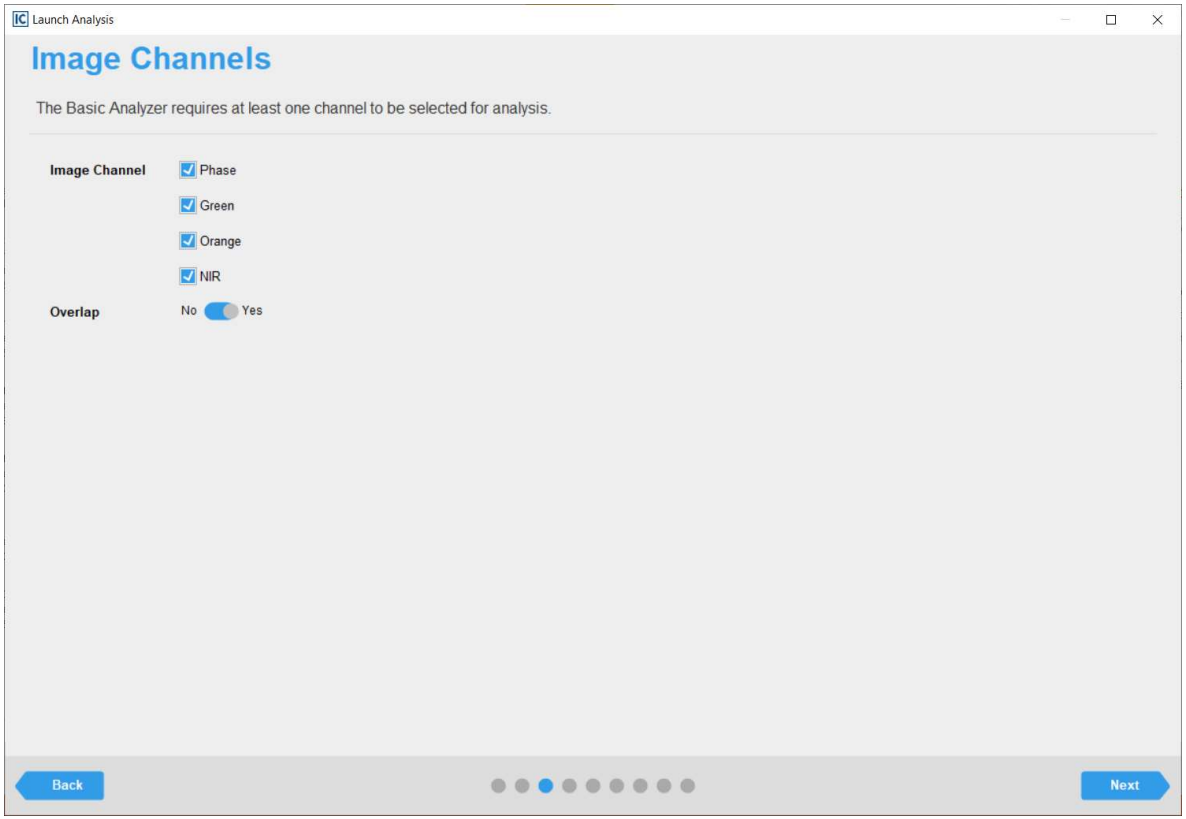
Image Processing: Analysis Type



Choose the Appropriate Analysis Type

- The Basic Analyzer can be used to analyze assays such as proliferation, cell health and immune cell killing
- Cell-by-Cell can also be used for these assays yielding per cell information
- Certain imaging modes will predetermine the analysis type, e.g. Spheroid

Image Processing: Select Image Channels

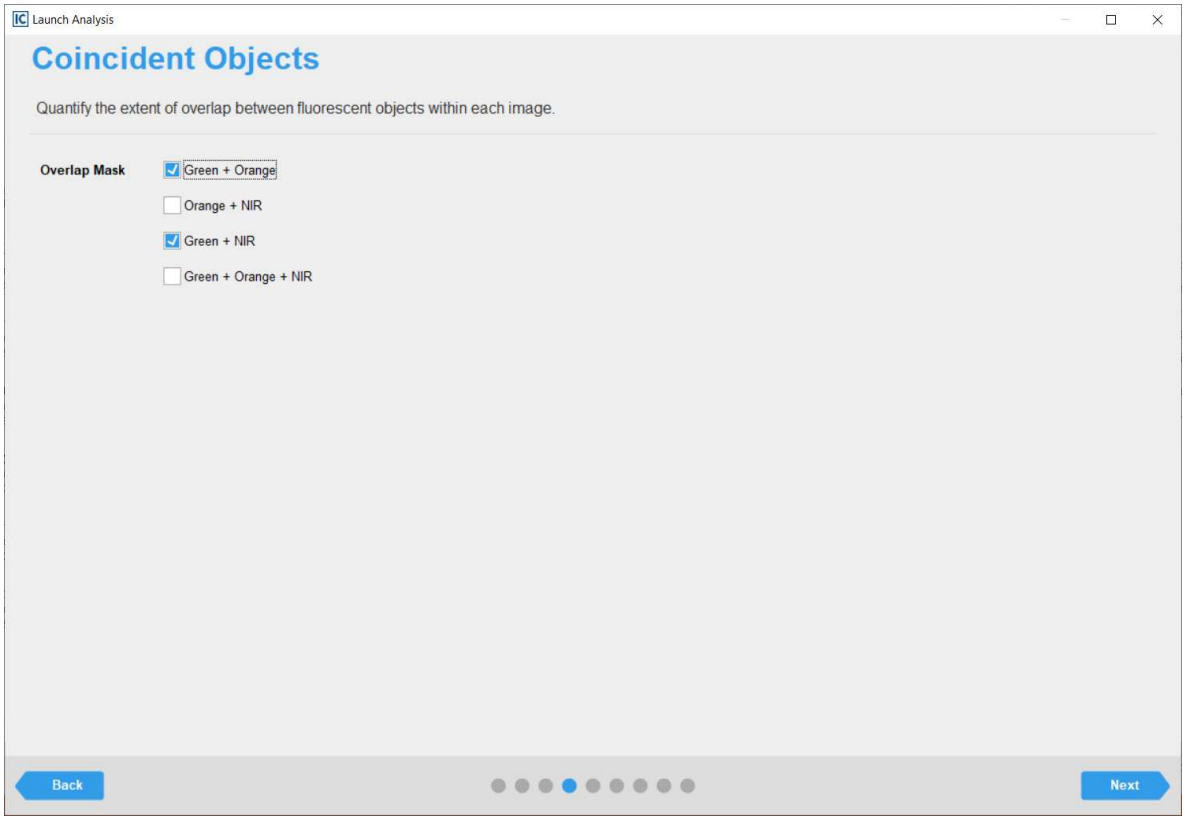


Choose Appropriate Channels

- You do not have to analyze all channels, e.g. phase for ICK
- Overlap option quantifies fluorescent coincidence of objects positive for two or more fluorescent channels

Process

Image Processing: Select Channels for Coincident Object Analysis

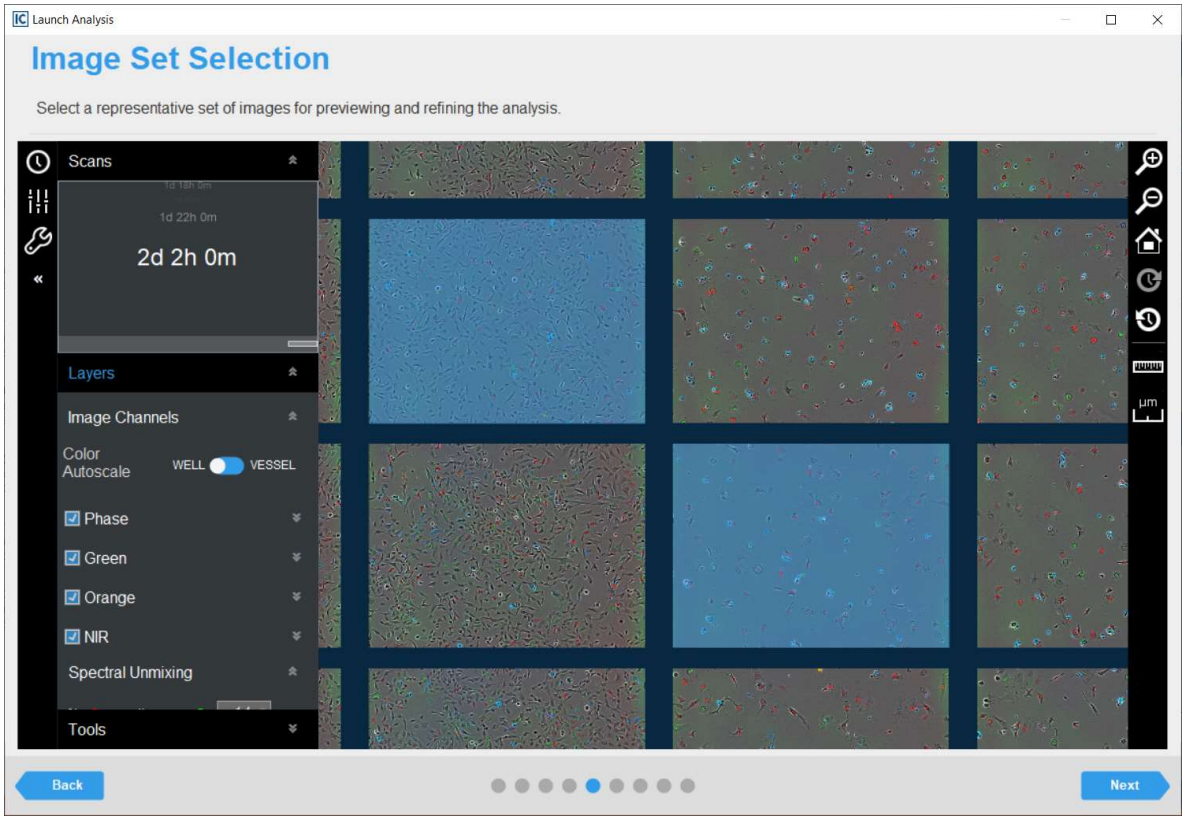


Quantify Fluorescent Overlap

- If desired select relevant channels for coincident object analysis
- Coincident object analysis is dependent on the initial analysis of each independent channel

Process

Image Processing: Choose an Image Set



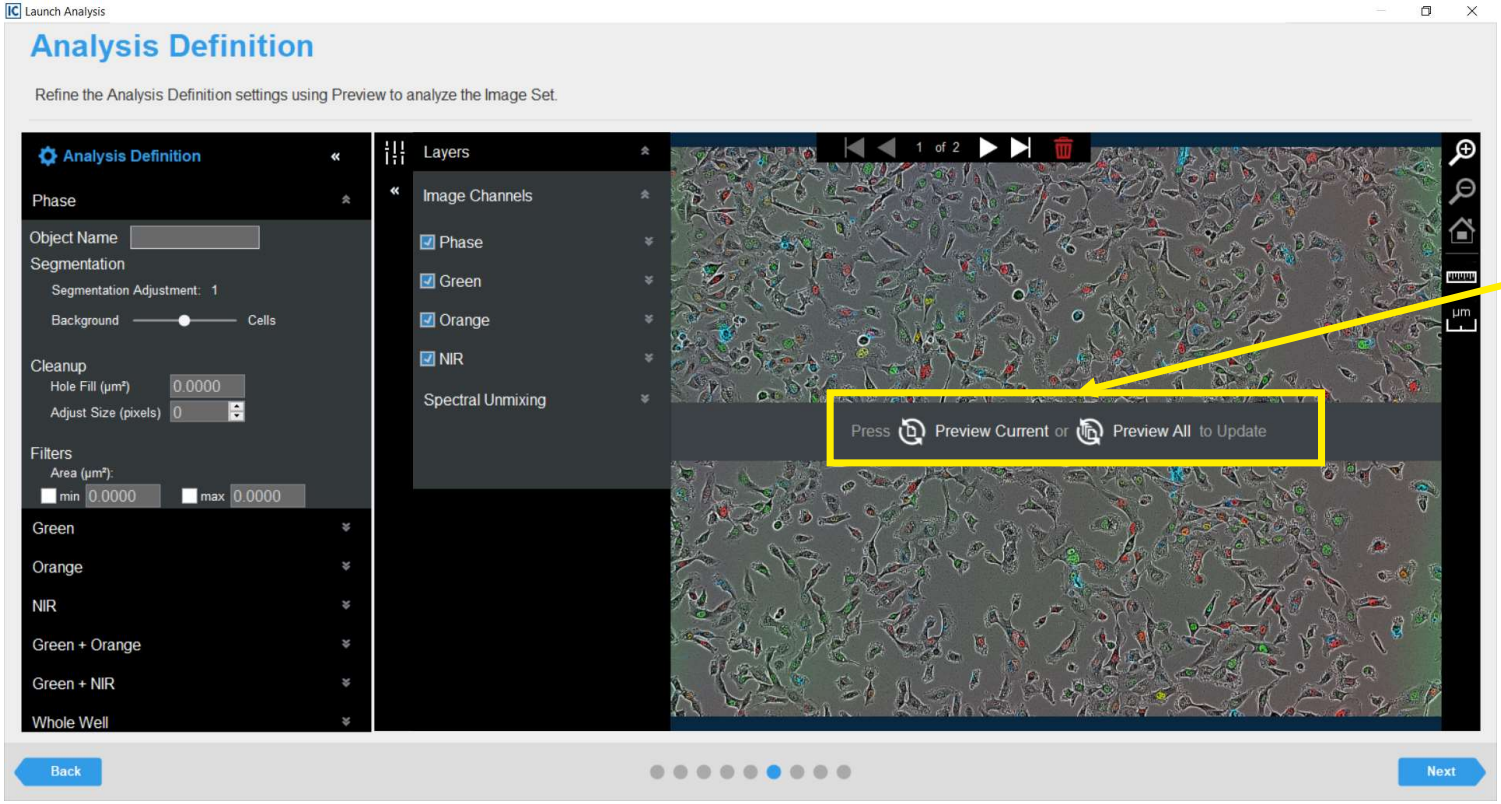
Select Appropriate Images

- Select a range of images
- Should include the extremes of your experiment and in between

Image Processing: Selection of Representative Images

- Images representing the variability inside an experiment (cell morphology, density, fluorescence intensity, background)
- HD-phase
  - Different confluency level (10-80%range)
  - Different cell morphology (differentiation, spreading, death)
  - Images with debris/scratches if representative
- Brightfield (Spheroid and Organoid scan types)
  - Different size and density
  - Different background
- Fluorescence
  - Different intensity after correct setting of Min/Max
  - Different background

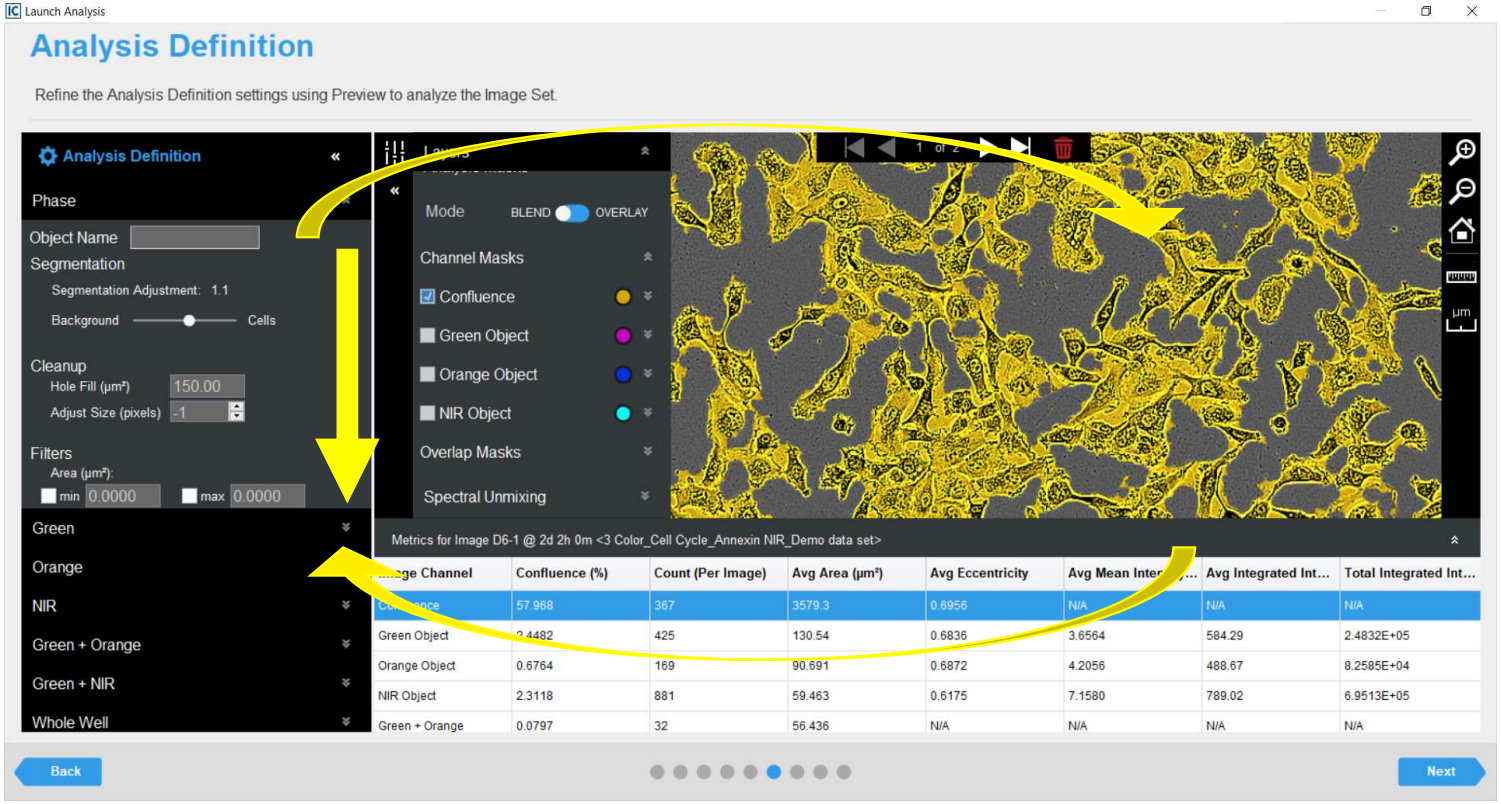
Image Processing: Preview the Current Image



Always Preview First

- The software uses the images to train a mask
- The user can then make manual adjustments

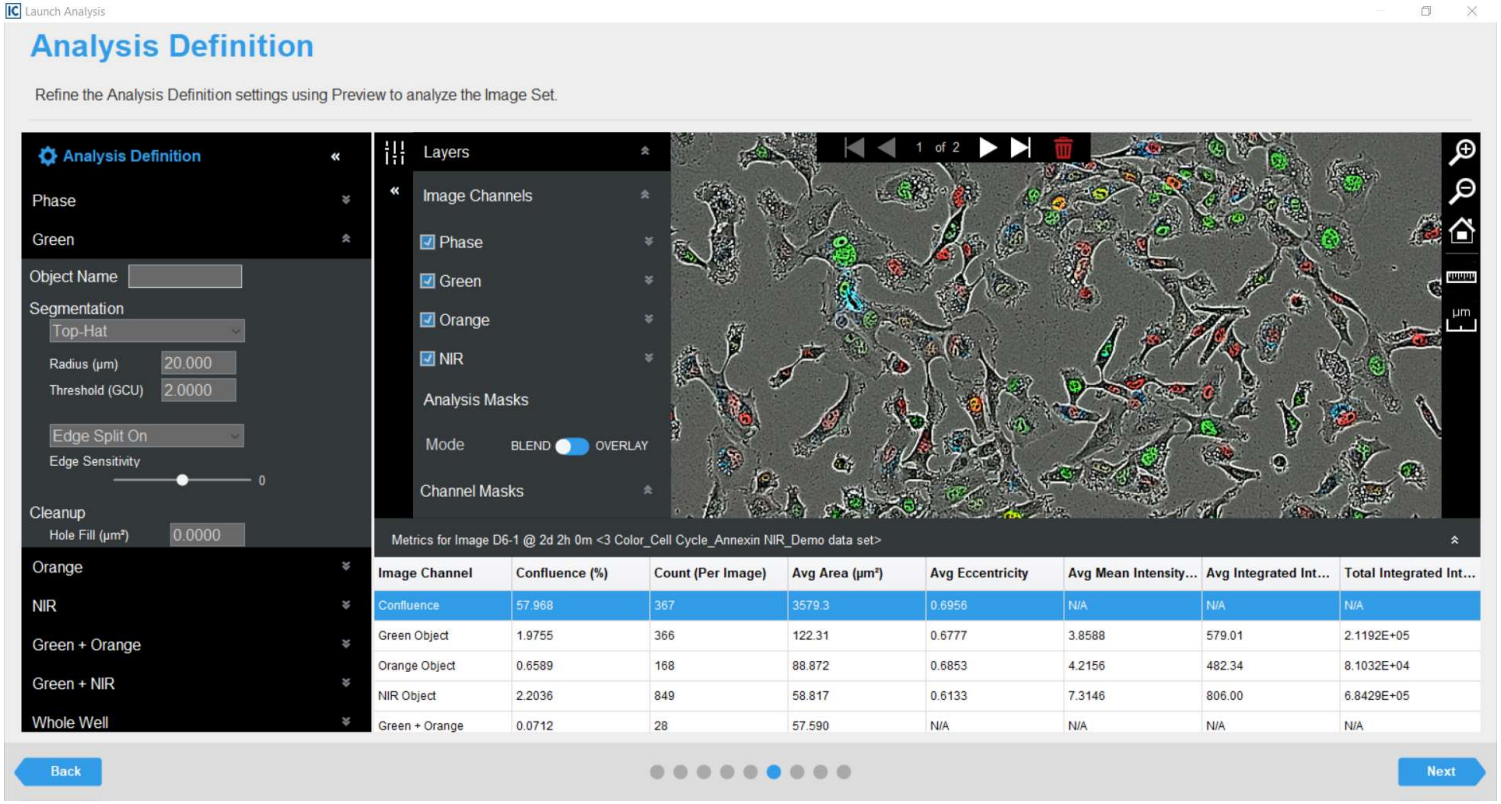
Image Processing: Adjust Masking



Manual Changes

- Go from top to bottom, changing parameters sequentially and checking
- Eccentricity is most often used when filtering clusters, not monolayers, due to variance in cell shape

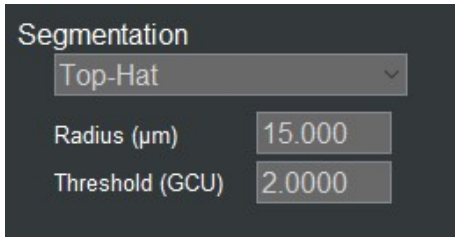
Image Processing: Fluorescence Analysis



Increased Parameters

- Always start with Top-Hat, selecting an appropriate radius
- Select a threshold e.g., GCU or OCU based on an unlabeled control

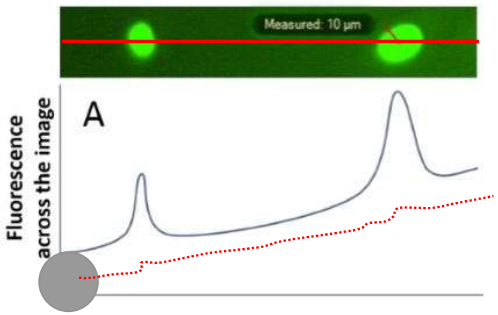
Image Processing: Top-Hat Background Subtraction for Fluorescence

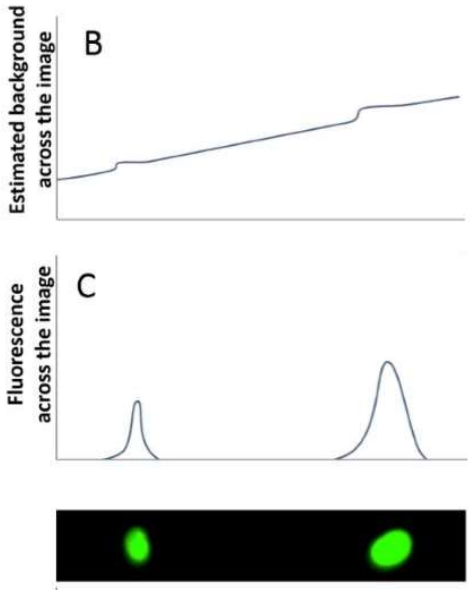


Top-Hat:

- Radius larger than that of the largest object of interest
- Separation into 2 components: background and real signal
- Threshold applied to include only real signal in the mask

How does it work:





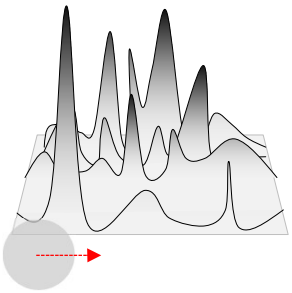
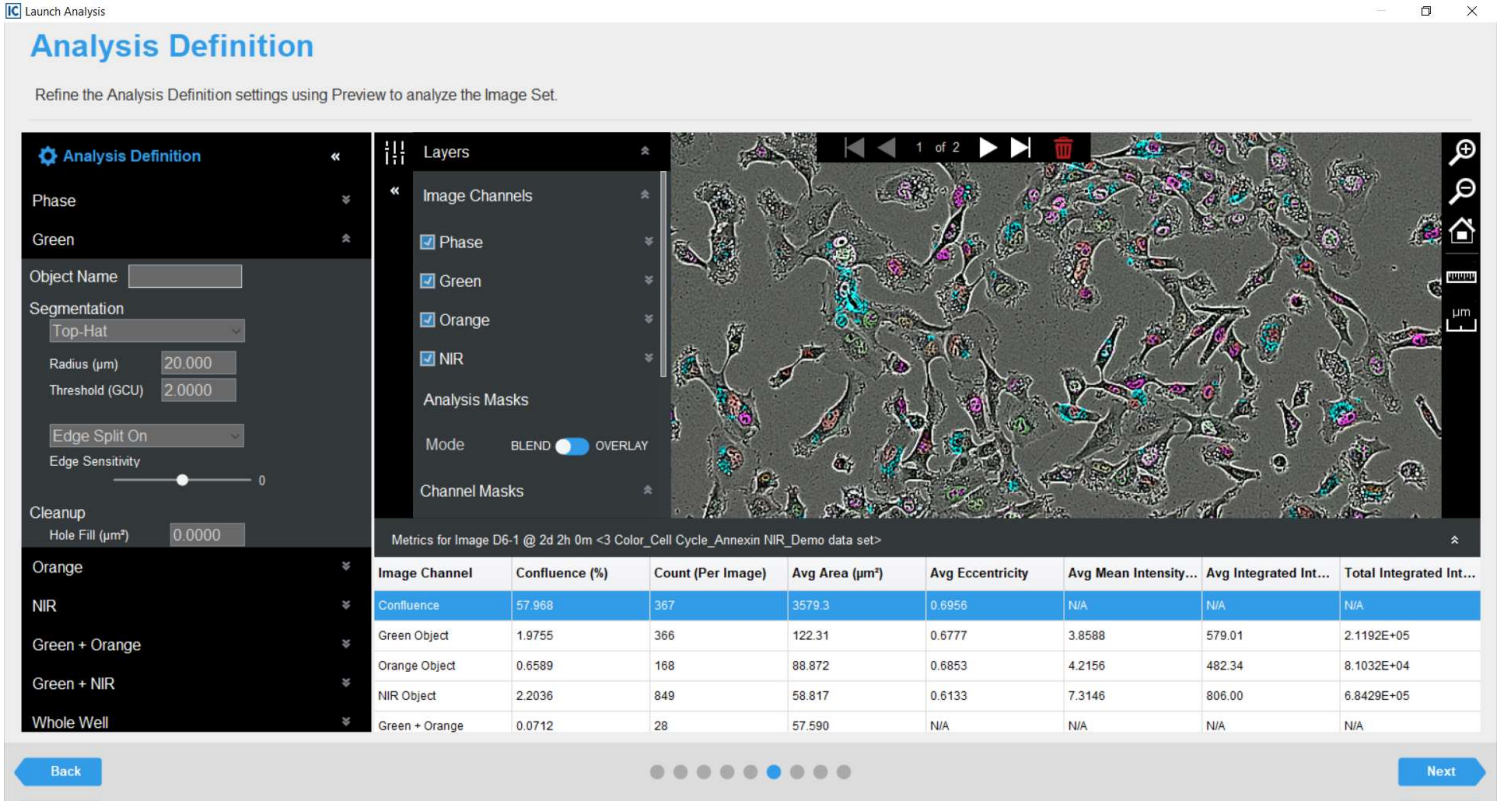


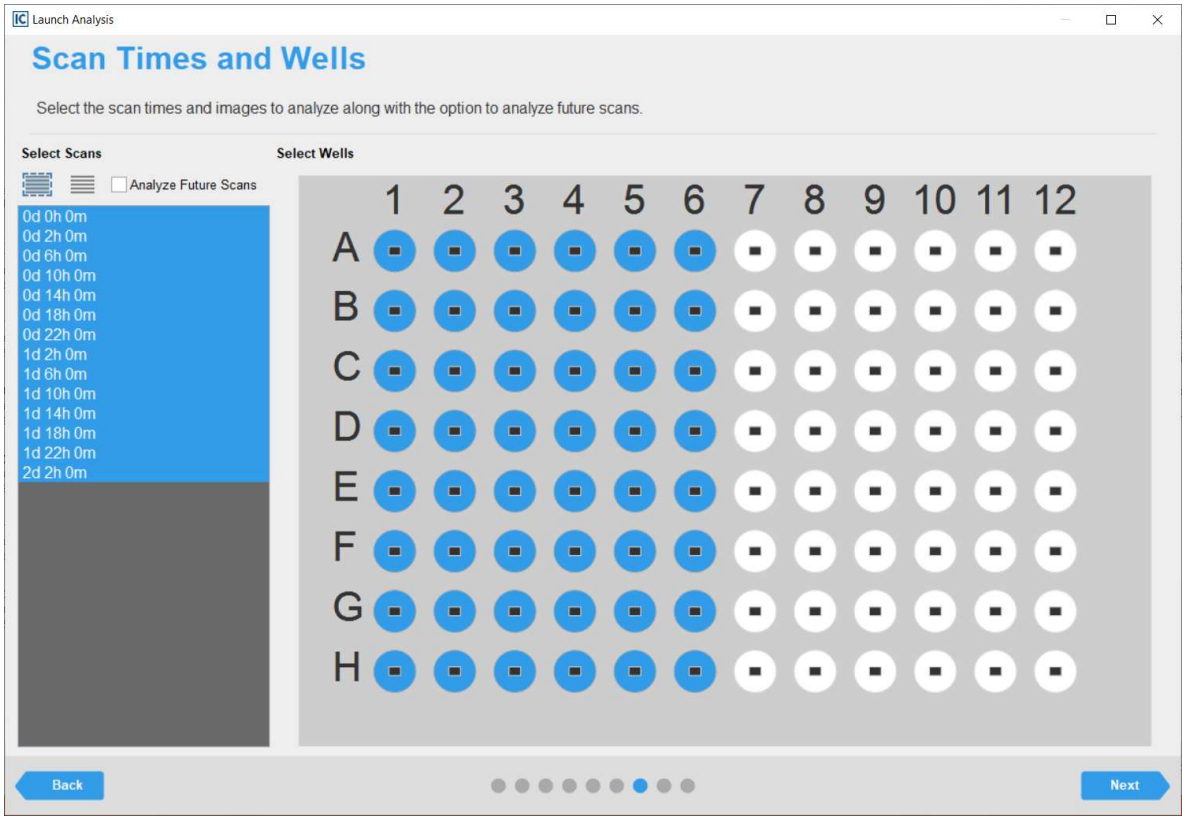
Image Processing: Fluorescence Analysis



Analyze each channel

- For nuclear labels keep Edge Split on
- For membrane and cytoplasmic labels turn Edge Split off
- For nuclear counts a size filter is essential to exclude vesicles and debris

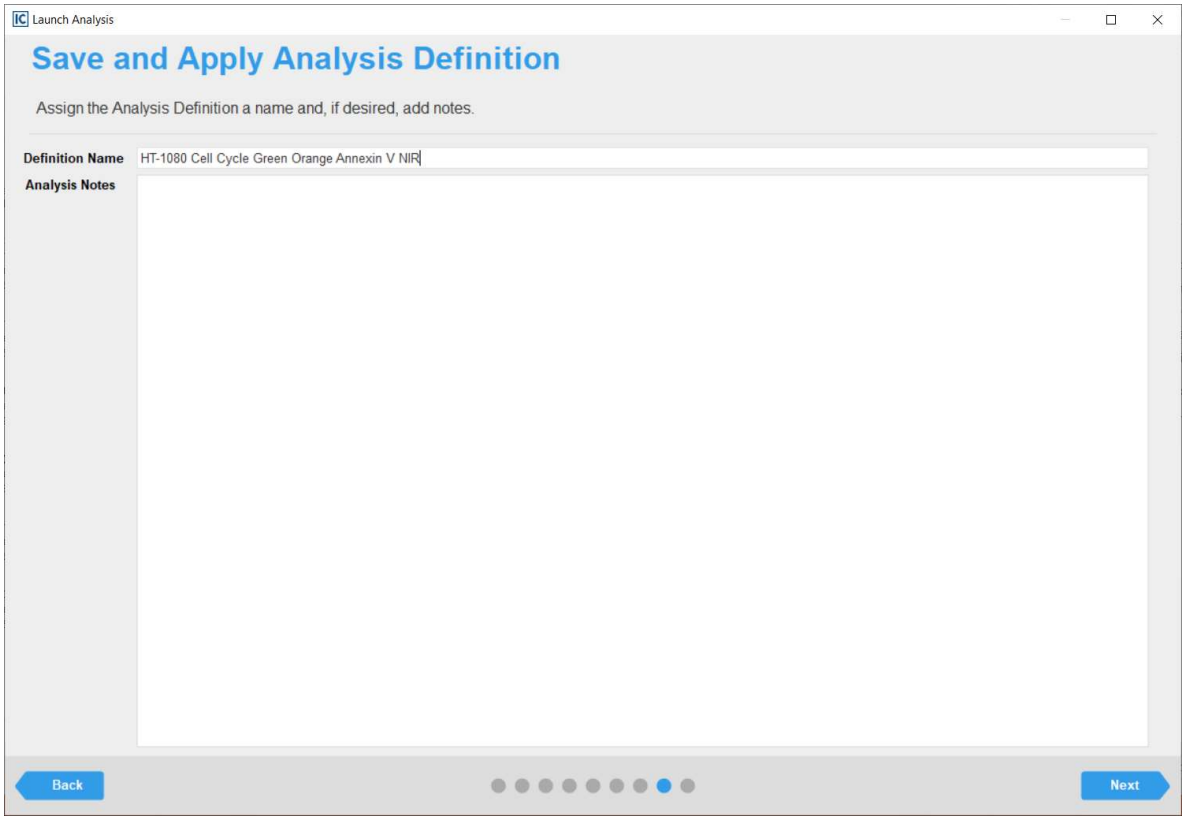
Image Processing: Select Wells and Times to Analyse



Choose and Include Appropriate Wells

- Analyse wells of interest
- Exclude individual timepoints if necessary

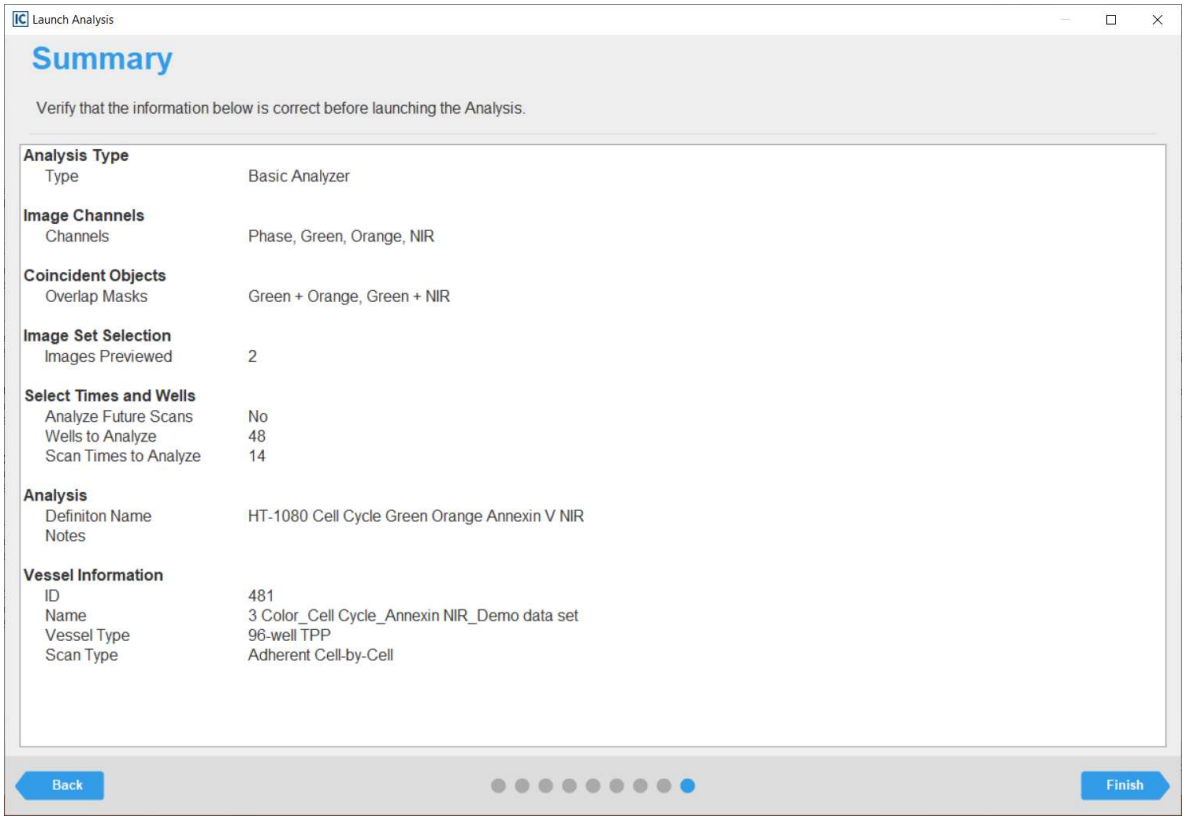
Image Processing: Name the Analysis



Descriptive Name

- Try to be clear with naming and contain details about the analysis
- Possible to share analyses between users

Image Processing: Summary Screen



Check the Analysis Looks Correct

- If changes need to be made, click the tab and it will link back
- If all is correct click Finish and the analysis will start

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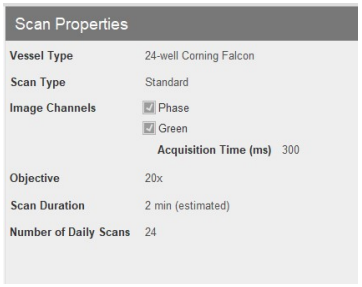
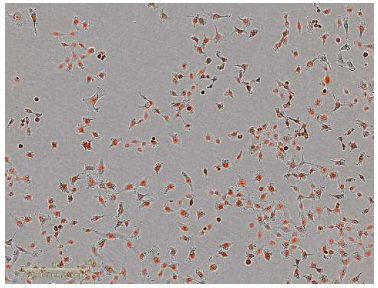
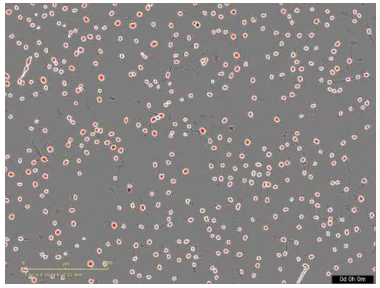
Acquire Images



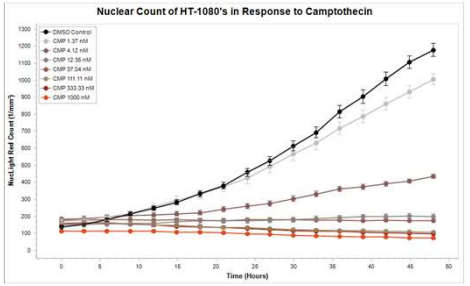
View Images



Process Images

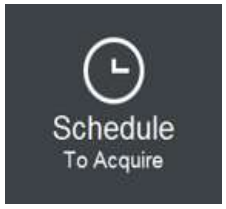


Export Data



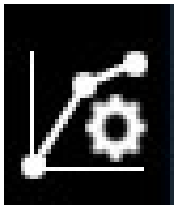
Incucyte® : A Guided Interface

Schedule Scans

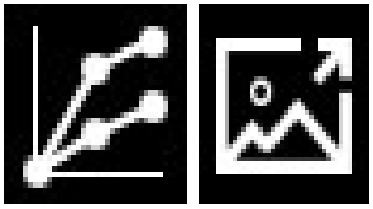


Vessel View

Analysis Definition



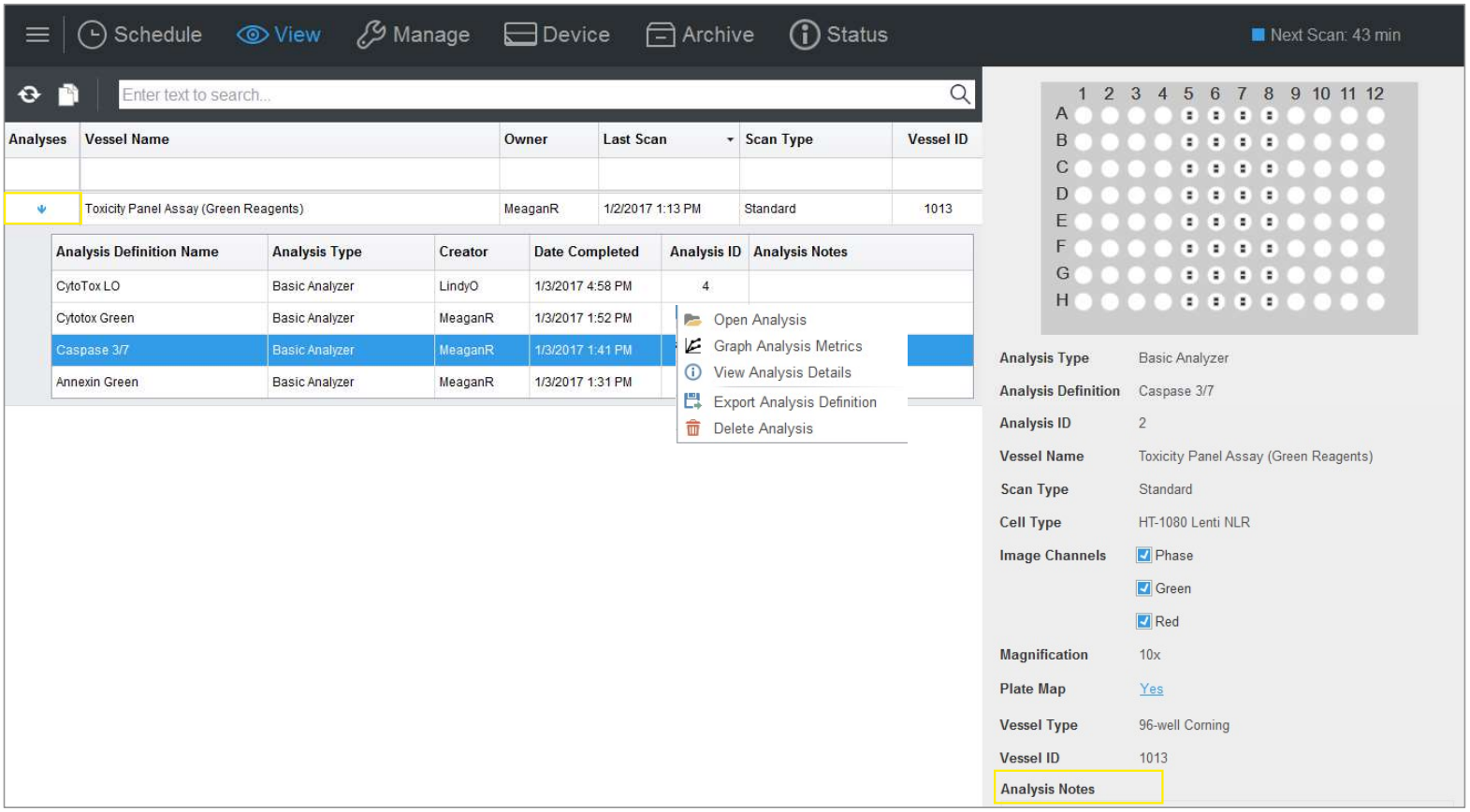
Graphing and Exporting



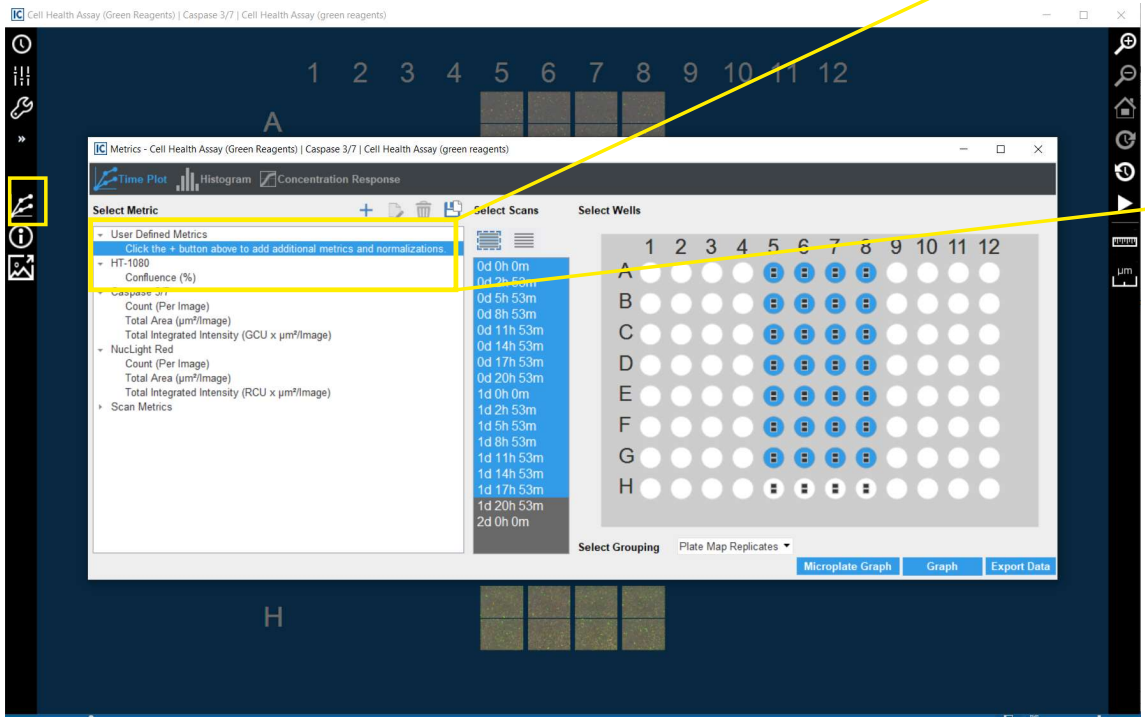
SARTORIUS

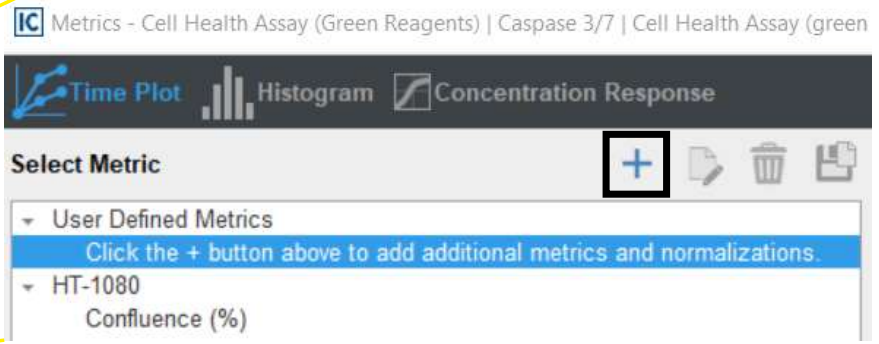
Export: Search Analyses

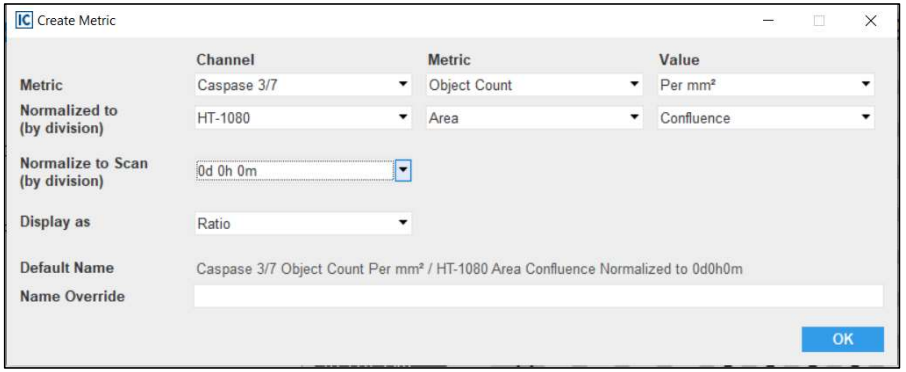
- Analyses associated with a vessel are indicated by a blue arrow
  - Analyses saved by Analysis Definition Name
  - Right click on Analysis Definition to view details
  - Directly go to graphing window
- Analysis Notes specify details of analysis definition
- Details in the right pane show wells/image channels analyzed



Export: Graph – View/Export

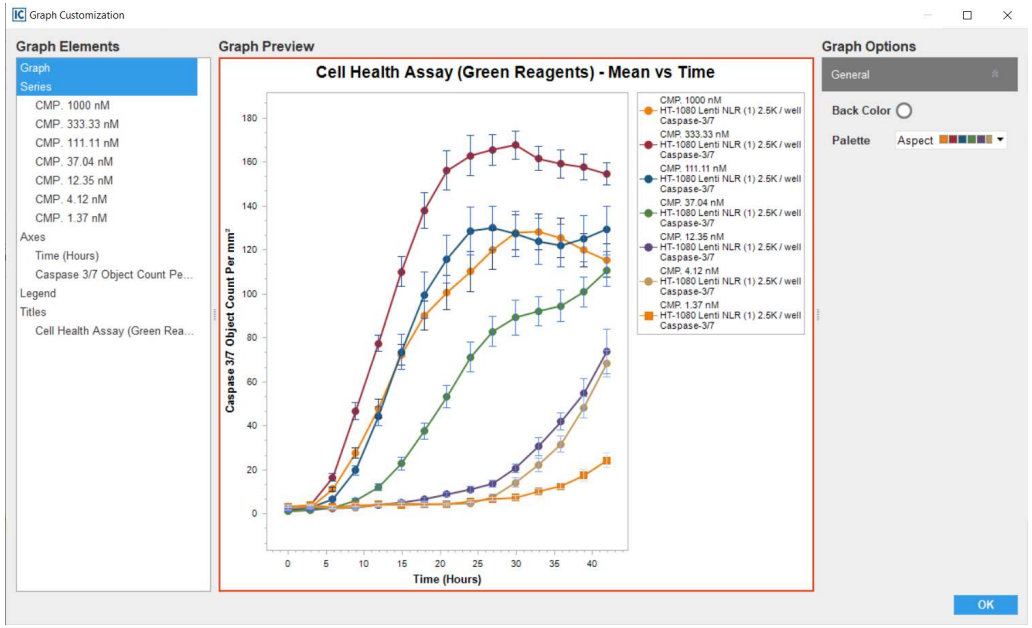


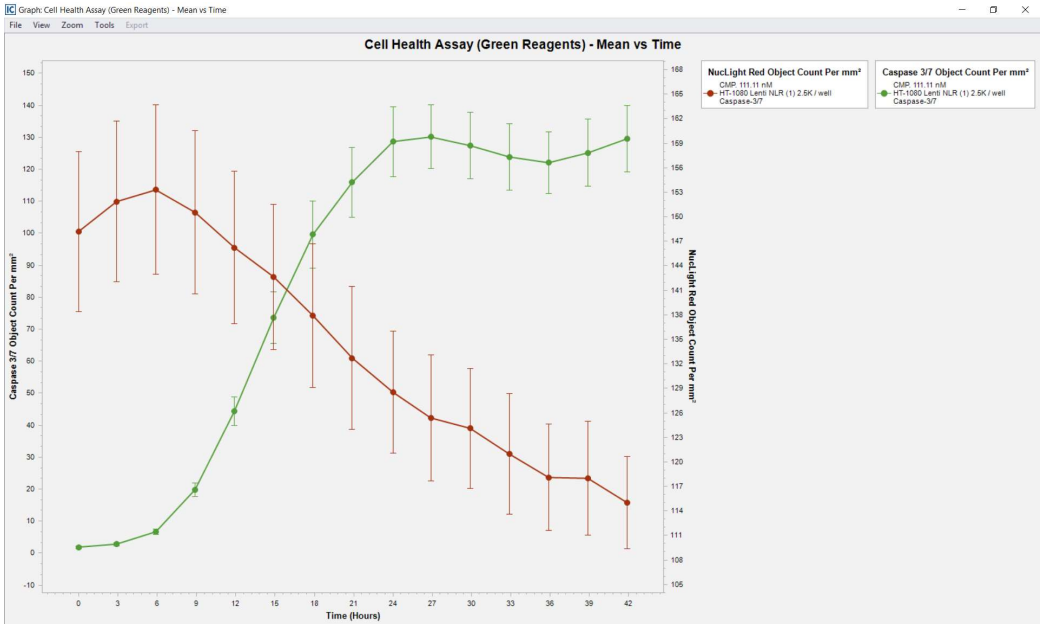




- Select time points, wells, metric and grouping of interest
- Possible to build custom metrics, including data normalisation

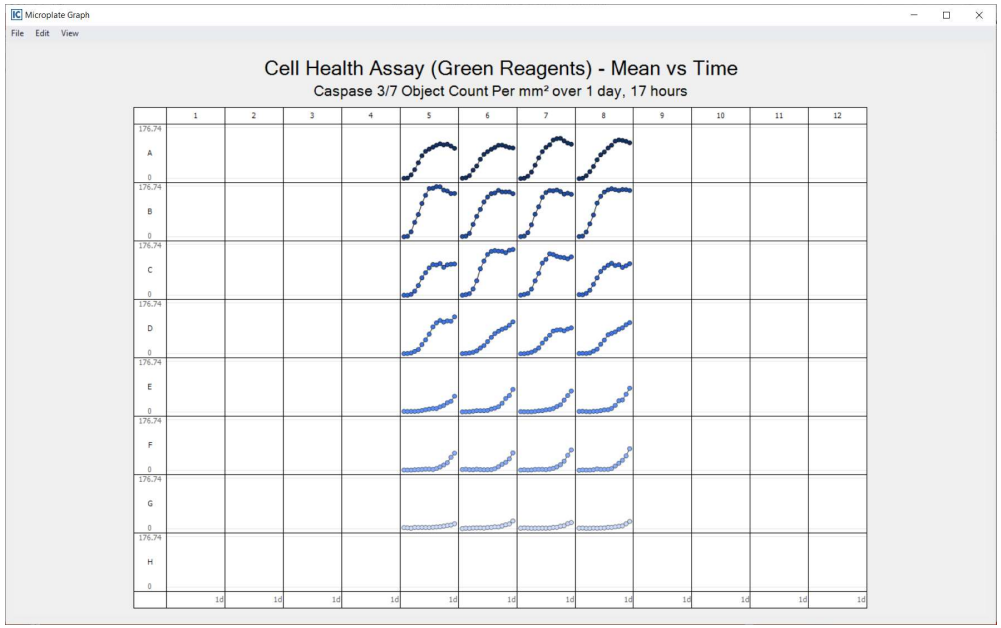
Export: Graph – View/Export

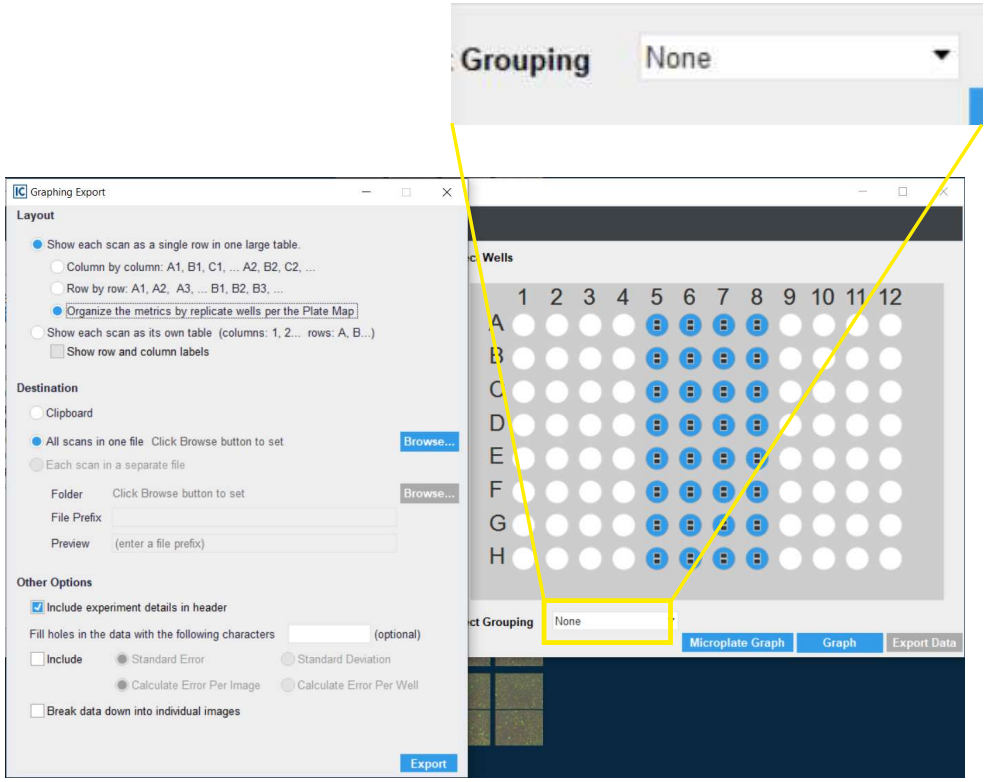




- Customize graphs to adjust colors, legends, axes etc.
- Combine graphs and compare datasets using Drag and Drop

Export: Graph – View/Export



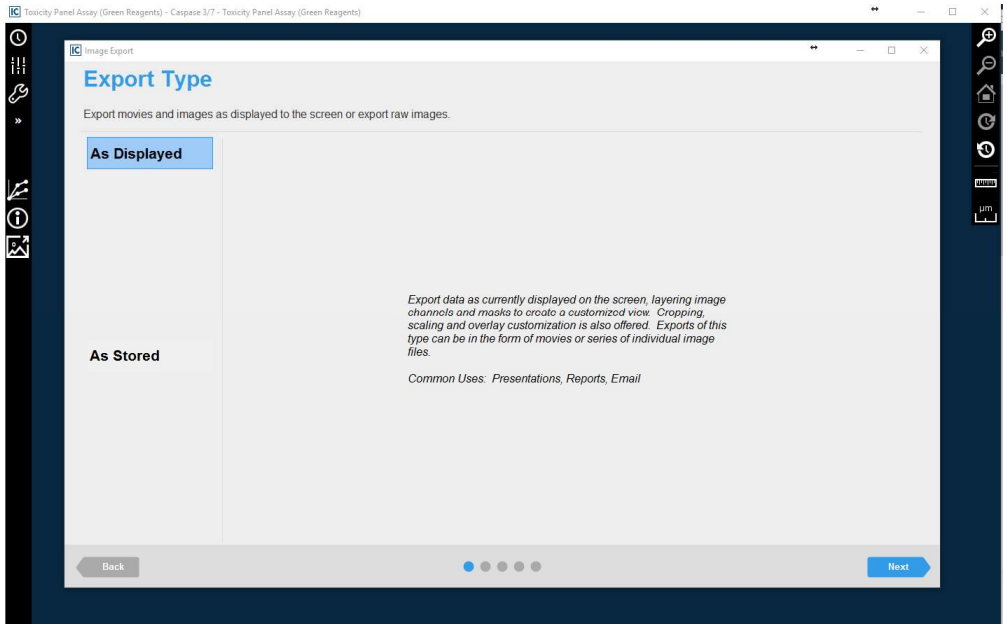


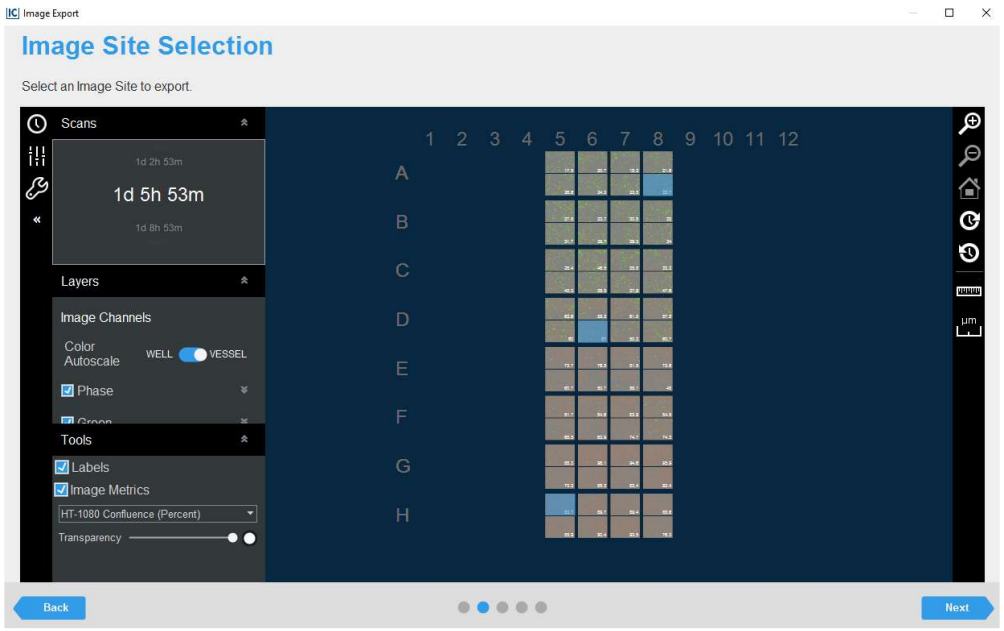
- Export Microplate Graphs to assess data trends and outliers
- Export data for 3rd party statistical analysis, select grouping as ‘None’

Export: Images and Movies

- Export “As Displayed”
  - Presentations/Publications
  - Adjust Brightness/Contrast for phase-contrast
  - Adjust min/max intensity settings for fluorescence (remove autoscale)
- Export “As Stored”
  - Analyze images for 3rd Party analysis packages
  - Phase: 8-bit raw
  - Fluorescence: Uncalibrated raw 16-bit or calibrated 32-bit floating point

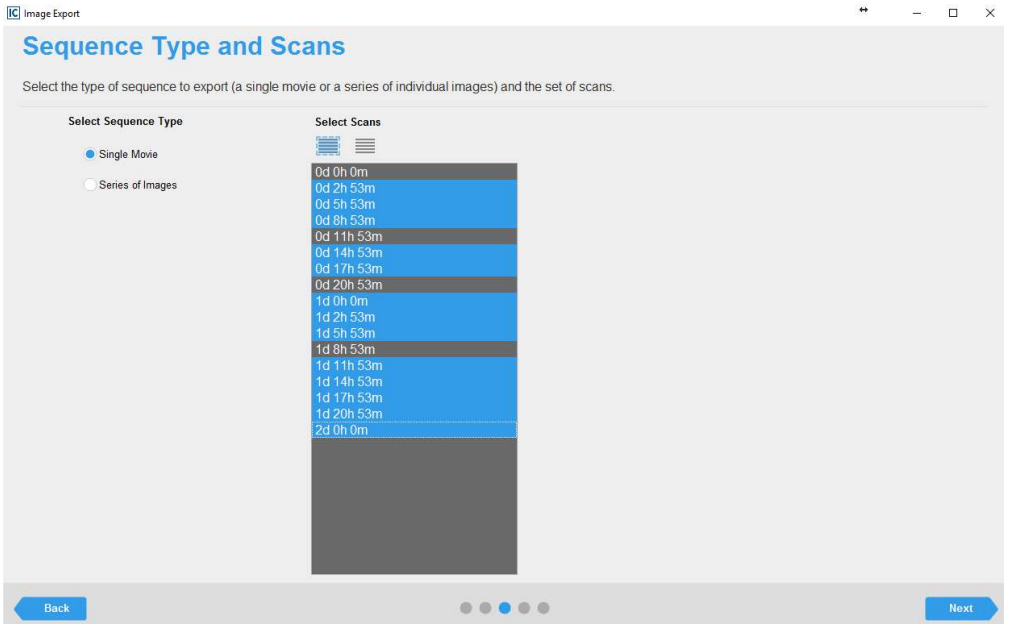
Export: Images and Movies

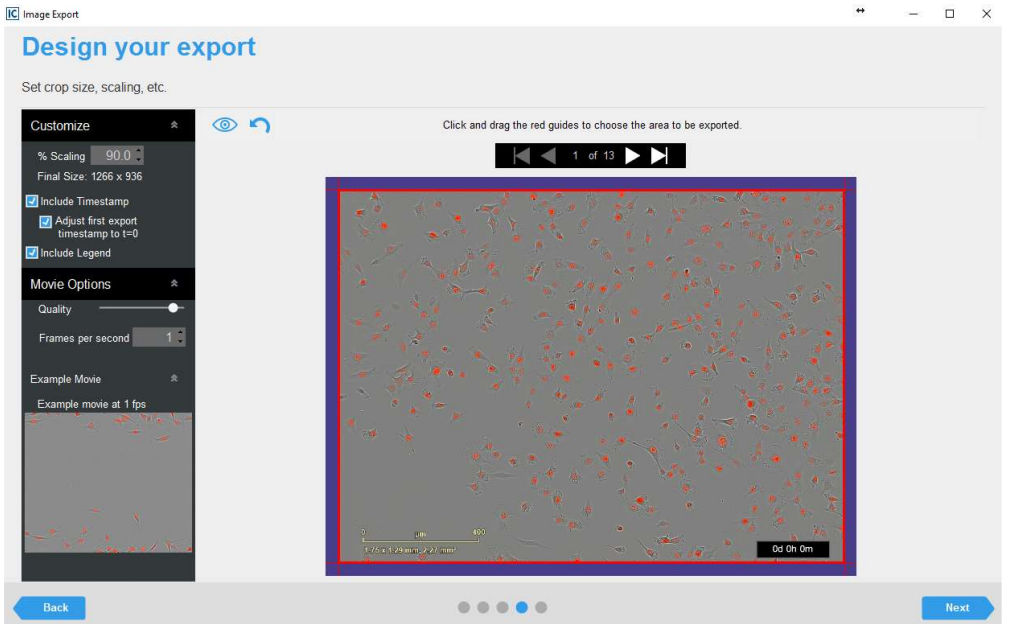




- Enter Guided Interface and select Export Type
- Select images of interest, channels and masks and adjust fluorescence scaling

Export: Images and Movies





- Select Single Movie or Series of Images and select timepoints
- Adjust scaling, frame rate, timestamp and legend

Agenda

Introduction and Applications

Incucyte® Hardware and Best Practices

Software Overview

Acquire

Visualize

Process

Data Export

Conclusion

Resources – Publications Library

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Incucyte® Publication Library

Conclusion

Incucyte® Support

Mathias Lucas
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mathias.lucas@sartorius.com
+33 6 42 02 62 28

Additional Resources:

- Quotes, Orders:
  Olivier Bruno
  olivier.bruno@sartorius.com
  +33 33 6 60 27 46 53

- Assays, Reagents, Protocols, Software:
  www.essenbioscience.com
  askascientist@sartorius.com

- Technical Support and Hardware:
  EUincucyte.support@sartorius.com

Thank You

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SARTORIUS

Main sources of pipetting errors according to ISO 8655-2

Greatest impact on the pipetting results - up to 50%:

- Leaky piston and cylinder system of the pipette
- Poor tip fitting (tip is leaking)

Medium impact to the pipetting results - up to 4%:

- Re-use of the tip
- Failure to wipe the tip against the vessel wall (10-15mm upwards)
- Unstable humidity of the pipetting environment
- No pre-rinsing of the tip
- Uneven rhythm and timing of pipetting
- Immersion depth of the tip (2-3mm) and angle of the pipette during pipetting

Minor impact on the pipetting results, up to 0,5%:

- Inconsistent piston movement
- Difference in temperature between the pipette, tip, liquid and room temperature (up to 0,3%/C°)