sartorius

IncuCyte® S3 Training

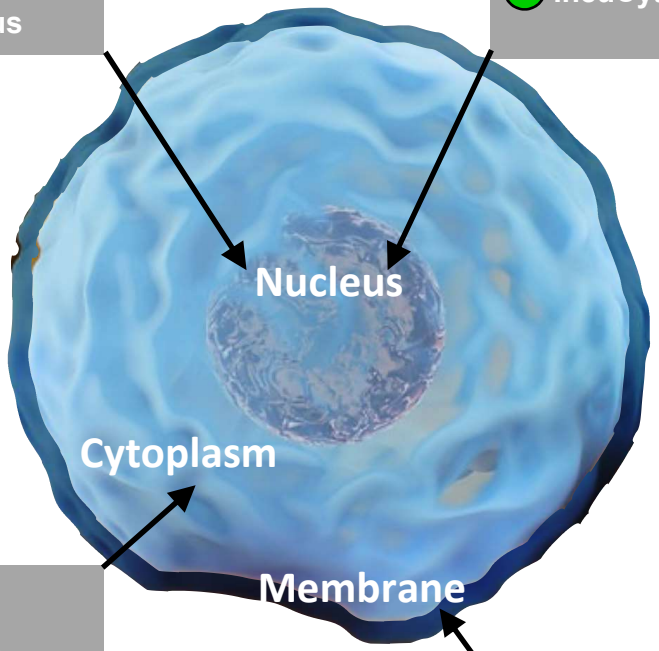
IncuCyte®

Applications and Reagents Discussion

Cell Health and Viability

- IncuCyte Nuclight Rapid Red Reagent
- IncuCyte® NucLight Green Lentivirus
- IncuCyte® NucLight Red Lentivirus

- IncuCyte® Cytotox Red Reagent
- IncuCyte® Cytotox Green Reagent
- IncuCyte® Caspase-3/7 Red Apoptosis Reagent
- IncuCyte® Caspase-3/7 Green Apoptosis Reagent



- IncuCyte® CytoLight Rapid Green
- IncuCyte® CytoLight Red Lentivirus
- IncuCyte® CytoLight Green Lentivirus

- IncuCyte® Annexin V Red Reagent
- IncuCyte® Annexin V Green Reagent

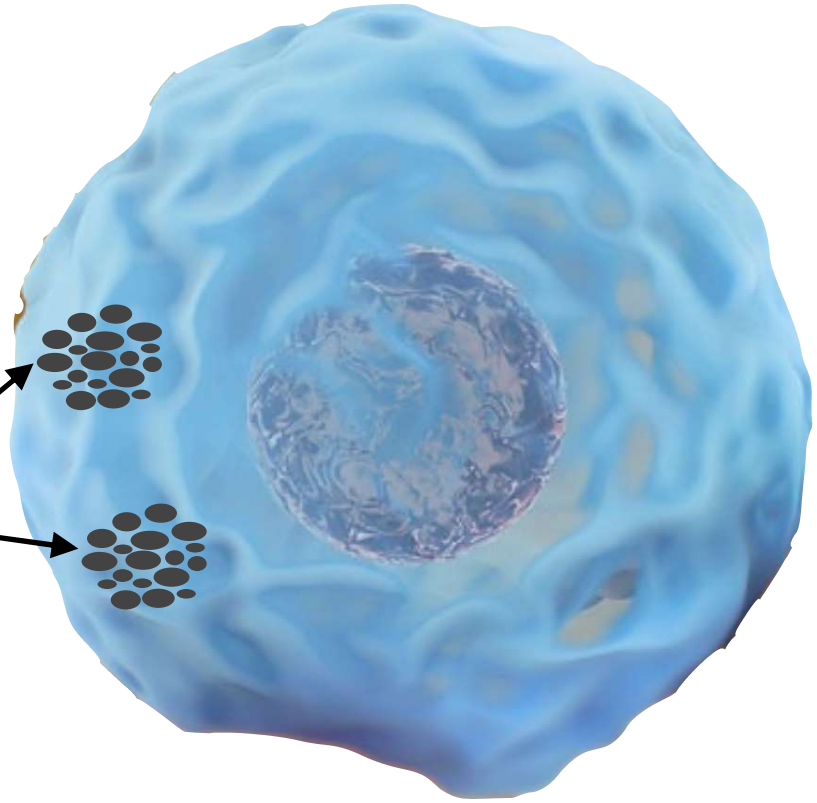
Live Cell Assays

Phagocytosis

- IncuCyte® pHrodo® Red Cell Labeling Kit
- IncuCyte® pHrodo® Red E. coli Bioparticles®
- IncuCyte® pHrodo® Green E. coli Bioparticles®
- IncuCyte® pHrodo® Red Zymosan Bioparticles®
- IncuCyte® pHrodo® Green Zymosan Bioparticles®
- IncuCyte® pHrodo® Red S. aureus Bioparticles®
- IncuCyte® pHrodo® Green S. aureus Bioparticles®

Antibody Internalization

- IncuCyte® Human FabFluor Red Antibody Labeling Reagent
- IncuCyte® Mouse IgG1 FabFluor Red Antibody Labeling Reagent
- IncuCyte® Rat FabFluor Red Antibody Labeling Reagent



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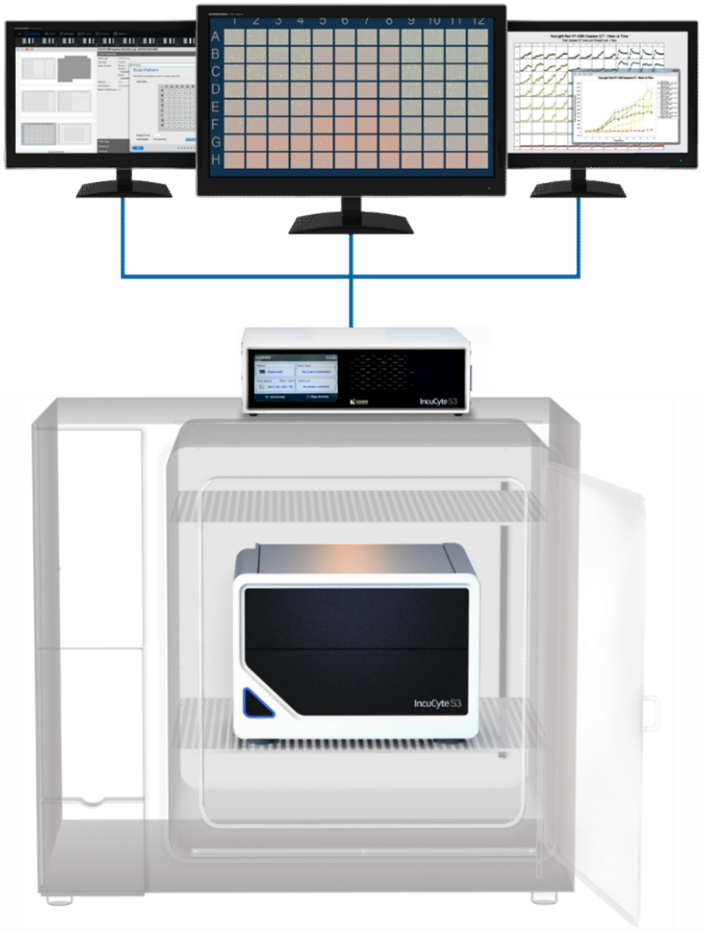
IncuCyte Overview and Best Practices

IncuCyte®

Overview of IncuCyte

- **Image Channels**
  - HD Phase (brightfield with spheroid scan)
  - Green
    - Ex: 440-480nm
    - Em: 504-544nm
  - Red
    - Ex: 565-605nm
    - Em: 625-705nm
- **Objectives**
  - 4x, 10x, 20x on automated turret
- **Compatible with most tissue culture vessels**
- **Software**
  - Remote Access
  - Guided interface
  - Unlimited user license



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 15 minutes before placing into the incubator for optimal cell distribution • Non-adherent may require a coating for optimal cell distribution

IncuCyte functions Inside your Incubator

- **IncuCyte is NOT an incubator and will not regulate the environment**
- **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
  - Read the manufacturer's recommendations for routine incubator maintenance

Automated Image Acquisition and Auto-Focus

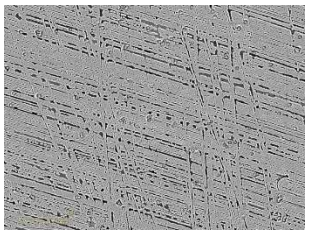
Manual Focusing Analogy

- 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

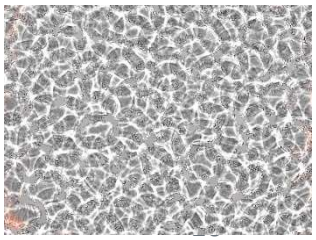
Best Practices for Image Acquisition

Image Artefacts to Avoid	How to get best Image
Empty wells (no cells)	Edit scan pattern to not include wells without cells. Make sure there is >10% confluent cells in all wells (unless scanning in dilution cloning mode).

Fingerprints



Condensation



The Bubble Buster

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 an 30 – 45° angle
- Pipette against the inside wall of the receiving vessel
- Operate with smooth and consistent thumb action

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

Cell Culture should be Free of Contaminants

Technique is Key for Accurate Results

Microsart® AMP Mycoplasma Kit

MD8 Air Samplers

MYCAP™

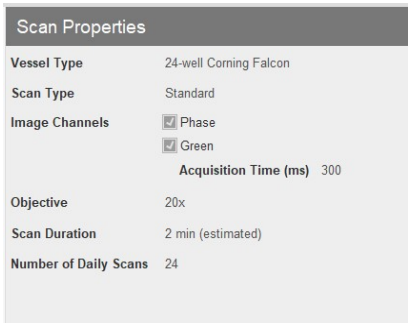
- **Check cell culture routinely for mycoplasma contamination**
- **Ensure cell culture environment has the lowest concentration of airborne contaminants**
- **Aseptically remove samples and close containers without contaminating contents**

Breaking Point to go into Lab

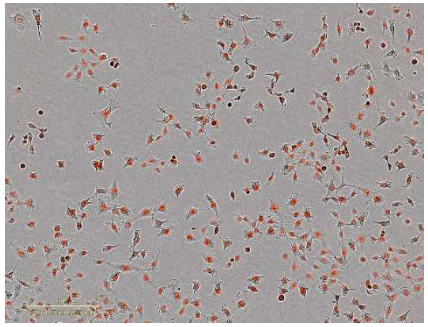
Live Cell Analysis Software Training

4 Steps to Image Analysis

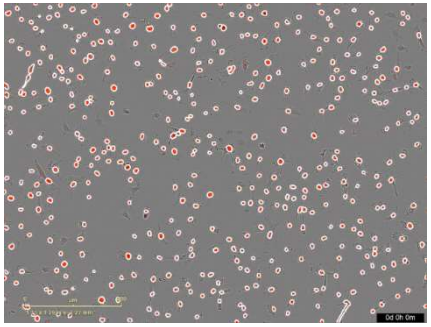
Acquire Images



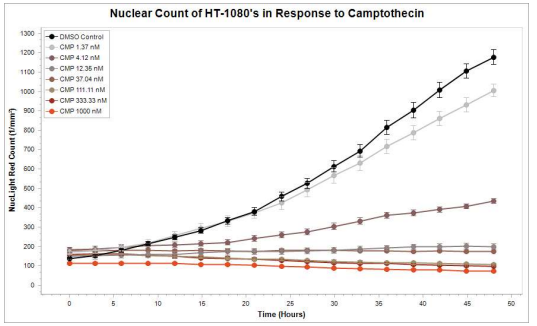
View Images



Process Images

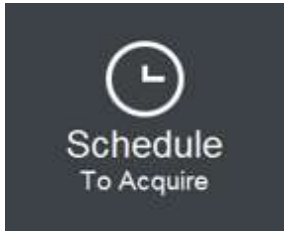


Analyze Images



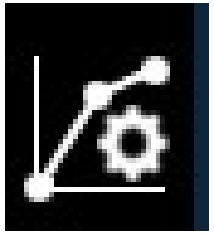
IncuCyte: A Guided Interface

Schedule Scans

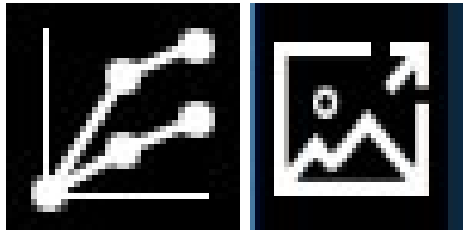


Vessel View

Analysis Definition



Graphing and Exporting



Logging into IncuCyte® S3

- IncuCyte: Instrument identifier assigned by your FSE at installation (e.g. IC50014 or an IP address)
- User Name and Passwords can be set up by the Admin
- Main Window opens up to guide you through the interface

User Accounts

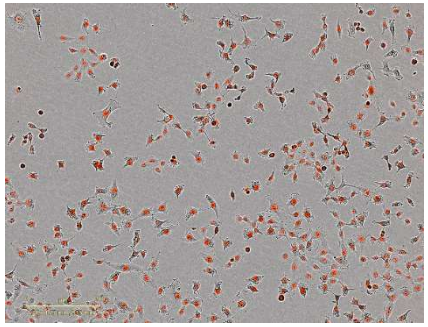
Function	Admin	Standard	Limited	Guest
Add/Remove/Edit Scheduled Scans	Any	Any	No	No
View Vessels	Any	Any	Any	Any
Create Analysis Definitions	Any	Any	Any	No
View Analysis Definitions	Any	Any	Any	Any
Edit/Delete Analysis Definitions	Any	User Owned	User Owned	No
Edit Vessel Documentation	Any	Any	Any	No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

*Limits are placed on user accounts to prevent mistakes, while also providing a collaborative environment for colleagues/peers to view and interact with data. Additional scheduling scans functions and log files help restore or diagnose mistakes if they happen to occur, which is rare.

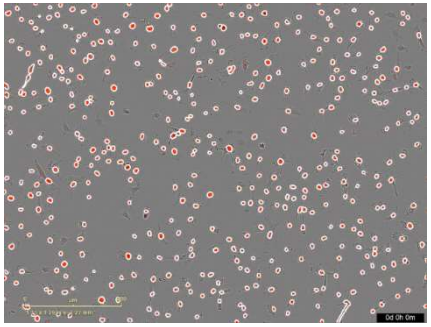
Scheduling Scans

Acquire Images

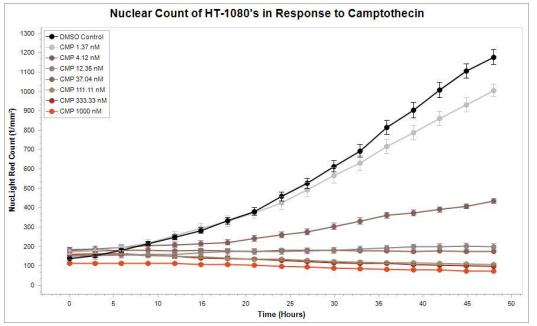
View Images



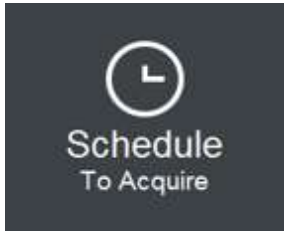
Process Images



Analyze Images

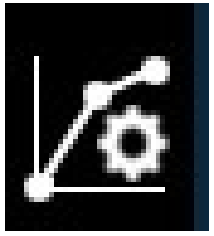


Schedule Scans

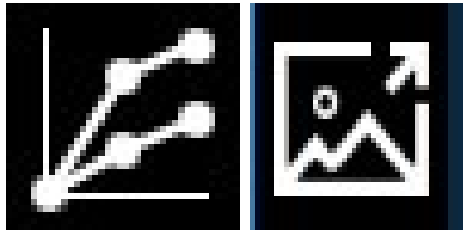


Vessel View

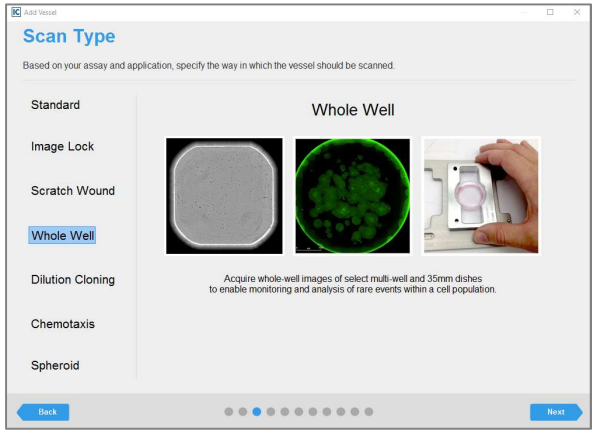
Analysis Definition



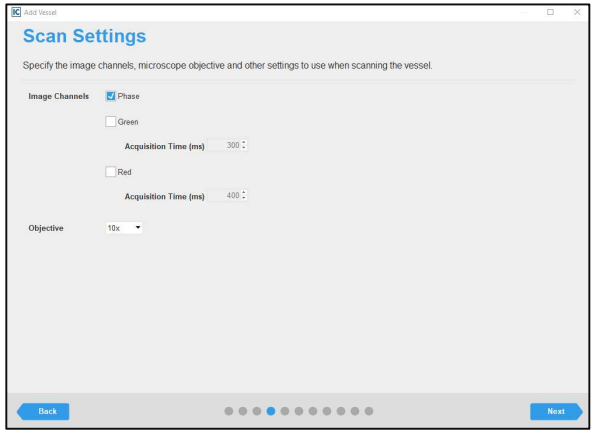
Graphing and Exporting



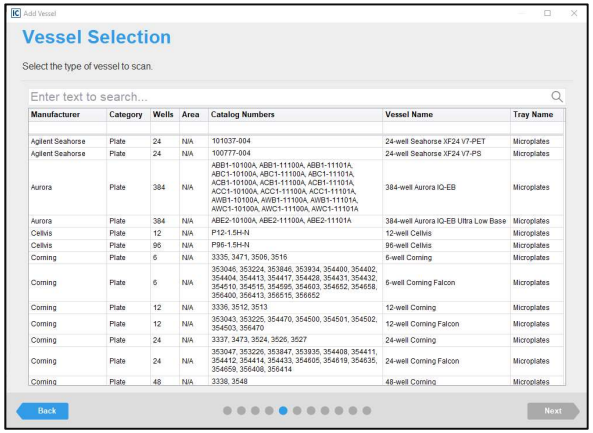
Schedule Scans – Guided Interface



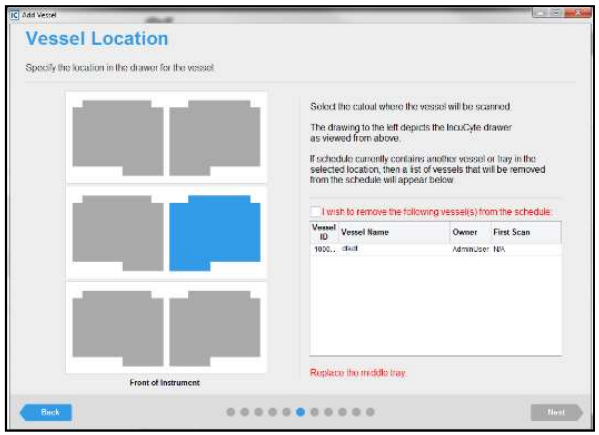
Pre-set scan types will automatically select objectives, plates, scan patterns downstream. Most experiments = standard



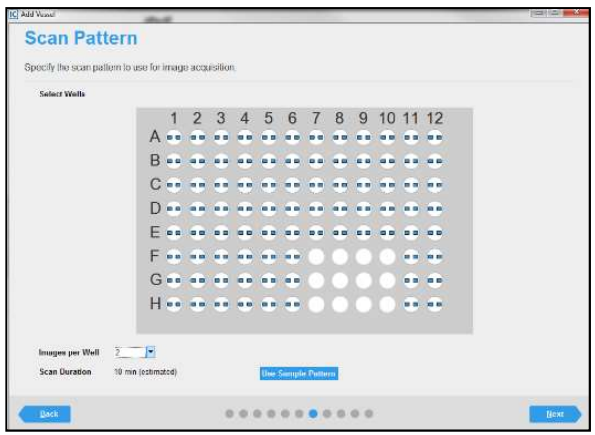
Select image channels and objective. Default acquisition times work for most applications.



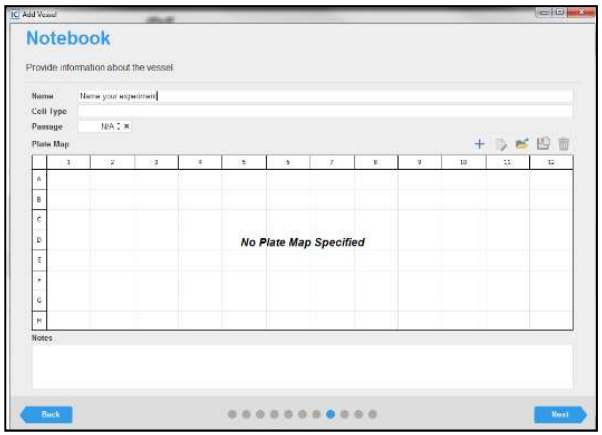
Search for plate by catalog # to ensure proper focusing and alignment of images



Physically place plate in instrument before selecting cutout in software. Warning will appear if someone else's plate is occupying your location of choice.



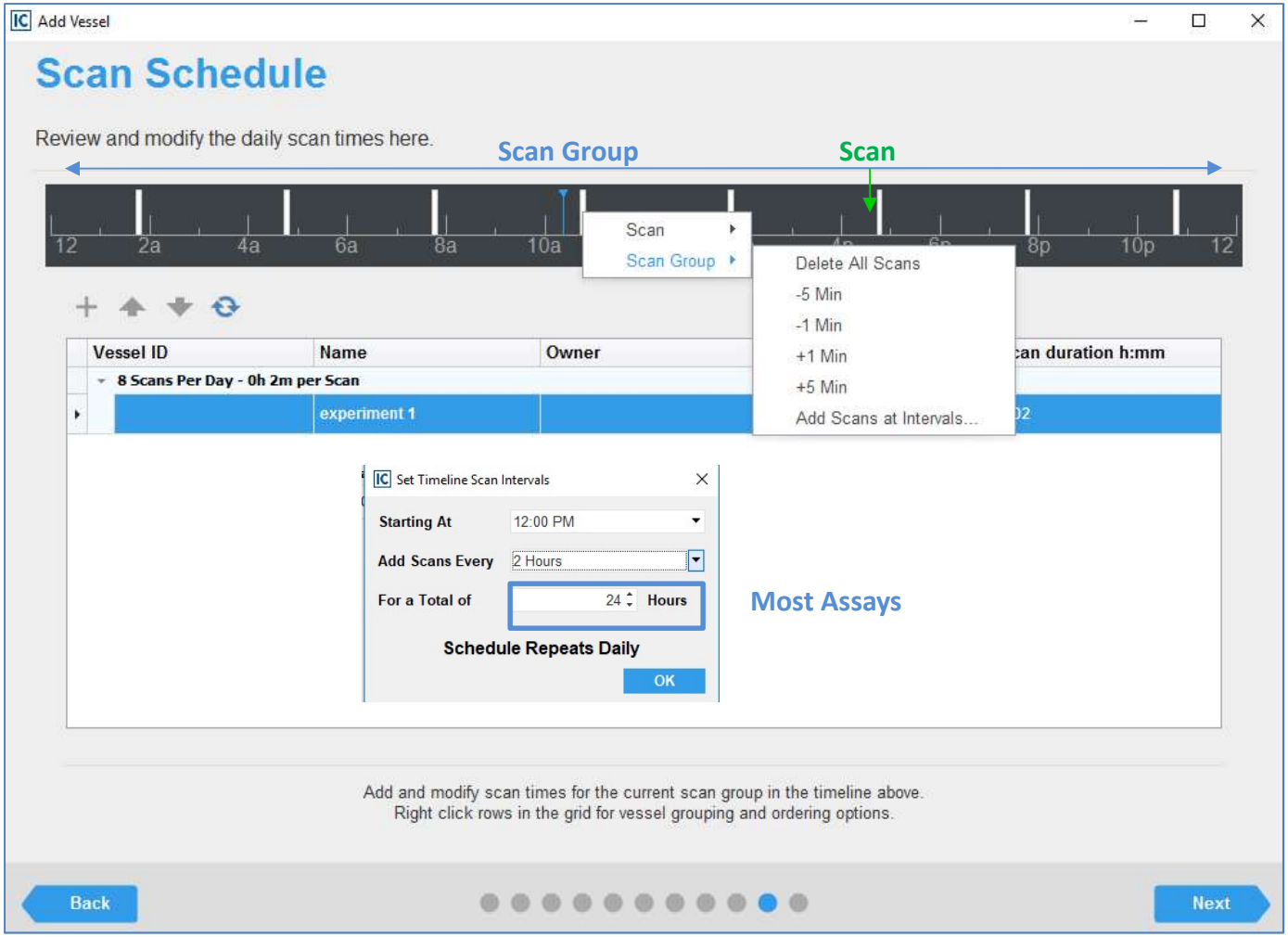
Do not scan empty wells. Consider the health of sensitive cells when deciding # of images per well and frequency of scanning.



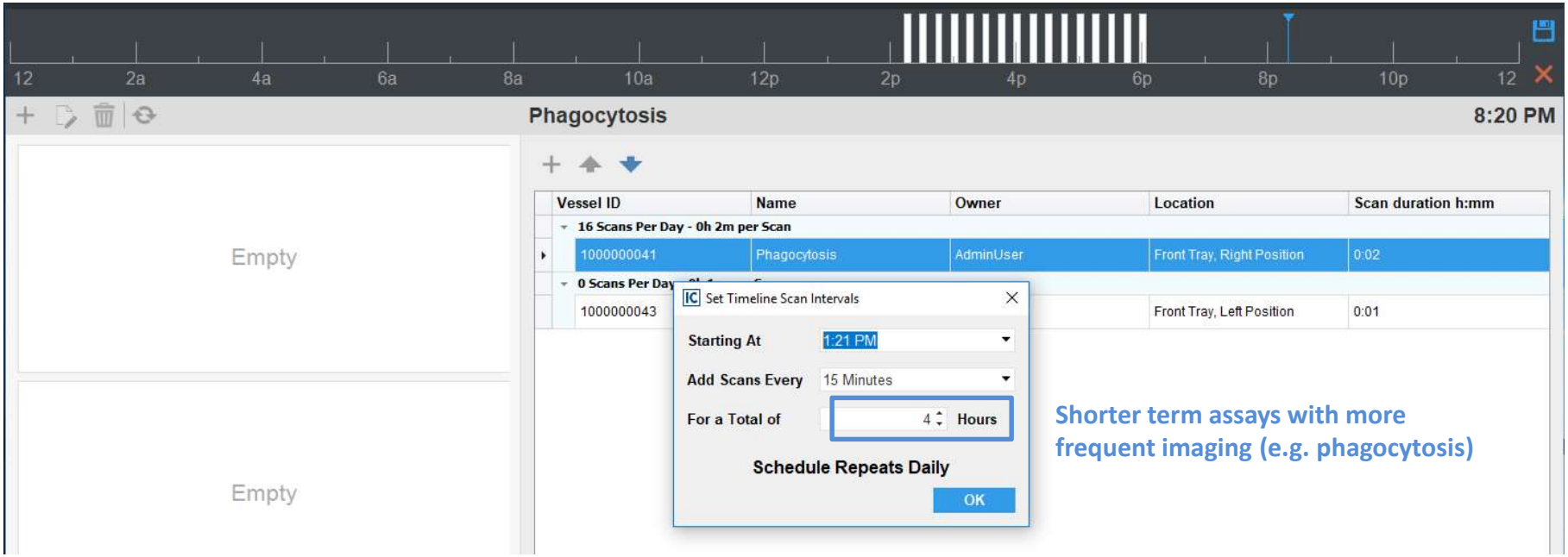
Include details about experiment including a platemap which can be mapped to data

Set the Scan Frequency

Set interval by right clicking in black timeline



Schedule Repeats Daily

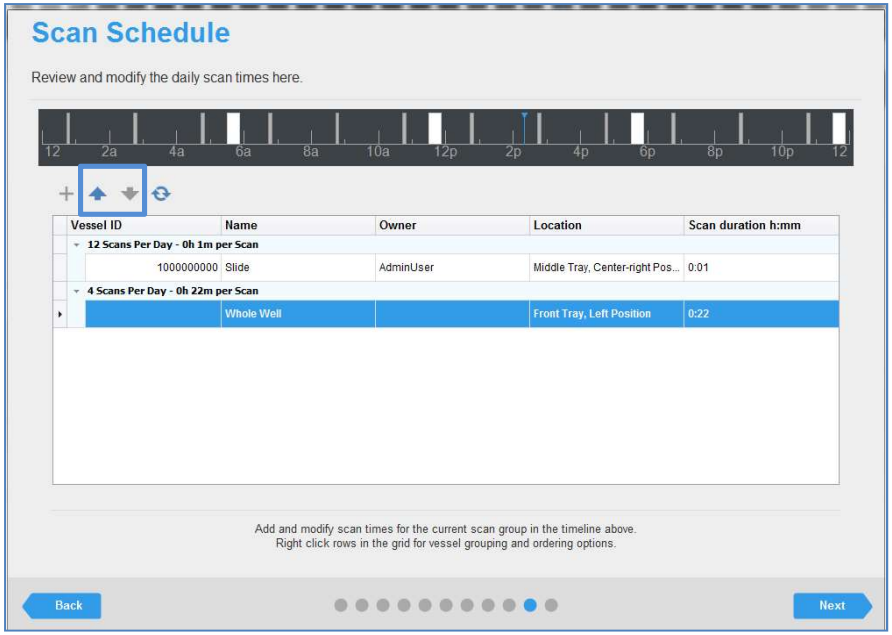


Shorter term assays with more frequent imaging (e.g. phagocytosis)

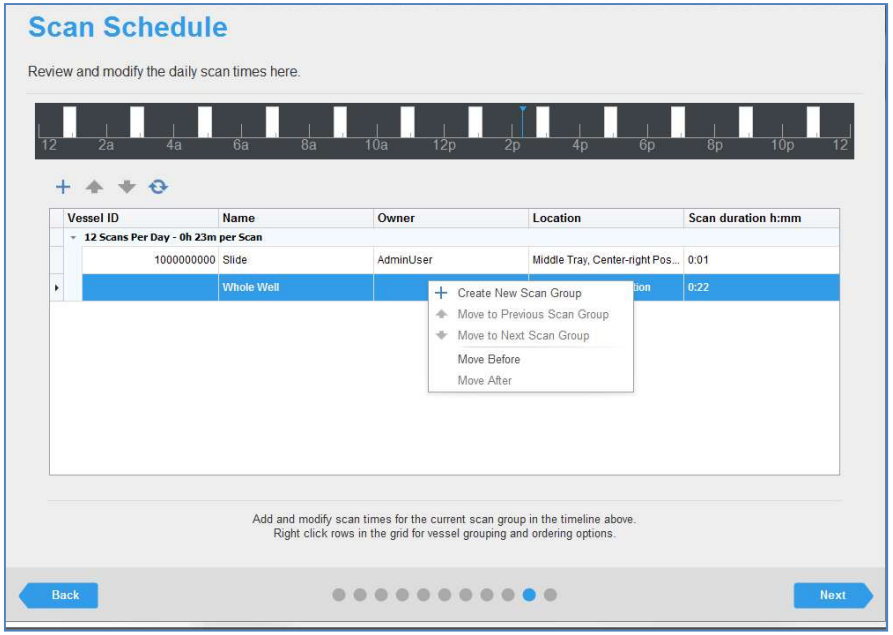
Adding Multiple Vessels

Additional Vessels Added

Set interval by right clicking in black area



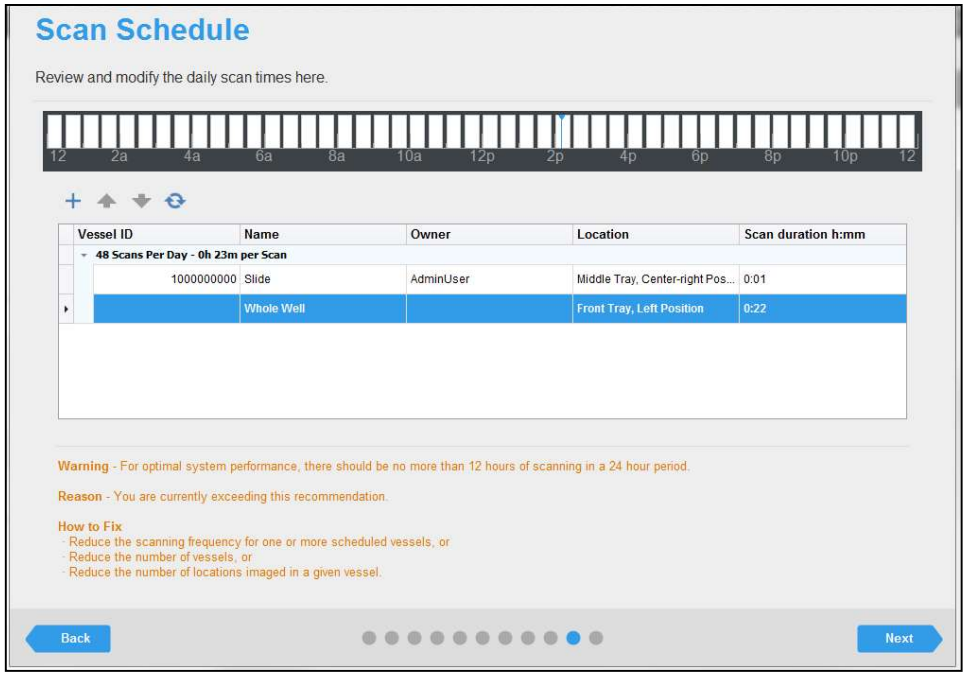
Add to another person's scan group by using the arrows



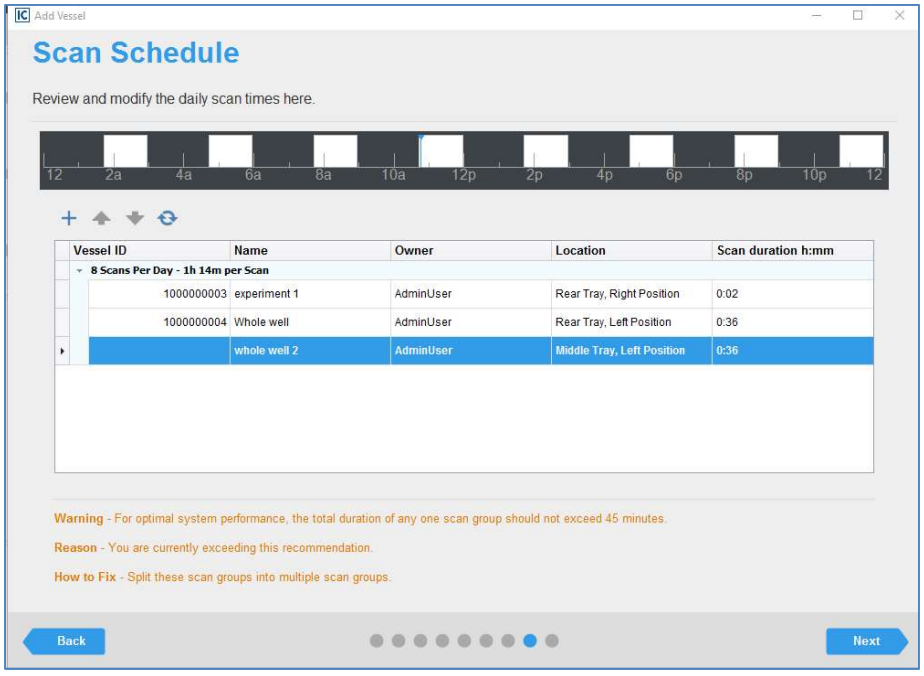
2 Heat Threshold Warnings

Maintain Cell Health with Optimal Temperature Conditions

For every 1 minute of scanning, there should be at least 1 minute of non-scanning



Scan groups should not exceed 45 minutes

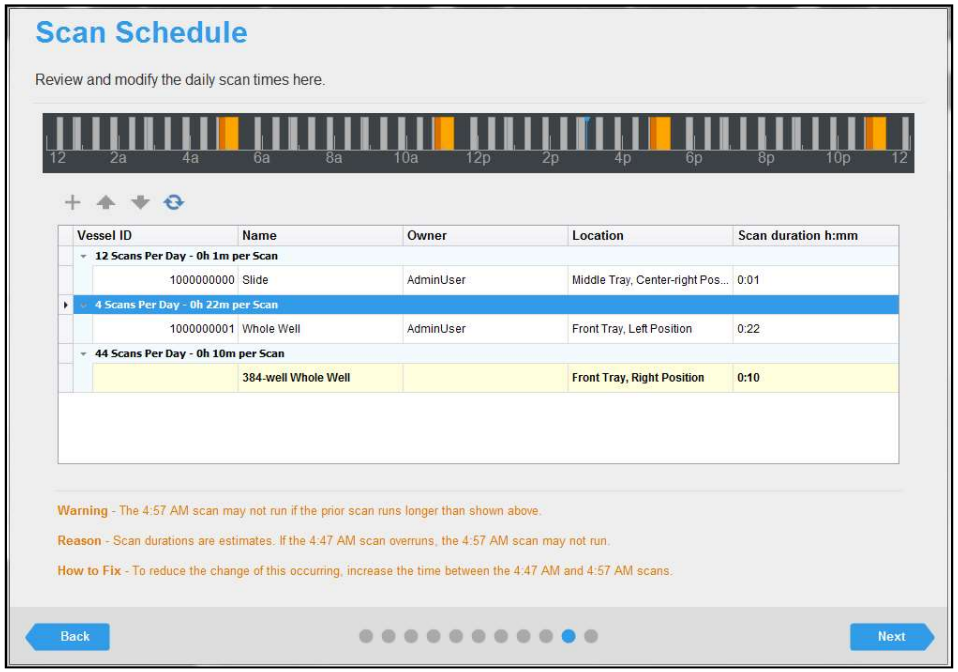


Recommendations in Orange
Do NOT Ignore

2 Scan Overlap/Duration Warnings

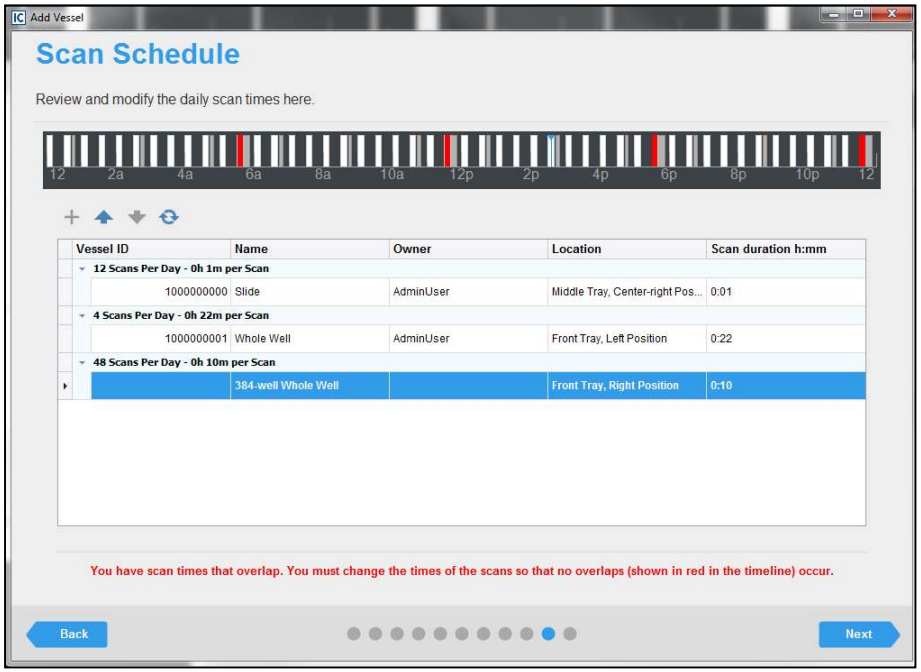
Avoid Conflicts in Scheduling

Scan Durations are Estimates



Scans Overlap

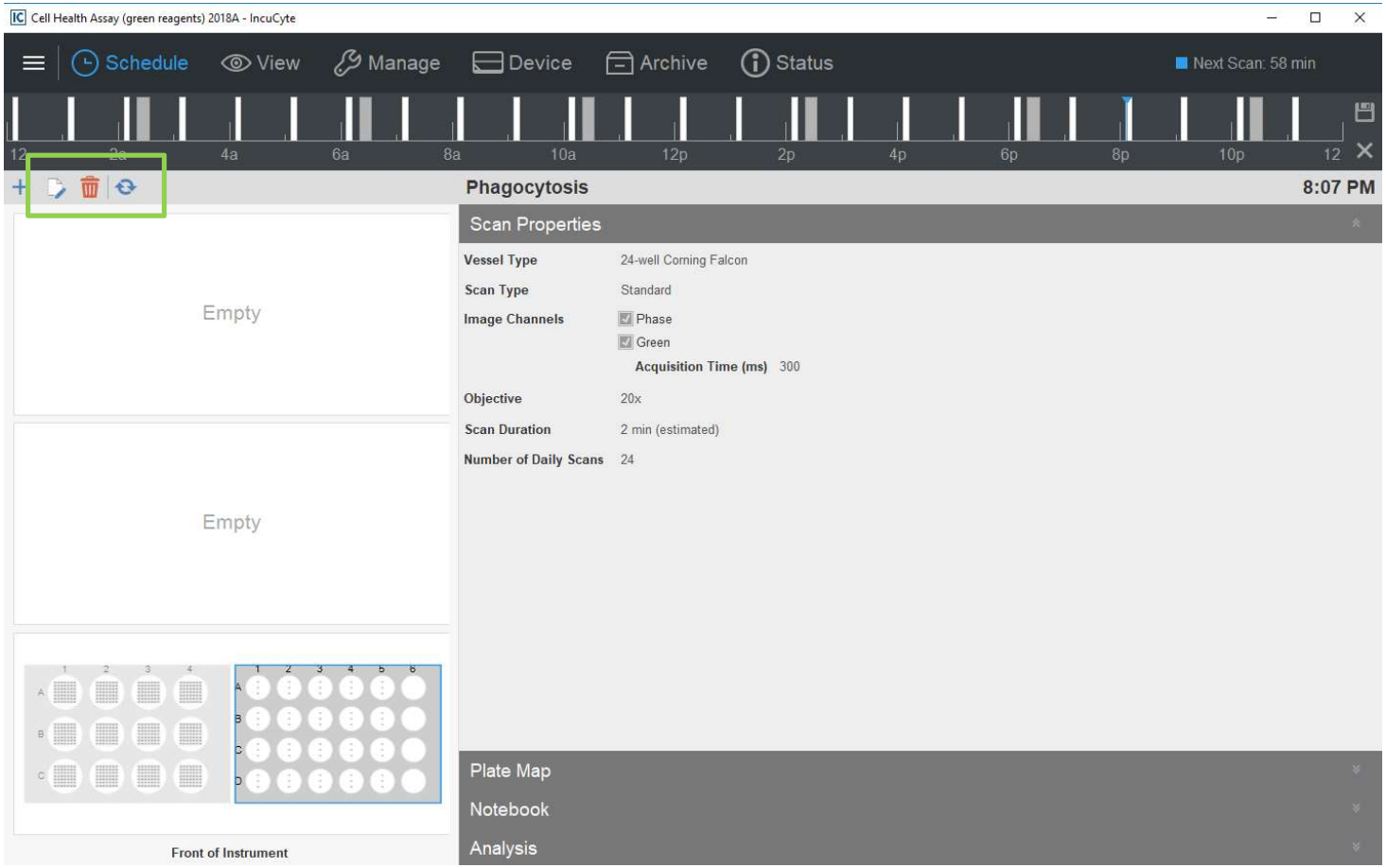
May occur with multiple scan intervals



Drag ‘red/orange’ bars to add more time in between scans or right click on individual scans to delete some time points

Will not let you ignore

Drawer Layout Summary

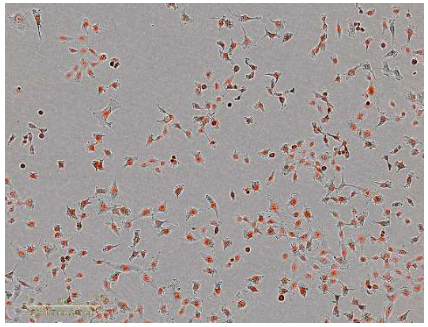


- Vessel selected in ‘blue’
    - View Scan Properties/Notebook/Analysis
    - Schedule is white (e.g. every hour; other plate in drawer is gray at every 4h)
- Edit or Remove Vessels from the schedule

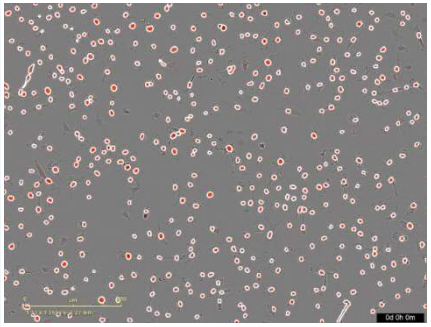
Vessel View

Acquire Images

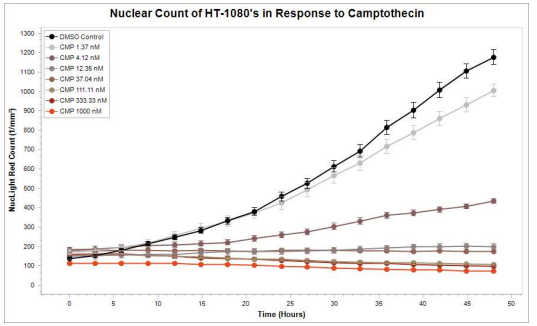
View Images



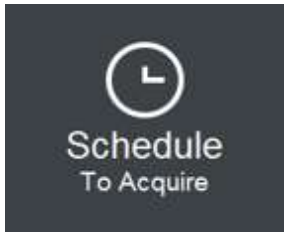
Process Images



Analyze Images

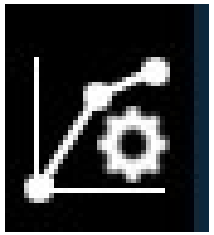


Schedule Scans

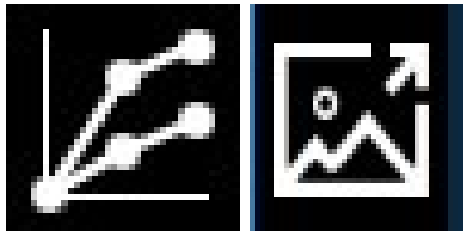


Vessel View

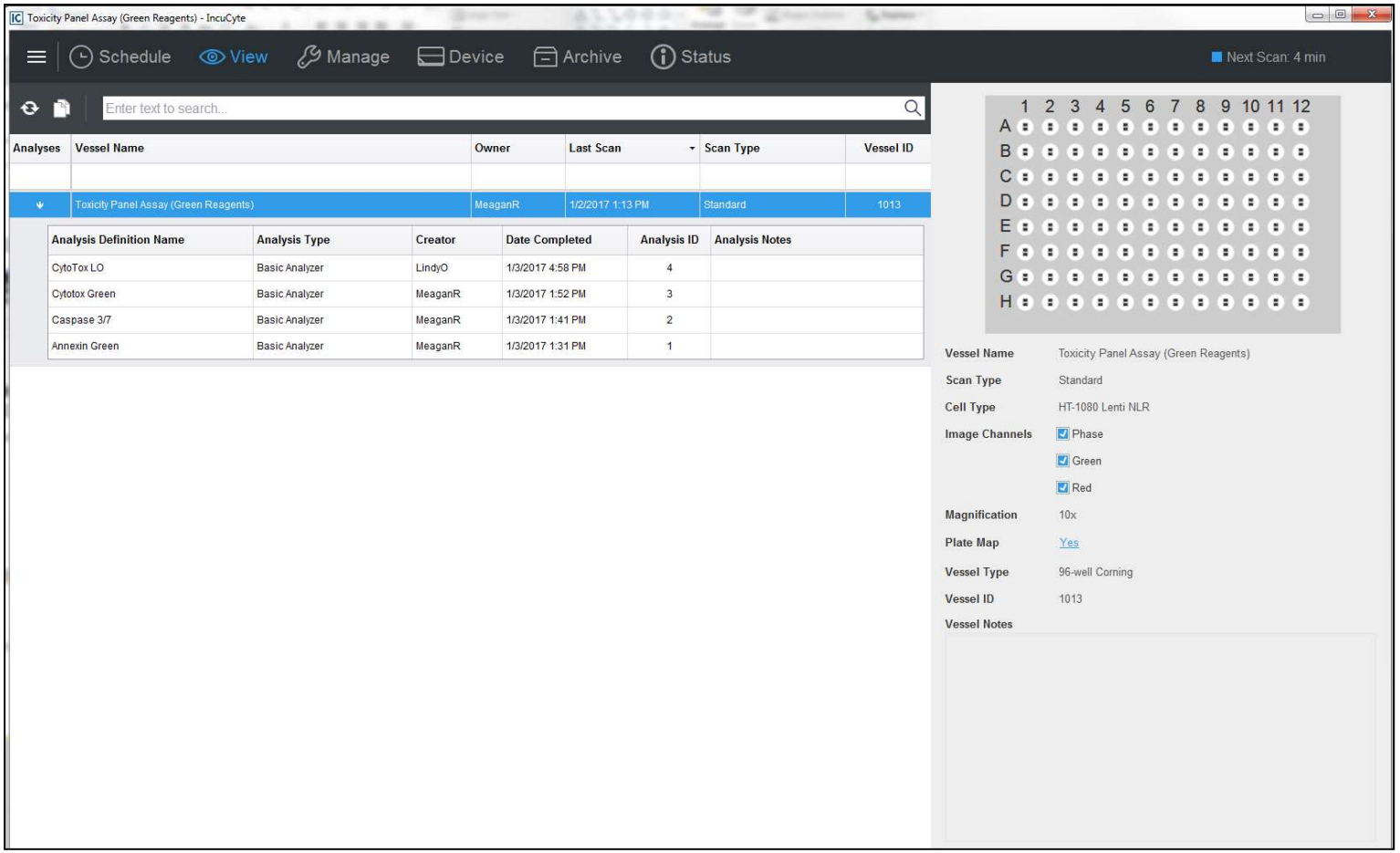
Analysis Definition



Graphing and Exporting

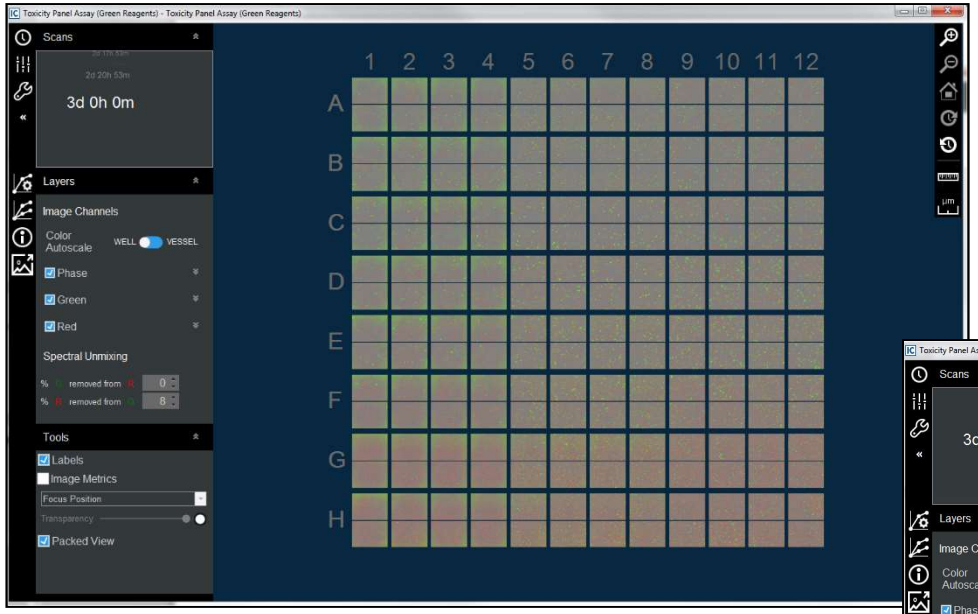


Search for Experiments by Label/user/etc.



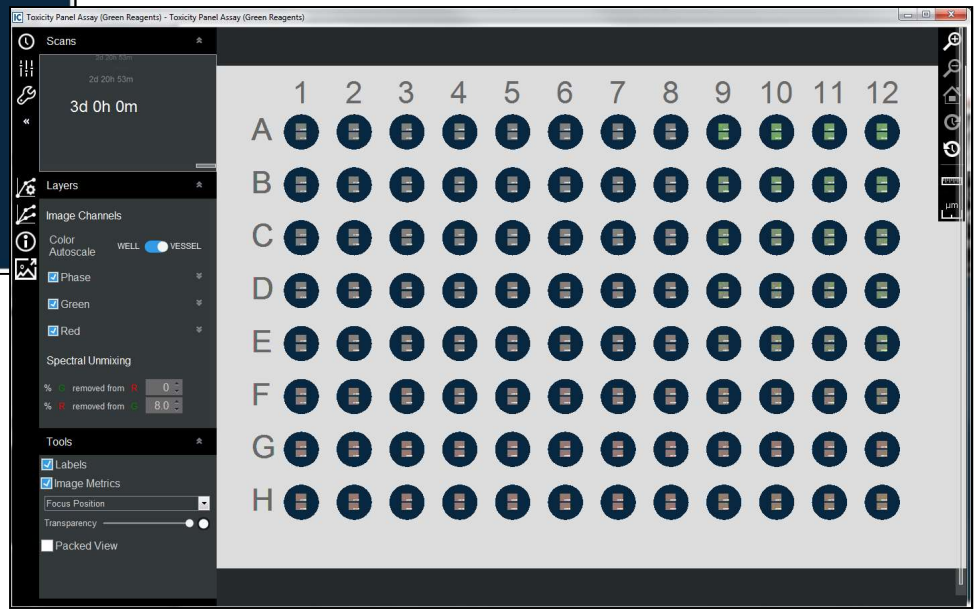
Vessel (Image) Display

Packed View



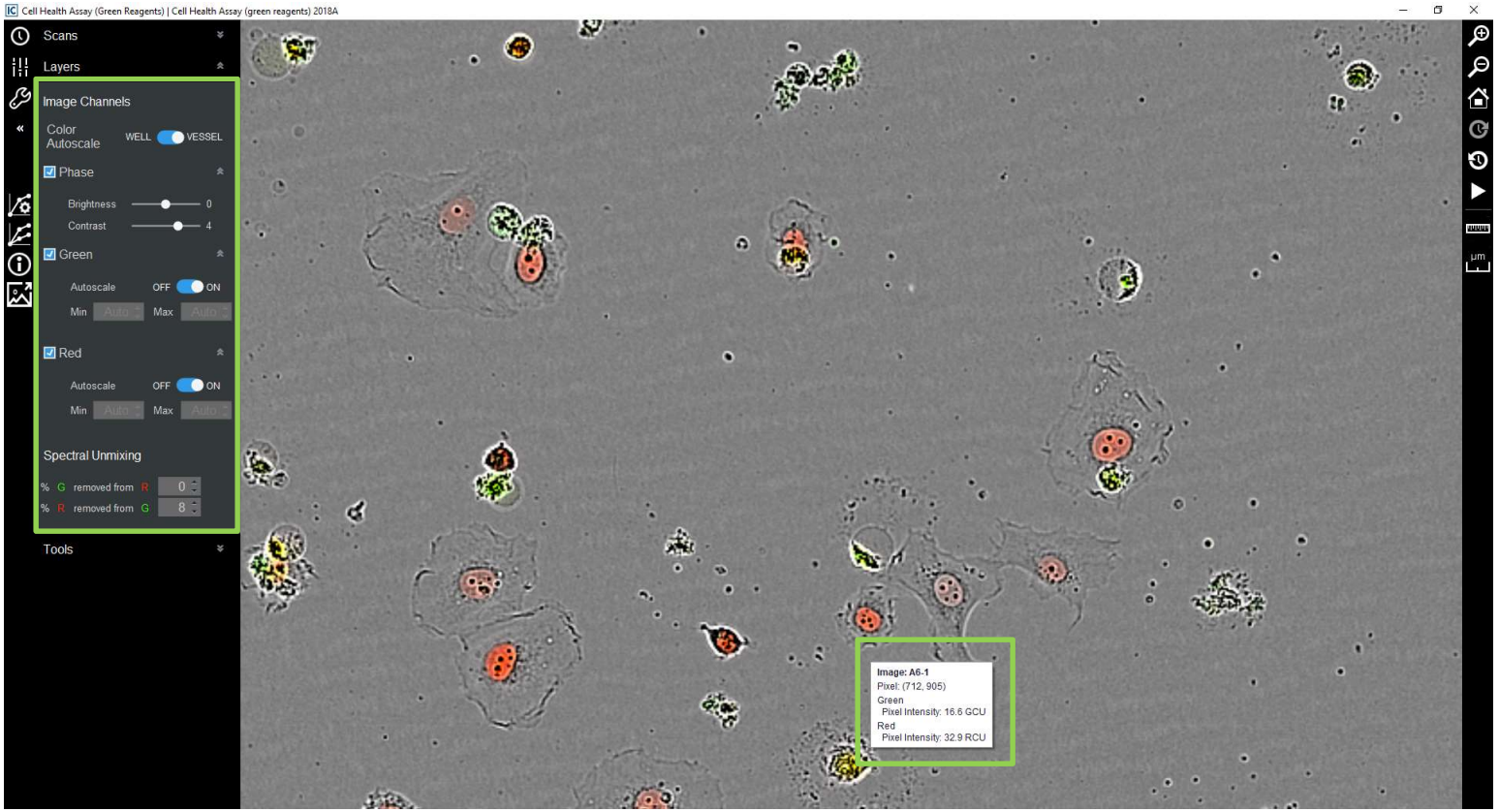
Quickly assess trends in data in default 'Packed View'

Unpacked View



Visualize location of images in the well

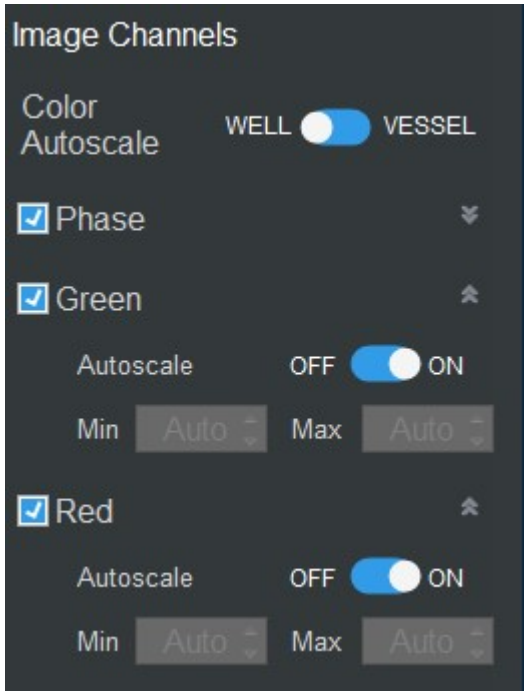
View and Interact with Images



- **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 GCU (Green Calibrated Unit) and RCU (Red Calibrated Unit). Hover over any pixel to find these values.
- **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

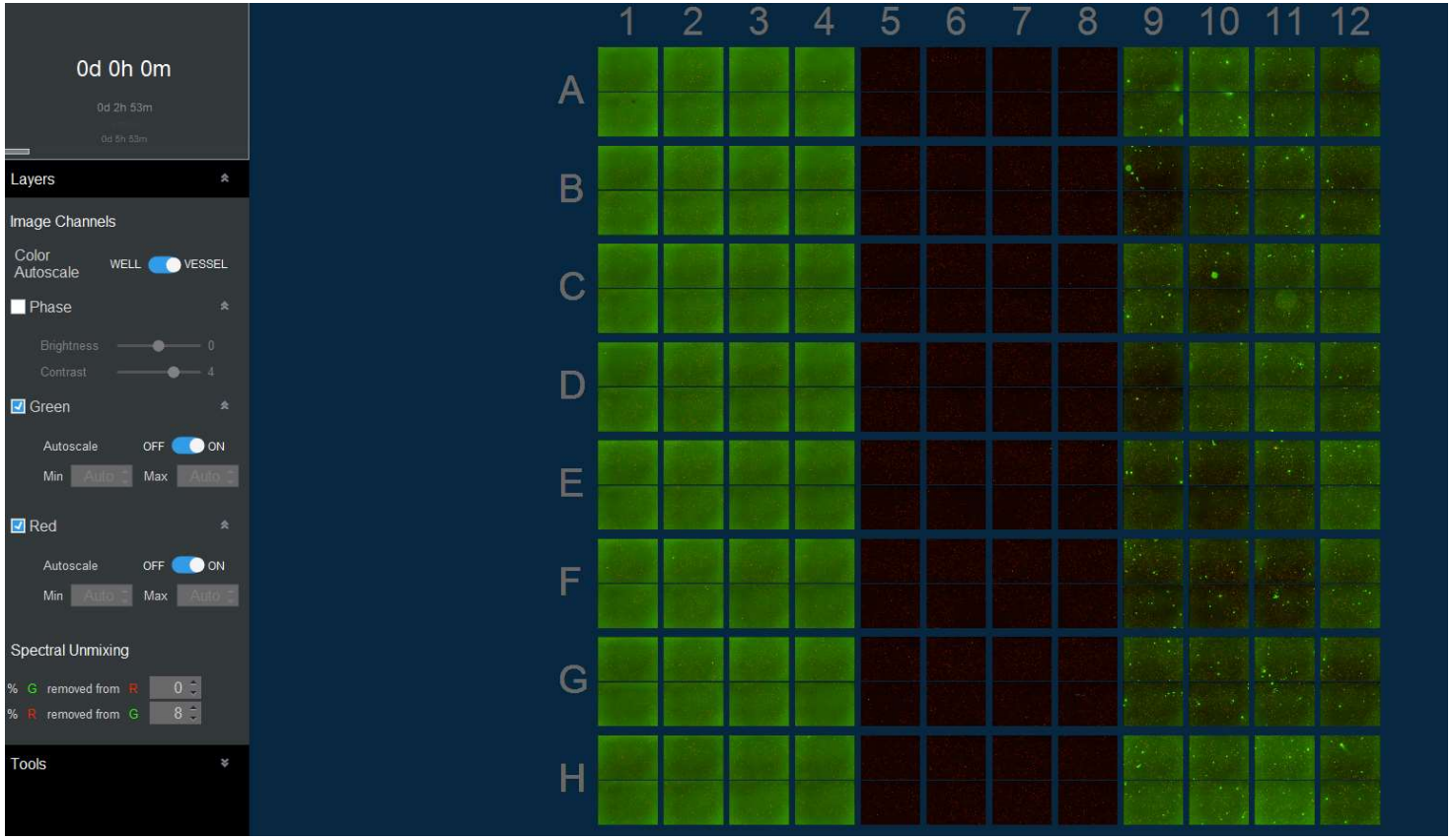
Autoscale

- **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



Vessel – Autoscale (default)

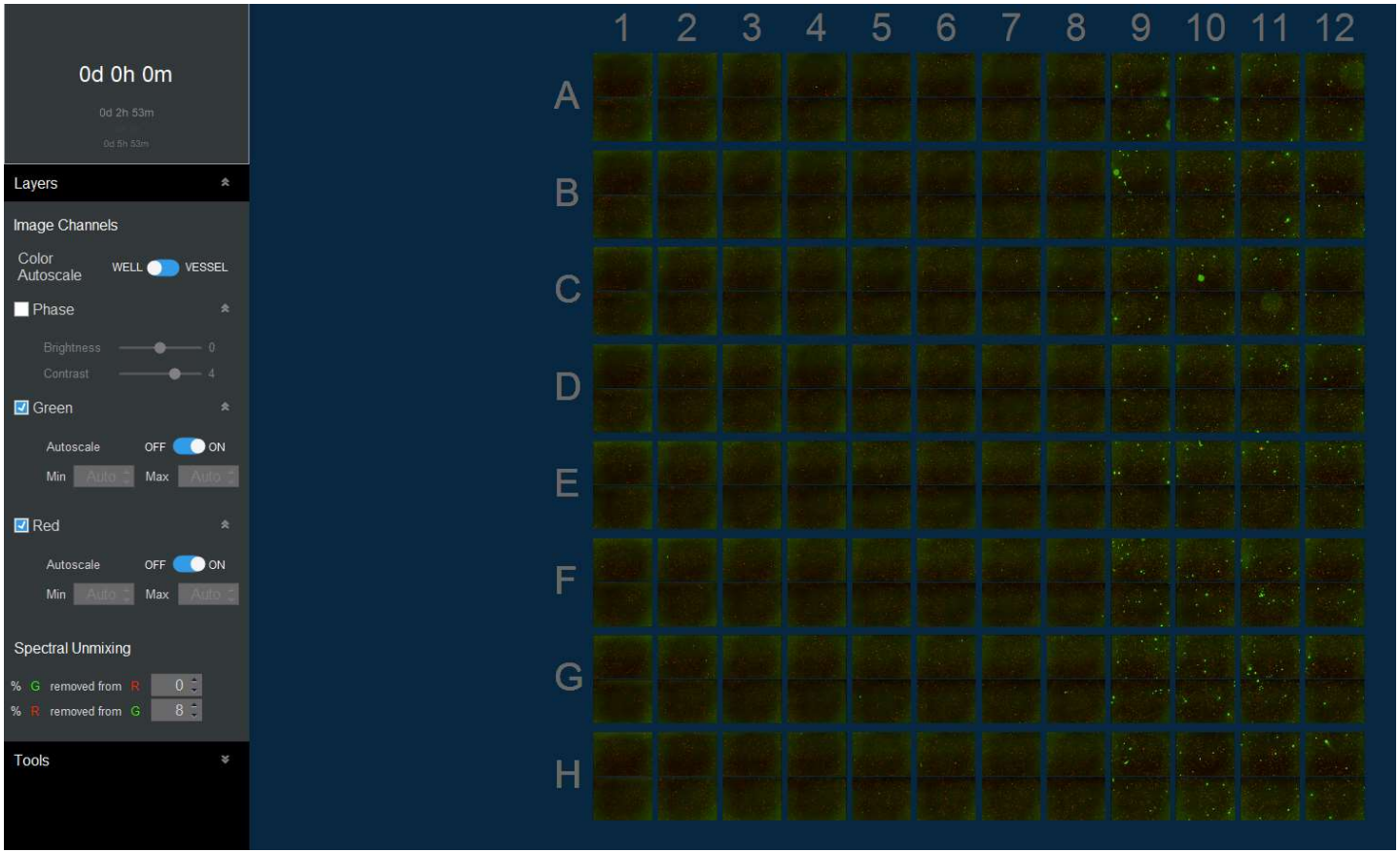
Applies an autoscale across entire vessel based on min/max brightness across entire plate



Ideal for experiments with the same reagent. In this experiment, 3 sections of the plate were treated with different reagents of different fluorescent intensity; therefore, vessel autoscale is not recommended

Well – Autoscale

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

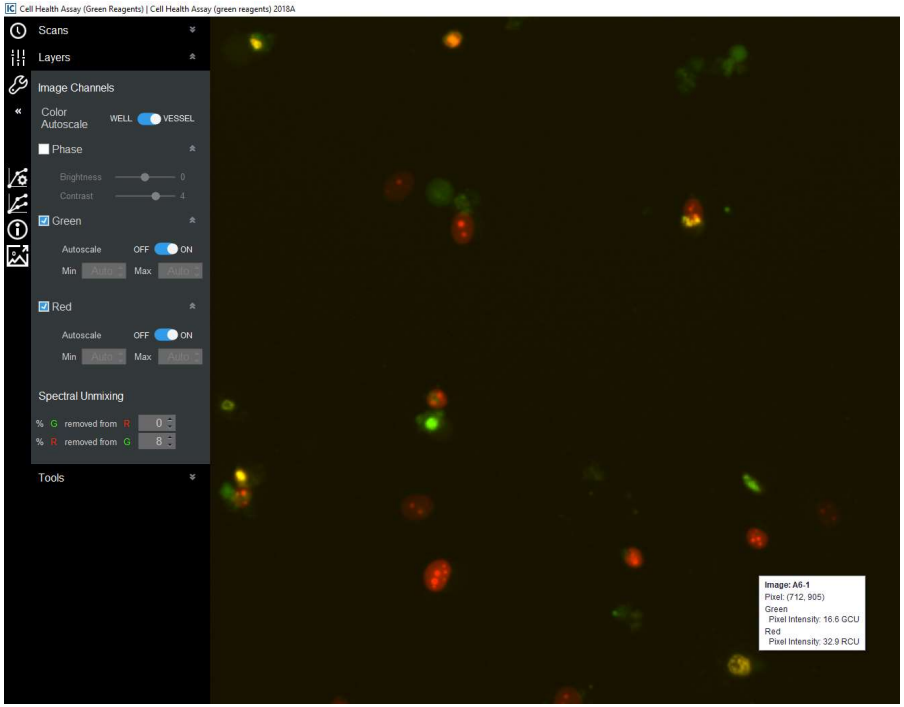


Ideal for experiments with different reagents and to determine the baseline intensities of individual wells. In this example, well autoscale is recommended

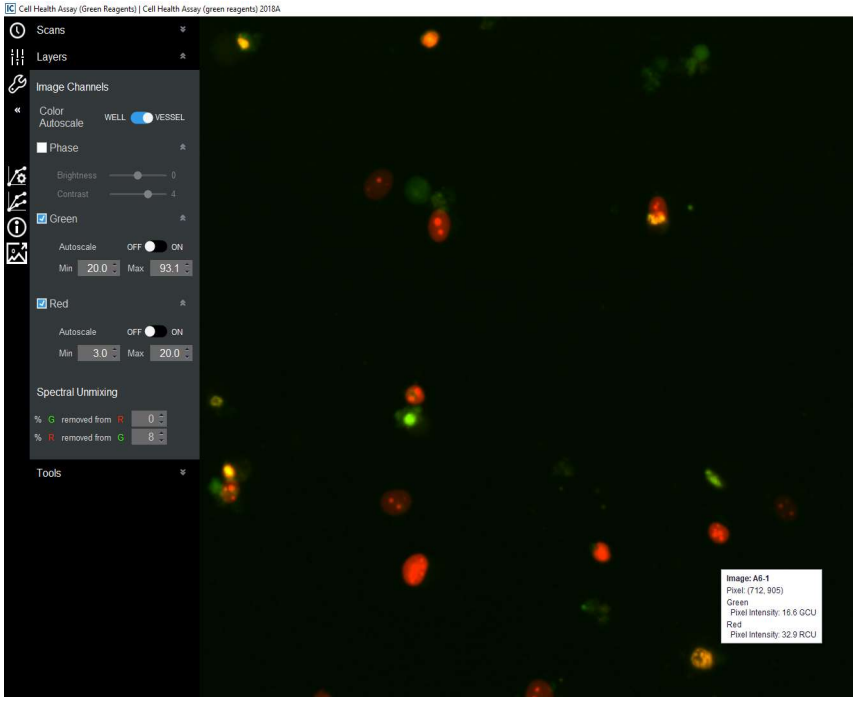
Turn off Autoscale (recommended)

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



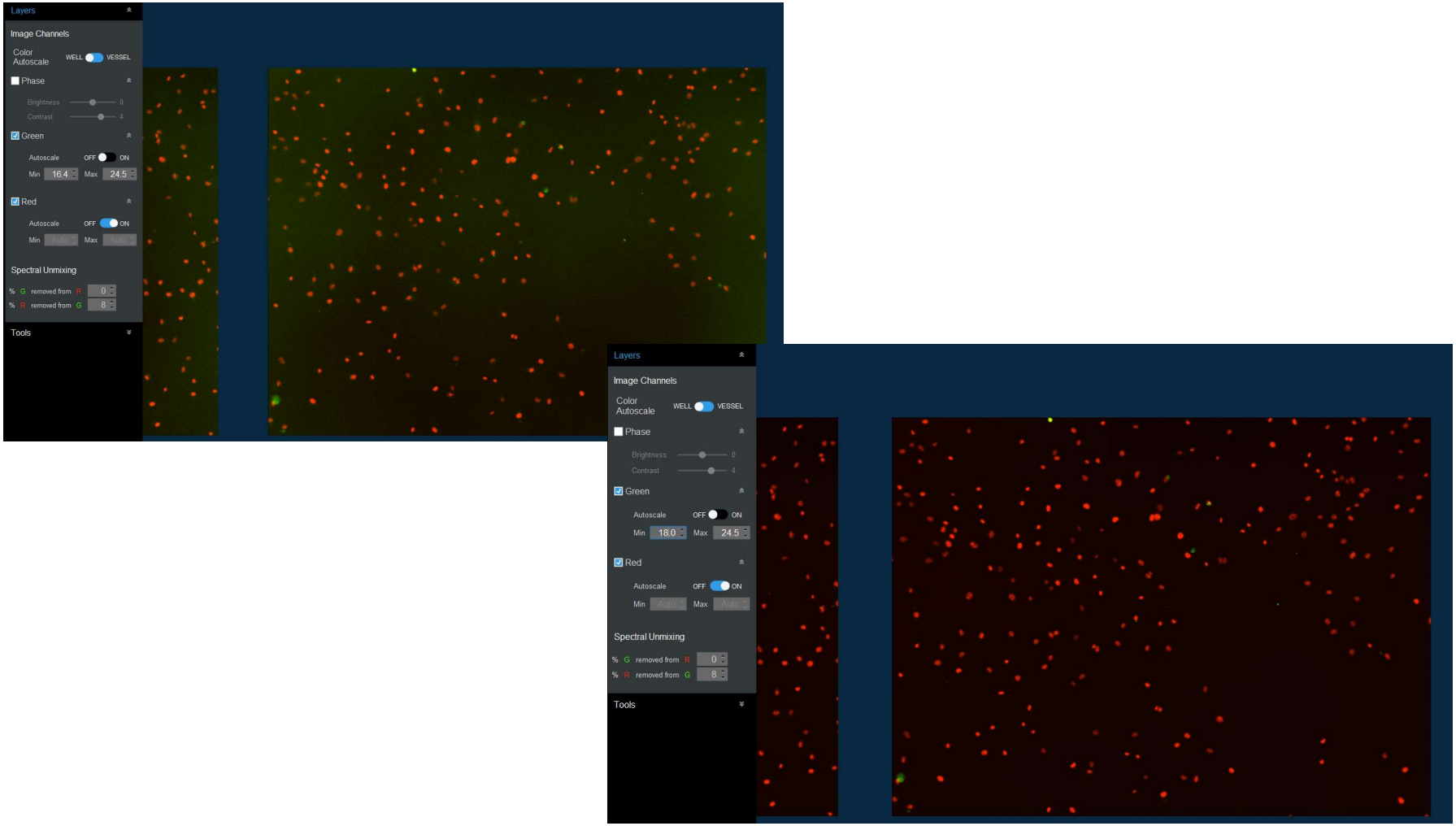
Manually adjusted min/max settings



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.

How would you adjust the min or max settings in this image for the green channel to remove background?

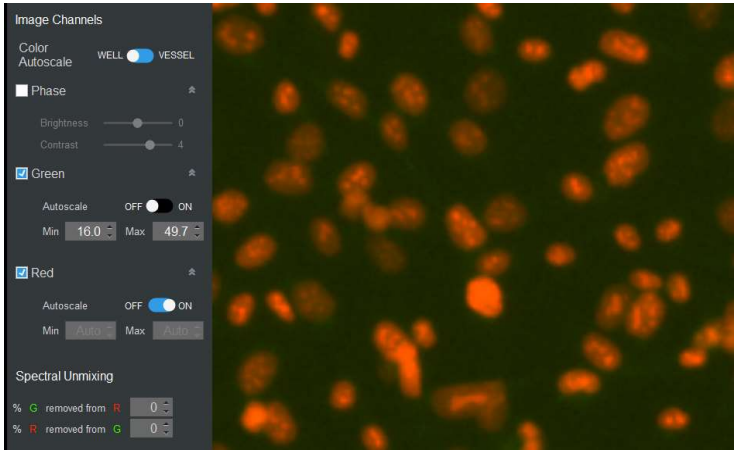


Spectral Unmixing

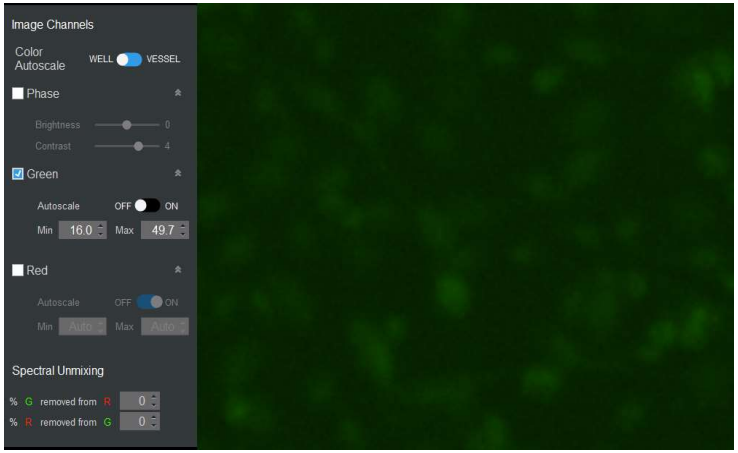
- **Some fluorophores may be excited by the opposite channel's LED (e.g. a red fluorophore may show up with a dim green signal)**
- **Determine spectral unmixing for your fluorophore only the first time you run the experiment and apply to all future experiments**
  - Set-up an experiment with only one fluorophore per well
  - Image the well in both channels

Example of how to adjust spectral unmixing

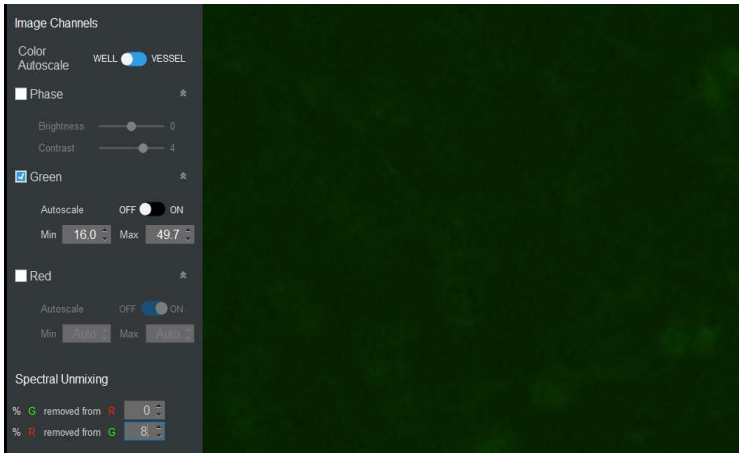
Red only fluorophore – imaged in both red and green channel



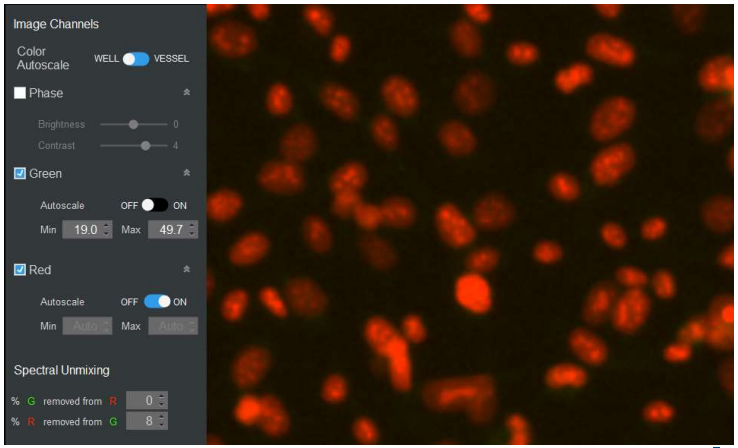
Turn off the Red channel and determine if there is any green signal



Increase the %R removed from G until there is little/no green signal left



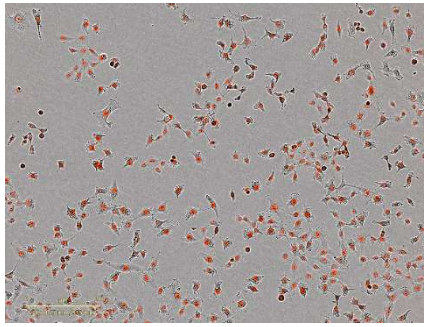
Adjust the min/max intensities to remove remaining green background



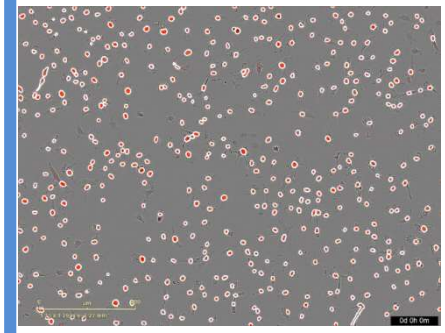
Create an Analysis Definition

Acquire Images

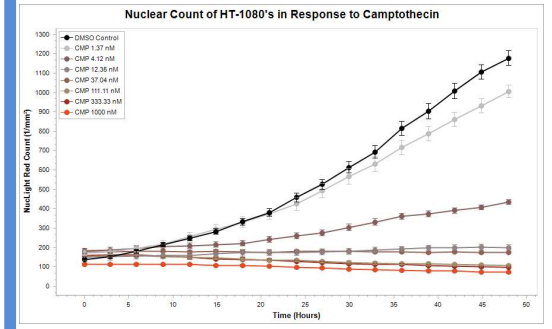
View Images



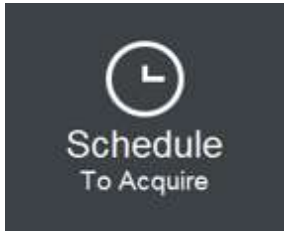
Process Images



Analyze Images

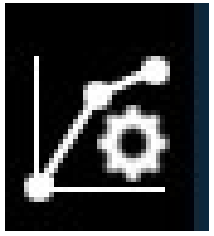


Schedule Scans

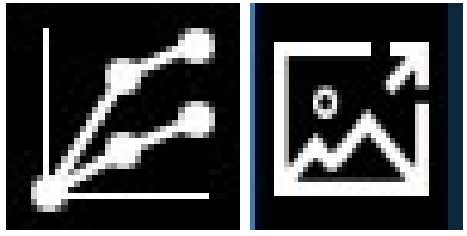


Vessel View

Analysis Definition



Graphing and Exporting

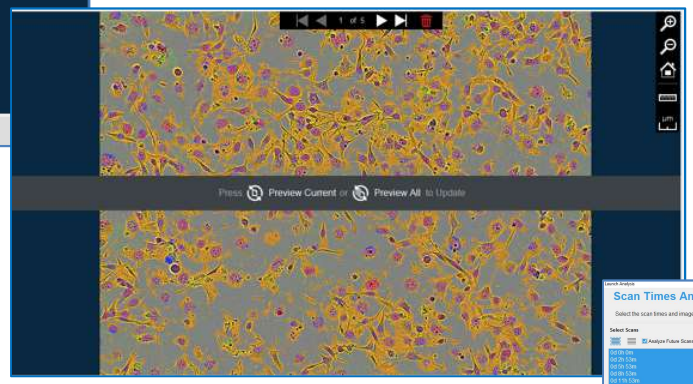


3 steps to run an Analysis on the IncuCyte S3

1. Select representative images

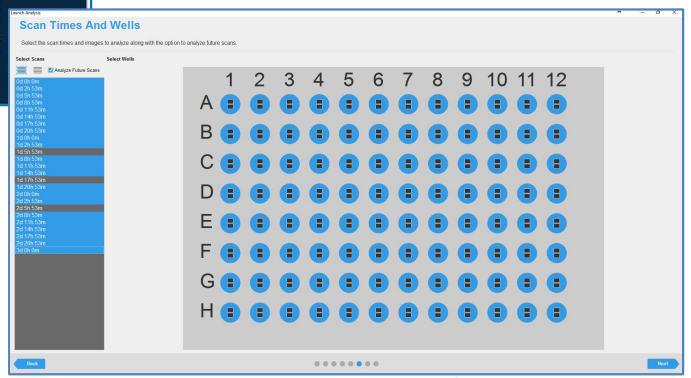
Include images from beginning/end, untreated/treated, dim/bright

2. Preview Analysis Definition



Adjust parameters to create mask

3. Select Time Points/Wells



Run analysis definition on all images/time points of choice

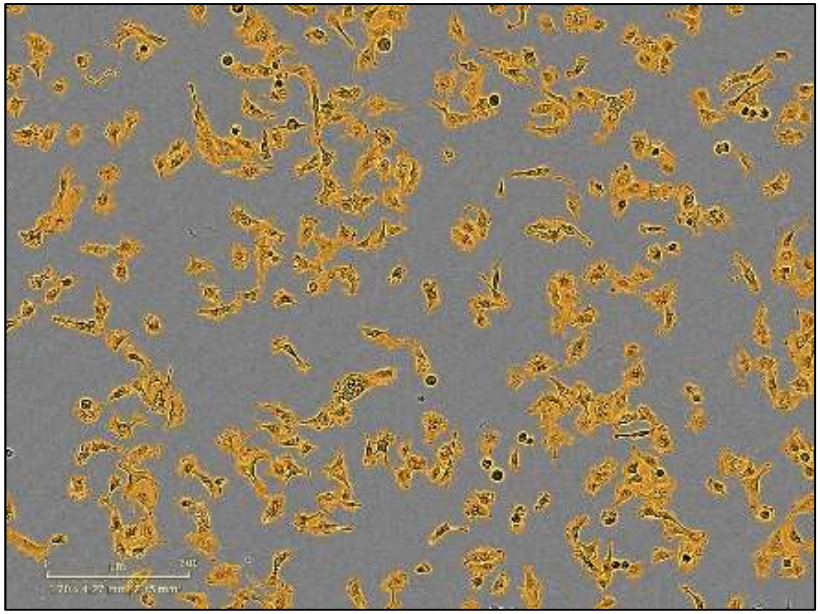
Selection of representative images

- **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)**
  - Different size and density
  - Different background
- **Fluorescence**
  - Different intensity after correct setting of Min/Max
  - Different background

Define Masks and use them for analyses

What is a mask?

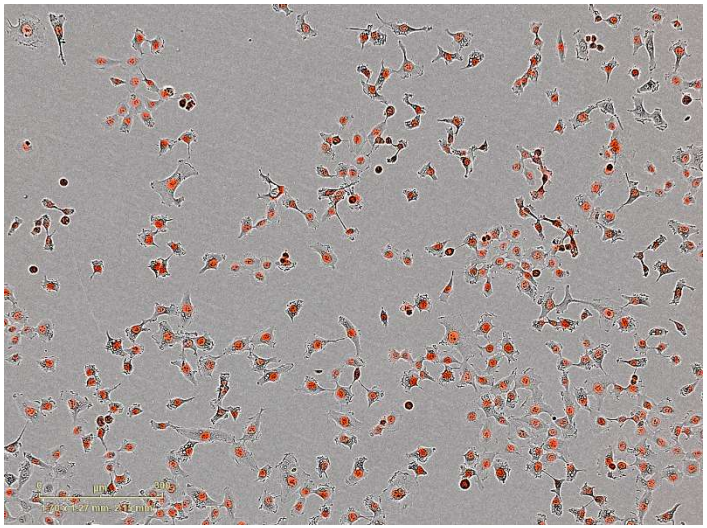
- Indication of areas of interest (cells) within an image
- Define which pixels in image correspond to cells (by eye)



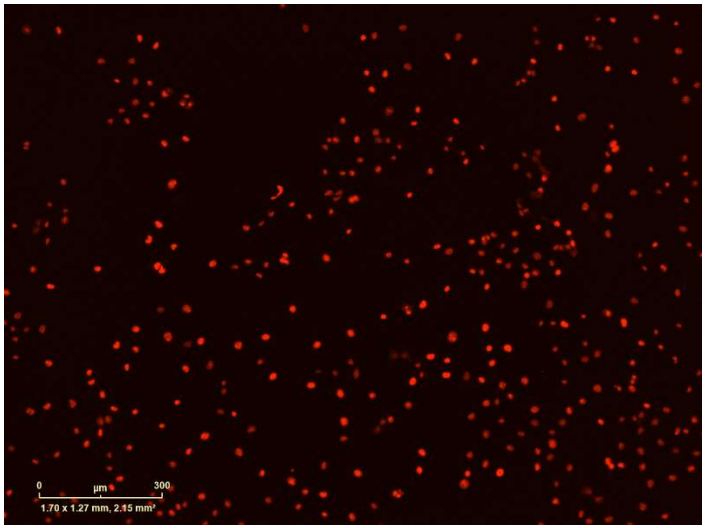
The pseudo yellow color in this image indicates what phase areas of the image are of interest (cells) and should be quantified in the data analysis.

Different Masks of the same Image

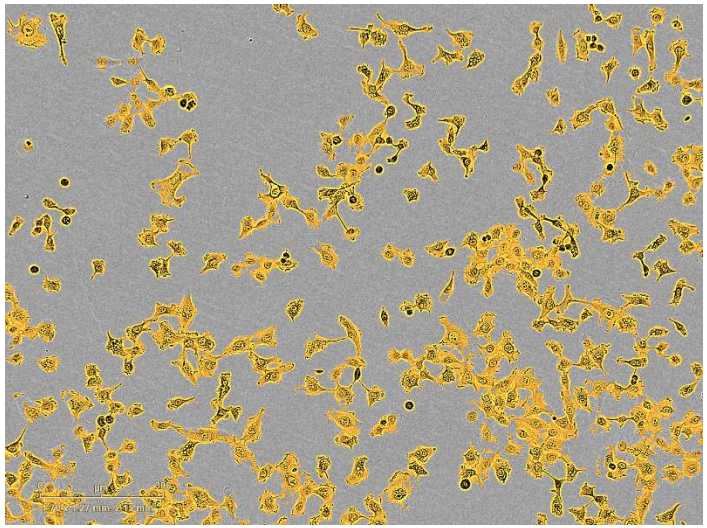
Phase and Red Blend Image – No Mask



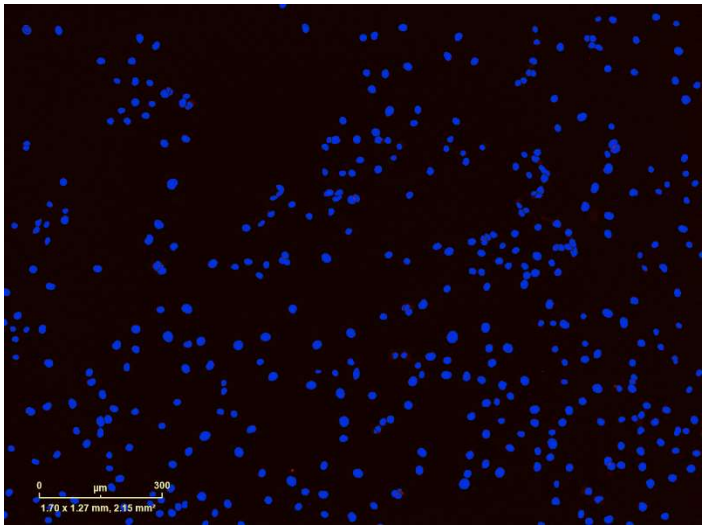
Red Image – No Mask



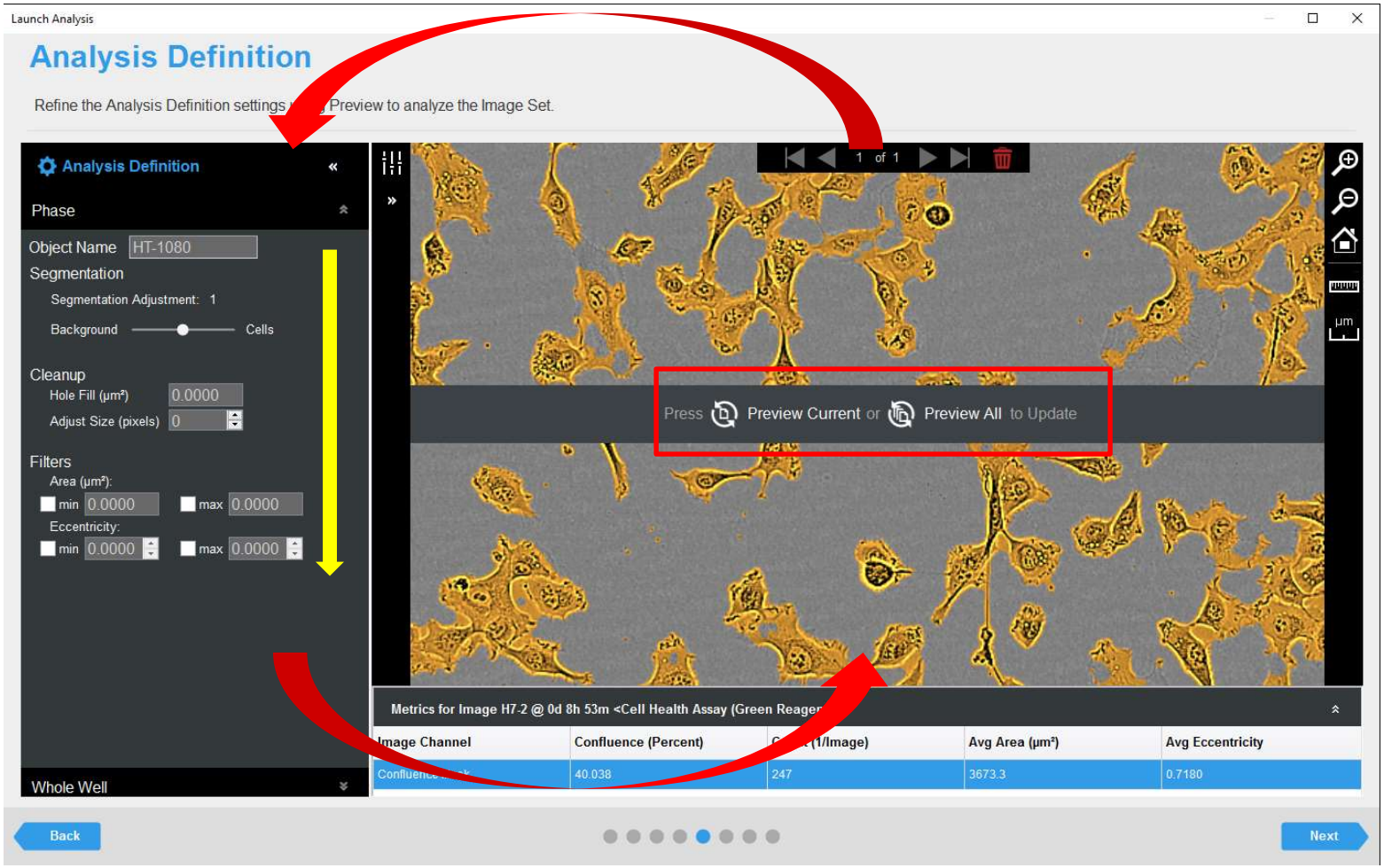
Phase Image – Blend Phase Mask (yellow)



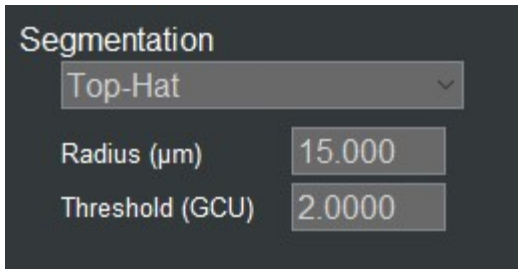
Red Image – Overlay Red Mask (blue)



Analysis definition



Top-Hat background subtraction for fluorescence

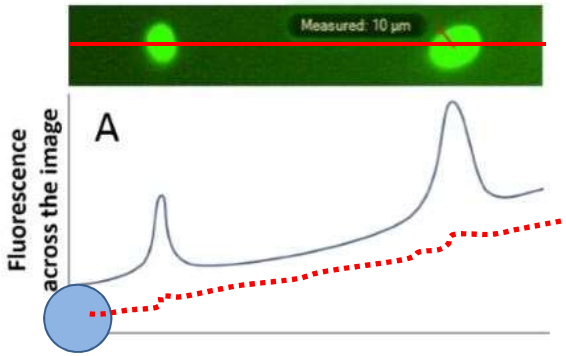


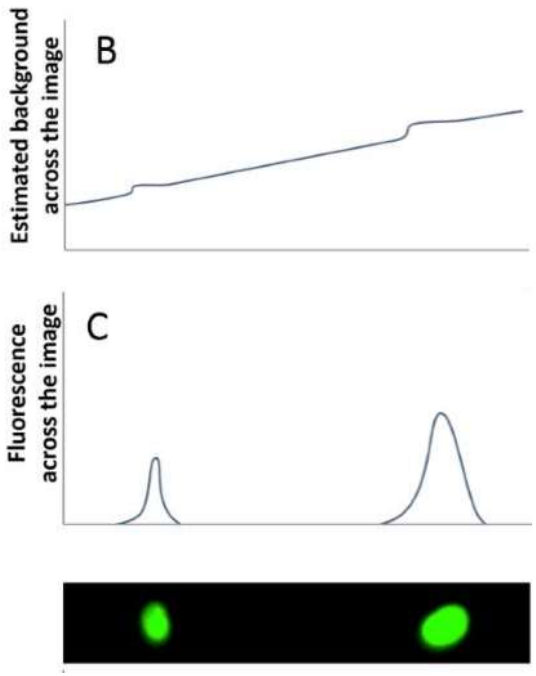
Top-hat:

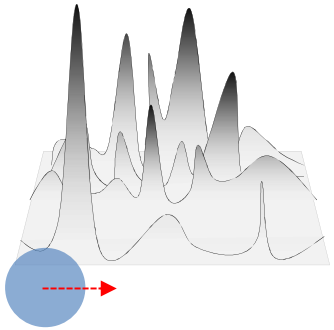
- radius larger than the one of the largest object of interest
- Separation into 2 components: background and real signal

Threshold applied to include only real signal in the mask

How does it work:



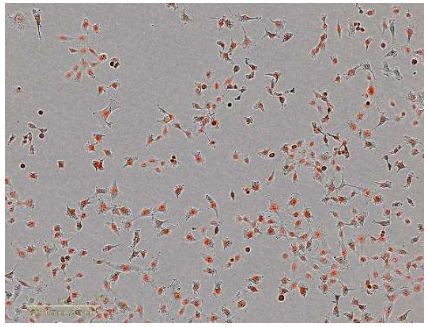




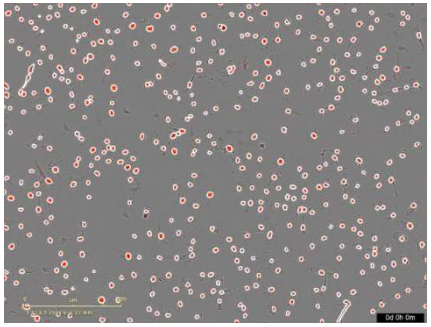
Analyze Images

Acquire Images

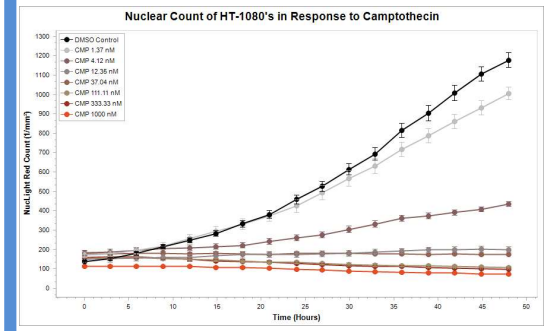
View Images



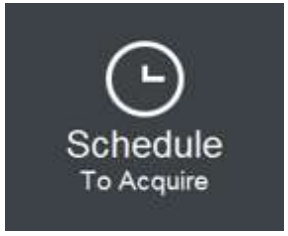
Process Images



Analyze Images

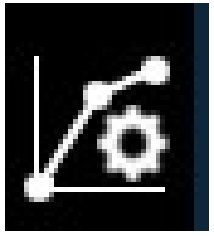


Schedule Scans

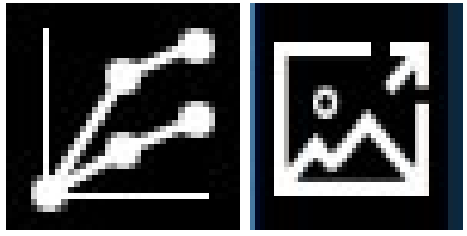


Vessel View

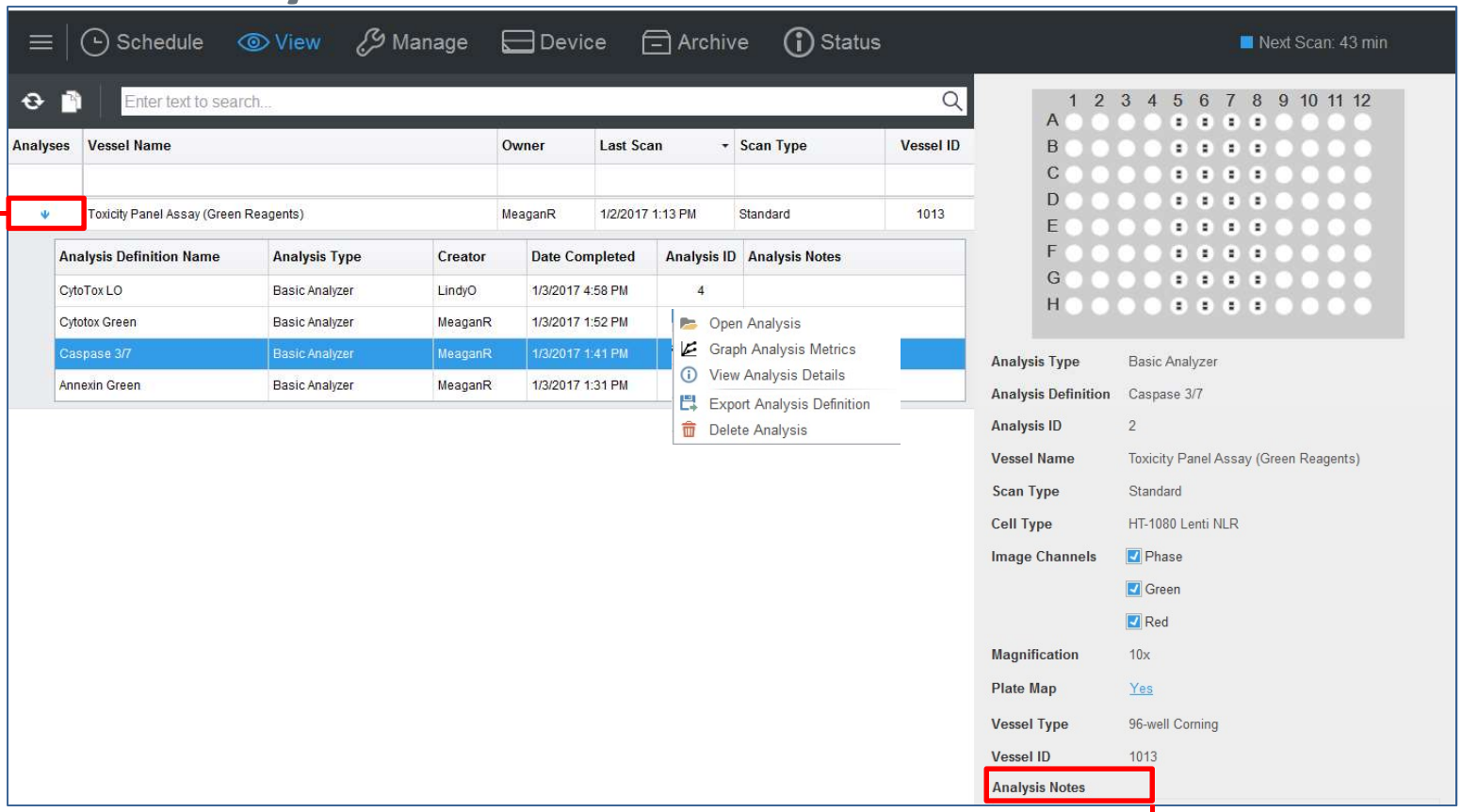
Analysis Definition



Graphing and Exporting



Search Analyses



Analyses associated with vessel indicated by blue arrow

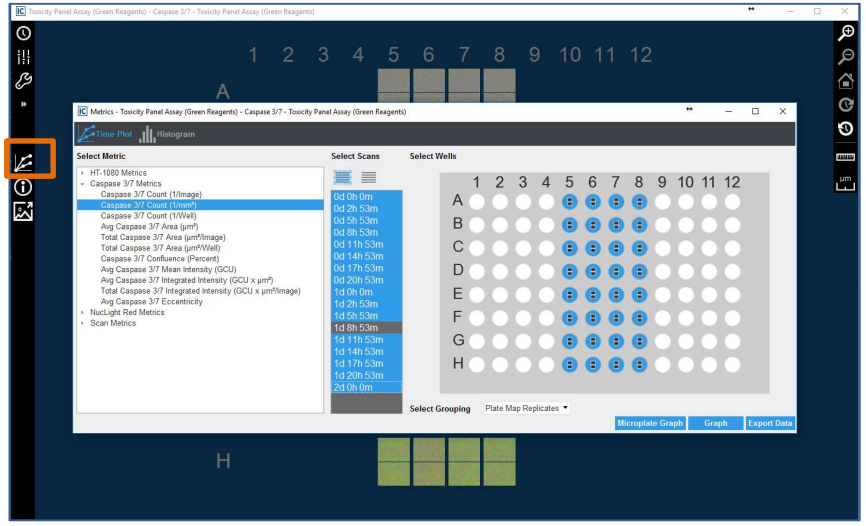
- Analyses saved by Analysis Definition Name
- Right click on Analysis Definition to view details
- Directly go to graphing window

Analysis Notes specify details of analysis definition

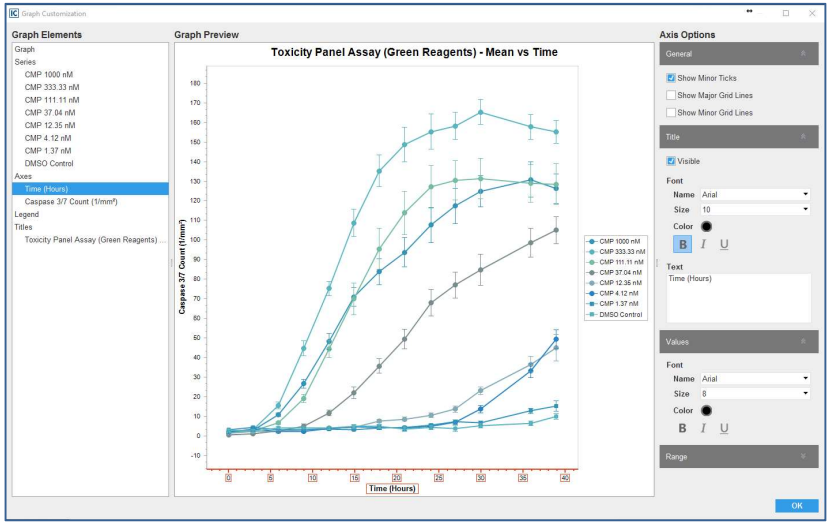
Details in the right pane show wells/image channels analyzed

Graph – View/Export

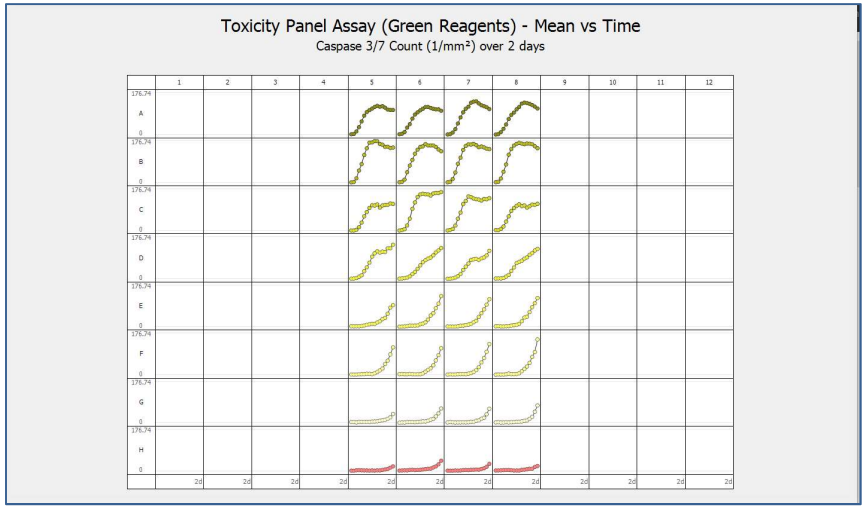
Select wells/time points/metric/grouping



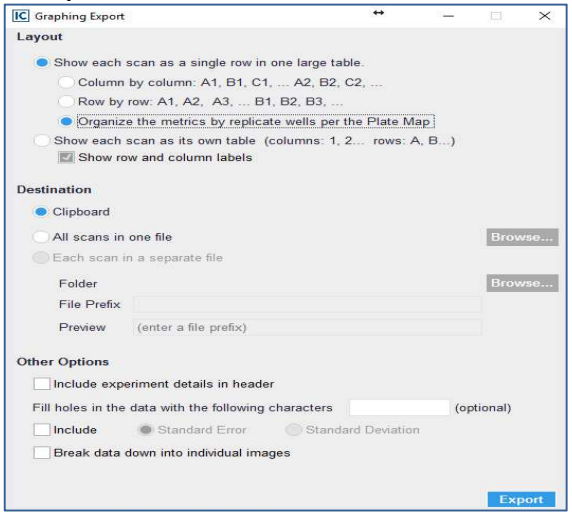
Tools – Customize Chart to edit colors/layout



Microplate Graph

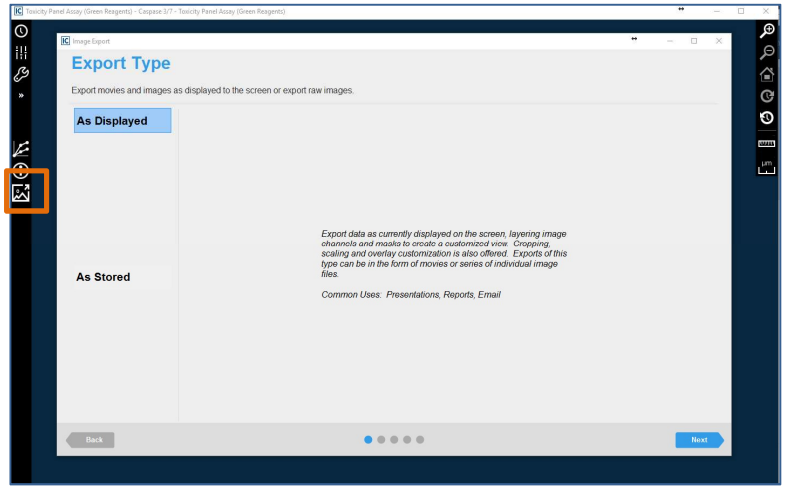


Export Data

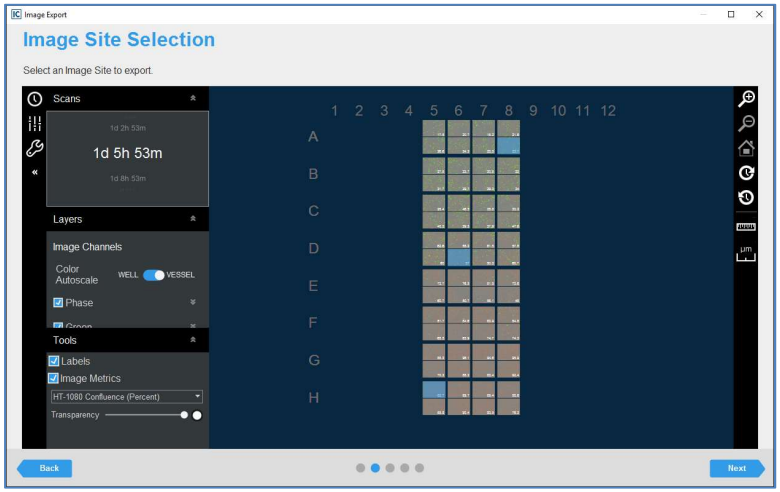


Select/Edit Images & Time Points

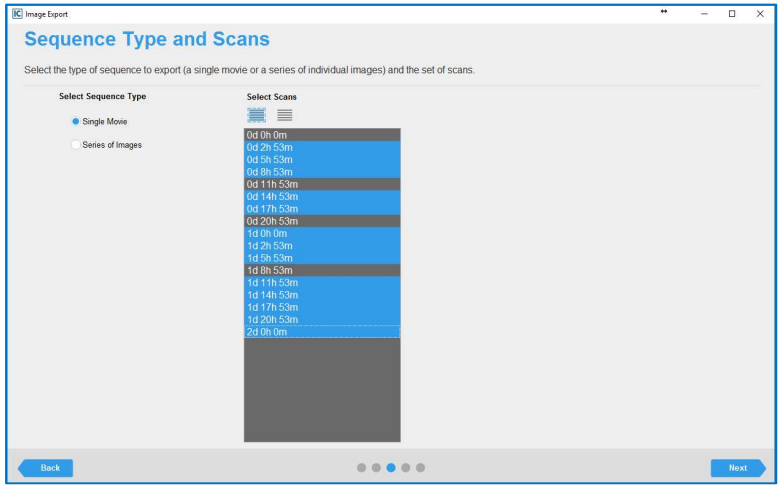
Enter guided interface from current image/time point



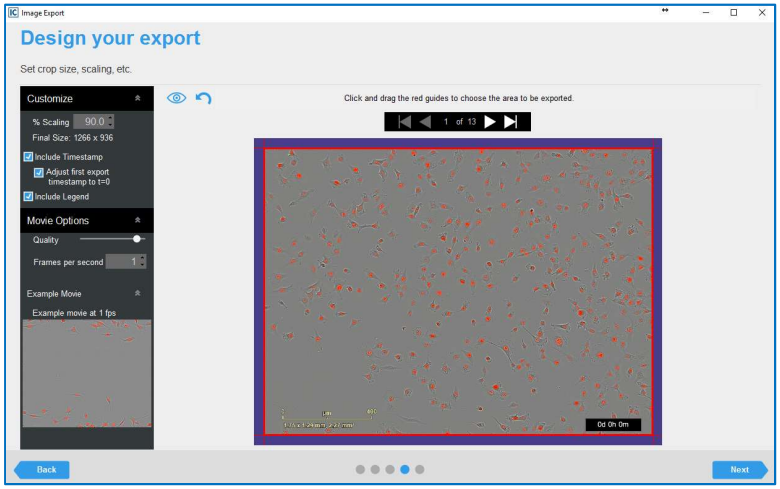
Select well/s and adjust auto-scale/image channels



Select time points of interest



Scaling/Timestamp/FPS and Preview before Export



Export Images/Movies

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

Software Demo – Schedule Scans

Practice Sets for Trainees

Experiment 1

- Phase/Green/Red – 4x whole well
- Corning 6-well 3335
- Front Right
- Use sample pattern (all 6 wells)
- Name: Experiment 1
- Every 6h starting at 12pm

Experiment 2

- Scratchwound
- Phase – 10x
- Wide Mode
- Front Left
- Whole Plate/1 image per well
- Name: Experiment 2
- Create a new scan group independent from Group 1: Every 1h starting at 1pm
- *How would you adjust the collisions at 12pm, 6pm, 12am, and 6am so all of the scans in the Scratchwound expt [Experiment 2] scan 10 minutes before the hour instead of on the hour?*

Experiment 3

- General
- Phase/Green – 20x
- 24-well Corning plate (3337)
- Middle Right
- Columns 1-4 (25 images per well)
- Name: Experiment 3
- Move the 24-well plate [Experiment 3] to the scan group for Experiment 1
- *How would you adjust Experiment 2 so there is enough time in between scan groups? Hint: The lab tech who setup Experiment 2 is okay with moving their 6:50 am, 12:50pm, 6:50pm, and 12:50 am scans to start at 7:00 am, 1:00 pm, 7:00 pm, and 1:00 am*
- *How would you adjust Experiment 3 to only scan every 6h but not exceed the 45 minute threshold? Hint: You prefer to start Experiment 3 at 1:30pm*

Experiment 4

- Copy Experiment 3
- Front Right (Hint: Experiment 1 is done so you can remove it)
- Add columns 5-6 to the scan pattern (i.e. scan the whole plate with 25 images per well)
- Name: Experiment 4
- Add this plate to the same interval as Experiment 2, adjust this scan group to scan only every 2h instead of every 1h starting at 2pm (Hint: you might need to delete the scan interval and set a new interval)

Software Demo - Vessel View

Practice Sets for Trainees (Cell Health Assay)

- **View in Packed View**
  - Go to the first time point
  - Turn off the phase
  - Why is there a green haze in columns 1-4 and 9-12?
    - Adjust the autoscale to each individual well
    - What are the green min/max settings of well A5?
- **View in UnPacked View**
  - Go to the last time point
  - Turn on the autoscale for each individual well
  - What are the green min/max settings of well A9
- **Additional Questions**
  - Keep the min/max settings for well A9 at the last time point
  - Return to packed view
  - Return to the first time point and notice how the haze is no longer there

Software Demo - Analysis Definition

Practice Sets for Trainees (Cell Health Assay)

- **Create a new basic analyzer definition analyzing phase, green, red, and overlap**
- **Select 6 images from wells containing Caspase-3/7 (Columns 5-8)**
  - 2 from late time point (rows C and H)
  - 2 from middle time point (rows A and F)
  - 2 from early time point (rows A and B)
- **Preview All using the default settings**
  - Adjust the phase mask to fit the cell area
  - Adjust the green mask to fit the dying cells
  - Adjust the red mask to fit all cells
  - Adjust the overlap mask to fit green/red cells
- **Select Columns 5-8 and all time points**
- **Save Analysis Definition as 'Caspase 3/7'**

Software Demo - Graph Export

Practice Sets for Trainees (Cell Health Assay – Caspase-3/7)

- **Graph Caspase 3/7 Count (1/mm^2)**
  - Remove time points (1d 8h 53min and the last 3 time points)
  - Remove the 1000nM Series from the Graph
  - Keep only the compound information in the series title
  - Change the Color Palette to Green
  - Change the Title to “Caspase-3/7 Response to Camptothecin Treatment”
  - Drag and Drop into Powerpoint
- **Graph NucLight Red Count (1/mm^2)**
  - Change the Color Palette to Red Orange
  - Keep only the compound information in the series title
  - Edit the color of DMSO Control to be black and circle, edit the color of CMP 1.37nM to be light gray and circle
  - Reverse the order of the legend so that DMSO is on top
  - Move the legend to the top left corner inside the graph
  - Change the Title to “Nuclear Count of HT-1080’s in Response to Camptothecin”
  - Drag and Drop into Powerpoint

Software Demo - Image Movie Export

Practice Sets for Trainees (Cell Health Assay - Caspase 3/7)

- **Export Movies from well A6**
  - Set the min setting to 1.0 and the max setting to match the auto-scale max of the last time point for well A6
  - Green only (no mask)
  - Remove time points (1d 8h 53min and the last 3 time points)
  - Include timestamp
  - 2 fps
  - Windows AVI Video (MPEG-4 compression)
- **Export Images from wells A6 and C6 (img 1 of 2)**
  - Red and Phase only + Red Object Mask (Outlines on, width = 4, mask color white)
  - Select time point 0 and 1d 14h 53m
  - Include timestamp
- **Use the right click – copy to clipboard to export the field of view of rows A-C (5-8)**
  - Make 2 fields of view: 1) Red only and 2) Phase + Blend Phase Mask
  - Show the 1) Image Metrics of the NucLight Red Count (1/Image) and 2) Confluence values from these wells
  - Select time point 0

IncuCyte Support

Field Application Scientist
Luca.Caneparo@Sartorius.com

Additional Resources:

- **Quotes, Orders:**
  Claudio Bencivenga
  Claudio.Bencivenga@Sartorius.com

- **Assays, Reagents, Protocols, Software:**
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