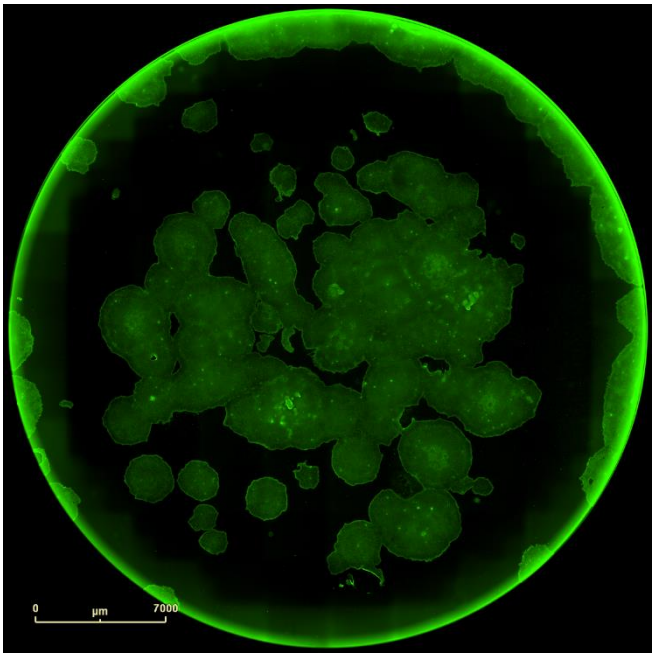
ESSEN BioScience

TECHNICAL NOTE

IncuCyte ZOOM™ Whole Well Imaging Overview

With the release of the 2013B software, IncuCyte ZOOM users will have the added ability to image the complete surface of select multi-well plates and 35 mm dishes using two new imaging modes: 1) Whole Well, and 2) Dilution Cloning. As with Standard imaging on the IncuCyte ZOOM, all three acquisition channels (Phase, Green, Red) can be used to acquire whole well images. Scenarios that require the whole well imaging strategy include monitoring or tracking rare events within a population of cells (e.g. induced pluripotent stem cell reprogramming), and making observations around well and/or dish edges. In addition to new whole well image acquisition modes, this software upgrade also includes redesigned image processing tools that provide a simplistic approach to deriving quantitative information from all three acquisition channels. Additional navigation tools, point of interest markers, as well as digital and physical markers for labeling plates provide new ways for researchers to interact with the IncuCyte ZOOM system to inspect and monitor cell biology over the course of time. Regardless of the application, integrating "Whole Well" and "Dilution Cloning" scan modes into Essen BioScience's Live Content Imaging approach to quantitative kinetic biology provides yet another powerful new tool to interrogate complex biological models. The purpose of this technical note is to discuss the whole well imaging capabilities of the IncuCyte ZOOM system, to provide examples of how to use the software most effectively, and to describe how to process whole well images to take full advantage of this new imaging technique.



In this whole well image, induced pluripotent stem cells (iPSCs) in one well of a 6-well plate were stained with SSEA4 antibody followed by an Alexa Fluor 488 secondary antibody. The image was taken using 'Whole Well' scan mode on an IncuCyte™ ZOOM.

Before you begin…
"Whole Well" and "Dilution Cloning" scan modes:

- *Support Phase, Green, and Red image acquisition*
- *Require an update to IncuCyte™ ZOOM 2013B software*
- *Require a 4x microscope objective*
- *Require a good computer to effectively work with the images. A 64-bit operating system and 8 GB of RAM are imperative*
- *Require the use of supported tissue culture vessels. Select multi-well plates (96, 24, 12, 6-well plates) from Corning, Falcon and TPP and 35 mm dishes from Corning, Falcon and Greiner are supported.*
- *Require additional hardware (Tray 25) ONLY if scanning 35 mm dishes*
- *Should be used only when necessary: To track rare events within populations of cells (e.g. iPSC reprogramming) or to visualize cell behavior at the edge of the well.*

Scheduling

To schedule a "Whole Well" scan, simply change the "Scan Type" to "Whole Well" following the addition of a "Whole Well" supported vessel into the scan schedule. To identify vessels that are supported in the 'Whole Well' scan mode, use the **Help | Supported Vessels** menu option and search the "Scan Types" column for "Whole Well". Alternatively, using the "Add Vessel" function, search the "Scan Types" column for "Whole Well". Remember, your IncuCyte™ ZOOM must be configured with the 4x objective to scan vessels in "Whole Well" mode.

*Use the **"Whole Well"** scan type when monitoring rare events within a population of cells with an initial seeding density ≥ 5% confluence. (96, 24, 12, 6-well plates and 35 mm dishes)*

*Use the **"Dilution Cloning"** scan type when monitoring rare events in wells that have a very low density of cells, or might be devoid of cells altogether. (96-well plates only).*

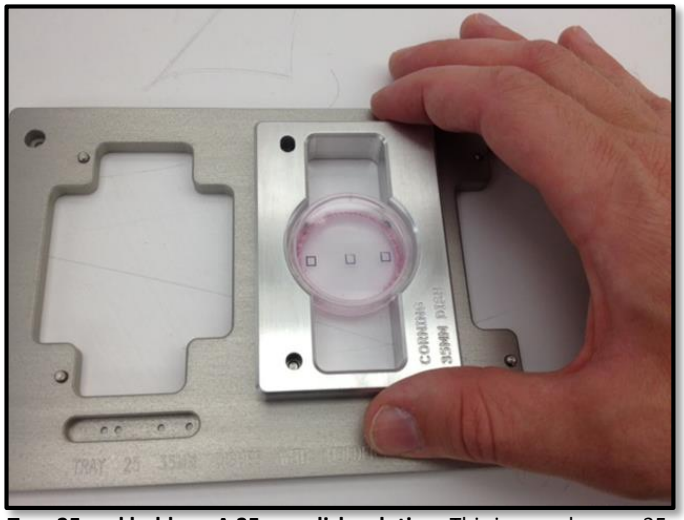
Select 35 mm dishes are also supported for 'Whole Well' scan mode. To scan 35 mm dishes, a unique tray (Tray 25) and holders are required. Designed specifically for the tissue culture vessel you are working with (Corning, Falcon, or Greiner), the combination of Tray 25 and holders are required to prevent rotational variations following manual manipulation of the culture (e.g. cell feeding), and to keep the vessel in place during physical marking. The holders are crafted to allow researchers to easily access cultures while maintaining integrity of imaging.

	Phase	Phase/1-color	Phase/2-color
96-well Plate	20	26	34
24-well Plate	26	35	47
12-well Plate	22	29	39
6-well Plate	26	36	48
35 mm Dish	5	6	8

Table 1: Scan Time Estimates for the Whole Well Scan Type (minutes). This table describes the estimated scan time for whole well image collection in each of the listed vessel formats. *While scan times might be slightly longer compared to other imaging systems, vessels remain in the incubator during scanning protecting cultures from environmental perturbations.

	Phase	Phase/1-color	Phase/2-color
96-well Plate	36	43	52

Table 2: Scan Time Estimates for the Dilution Cloning Scan Type (minutes). This table describes the estimated scan time for Dilution Cloning image collection in 96-well plates.

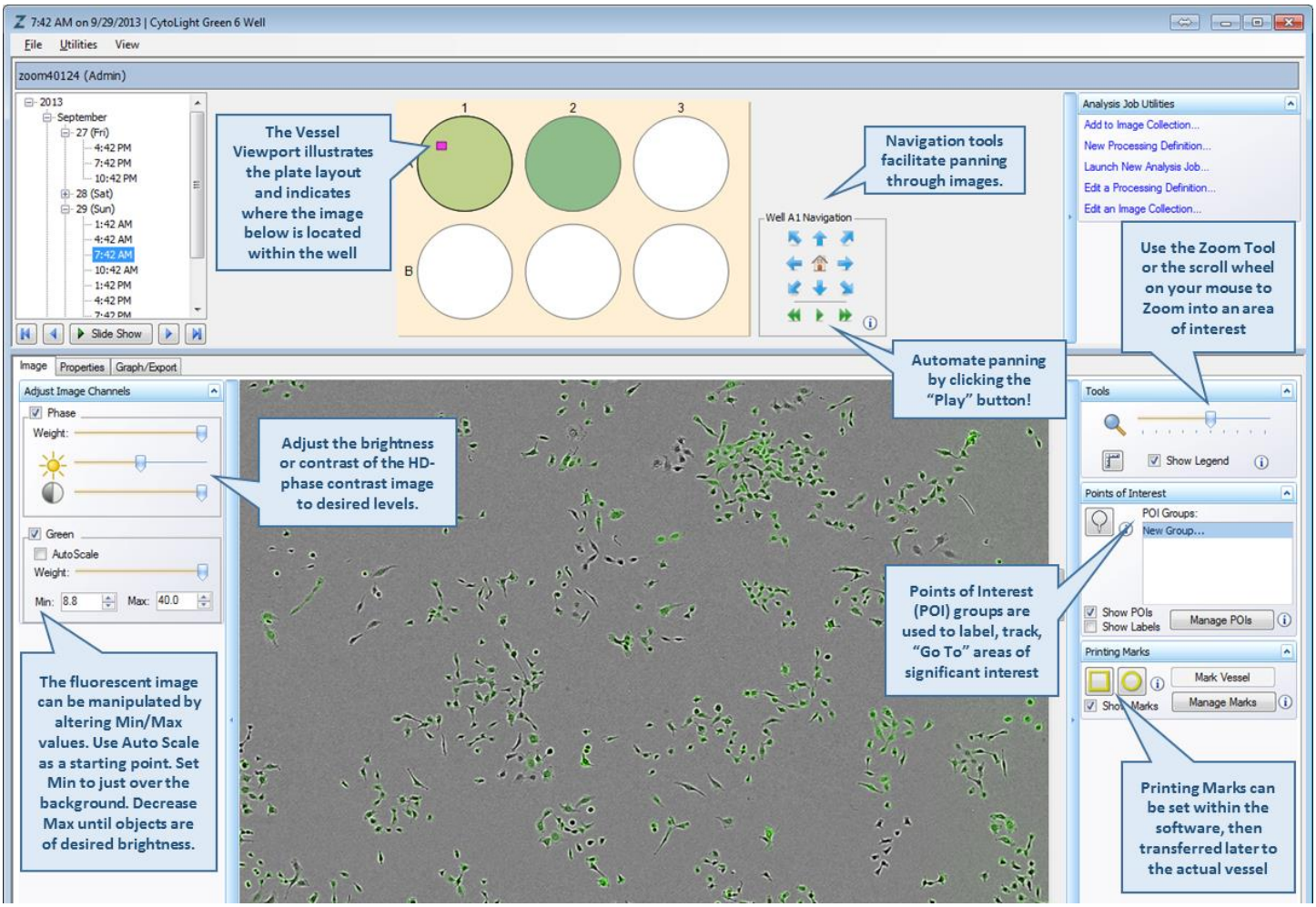


Tray 25 and holders: A 35 mm dish solution. This image shows a 35 mm Corning tissue culture dish in the Tray 25 Corning holder. The holder locks the vessel into place to prevent rotation variations when imaging, and to keep the vessel in place during physical marking.

Additional Considerations

Most experiments do not require imaging the whole well. The natural tendency is to image as much surface area as possible, increasing the sampling of the biology and thus increasing the statistical power of the assay. However, we have found empirically that with proper distribution of reagents and cells, sampling only a portion of the well (e.g. 2 images per well with a 10x objective in a 96-well plate) is sufficient for most applications. Why is this an important consideration? Whole Well scans are considerably longer than standard scans (See Tables 1 and 2 for scan time estimates), the size of the image files are significantly greater, image processing times are longer, and the burden on the client computer is higher when imaging in Whole Well. Use discretion and image the whole well only when necessary.

Viewing Whole Well Images



The "Vessel View": Once scans have been acquired, the "Vessel View" can be opened by double clicking on the vessel itself in the "View Scans" screen, or by double clicking the vessel information within the "Search" grid. The "Vessel View" is an interface that provides researches their first opportunity to interact with images from their experiment. Contrast and/or brightness of the phase image, and fluorescent channel viewing thresholds can be simply manipulated using image channel adjustors. The Viewport and Navigation tools have been designed to facilitate moving around the image with ease, updating image location while panning. Using the zoom tool or the scroll wheel on the mouse provides a means for zooming in on specific areas of interest. Points of Interest (POIs) are used to label objects (e.g. cells, colonies, etc.) in order to make taking notes, labeling and returning to the same location easy. The lifetime of a POI can also be defined chronologically or at user defined intervals. Printing Marks can be set within the software, then transferred to physical plates with a simple click of the button.

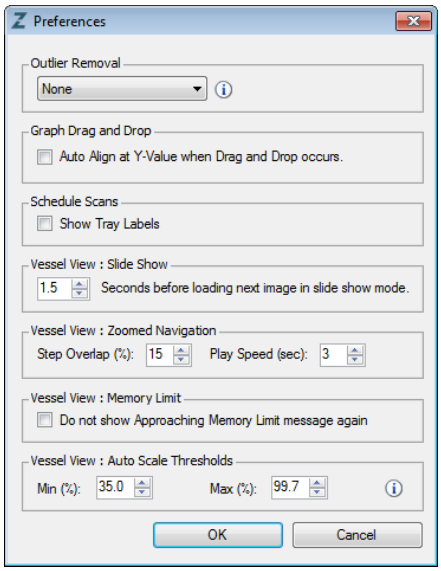
Image and Movie Export

The export of images and movies of whole well images follows the same rules as exporting standard images. Using the "Utilities" menu, select "Export Current Image…" or "Export Movie or Image Set…", set the Sequence Type to "Image Set via Image Designer" or "Movie" and use the Designer tools to set Cropping and Scaling. It should be noted that images acquired using "Whole Well" and "Dilution Cloning" scan modes are much larger than standard ZOOM images. Because the full-size image is seldom useful when creating a movie or presentation materials, we recommend that the Designer Tool be used to crop and/or scale exports to a smaller size. This will also lead to smaller exported files.

Navigation Tools

The Vessel View Viewport displays the vessel layout with a pink indicator highlighting the location within the well that corresponds to the image in the viewer. Navigation tools accompany the Viewport to facilitate panning through images. When zoomed in, these controls are used to systematically navigate within the image. Navigation tips:

- Use the Home button to jump to the upper left
- Use the arrows to jump in the designated direction
- Use the set of controls at the bottom to auto-navigate in Play mode, or to advance/back up manually.
    - Set the Step Overlap and Play Speed using the Preferences menu in the Main IncuCyte Screen (shown at right).



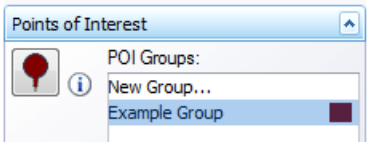
Researchers can also navigate with the keyboard [Arrow keys, 'Home', '+', '-', etc.]

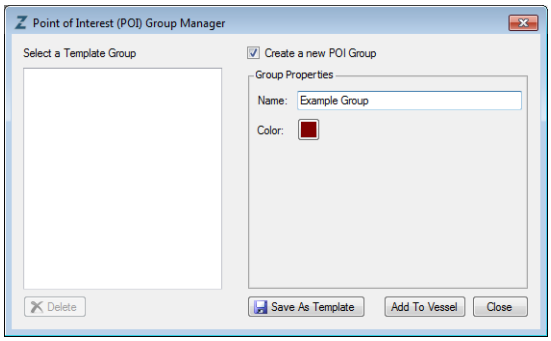
- Click anywhere in the image
- Use the keyboard to navigate

Points of Interest (POIs)

Rare events or objects of interest can be labeled as Points of Interest (POIs). Each POI belongs to a specified POI Group. To add a Point of Interest, you must first make a "New Group".

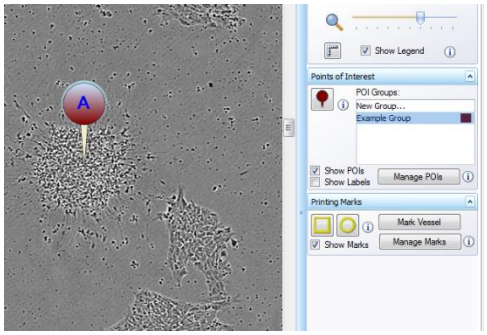
- Click the "New Group" list item to add a group (shown at right)
- To edit or delete a listed POI Group, Right Click and delete/edit
- "Create a new POI Group" within the POI Group Manager or select a Template Group that has been made previously.
    - Use the "Add to Vessel" button to create a group for only the vessel currently in use or to add an existing Template Group to the vessel in use.
    - Use the "Save as Template" button to save a group for use in other vessels





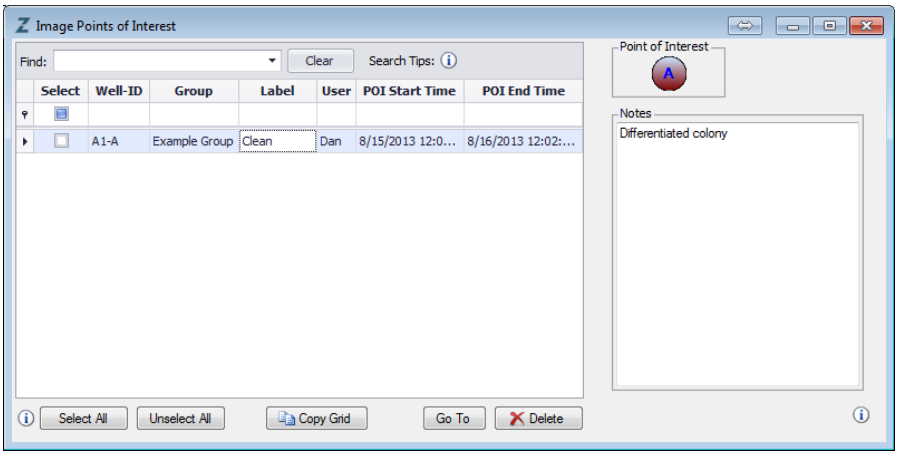
To Add a POI to an Image

- Select a Group from the POI Group list
- Click the button in the upper left area of this section
- Click on the image at the desired location
- Click/Hold/Drag to move the POI
- Right-click on the POI to:
    - Assign the Start or End time to the current scan time
    - Clear the POI's Start and End time
    - Delete the POI



Researchers can manage their POIs using the "Manage POIs" button. Use the "Manage POIs" button to:

- View all POIs across the vessel
- Add labels and notes to POIs
- Filter out POIs
- Jump to POIs using the "Go To" button
- Delete POIs



Tips for Managing Points of Interest

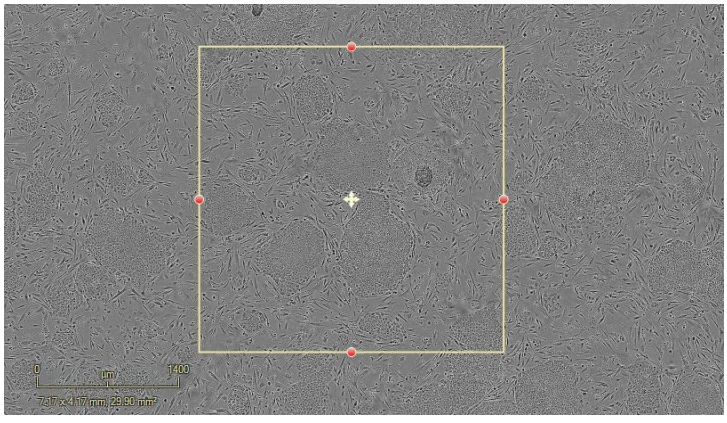
- Click on a row in the table to interact with a POI:
  - Enter a Label or add Notes
  - Navigate to it in the Vessel View using the "Go To" button
  - Select the POI for deletion
  - Note: Start/End times cannot be edited in this table
- Filter POIs from the table. If a POI is not displayed in the table, then:
  - It will not appear in the Vessel View image
  - It will not appear when an image is exported.
- POI filters are automatically removed when this window is closed
- Use the Select Boxes to delete POIs. POI deletion permissions depend on User Account permission level (unless you have administrator permission, you may only delete those POIs that belong to you).

The accumulation of POIs can be graphed over time. Use the Graph/Export function to generate a kinetic graph of POI accumulation.

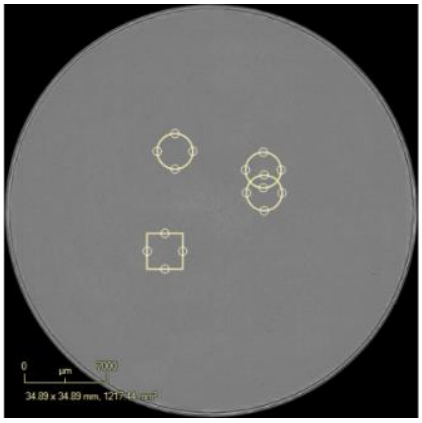
Printing Marks

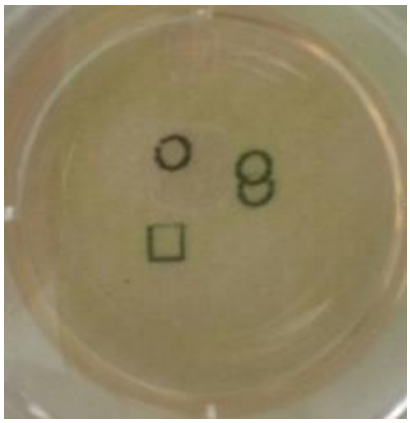
Evaluation of whole well images can lead to a decision to manually manipulate areas within a tissue culture dish/well (e.g. cleaning differentiation, picking a clone). The IncuCyte ZOOM software provides a method for transferring marks made within the software directly onto tissue culture vessels using the "Printing Marks" tool. To draw marks using the ZOOM Marking Tool:

- Designate Mark Locations
  - Select either a Square or Circular mark using the buttons in the upper left area of the "Printing Marks" section.
  - Click on the image at the desired location.
  - The size cannot be changed
  - Move marks by using grab points to click/hold/drag
- Mark with the ZOOM Marking Tool
  - Remove the ZOOM Marking Tool cap
  - Install the ZOOM Marking Tool, replacing the objective
  - Click "Mark Vessel" to initiate printing



Printing Marks: The square printing mark in this image encloses some stem cell colonies that will be manually removed from the well. The diameter of the printing marks is 3 mm. Printing marks cannot be resized.





Marking a Vessel: Once the objective within the IncuCyte has been replaced by the IncuCyte ZOOM Marking Tool, Printing Marks applied to the image within the IncuCyte ZOOM software can be transferred directly to the culture vessel by clicking the "Mark Vessel" button within the Vessel View.

IncuCyte ZOOM Marking Tool: Shown here is the IncuCyte ZOOM Marking Tool next to a standard 20x microscope objective. The ZOOM Marking Tool is screwed into the place of the objective and must be removed following marking.

- Use the "Manage Marks" button to:
  - View all Printing Marks across the vessel
  - Jump to a Printing Mark
  - Delete Printing Marks

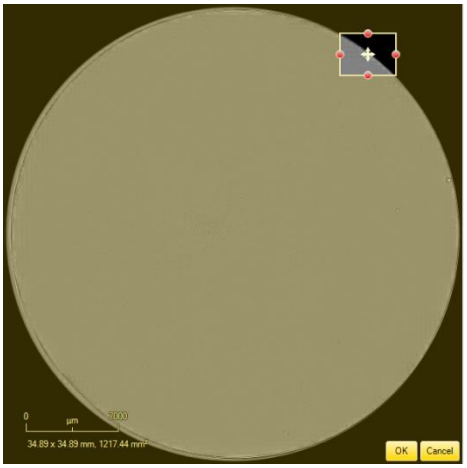
After using the IncuCyte ZOOM Marking Tool, be sure to replace it with the appropriate objective or you will negatively affect subsequent scans.

Processing Whole Well Images

Making Image Collections

As with Standard Scans, discovering processing parameters for whole well images requires generation of image collections. With very few exceptions (e.g. "Keep-out" masks discussed below), the rules and/or suggestions for processing whole well images are the same as the rules for processing images collected from standard scans. For an in-depth discussion and additional information, refer to the following technical notes: ZOOM Confluence Processing and ZOOM Fluorescence Processing. For 96-well plates, whole well images can be added directly to a new image collection. For any other multi-well plates and dishes, the whole well images are simply too large to add to image collections. Consequently, whole well image collections are generated from carefully selected Regions of Interest (ROIs) within the image. To initiate this process, click the "Add to Image Collection…" link within the Vessel View. This will activate the ROI Extraction Tool to add a sub-image.

- The ROI is shown as a rectangle and cannot be resized
- "Grab points" are found along the perimeter and at the center of the ROI
  - Click and drag on a grab point to reposition
- Click the "OK" button in the bottom right corner of the image to finalize the ROI
- Select 3-5 representative images to add as part of an image collection.
- Select 2-3 that straddle the perimeter of the well (as shown at right) to design a processing "Keep-out" region, the region of the edge of the well that should be excluded during image processing.



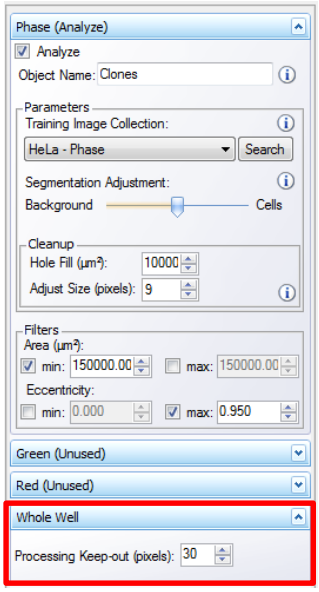
ROI Extraction Tool: In multi-well plates with large wells, sub-images are used to develop image collections. Select representative images of interior regions as well as regions around the well edge. Reposition the tool using "Grab Points". Click OK to proceed.

Generating Processing Definitions:
Researchers can process whole well images in a similar fashion to standard scans. Refer to ZOOM Confluence Processing and ZOOM Fluorescence Processing technical notes for detailed discussions of how to define Phase Contrast and Fluorescence image processing parameters. The only feature added to the Processing Definition editor that is unique to whole well images is the "Processing Keep-out" parameter.

> ***The "Processing Keep-out" is used to adjust the area over which the whole well images are analyzed. A positive value increases the "Keep-out" region, thereby decreasing the diameter of the analyzed area. A negative value has the opposite effect.***

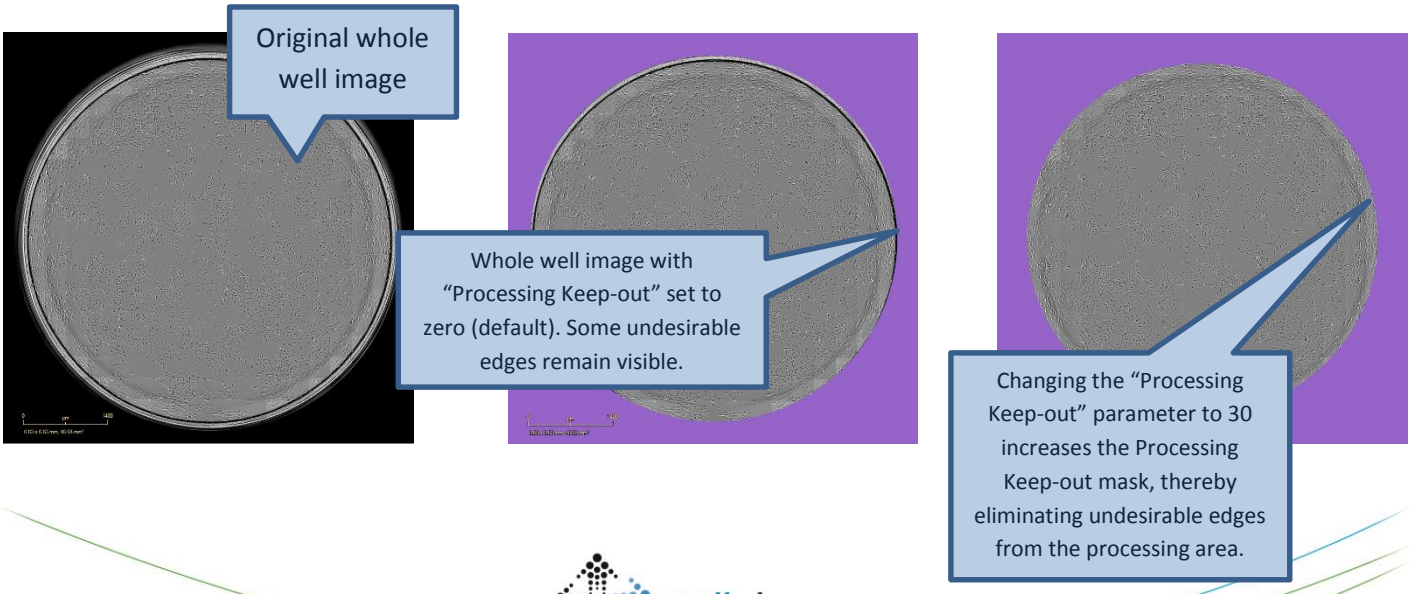
The Processing Keep-out Mask can be viewed after a Preview is run on a Whole Well image collection of:

- Full-sized Images, or
- Regions of Interest that straddle the perimeter of a well



Within the Processing Definition editor, the default value for the "Processing Keep-out" parameter is set to zero. For many cases this value is sufficient as it is designed to exclude all areas of the image outside of the manufacturer specified well area. However, there might instances when a researcher will want to decrease the diameter of the processed area (see below). This is achieved by adding pixels to the "Processing Keep-out" mask. A positive value increases the "Keep-out" region, thereby decreasing the diameter of the analyzed area. A negative value has the opposite effect (increases the diameter). A negative value will only be used in rare instances.

The example below illustrates how researches can adjust the Keep-out processing mask. In this case, a value of 30 pixels was added to the mask in order to "Keep-out" undesirable plate artifacts at the edge. A similar work flow can be used using Region of Interest sub-images taken from whole well images of multi-well plates >96-wells by creating an image collection of sub-images that straddle the perimeter of the well or dish.



Summary

The "Whole Well" and "Dilution Cloning" imaging modes provide a way to monitor rare events over the course of time. Essen BioScience has exemplified the utility of these imaging modes in two applications notes: CellPlayer™ Kinetic Stem Cell Application and CellPlayer™ Dilution Cloning Application (coming soon). There is no question that the growing IncuCyte ZOOM user base will continue to find exciting new ways apply the Live Content Imaging approach to generating cutting-edge biological models to interrogate cell biology.

Frequently Asked Questions

I have already developed processing definitions for images acquired at 4x. Can I use those definitions to process whole well images?
Yes, with the caveat that the "Processing Keep-out" parameter will be set to the default value (0). We think this value will be sufficient for most cases. However, you might find that you are segmenting portions of the edge of the well that you would rather eliminate. In this case, we recommend that you make a separate image collection to discover the appropriate "Processing Keep-out" parameter. In this way, you can test your existing Processing Definition on the new image collection containing whole well images (96-well) or whole well sub-images (other multi-well plates/dishes) as described on page 7.

Can I add whole well images (96-well) or whole well sub-images (other multi-well plates/dishes) to existing 4x image collections?
No. There are two good reasons why:

1) Our software needs to know when to use a Keep-out region during parameter discovery. When previewing with an image collection containing whole well images or whole well sub-images, it tells the Processing Definition Editor to use Keep-out regions. Adding whole well images to existing collections will not trigger the software to look for Keep-out regions, therefore a new image collection must be made.
2) If a researcher is using a phase image collection to train the phase processor, adding images to existing image collections for the single purpose of discovering the Keep-out parameter would alter that training model. In this way, all future data analyzed with this model would be processed differently than prior data. We did not want to put a researcher in a position to make that mistake.

Does adding the Keep-out parameter to my Processing Definition alter how images acquired in Standard Scans are processed?
No. The Keep-out Parameter is only used when processing whole well images.

I can acquire whole well images, but I'm unable to open the Vessel View, or Processing Definition editor without an error. Why is that?
You are working on a computer with a 32-bit operating system. In order to handle the data and operations required of whole well images, we had to make a tough decision: to only support full functionality of whole well imaging on computers with 64-bit operating systems. However, we did not want force this requirement on all users. Some of our users have 32-bit systems in their labs. These computers are used to quickly schedule scans and perform basic functions. We did not want to take that away. So, you can perform basic whole well functions on 32-bit systems, but can only perform advanced functions (e.g. generating Processing Definitions) on 64-bit systems.

When working with whole well images, the software seems slow. Why?
Whole well images are really big! Especially those acquired from a 6-well plate or a 35 mm dish. The size of one image alone is on the order of 50 MB. Remember the floppy disk days? Each floppy could hold about 1.44 MB of information. That means you would need 35 floppy disks just to save one whole well image. Multiply that by six wells, 4 time points per day, over the course of two weeks… that's 16.8 GB of data (or 11,667 floppys!). The speed of the software can vary based on the speed of the network at your facility, the specifications of your computer, the number of other applications running simultaneously on your system, and the size of the images. Images from 96-well plates are much smaller, and so the software is much snappier when working with them. The software provides several tips when working with whole well vessels. Please read them carefully and be patient.

What happens if I forget to remove the ZOOM Marking Tool, or replace the wrong objective?
If you forget to remove the ZOOM Marking Tool, the instrument will fail to scan all future time points until the objective is replaced. If you put in the incorrect objective, an array of different issues are possible…. None of which are good. Do not make these mistakes! We suggest that you place the removed objective next to the IncuCyte ZOOM within the incubator so you know which objective to put back in following vessel marking.

Why do I need a special tray and holders to image in 35 mm dishes?
If you didn't have a way to take-out and put-back the dish in the exact same orientation every time, following an object over time would be very difficult. The 35 mm dish holders are designed to prevent such rotational variations, and to hold the dish in place during vessel marking.