IncuCyte ZOOM™ Fluorescent Processing Overview

The IncuCyte ZOOM offers users the ability to acquire HD phase as well as dual wavelength fluorescent images of living cells—producing multiplexed data that can kinetically measure cell proliferation, apoptosis, cytotoxicity, and other cell biology applications. The IncuCyte ZOOM's refined processor is capable of accurately distinguishing between background and positive fluorescent objects across a wide range of cell types and fluorescent intensities. With this feature, an IncuCyte ZOOM user has the ability to tailor **Processing Definitions** to optimally analyze a specific phenotype of cells. This requires 3 simple steps as described below: 1) creating an **Image Collection**, 2) creating a **Processing Definition**, and 3) **Launching an Analysis Job**. As illustrated in Figure 1, the data processing flow is separated into two distinct phases, the **Assay Development Phase**, and the **Established Assay Phase**
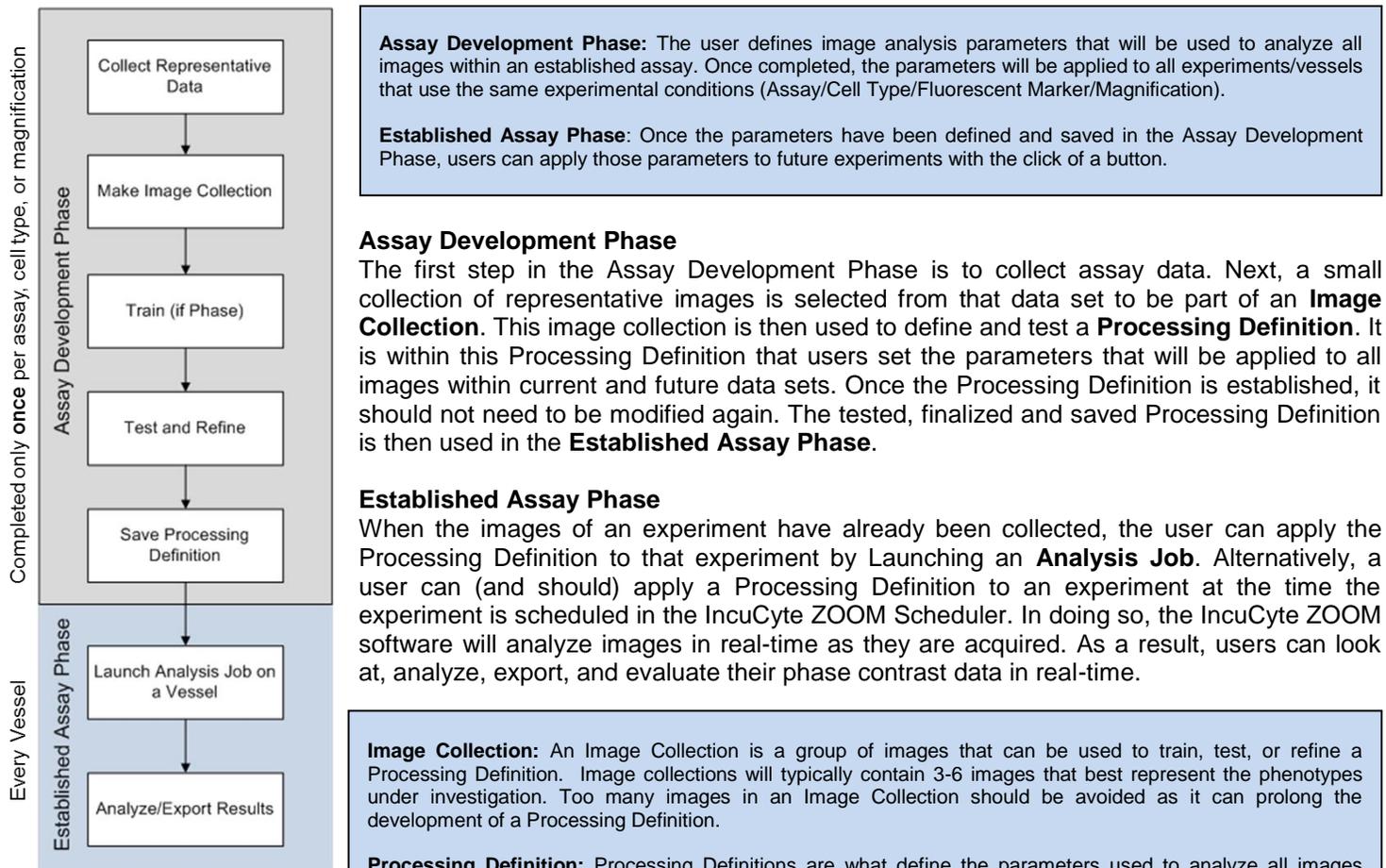

Assay Development Phase: The user defines image analysis parameters that will be used to analyze all images within an established assay. Once completed, the parameters will be applied to all experiments/vessels that use the same experimental conditions (Assay/Cell Type/Fluorescent Marker/Magnification).

Established Assay Phase: Once the parameters have been defined and saved in the Assay Development Phase, users can apply those parameters to future experiments with the click of a button.

Assay Development Phase

The first step in the Assay Development Phase is to collect assay data. Next, a small collection of representative images is selected from that data set to be part of an **Image Collection**. This image collection is then used to define and test a **Processing Definition**. It is within this Processing Definition that users set the parameters that will be applied to all images within current and future data sets. Once the Processing Definition is established, it should not need to be modified again. The tested, finalized and saved Processing Definition is then used in the **Established Assay Phase**.

Established Assay Phase

When the images of an experiment have already been collected, the user can apply the Processing Definition to that experiment by Launching an **Analysis Job**. Alternatively, a user can (and should) apply a Processing Definition to an experiment at the time the experiment is scheduled in the IncuCyte ZOOM Scheduler. In doing so, the IncuCyte ZOOM software will analyze images in real-time as they are acquired. As a result, users can look at, analyze, export, and evaluate their phase contrast data in real-time.

Figure 1: Flow chart illustrating the IncuCyte ZOOM assay development strategy.

Image Collection: An Image Collection is a group of images that can be used to train, test, or refine a Processing Definition. Image collections will typically contain 3-6 images that best represent the phenotypes under investigation. Too many images in an Image Collection should be avoided as it can prolong the development of a Processing Definition.

Processing Definition: Processing Definitions are what define the parameters used to analyze all images within an experiment. Each new Assay, Cell Type, and/or magnification will likely require a new Processing Definition, but once established, will not have to be created again. Once finalized and saved, Processing Definitions can be applied to vessels/experiments at the time of scheduling such that images are analyzed in real-time.

Analysis Job: An Analysis Job is launched to analyze images and produce metrics based on the parameters contained within the saved Processing Definition. The scope of the job is specified by the user when the job is launched. This scope includes controls for a single time point, over a defined time frame, or open ended if the Vessel/Experiment is still being actively scanned.

Fluorescent Processing: Metrics

IncuCyte ZOOM provides the following set of metrics relevant for fluorescent object analysis:

- Count
- Confluence
- Average Area
- Average Eccentricity
- Average Mean Intensity
- Average Integrated Intensity
- Total Integrated Intensity

To accurately quantify these metrics, a user creates a Processing Definition well-suited to the cell type, assay conditions, and magnification. The following section will guide you through the exact steps you need to complete the Assay Development Phase for your specific assay. Screenshots are included for further clarity, and definitions of all the IncuCyte ZOOM terms can be found in blue boxes.

> ### *Fluorescent Processing Metrics Defined*
>
> **Count (1/Image or 1/mm^2 or 1/well)**: The number of objects per image, per mm^2, or per well.
>
> **Confluence (%):** The percentage of the image area occupied by objects.
>
> **Avg. Area (µm^2):** The average area of the objects in the image.
>
> **Avg. Eccentricity:** The average of how round or compact the objects are. Ranges from 0 to 1 with a perfect circle having a value of 0.
>
> **Avg. Mean Intensity (CU):** The image average of the objects' mean fluorescent intensity.
>
> **Avg. Integrated Intensity (CU x µm^2/Image):** The image average of the objects' summed fluorescent intensity.
>
> **Total Integrated Intensity (CU x µm^2/Image):** The total sum of the objects' fluorescent intensity in the image.

Fluorescence Processing: Assay Development Phase

Step 1. Determining the Spectral Unmixing

1. Image wells containing cells that express the green signal ONLY or wells containing cells that express the red signal ONLY (depending upon the reagent being used). These images MUST be acquired using both the red and green channel to evaluate if the fluorophore produces signal in both channels.
    NOTE: It is rare that green fluorescence is detected by the red channel.

2. Once a few images have been collected, the User can visualize how much of the red fluorescence is detected by the green channel (and vice-versa) by toggling between Image Channels **(Screenshot 1)**.

3. The user can then adjust the "% of Red removed from Green" by increasing the values on the right under "Spectral Unmixing" until no red image is visualized in the green channel. Too high a percentage of Red removal from Green may result in overcorrection and the appearance of "holes" within the image.

Red Channel   **Green Channel**

> **Screenshot 1: Spectral Unmixing**
> The red fluorophore in this screenshot produces a signal in both the red and green channel (top row) The red channel is unmixed from the green channel by 8% to better distinguish between the 2 fluorophores (bottom row).

NOTES: Spectral unmixing should be determined for all new fluorescent proteins or dyes. Once established, the percentage can be applied at time of vessel scheduling in future assays containing that fluorophore. (i.e. 8% of Red removed from Green is optimal for Essen's NucLight and CytoLight Red Reagents/Cell Lines). The User must be aware that overcorrection using the spectral unmixing tool may affect assay metrics as well as the loss of detection of true green objects.

4. Click "Save" to apply the spectral unmixing to the current vessel.
   IMPORTANT: It is imperative that spectral unmixing is saved before any image collections, processing definitions, or analysis jobs are created.

Step 2. Creating an Image Collection

1. Collect Dual-Fluorescent images of your cells every 2-6 hours until assay is complete.

2. Open the Vessel View. If the appropriate spectral unmixing was not applied at the time of Vessel Scheduling, use the Spectral Unmixing tool in the Vessel View and click "Save".

3. Review images and select an image you want to add to the Image Collection by clicking on the "Add to Image Collection" link **(Screenshot 2)**.

4. Select "Basic Analyzer" from the Analysis Job Type drop-down menu.

5. When adding the first image of a collection, select "New" and assign a unique name.

6. Choose the Required Image Channels. For a 2-color assay, both green and red channels are required.

7. Continue to add images to the image collection just made by clicking on the "Add to Image Collection" link (**Screenshot 2**). The image collection named in Step 5 will now be listed as an "Existing" image collection. Choose images that represent the objects at their dimmest, brightest, smallest, and largest.
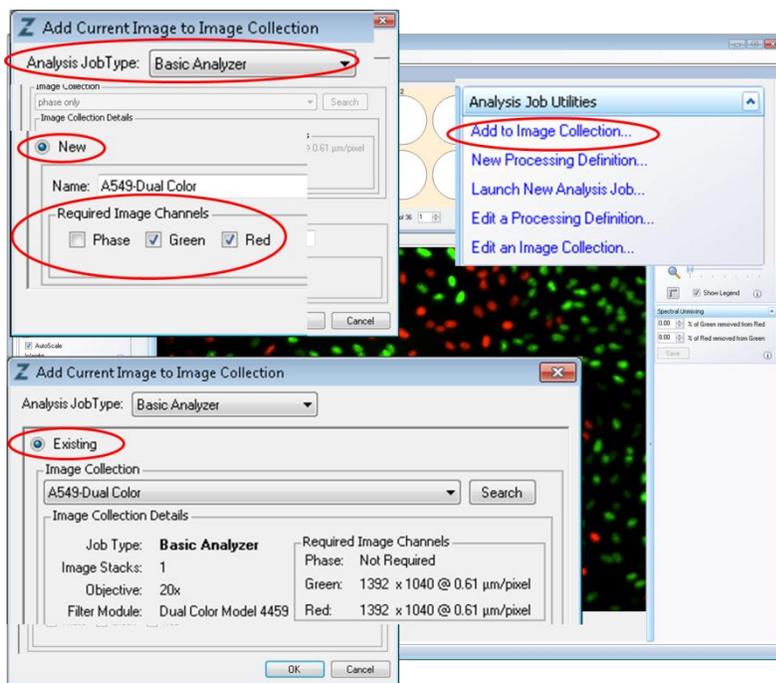
Screenshot 2. Adding Images to an Image Collection

NOTES: An Image Collection can only contain images taken at the same magnification. An Image Collection may contain images from multiple tissue culture vessels. The collection should not contain too many images as it will prolong the development of the Processing Definition. We recommend adding images to a collection without regard to how many you've added until you feel you have the range of images you want. Then, go to "Edit image collection" link (located under Analysis Job Utilities in **Screenshot 2**), and evaluate which images are needed to make a representative set. Then remove redundant images until only 4-8 representative images remain.
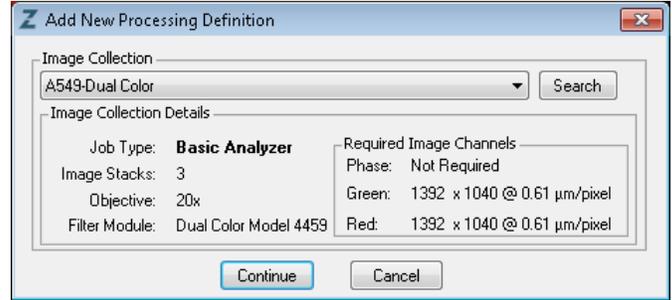
Step 3. Creating a Processing Definition

1. Start a new Processing Definition by clicking on the "New Processing Definition" link within the Vessel View (found underneath "Analysis Job Utilities" in **Screenshot 2**).

2. Select the proper Image Collection and click "Continue" **(Screenshot 3)**.

3. The Basic Analyzer Processing Definition Editor will open displaying the "Preview Image Collection" drop down menu **(Screenshot 4)**. This will default to the image collection selected in the previous step (**Screenshot 3**), but it is advised to check before changing parameters.

    NOTE: You may preview a processing definition on multiple image collections, if desired.

4. Check the boxes which you wish to analyze. For instance, if you are only analyzing Green and Red data it is unnecessary to check the Phase box. This will save time in previewing and running jobs.

5. Assign phenotypic "Object Names" (for example, if your green objects represent nuclei, you will label them "Nuclear", and the metric would appear as "Nuclear object per image").

6. Choose a "Preview Image Collection" on which to test your processing definitions. This will default to the image collection selected in step 2, but it is advised to check before changing parameters.

    NOTE: You may preview a processing definition on multiple image collections, if desired.

7. Determine the brightness of objects and background **(Screenshot 5)**.

    a. For each channel, find the dimmest object which you wish to count in your analysis by scrolling your cursor over dim objects to see their pixel intensity (See left and right boxes in **Screenshot 5**). You may find dimmer objects more easily by clicking off the auto scale under "Adjust Image Channels" and decreasing the max intensity value (red circles in **Screenshot 5**).

    b. Next, find the average background intensities for each channel (center box, **Screenshot 5**)

    c. Determine whether the difference between background and positive object intensity is high or low. In this example, the difference is low for both channels.

Screenshot 3: Starting a New Processing Definition
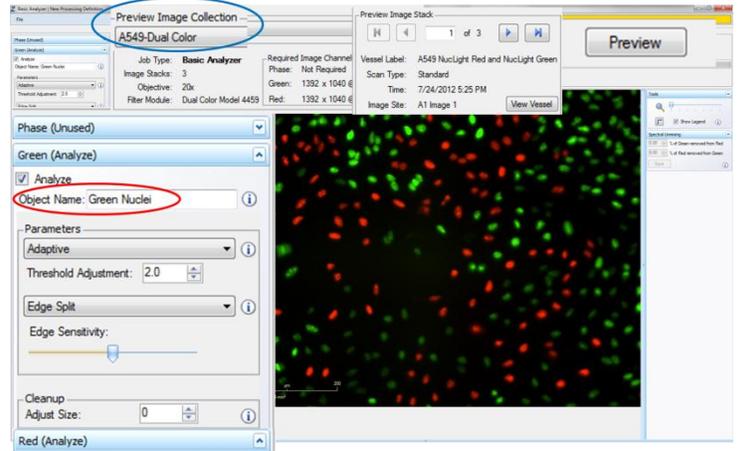
Screenshot 4: Basic Analyzer Processing Definition Editor
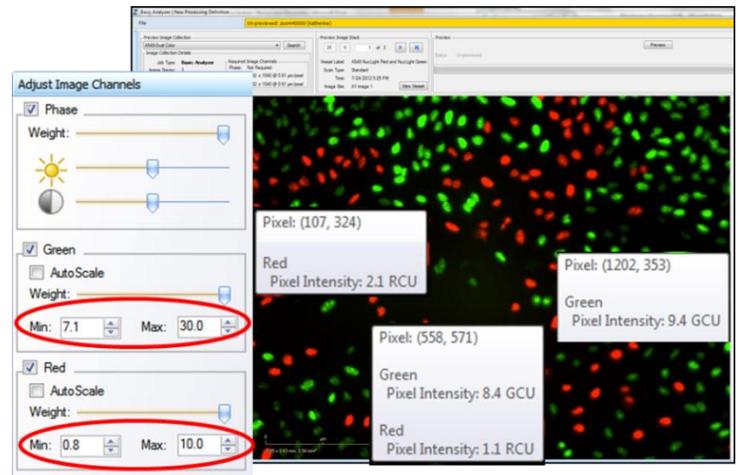
Screenshot 5: Determining the Positive Object and Background Intensities
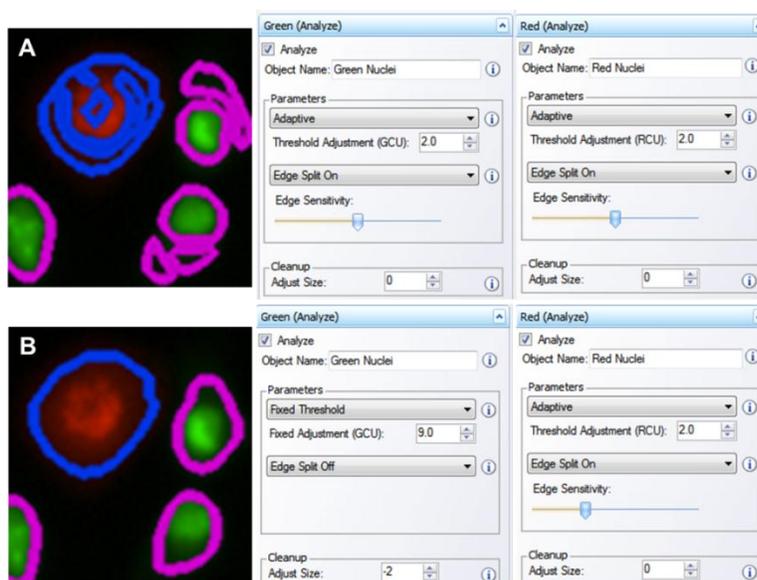
8. Using these values found in step 6, Color Parameters can be adjusted to mask objects in 2 different ways ("Parameters", **Screenshot 6**)

   Adaptive Segmentation – A local background level (LBL) across each processed image is automatically determined and a threshold adjustment is set above (if positive) the LBL. It is advised to preview the default threshold adjustment of 2.0 first (Parameters for both channels in row A of **Screenshot 6**).

   Fixed Threshold – A single threshold level in calibrated fluorescence units is used across the image. This number can be set as a number near or in between the dimmest positive object and the brightest background area (Parameters for Green channel in row B of **Screenshot 6**).

9. After determining the Color Parameters, click "Preview".

10. Make sure that both the correct fluorescent image channel box and the "Mask" box are checked and evaluate your mask.

11. Use the "Blend Mode", "Weight", "Outlines", and "Color" selection options to help you to assess the mask (**Screenshot 7**). Changing these will not affect the processing definition. To clearly determine the mask, you can zoom in on the network using the tool slider followed by the weight slider located under the mask section.

12. If necessary, increase the threshold to eliminate masking of background or decrease the threshold to include dimmer objects. Click "Preview" to assess the changes.

13. Further refine the mask by modifying the Edge split, Cleanup, and Filters.

    a. Select "None" for Edge Split if objects are not closely spaced. The user also has the option to finely tune the Edge split by moving the edge sensitivity bar to the left to minimize the number of splits (Red channel, Image B of **Screenshot 6**) and to the right to maximize the number of splits.

    b. Decrease the size of the mask by entering a negative "Cleanup" value (Green channel, Row B of **Screenshot 6**) or increase the size of the mask by entering a positive "Cleanup" value.

    c. Apply filters for area, eccentricity, mean intensity, and integrated intensity to eliminate dead cells, debris, or background from being masked as objects. (See Fluorescent Processing Parameters on next page for definitions.)

Screenshot 6: Adjusting Parameters
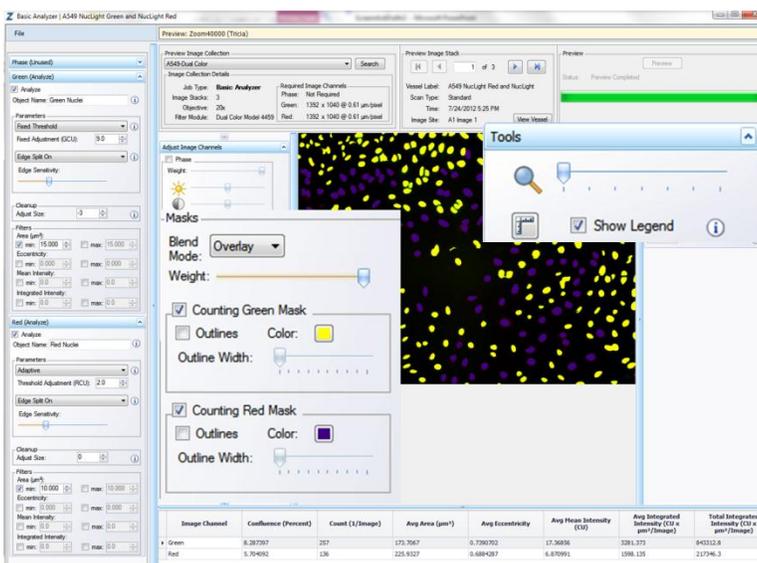
Screenshot 7: Previewing Object Masks

14. Once you have previewed the complete Image Collection and are satisfied with the parameters, save the Processing Definition (File > Save) and give it a unique name.

15. You may also "Save as" only green parameters or only red parameters at this time by un-checking the "Analyze" box of the color you wish to no longer include, clicking "Preview", and clicking File > Save as.

16. If you need to modify your processing definition for a new assay (e.g. new cell type or reagent), click on "Edit a Processing Definition" link in the Vessel View. Adjust the necessary parameters, filters, and cleanup, then "Preview" the changes. After you are satisfied with your changes, choose File > Save to replace the original definition, or File > Save As to save a new processing definition and keep the original.

NOTE: A Processing Definition can only be modified for a new assay if the new assay is imaged at the same magnification (i.e. you may only preview image collections taken at the same magnification as the image collection chosen in **Screenshot 3** under "Creating a Processing Definition", pg 3 of this Technical Note).

| *Fluorescent Processing Parameters*	
Adaptive Threshold:	A local background level (LBL) of fluorescent intensity is automatically determined across each processed image.
Threshold Adjustment:	A threshold is set this far above (if positive) the LBL in calibrated fluorescence units. Any object that is not higher than the background by this value will be excluded from the mask. The threshold is never set below (negative) the LBL.
Fixed Threshold:	A single threshold level in calibrated fluorescence units is used across the image, so all objects below this level will be excluded.
Edge split:	Edge split is recommended for separating closely-spaced objects at the weak signal points between. Making the Edge Sensitivity number larger will cause more splits to be made.
Adjust Size:	Adjusts the size of your mask in pixels by either shrinking the mask (if negative) or growing the mask (if positive).
Area Filter:	Defines a range of sizes (in μm^2) that the object can be, and eliminates objects that lie outside this range
Eccentricity Filter:	Defines how round or compact the nucleus can be, and eliminates objects that lie outside this range. Eccentricity ranges from 0 to 1 with a perfect circle having a value of 0.
Mean Intensity Filter:	Defines the limits of mean intensity of an object, (the average pixel intensity in calibrated units), and eliminates objects that lie outside this range.
Integrated Intensity:	Defines the limits of integrated intensity of an object, (the summed pixel intensity in calibrated units), and eliminates objects that lie outside this range.

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Fluorescent Processing: Established Assay Phase

## Option 1. Launch an Analysis Job for an existing vessel

1. Open the Vessel View for the vessel you wish to analyze.
2. Click "Launch New Analysis Job" under "Analysis Job Utilities" (**Screenshot 7**).
3. Select "Basic Analyzer" for Job Type
4. Choose the "Processing Definition" you wish to use from the drop down menu.
5. Assign a unique name to the Job.
6. You may select a "Time Range", "Single Time", or "Open Ended" to analyze.
7. Select the wells you wish to analyze and click "OK".
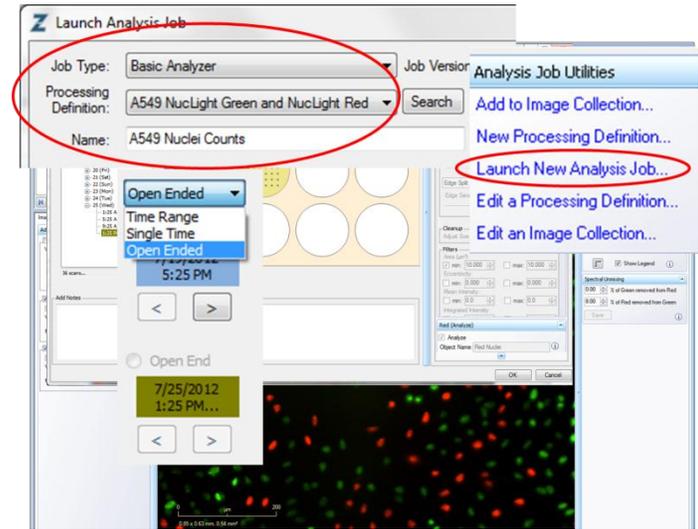
Screenshot 7: Launching an Analysis Job for an existing vessel

Option 2. *(Recommended)* Launch an Analysis Job at time of Vessel Scheduling

1. Add a New Vessel and select the appropriate channels required for imagin (**Screenshot 8**).
2. Under Spectral Unmixing, enter your pre-determined and optimized %.
3. Under Analysis Job Setup, select "Basic Analyzer" for Job Type.
4. Under the same heading, select your Processing Definition.
5. Name your vessel, add notes, determine the frequency of scans, and click Apply.
6. Data will be processed following each scan to provide fluorescent object metrics in real-time.
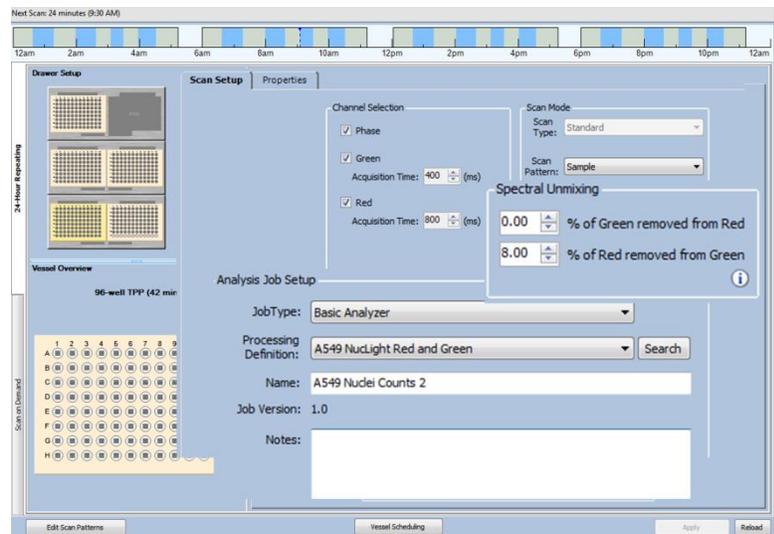
Screenshot 8. Launching an Analysis Job at the time of Vessel Scheduling

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