IncuCyte ZOOM™ Confluence Processing Overview

The IncuCyte ZOOM's Confluence Processing analysis tool (Basic Analyzer) allows users to tailor phase segmentation parameters to individual cell types and experimental conditions. The Basic Analyzer calculates and provides cell confluence metrics in real-time, allowing for optimal analysis and characterization for a wide range of cell types. 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 described in Figure 1, the data processing flow is separated into two distinct phases, the **Assay Development Phase**, and the **Established Assay Phase**.
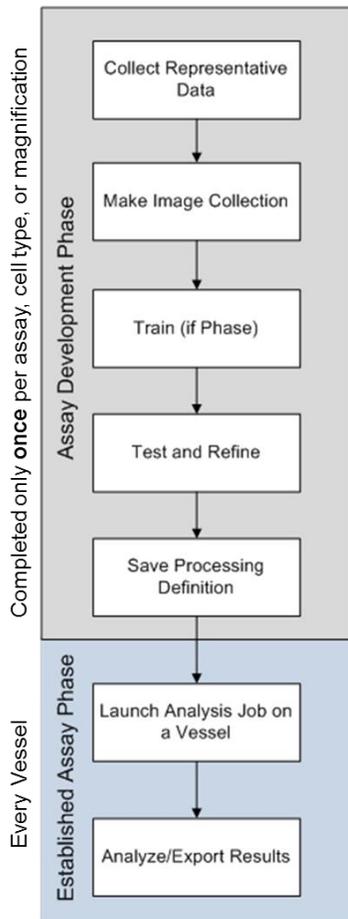

Figure 1: Flow chart illustrating the IncuCyte ZOOM assay development strategy.

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/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. One Processing Definition will likely be needed for each cell type in use and/or for each assay being run. 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.

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.

Confluence: Metrics

IncuCyte ZOOM provides the following set of basic metrics relevant for phase object analysis:
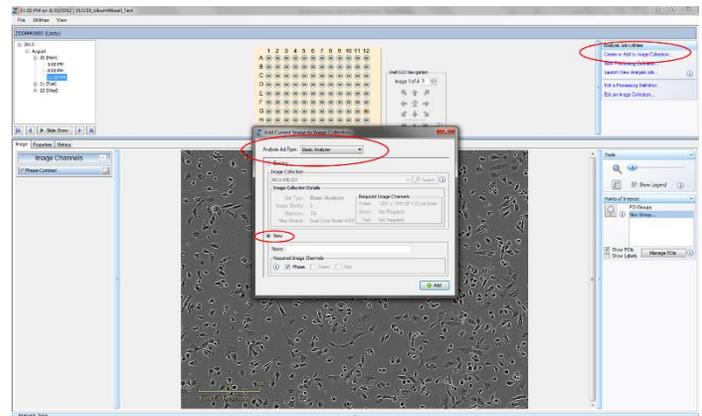
- Count
- Confluence
- Average Area
- Average Eccentricity

To accurately measure 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 phase processing terms can be found in blue boxes.

> ### *Confluence Processing Metrics Defined*
>
> **Count (1/Image or 1/mm² or 1/well):** The number of objects per image, mm^2, or well.
>
> **Avg. Area (μm²):** The average of the area of the objects in the image.
>
> **Confluence (%):** The percentage of the image area that is occupied by objects.
>
> **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.

Confluence: Assay Development Phase

Step 1. Creating an Image Collection

1. Collect HD-Phase Contrast images of your vessel every 2-6 hours over the course of 3-5 days.

2. Open the Vessel View and select 3-6 representative images of your cells. Save them in an Image Collection by clicking on the "Create or Add to Image Collection" link **(Screenshot 1)**.

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

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

5. Since Phase Confluence is of primary interest, only the Phase image channel is required to be part of the Image Collection.

6. Continue to add images to the image collection just made by clicking on the "Create or Add to Image Collection" link (**Screenshot 1**). The image collection named in Step 4 will now be listed as an "Existing" image collection.

Screenshot 1: Adding Images to Image Collections

NOTES: The total cell confluence over the complete collection should be between 30-70%. 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 1**), and evaluate which images are needed to make a representative set. Remove redundant images until only 3-6 representative images remain.

Step 2. Creating a Processing Definition

1. Start a New Processing Definition by clicking on the "New Processing Definition" link within the Vessel View.

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

3. The Basic Analyzer Processing Definition Editor will open displaying the "Preview Image Collection" drop down menu **(Screenshot 3)**. This will default to the image collection selected in the previous step (**Screenshot 2**), but it is advised to check before changing parameters (red circle in **Screenshot 3**).

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

4. Choose a "Training Image Collection" (blue rectangle in **Screenshot 3**) to determine the parameters that will differentiate between the "Background" and the "Cells" within the phase image. The "Training Image Collection" should be the Image Collection that you made containing 3-6 representative images of your cells of interest. In this case, HT-1080 cells. It is often the same as the "Preview Image Collection".

5. Give the phase analysis an Object Name (green circle, **Screenshot 3**). For example "HT 1080". When graphing, HT1080 will be listed in front of any graphed confluence metric.

6. Click the "Preview" button.

   NOTE: The best way to begin setting up the Processing Definition is to use the preset values already contained within the Processing Definition Editor, therefore do not change "Segmentation Adjustment", "Cleanup", or "Filters" at this time.

7. Make sure the "Confluence Mask" box is checked and evaluate your phase segmentation **(Screenshot 4)**.

8. To refine the mask, move the "Segmentation Adjustment" slider more towards "Background" to eliminate background texture and debris, or towards "Cells" to include more cell area and click "Preview".

9. Use the "Cleanup" options to further manipulate your mask. "Hole Fill" masks as confluence any holes that are smaller than the area specified. "Adjust Size" grows or shrinks the mask by the number of specified pixels. Click "Preview" to evaluate the refined mask.
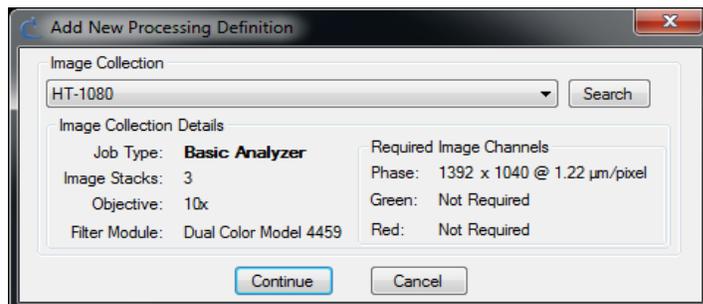
Screenshot 2. Starting a New Processing Definition
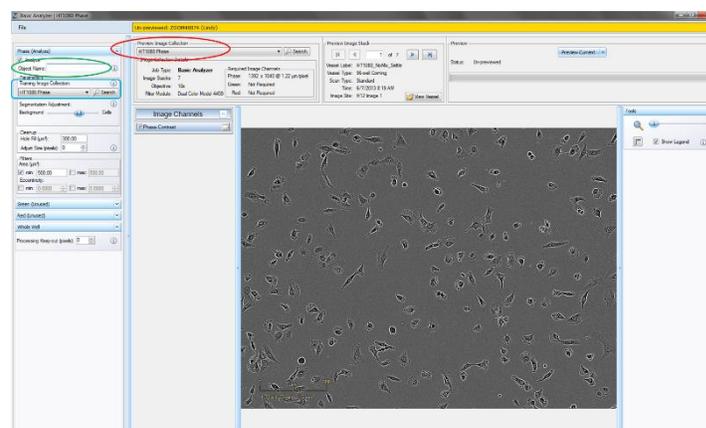
Screenshot 3. Basic Analyzer Processing Definition Editor
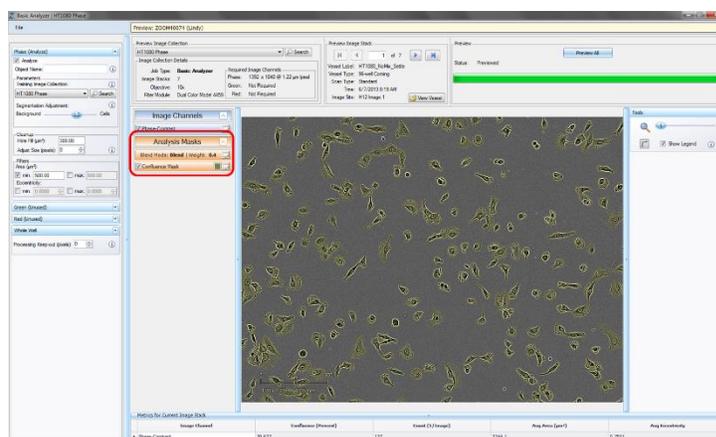
Screenshot 4. Confluence Mask

10. 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, in this example, HT-1080.

11. 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. Make your changes. Be sure to preview your latest changes before saving. 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 2** under "Creating a Processing Definition", pg 3 of this Technical Note).

Confluence Processing Parameters, Cleanup, and Filters

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Training Image Collection:	An image collection required for phase analysis. An algorithm analyzes these representative images and uses the information to differentiate between background and cells. Using a different image collection will alter the phase segmentation of the processing definition.
Segmentation Adjustment:	A slider that adjusts the parameters to bias the segmentation mask toward more background or more cells.
Hole Fill:	Masks areas between cells if the hole is smaller than the given area.
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 object 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.

Confluence: 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 **(Screenshot 5)**.

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".
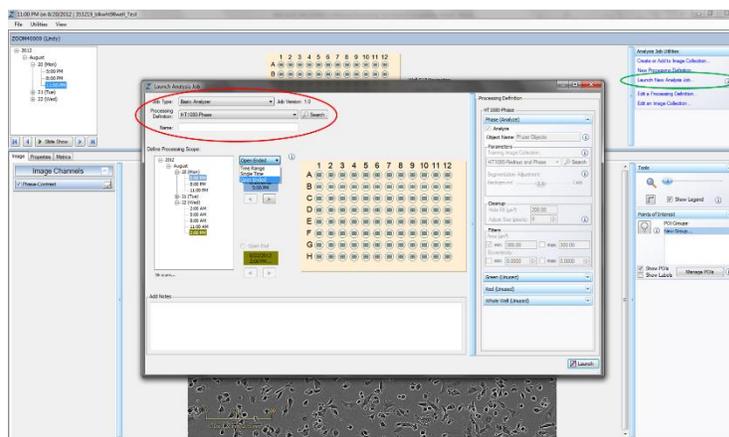
Screenshot 5. Launching an Analysis Job for an Existing Vessel

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

1. Add a New Vessel and select the appropriate channels required for imaging

2. Under Analysis Job Setup, select "Basic Analyzer" for Job Type.

3. Under the same heading, select your Processing Definition **(Screenshot 6)**.

4. Name your vessel, add notes, determine the frequency of scans, and click "Apply".

5. Data will be processed following each scan to provide Confluence metrics in real-time.
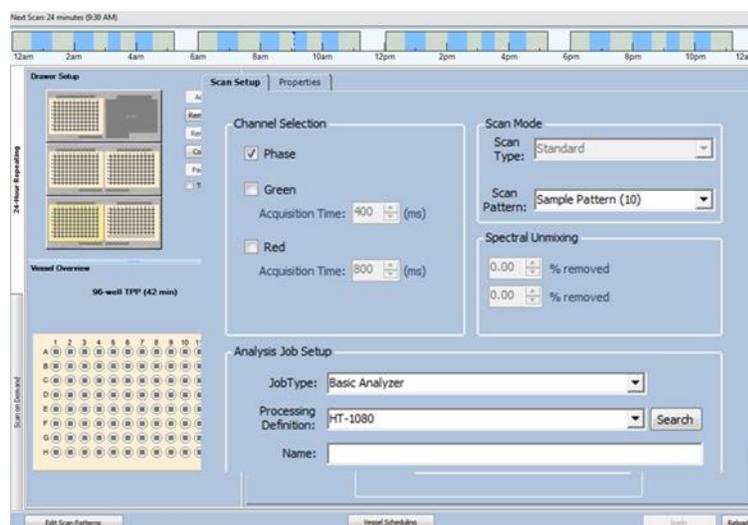
Screenshot 6. Launch an Analysis Job at the Time of Vessel Scheduling