

IncuCyte ZOOM™ Scratch Wound Processing Overview

The IncuCyte ZOOM Scratch Wound assay utilizes the WoundMaker-IncuCyte ZOOM-ImageLock Plate system to analyze both 2D-migration and 3D-invasion in label-free, live cells. The integrated processor applies an algorithm to HD-phase contrast images of migrating or invading cells to produce pertinent metrics that allow analysis of pharmacological or genetic effects on the migration and/or invasion potential of the cell type being assayed. Because the assay is compatible with primary and immortalized cells types, the processor has been designed to appropriately handle a wide range of phenotypes. To fully utilize this feature, the IncuCyte ZOOM user has the ability to tailor **Processing Definitions** that optimally analyze the cell types under investigation. This requires 3 simple steps: 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**.

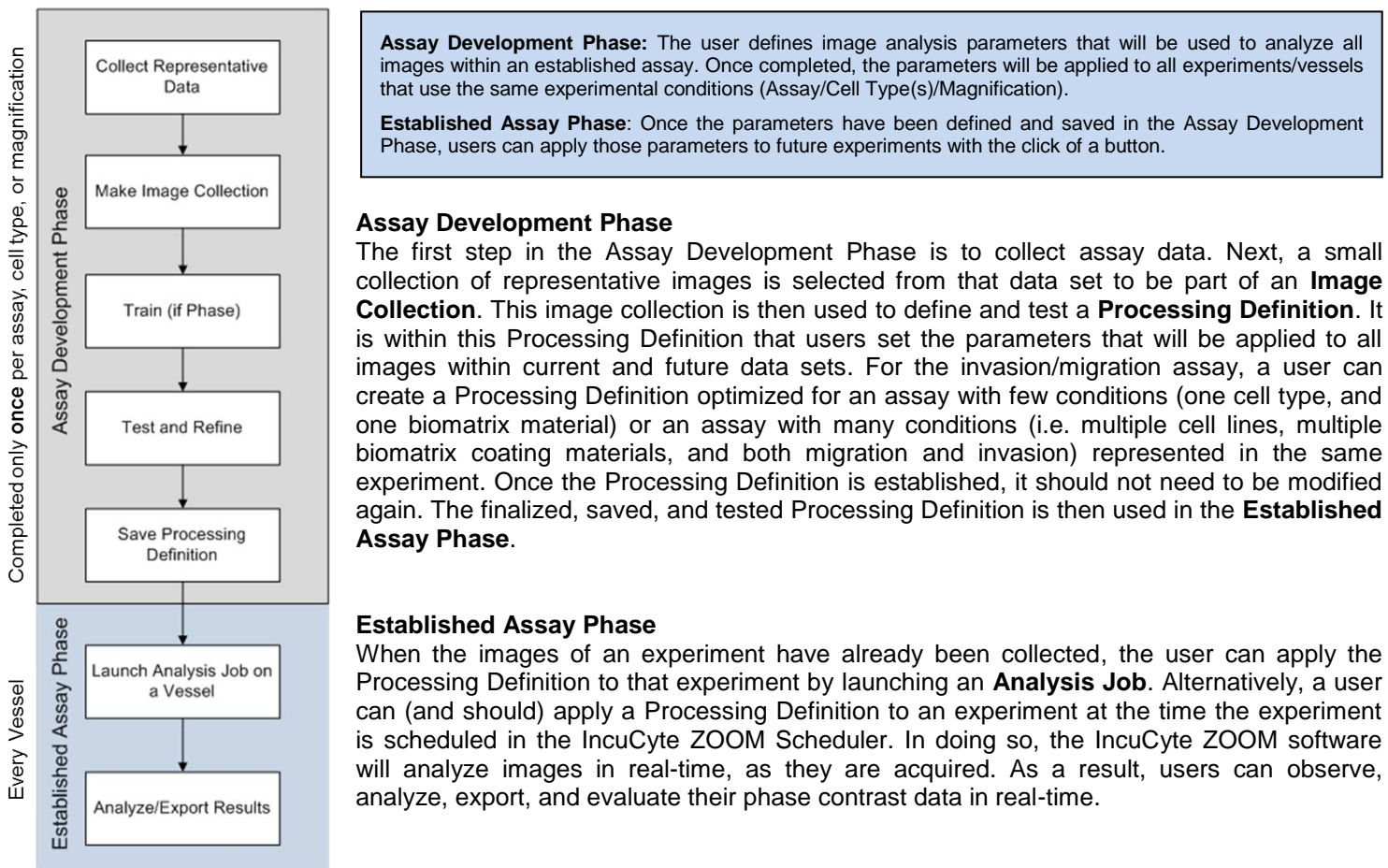


Figure 1: Flow chart illustrating the IncuCyte ZOOM assay development strategy.



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. It is recommended that every condition is represented in the image collection for Invasion/Migration studies. Depending on the experimental design, this may include multiple cell types, biomatrix materials, and both migration and invasion phenotypes. Up to 10 images is acceptable, but keep in mind that with more images, the Processing Definition will take a little longer to optimize.

Processing Definition: Processing Definitions are what define the parameters used to analyze all the 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.

Scratch Wound: Metrics

IncuCyte ZOOM provides the following three metrics relevant to cell migration analysis:

- Wound Width
- Wound Confluence
- Relative Wound Density

Invasion assays are analyzed using Relative Wound Density alone. 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 Scratch Wound terminology can be found in blue boxes.

Scratch Wound Metrics Defined

Wound Width (µm): The distance between the migrating/invading edges of the wound. In some cases, as cells migrate, the new boundaries that are formed remain relatively parallel to each other. If this is the case, then changes in wound width accurately report the time course and extent of cell migration.

Wound Confluence (%): This metric is a report of the confluence of cells within the wound region, given as the percentage of the wound region area occupied by cells.

Relative Wound Density (%): This metric relies on measuring the spatial cell density in the wound area relative to the spatial cell density outside of the wound area at every time point. It is designed to be 0% at t=0, and 100% when the cell density inside the wound is the same as the cell density outside the initial wound. In this respect, the metric is self-normalizing for changes in cell density which may occur outside the wound due to cell proliferation and/or pharmacological effects. Importantly, the RWD metric is robust across multiple cell types as it does not rely on finding cell boundaries.

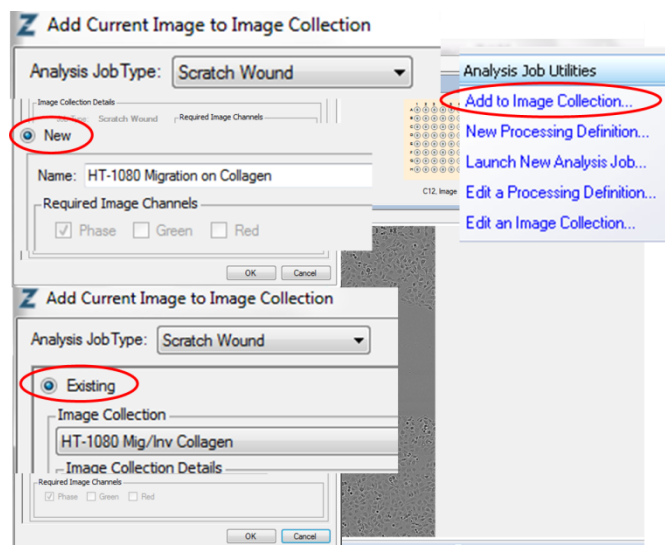
Scratch Wound: Assay Development Phase

Step 1. Creating an Image Collection

1. Collect HD-Phase Contrast images of your vessel every 1-hour for Migration Assays or 2-3 hours for Invasion Assays until assay is complete.
2. Open the Vessel View, view an image you want to add, and click on the “Add to Image Collection” link (**Screenshot 1**).
3. Select “Scratch Wound” from the Analysis Job Type drop down menu.



- When adding the first image of a collection, select “New” and assign a unique name.
- Since Phase Confluence is of primary interest, only the Phase image channel is required to be part of the Image Collection.
- Continue to add images to the image collection just made by clicking on the “Add to Image Collection” link (**Screenshot 1**). The image collection named in Step 4 will now be listed as an “Existing” image collection. An image Collection for “Scratch Wound” should include an image of cells immediately following wounding, as well as an image showing migration and/or invasion of cells into the wound area at ~10% and ~50% wound closure. If multiple cell types are on the same plate, or both invasion and migration conditions are present in the same experiment, representative images of each cell line and condition should be included.

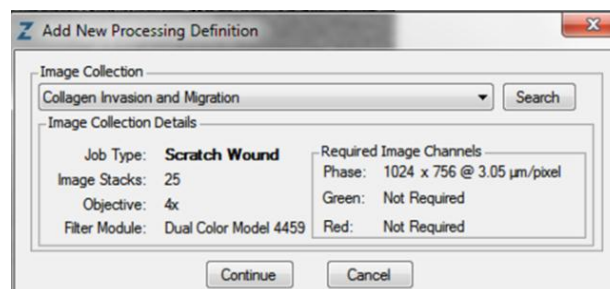


Screenshot 1: Adding Images to Image Collections

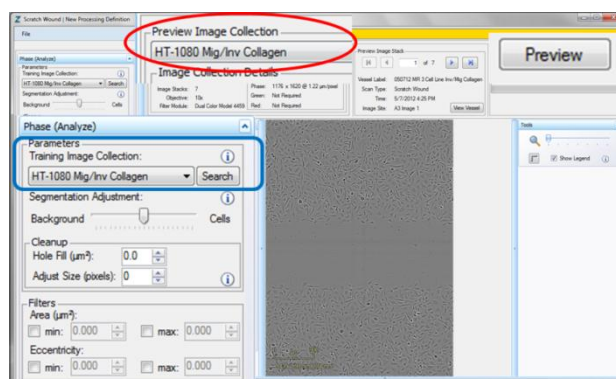
NOTES: An Image Collection can only contain images taken at the same magnification. An Image Collection may contain images from multiple tissue culture vessels. The collection should not contain too many images as it will prolong the development of the Processing Definition. We recommend adding images to a collection to represent all cell types present, all biomatrix materials used, both migration and invasion phenotypes, where relevant, and examples of any treatment added to the assay. Then, go to “Edit image collection” link, and evaluate which images are needed to make a representative set. Then remove redundant images until only 3-6 representative images remain.

Step 2. Creating a Processing Definition

- Start a New Processing Definition by clicking on the “New Processing Definition” link within the Vessel View (found under “Analysis Job Utilities” in **Screenshot 1**).
 - Select the proper Image Collection and click “Continue” (**Screenshot 2**).
 - The Scratch Wound 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.
- NOTE:** You may preview a processing definition on multiple image collections, if desired.
- Select the “Training Image Collection” (blue rectangle, **Screenshot 3**) that includes the 3-6 representative images of your cell type under investigation. The training image collection will determine the parameters that will differentiate between the “Background” and



Screenshot 2: Starting a New Processing Definition



Screenshot 3: Scratch Wound Processing Definition





the “Cells” within the image. This is often the same image collection as your Preview Image Collection.

5. 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.

6. Adjust “Blend Mode”, “Masks”, “Brightness” and Contrast to best view your image. Changing these will not change the Processing Definition but may help you to discern the image.

7. Check the “Scratch Wound Mask” and “Confluence Mask” box and evaluate that your phase segmentation masks your cells appropriately (**Screenshot 4**).

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

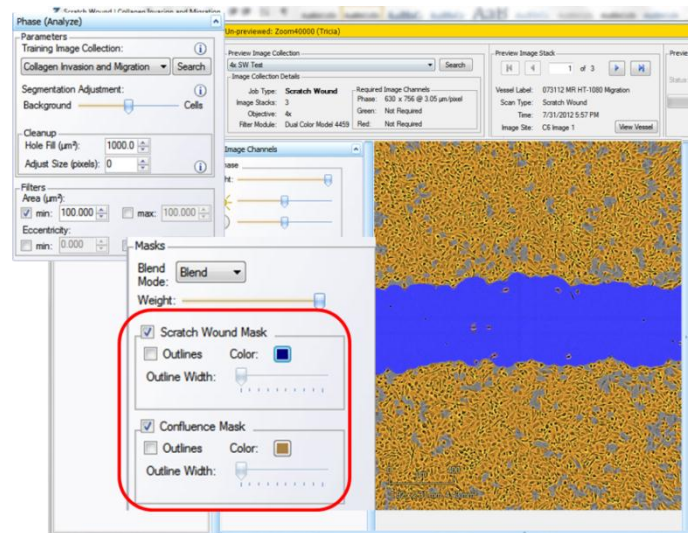
9. Use the “Cleanup” options to further manipulate your mask. “Hole Fill” removes any holes in the cell mask that are greater than the area specified. “Adjust Size” grows or shrinks the mask by the number of specified pixels. Click “Preview” to evaluate the refined mask.

10. Apply filters for area or eccentricity to exclude unwanted objects like debris or dead cells being masked. (See blue box labeled “Scratch Wound Processing Parameters and Masks, Defined” below, for a more detailed explanation.)

11. 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.

12. 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 2** under “Creating a Processing Definition”, page 3 of this Technical Note).



Screenshot 4: Previewing Segmentation and Scratch Wound Mask

Scratch Wound Processing Parameters and Masks

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 nucleus can be, and eliminates nuclei that lie outside this range. Eccentricity ranges from 0 to 1 with a perfect circle having a value of 0.
Scratch Wound Mask:	Identifies the leading edge of the population of migrating/invading cells within each image of the series.
Confluence Mask:	Represents the cell confluence outside of the wound region.

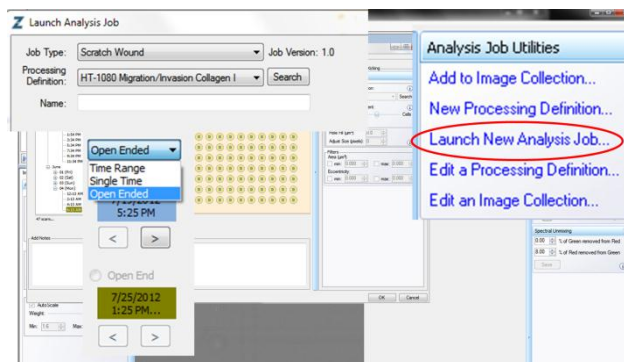




Scratch Wound: Established Assay Phase

Option 1. Launch an Analysis Job for an existing vessel

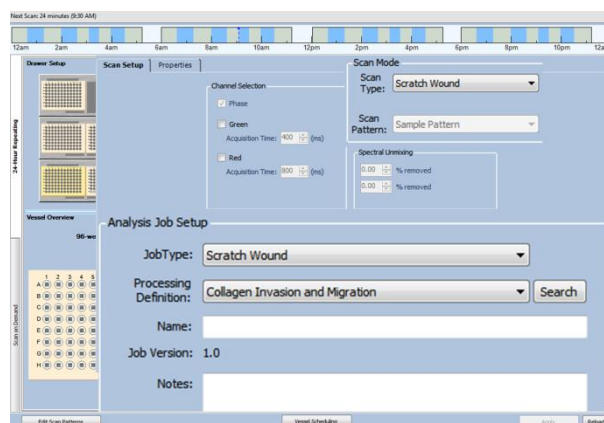
1. Open the Vessel View for the vessel you wish to analyze.
2. Click “Launch” under “Analysis Job Utilities” (**Screenshot 5**).
3. Select “Scratch Wound” for Job Type
4. Choose the Processing Definition you wish to use from the drop down menu (**Screenshot 5**).
5. Assign a unique name to the Job.
6. 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 “Scratch Wound” 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 Scratch Wound Metrics in real-time.



Screenshot 6: Launching an Analysis Job at Time of Vessel Scheduling

