

## CellPlayer™ 96-Well Cell Migration and Invasion Metrics

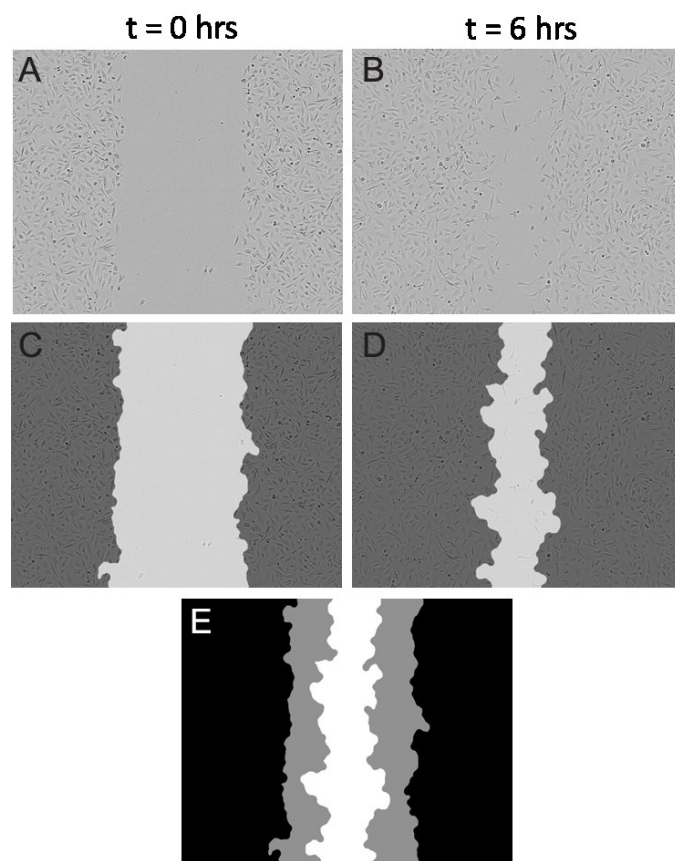
### Introduction

Cell migration and invasion are multistep processes that are fundamental components of many important biological and pathological processes, such as embryonic development, tissue re-organization, angiogenesis, immune cell trafficking, chronic inflammation and tumor metastasis. The Essen IncuCyte™ live-cell imaging system, coupled with the CellPlayer™ 96-Well Cell Migration/Invasion Assay Kit, is a powerful platform for completing high throughput, label-free cell migration and invasion assays. The IncuCyte™ is an automated microscope which resides inside a standard cell culture incubator. It is ideal for assays that benefit from longer-term kinetic read-outs, because cells are maintained at optimum physiological conditions for the duration of the experiment. This integrated solution for both cell migration and cell invasion combines the time-lapse microscope, automation, and quantitation in one system, alleviating the need to implement component solutions from multiple vendors. The analysis software provides multiple analytical metrics, including Wound Confluence v1.5, Wound Width, and Relative Wound Density v1.0. The purpose of this technical note is to describe each of these metrics in greater detail and to provide recommendations for their use in both Cell Migration and Cell Invasion applications.

### Automated image processing

To initiate a Cell Migration/Invasion assay, the user must select the “Scratch Wound” scan type in the correct tray position and cutout within the “Schedule Upcoming Scans” window found within the IncuCyte™ software. The 96-Well Cell Migration/Invasion software Application Module was designed to analyze each image as it is acquired. For both Cell Migration and Cell

Invasion applications, the first scan in the time course is used to generate the **initial scratch wound mask** (Figure 1C). The creation of the initial scratch wound mask is based on the Wound Confluence v1.5 algorithm, and is



**Figure 1.** Original HD Phase Contrast images (A-B); Initial Scratch Wound Mask (C); Scratch Wound Mask (D); and blended view (E).

designed to identify the borders of the wound region. This initial wound mask is used in subsequent quantification processes and is therefore critical to the success of the assay. A **scratch wound mask**, based on



the same algorithm, identifies the leading edge of the population of migrating cells within an image (Figure 1D). It is computed at all subsequent image acquisition time points beyond the first scan. In addition to the scratch wound mask, a **confluence mask** is also generated. The confluence mask represents the cell confluence of the wound region exactly as determined by the confluence algorithm. All of these masks can be viewed either alone or in a blended combination within the “Vessel View” window inside the IncuCyte™ software (Figure 1E).

**Initial Scratch Wound Mask:** Identifies the borders of the wound region using the first image in a temporal series

**Scratch Wound Mask:** Identifies the leading edge of the population of migrating cells within each image of the series

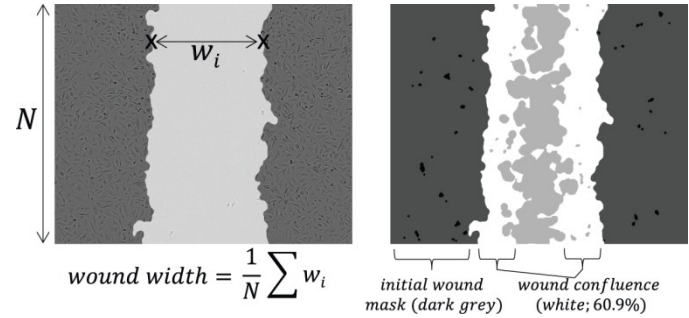
**Confluence Mask:** Represents the cell confluence of the wound region

### IncuCyte™ Software Integrated Analysis Metrics

There are three integrated metrics that are calculated based on the processed images and can be reported using the Cell Migration/Invasion Software Application Module.

#### Wound Width (Cell Migration Only)

Wound Width is equivalent to the average distance (microns) between the edges of the scratch wound mask in each line of resolution within an image (Figure 2A). This is the only metric of the three available metrics that does not rely on the initial scratch wound mask, as each image is analyzed independent of the starting point. It is important to note that the scratch wound mask identifies the boundary of the migrating population and, at times, will ignore a small number of cells that do not fit within that population.



**Figure 2.** Wound width (left) and wound confluence (right). N = 924 lines of resolution in a scratch wound image.

#### Wound Confluence v1.5 (Cell Migration Only)

Wound Confluence relies on the initial scratch wound mask to define the region of the image that is occupied by cells and differentiates that region from the wound region (Figure 2B). The Wound Confluence v1.5 algorithm is then applied, and only the confluence calculated within the wound region is reported. The resulting number represents the percentage of wound area that is occupied by cells.

#### Relative Wound Density v1.0 (Cell Migration and Cell Invasion)

Like Wound Confluence, **Relative Wound Density** also relies on the initial scratch wound mask to differentiate between cell-occupied and cell-free regions of the image. Once these regions are defined, a second image analysis algorithm based primarily on sharpness values, which is not viewable to the user and is completely independent of the confluence algorithm, is applied in order to calculate the density of both the cell region and the wound region as defined by the initial scratch wound mask. It is important to note that both the cell region and the wound region are variables in the Relative Wound Density v1.0 equation, and both are treated with equal importance. Specifically, the Relative Wound Density metric refers to the density of the wound region as a function of the density of the cell region and is defined by the following equation:





$$\%RWD(t) = 100 \cdot \frac{w(t) - w(0)}{c(t) - w(0)}$$

$w(t)$  = Density of wound region at time,  $t$

$c(t)$  = Density of cell region at time,  $t$

This equation highlights the fact that the initial density of the wound ( $w(0)$ ) is subtracted from the density of the wound at each subsequent time point ( $w(t)$ ). Importantly, this means that the value reported at each time point is background subtracted. The background subtracted wound density represents the numerator in the above equation. For the most part, the density of the cell region ( $c(t)$ ) represents the denominator in the above equation as the initial density of the wound ( $w(0)$ ) is typically very small. The reason  $w(0)$  is subtracted from  $c(t)$  is to guarantee that the relative wound density value is equal to 100% when  $w(t)$  is equal to  $c(t)$ .

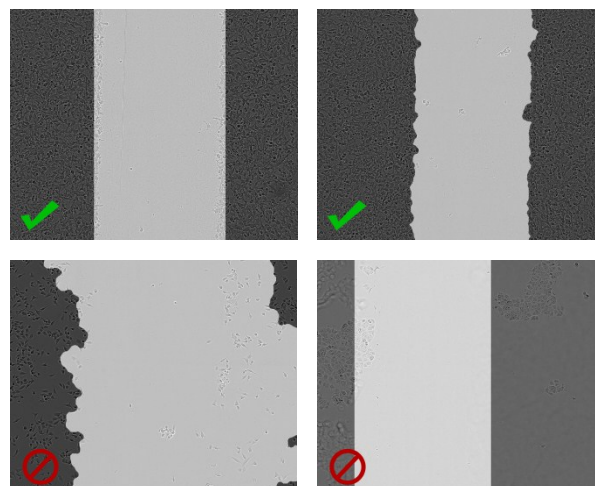
#### Important considerations for 96-well Cell Invasion

There are several differences between cell migration and cell invasion, both in experimental design and biological phenotypes. These differences have resulted in the qualification of Relative Wound Density as the only metric to be used in the 96-well Cell Invasion protocol. There are two reasons why this is the only metric utilized:

- 1) The morphology of invading cells in a 3D matrix is significantly different than migration in 2D. Cells with a mesenchymal invasion morphology typically display an elongated phenotype with lamellipodia that extend into the ECM. Furthermore, one leader cell is generally followed by numerous other cells, thereby forming a network of “tunnels”, as opposed to a leading edge of cells as is often observed in a cell migration assay in 2D. For this reason, the scratch wound mask does not always best represent the invading population and results in inaccurate measurements of wound width.
- 2) The invasion assay requires the addition of an ECM for cells to invade. The presence of the ECM can

**Relative Wound Density:** Measure of the density of the wound region relative to the density of the cell region

result in difficulty for the confluence algorithm in its ability to generate an initial wound mask that exactly fits the edges of the wound area. The initial scratch wound mask will also fail if cells are not plated at the appropriate density (for multiple examples, see Figure 3). Therefore, careful optimization is required for all cell types and experimental conditions. In most cases, the estimated wound region determined when exact borders are not identified is adequate. This estimated region can contain a small portion of cellular content within the wound region. However, the background subtraction built into the Relative Wound Density v1.0 metric subtracts this at each time point, thus making it statistically insignificant. Background subtraction is not performed within the calculations of either Wound Width or Wound Confluence v1.5 metrics, and they should not be used for measuring Cell Invasion.



**Figure 3.** Acceptable scratch wound masks (top) and unacceptable masks (bottom).



### Comparison of Relative Wound Density v1.0 metric to Wound Confluence and Wound Width

- 1) Relative Wound Density accounts for changes in both the density of the cell region, as well as changes in the wound region. This is especially important when experimental treatments alter cell morphology.
  - **Wound Confluence** – Morphological changes in the cell region are not taken into account.
  - **Wound Width** – The scratch wound mask identifies a migrating “population”. In doing so, some changes in cell content within the wound are ignored.
- 2) Relative Wound Density accounts for the background density of the wound at the initial time point, and subtracts that value from each subsequent time point resulting in an equivalent value of 0% RWD at the initial time point.
  - **Wound Confluence** - Cells or other matter within the wound are quantified as confluence and not background subtracted. Therefore, the wound confluence values at the initial time point are not 0, and they may be variable from well-to-well.
- 3) Relative Wound Density does not rely on the identification of individual cell borders. Because the RWD calculation computes the density of both the cell region and the wound region equivalently, and factors both into the final calculation, identification of the true borders of cells is not necessary. This is especially helpful when using cell types that are difficult for the Wound Confluence v1.5 algorithm to evaluate (e.g. HUVEC).
  - **Wound Confluence and Wound Width** – In order to get an accurate reading of the true confluence value of the wound, or to identify the leading edge of the migrating population, the cells used must be easily identified by the Wound Confluence v1.5 algorithm. For the majority of cell types, this is not a problem. However, very adherent cells with a flattened

morphology (e.g. HUVEC and other similar cell types, particularly those that have been over-passaged) can pose challenges for the Wound Confluence v1.5 algorithm. It is recommended that Relative Wound Density be utilized for these cell types to reduce errors caused by noisy confluence values and incorrect calculations of Wound Width.

