Tech Note

Measuring Confluence Using High Contrast Brightfield

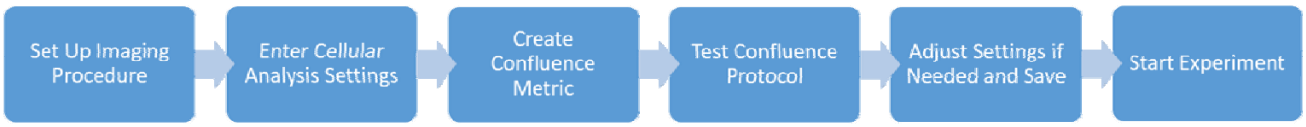
Introduction

Measuring confluence is a direct and straightforward approach to quantify a population of cells. However, manual methods for measuring confluence are subjective, while most automated approaches require fluorescent stains or dyes. Here we describe an automated label-free technique for measuring confluence using high contrast brightfield images and Gen5 image analysis. This robust and convenient method enables accurate quantification of cell proliferation over time and normalization of data across multiple experiments and time points.

Kinetic Proliferation Cell Handling

Confluence values over time are determined from high contrast brightfield images collected during a kinetic proliferation experiment. When seeding microplates, initial cell densities should take into account cell doubling time and duration of experiment. A four day experiment with 2000 cells seeded per well in a 96-well microplate is a good starting point for cell lines with a doubling time of around 24 hours. Allow cells ample time to adhere to the microplate before starting assay.

Gen5™ Workflow Overview



Gen5 Imaging Procedure

Images are acquired using a Cytation™ or Lionheart™ FX equipped with high contrast brightfield.

1. Select **4x PL FL** (4x PLN) objective.
2. Select **Bright Field** for channel 1.
3. Click channel 1  to view imaging settings.
4. Click **Find Image** to bring cells into focus and determine exposure settings.

   Note: Cells should appear in focus and darker than background (Figure 1). Auto-focus and auto-exposure settings in Gen5 work well for acquiring high contrast brightfield images for most cell types, although some applications may benefit from adjusting these settings.
5. Click **Save settings**

6. If using laser autofocus, capture a reference scan.

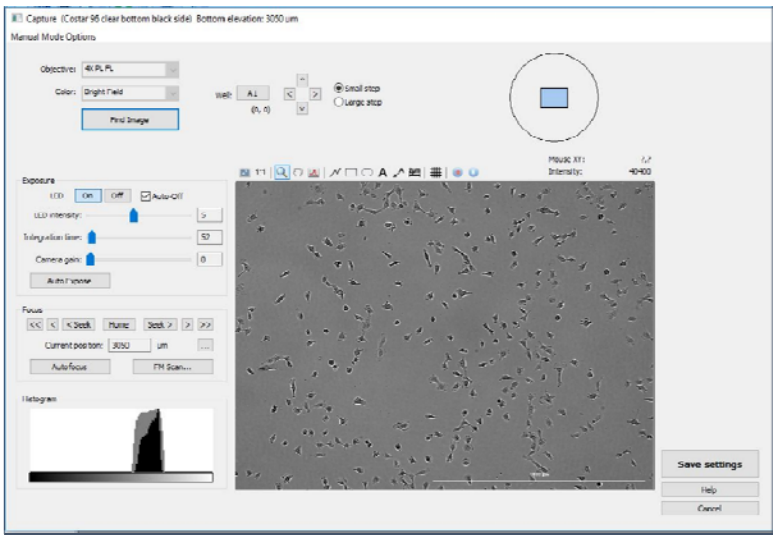


Figure 1. **Imaging setup window with representative exposure settings and in focus image for confluence evaluation.**

Gen5 Cellular Analysis Settings

Gen5 Cellular Analysis features enable accurate identification of cell outlines from high contrast brightfield images. The following settings have been verified on HT-1080, NIH3T3, HeLa, and HCT116, and are recommended starting values for analyzing other cell types (Table 1). Preprocessing of high contrast bright field images is not required for confluence measurements.

1. Open the **Data Reduction** window and add a new Cellular Analysis step.

2. Set **Primary Mask** around cells using the values from **Table 1.**

Cellular Analysis Parameters	
Channel	Bright Field
Threshold	1000
Background	Light
Split touching objects	Checked
Fill holes in mask	Checked
Min. object size	30 µm
Max. object size	3000 µm
Include primary edge objects	Checked
Analyze entire image	Checked
Advanced Analysis Parameters	
Rolling Ball diameter	20 µm
Image smoothing strength	5 cycle of 3x3 average filter
Evaluate background on	0%
Primary mask	Use threshold mask

Table 1. **Suggested Cellular Analysis settings for determining confluence.** Threshold value and Min. object size should be adjusted based on cell type and imaging conditions.

3. In the **Calculated Metrics tab**, click **None** to remove default well-level results, then click **Select or create object-level metrics of interest…**
4. In the **Manage Object-Level Metrics**, click **Insert custom metric…**
5. Create **Confluence Metric** (Figure 2).

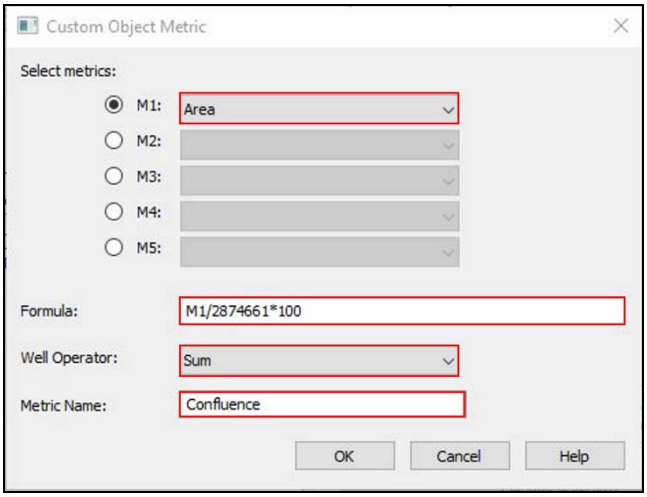


Figure 2. **Custom Object Metric Window for creating Confluence metric.** Confluence is calculated from total (Sum) masked area (Area) divided by the total image area (2874661 mm^2), and multiplied by 100 to yield percent.

Test Confluence Protocol

Test image acquisition and analysis settings by running the protocol on a few selected wells. Percent confluence is now available within the Plate matrix Data field, as well as displayed for individual wells in the Plate window (Figure 3A) and Analyze Tool (Figure 3B). Confirm that masks are accurately placed around all cells.

A. **B.**

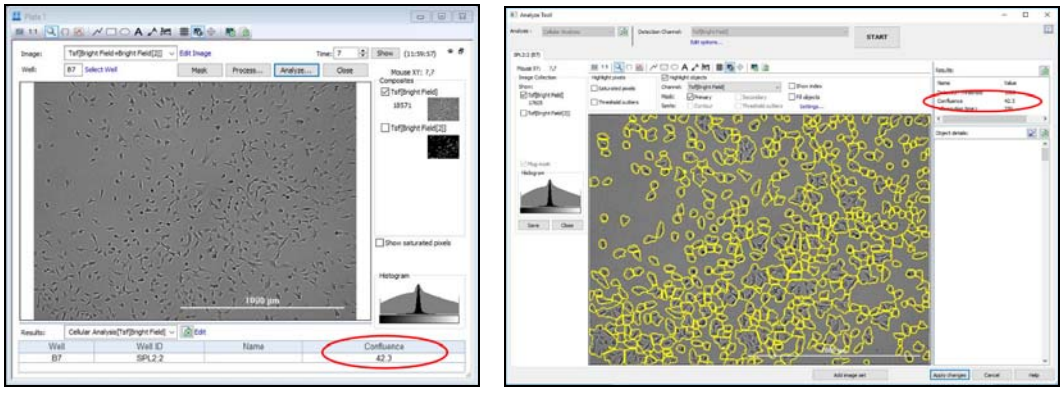


Figure 3. **Screen shots demonstrating percent confluence metric displayed in the (A) Plate window and (B) Analyze Tool.**

Adjusting Confluence Protocol Settings

If modifications to the Cellular Analysis settings are required, adjustments to the **threshold** value should be sufficient to address most conditional variations. Smaller cell types may require decreasing the **Min object size**, however adjustments to this setting should take into account the possibility of including debris into the analysis. All settings should be determined empirically by visually confirming accurate placement of outlines around cells (Figure 4). Once you are satisfied with the Cellular Analysis settings, the protocol is now ready to measure confluence for end point applications or kinetic proliferation experiments, and should be **saved** for future use with the same or similar cell types.

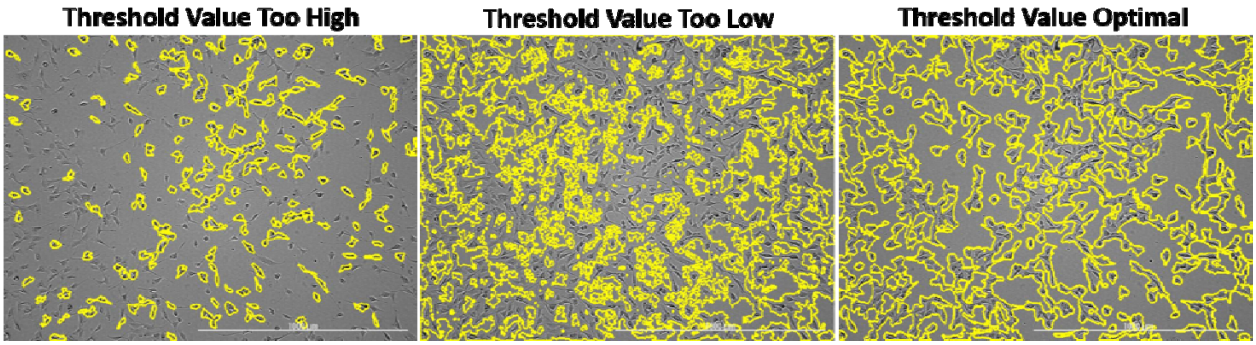


Figure 4. **Visual confirmation of placement of cell outlines.** Setting the threshold value too high will result in cells being excluded from the analysis. Conversely, a threshold value that is too low will include background in the analysis. Optimal settings will result in accurate placement of outlines around all cells while excluding background.

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