

High Contrast Brightfield

Enabling microplate-based automated label-free cell counting

Authors

Paul Held, Joe Clayton, and
Peter Banks
Agilent Technologies, Inc.

Introduction

Cell counting is a basic technique for cell culture propagation and establishing cell densities required for cell-based assay setup. The common hemocytometer that uses a defined small volume and grid pattern allows the scientist to count individual cells when magnified by brightfield microscopy. This can be an arduous and subjective task that would benefit significantly from an automated workflow. Automated cell counters are available, and some of the most popular are based on image analysis. These counters typically require cells to be placed in a disposable consumable that ensures that the same volume is analyzed each time, allowing accurate volumetric counts, much like the common hemocytometer. The consumable is designed to be exclusive to its counter and provide a limited number of experiments per consumable.

Cell counting is also used as a read-out for cell-based assays. The most common assay to use this read-out is cell proliferation, best performed in kinetic mode where cell proliferation can be monitored in real time. This is often done using genetically encoded fluorescent proteins that are expressed in the cell's nucleus, allowing a simple method for counting live cells using fluorescence microscopy. The assumption is that one cell has one nuclei. While automated cell counters have fluorescence channels that can perform these measurements, the required consumable make them impractical for conducting assays. Instead, cellular imaging instruments that allow the use of microplates are preferred due to their ability to rapidly assess many samples sequentially, in fixed volumes, at a relatively low recurring cost per assay point.

A preferred method for performing cell proliferation is the use of a nonperturbing detection mode. While the use of genetically encoded fluorescent proteins are preferred over chemical nuclear stains, the molecular biology required to express the proteins may perturb normal cell function. Brightfield and phase contrast microscopy both can image cells without the use of stains or fluorescent probes, but lack sufficient contrast to be able to count cells.

This technical overview describes the high contrast brightfield (HC brightfield) accessory kit that allows accurate brightfield cell counting by creating additional contrast and specimen depth of field. Refraction by each cell body generates a sharp, bright spot when the image is defocused. The technique allows robust, automated cell counting from a brightfield image using Agilent BioTek Gen5 image analysis software.

Materials and methods

Cell culture

HeLa, NIH 3T3, and U-2 OS cells were cultured in Advanced Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum and penicillin streptomycin. All cultures were maintained at 37 °C in 5% CO₂ and routinely trypsinized (0.05% trypsin-EDTA) at 80% confluency.

Cells were plated into Corning black-sided clear-bottom 96-well microplates (part number 3904) at the required cell density in a total of 100 µL growth medium.

Nuclear staining

Live cells were stained with Hoechst 33342 added directly to the medium. Hoechst 33342 dye working solution was made up as a 2x concentrate in complete medium and 100 µL was added to each well for a final concentration of 1 µg/mL. Fixed and stained cells were treated as follows: cells were washed once with 200 µL of PBS (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 7.4 mM) before fixation using the Agilent BioTek EL406 washer dispenser manifold, after which 100 µL of 4% paraformaldehyde (PFA) solution was added using the EL406 syringe pump dispenser manifold. Cells were fixed for 10 minutes at room temperature followed by two washes of 200 µL using PBS. Following fixation, cell nuclei were stained with 50 µL of working solution of 30 µM DAPI in PBS/0.1% Triton X-100. Cells were stained 10 minutes at room temperature followed by two washes with 200 µL of PBS. After aspiration, 100 µL of PBS was added to all wells and the plates were sealed using an optically clear TopSeal-A adhesive plate sealer (Perkin Elmer, Waltham, MA).

High contrast brightfield cell counting

Cells were imaged using an Agilent BioTek Lionheart FX automated microscope with brightfield and DAPI fluorescent light cube capabilities. The imager uses a combination of LED light sources, bandpass filters, and dichroic mirrors to provide appropriate wavelength light. The DAPI cube uses a 377/50 nm excitation filter and a 447/60 nm emission filter. Brightfield measurements were made using a dedicated white light LED positioned above the sample, along with the brightfield accessory.

Determination of offset value

Cells were brought into focus using the HC brightfield accessory and the "in focus" focal height recorded. The View Line Profile tool was then used to draw a line that crossed cells and background sections of the imaging field without cells present. The focal height was then decreased while observing the line profile to determine the focal height at which maximum contrast between cell and background brightness was achieved. This "High Contrast" focal height was then subtracted from the "in focus" height to determine the offset value.

Comparison measurements

HC brightfield cell counting was compared to conventional object counting of stained cell nuclei, which requires two separate read channels. Standard fluorescence imaging was performed, with the additional counting channel set to brightfield and the focus method is an offset read from the image channel. Exposure settings such as LED intensity, integration time, and camera gain were determined using slight manual adjustments from those determined using the Auto software feature. Focus for the primary channel was obtained using the image autofocus routine in the Gen5 software. The brightfield signal was then defocused using an offset based on the focal height of the primary channel. Figure 1 demonstrates the contrast enhancement this provides by comparing an intensity profile of a portion of an image showing three cells using first a zero offset (image plane established by autofocus) followed by defocused using an offset of -220 nm. Note the significant increase in intensity through the cells in the defocused line profile, which makes cell counting possible by thresholding the brightfield signal.

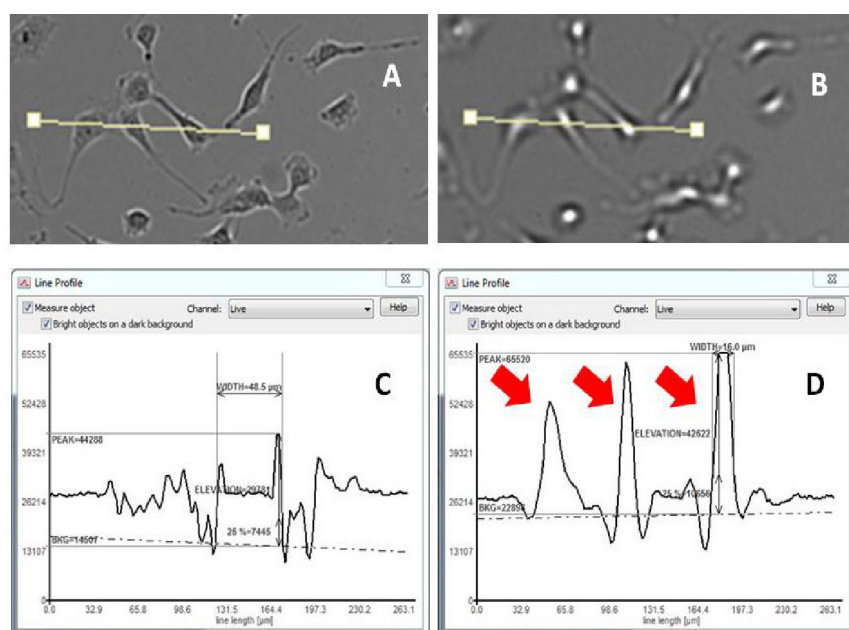


Figure 1. Enabling high contrast brightfield cell counting by defocusing. NIH3T3 cells were brightfield imaged using the HC brightfield accessory with an image plane set by autofocus (A); and defocused by setting an offset of -220 μm (B). (C) Intensity profile of three cells selected with the Line Profile tool with focused image plane (-220 μm offset) demonstrates satisfactory intensity changes (marked by red arrows) suitable for cell counting.

Analysis settings

Object masks were counted using Gen5 3.0 software. Object masks in the DAPI channel were defined using a threshold value of 18,000 and a minimum and maximum size exclusion of 10 μm and 100 μm, respectively. Background was set to "Dark" with flattening size was set to auto and evaluated on the 5% lowest pixels. Object masks for brightfield were also defined, based on a threshold of 18,000 and the same size exclusion as DAPI. Background was defined as "Light" with flattening size on auto and evaluated on the 50% lowest pixels. Identified objects were then counted as the basis for cell counting.

Cell proliferation assay

The Agilent BioTek BioSpa 8 automated incubator was connected to the Agilent BioTek Cytation 5 cell imaging multimode reader. This allowed long term kinetic measurements of cell proliferation of NIH 3T3 cells extending to 96 hours where microplates were shuttled from the controlled environmental conditions of the BioSpa 8 (37 °C, 5% CO₂, >90% humidity) to the Cytation 5 for HC brightfield cell counting every two hours.

Results and discussion

Cell counting

The effect of the HC brightfield accessory defocused imaging is shown in Figure 2. Refraction by each cell body generates a sharp, bright spot when the image is defocused. Each of these spots can be discerned as a discrete object when subjected to threshold mask cellular analysis.

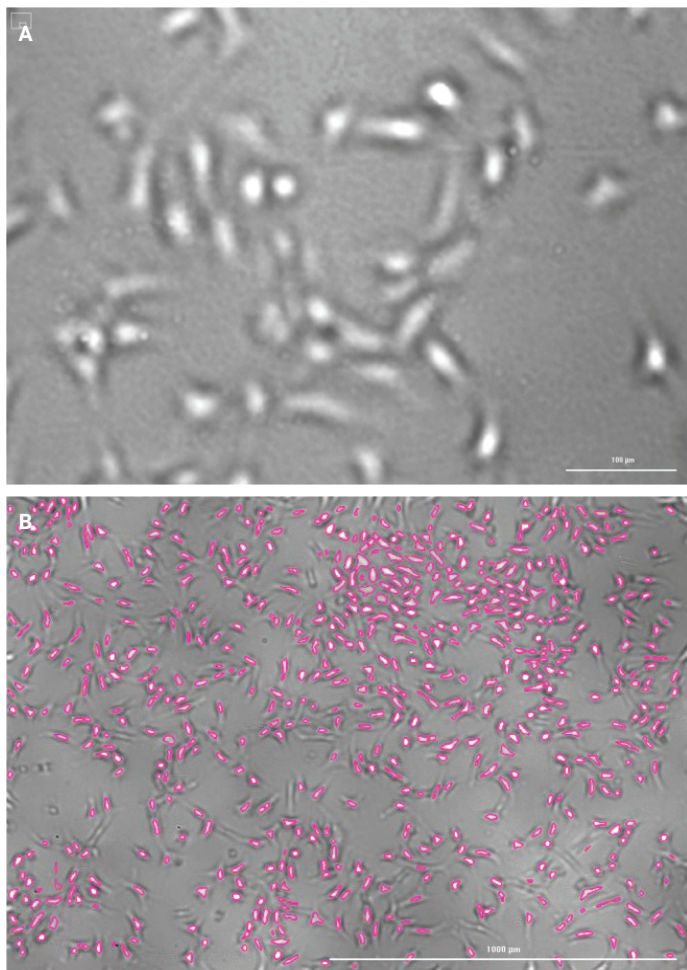


Figure 2. HC brightfield image analysis. NIH3T3 cells were brightfield imaged using the HC brightfield accessory with a focus point set 350 µm below true focus. (A) Digitally zoomed image of brightfield objects. (B) Masking of cellular objects through digital image analysis to provide cell counts.

Comparison of cell counts obtained using object mask cellular analysis of both the DAPI and HC brightfield channel show very good correlation with fixed and stained NIH 3T3 fibroblast cells (Figure 3) across a large cell titration. The slope of 1.036 computed by linear regression suggests that HC brightfield overestimates cell counting by an average of only 3.6% relative to DAPI staining over the range of cell densities tested, which suggest a high degree of equivalence between the two counting methods.

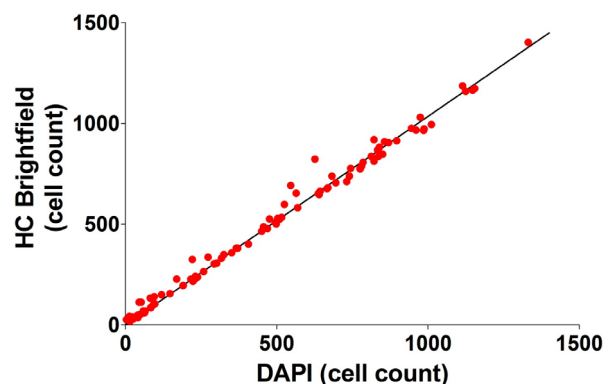


Figure 3. Comparison of HC brightfield and DAPI nuclear staining cell counting of fixed and stained cells. Paraformaldehyde-fixed NIH 3T3 cells stained with DAPI were imaged with a 4x objective. Each data point represents the mean of eight determinations.

Analysis of live NIH 3T3 cell cultures demonstrates very similar results as those obtained with fixed and stained cells. These cultures, which appeared denser than the fixed plate, were effectively confluent at the high end of the cell titration, but showed very similar results with either Hoechst 33342 stained nuclei (DAPI channel) or with the HC brightfield channel (Figure 4). The slope of the linear regression of 0.983 is indicative of a high degree of equivalence between the two methods of cell counting with HC brightfield underestimating cell counts by only 1.7% over the range of cell densities tested.

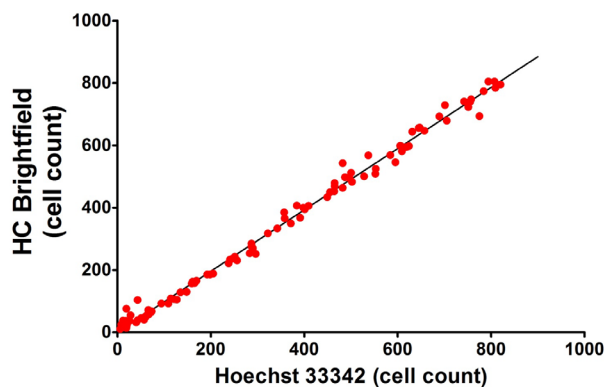


Figure 4. Comparison of HC brightfield and Hoechst 33342 nuclear staining cell counting of live cells. Live NIH 3T3 cells stained with Hoechst 33342 were imaged with a 4x objective. Each data point represents the mean of eight determinations.

Other immortal cell lines were also tested using a similar procedure. HeLa and U-2 OS cell titrations that have been paraformaldehyde fixed and DAPI stained demonstrated that HC brightfield underestimated cell counts by an average of 7.5% for HeLa and 5.7% for U-2 OS demonstrating a similar performance for cell counting relative to NIH 3T3 cells.

Cell proliferation

Figure 5 shows images of NIH 3T3 cells under HC brightfield at the beginning and end of a 96-hour cell proliferation experiment. Figures 5A (t = 0 hours) and 5B (t = 96 hours) demonstrate the contrast enhancing capabilities of HC brightfield where the NIH 3T3 cells appear as bright objects. Gen5 image analysis can then routinely place primary masks around these bright objects as shown in Figures 5C (t = 0 hours) and 5D (t = 96 hours) and thus count the bright objects or cells without the need for any nuclear stain.

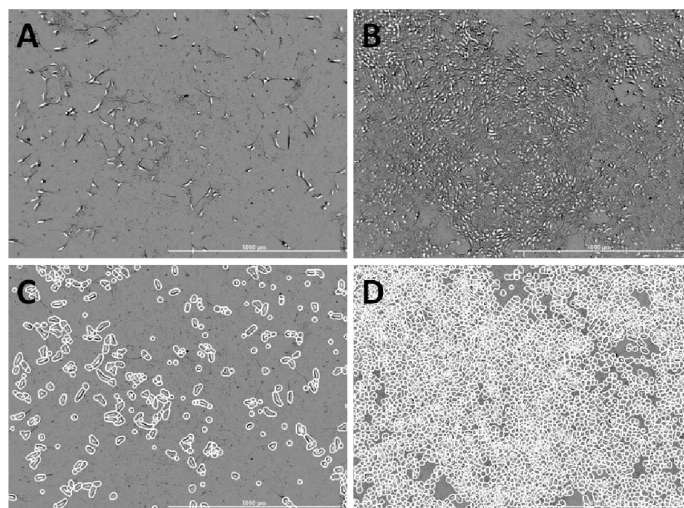


Figure 5. HC brightfield images of NIH 3T3 cells in a cell proliferation experiment. (A) Initial image at t = 0 hours; (B) Final image at t = 96 hours; (C) Cell counting masks applied to initial image at t = 0 hours; (D) Cell counting masks applied to final image at t = 96 hours.

Figure 6 depicts the full kinetic profile of NIH 3T3 cell proliferation over four days with a time resolution of 2 hours. The cell population doubles roughly every day indicative of robust growth until the wells become confluent.

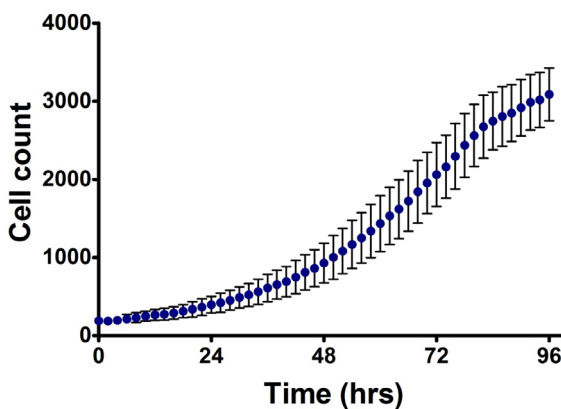


Figure 6. Proliferation of NIH 3T3 cells. Each data point represents an average of 16 individual measurements of cell count.

Conclusion

When adherent cells are imaged in brightfield, they generally lack contrast and are difficult to count using most automated cell counting analysis algorithms. This technical overview shows that the HC brightfield accessory, in conjunction with defocused imaging, can improve the ability to count cells through contrast enhancement. This technique was applied to both live and fixed cells, as well as a number of different commonly used tissue culture cell lines. With this method, the cell body acts as a focusing lens, creating a bright spot in the cell. Agilent BioTek Gen5 image analysis software can use this bright spot to create an object mask allowing facile cell counting. This cell counting method can also be used for mammalian cells grown in suspension or newly seeded adherent mammalian cells. Newly seeded adherent cells are still round in shape prior to attaching to the plate substrate and extending outward. Likewise, suspension cells are generally round and, if allowed to settle or centrifuged to the plate bottom, can be imaged successfully. Yeast cells have also been successfully counted. These round cells make good targets for accentuation by the pinhole defocus method.

There are a few limitations to this cell-counting method. Because it uses light refraction to create a lens-effect bright spot to enhance the contrast, fixative methods that reduce refraction are not compatible. For example, the mounting medias used to permanently mount and seal fixed cells on slides is designed to prevent refractive alterations are not compatible. Cells embedded in tissue or tissue slices are also not good candidates, as the variable Z-axis positioning of the cells precludes any sort of uniform defocusing. While the method described uses wet mounted fixed cells in microplates, older specimens begin to flatten over time, which reduces the accuracy of the label free method as compared to nuclei staining as a means to count cells.

As a result of the defocused imaging, there is a certain amount of aberration to the image, similar to that seen with a starfield. The most significant issue is that the counting mask with defocused imaging does not necessarily align with the mask from other channels, and therefore cannot reliably be used as a primary mask for secondary mask designation or as a means to overlay other channels that were obtained in focus. The optimal offset settings from true focus for the most precise cell counting will vary based on different cell types. This study used three different commonly used cell lines, but there are numerous other cell types that have markedly different morphology. For best results, different cell lines will require some optimization.

Reference

1. Drey, L. L.; Graber, M. C.; Bieschke, J. Counting Unstained, Confluent Cells by Modified Brightfield Microscopy. *BioTechniques* **2013**, 55, 28–33.