Application Note

Cell Culture QC

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Automated Hemocytometer-Based Live/Dead Cell Counting Using Phase Contrast and Color Brightfield Imaging

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Introduction

Cell counting is one of the most common laboratory tasks in the life sciences. It is required for routine cell culture propagation and as a starting point for any cell-based assay. While there are many instruments available for automated cell counting, the reusable hemocytometer remains the inexpensive tool of choice. It is used with another common tool of the laboratory, a brightfield optical microscope such that cells can be visualized and counted on a grid of squares. Figure 1 demonstrates both side and top views of the hemocytometer and the magnified view of the counting grid as seen by an optical microscope.

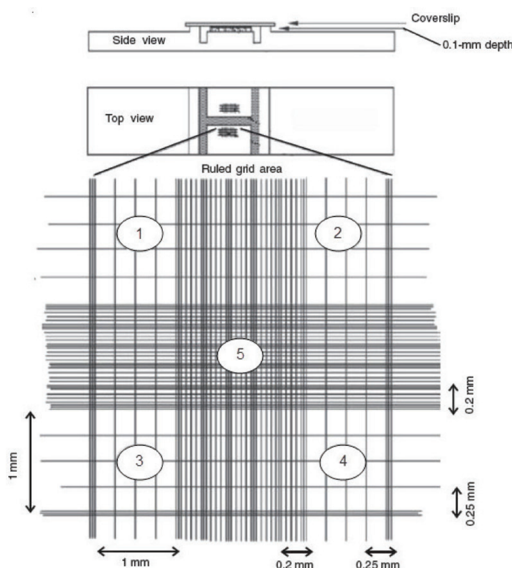

Figure 1. Hemocytometer, top, side, and ruled grid area under high magnification. The 1 mm^2 areas labeled 1, 2, 3, and 4 have ruling separated by 250 µm, while region 5 has much tighter ruling of 50 µm.

The ruled regions labeled 1 through 4 are useful for counting mammalian cells which are on the order of 20 to 30 µm in diameter or about a tenth of the ruling spacing. A red blood cell is approximately one fifth the size of a mammalian cell, so the ruled spacing of region 5 is more suitable for counting purposes. The counting procedure is somewhat subjective as some users have different protocols on how to count cells that reside on the lines of the grid.

The complete ruled grid area is covered by a coverslip that provides a perpendicular dimension of 100 µm, which serves to keep cells roughly in the depth of field of the microscope used and define the volume of the squares used for counting. For regions 1 through 4, the 16 squares used for counting represent a volume of 0.1 mm^3, or 10^{-4} mL. Thus, the cell density of the original culture can easily be calculated knowing the dilution factor used to get the culture at an appropriate density for counting. Often, an exclusion dye such as trypan blue is used such that live cells can be differentiated from dead cells and the cell count reflects only viable cells.

This application note demonstrates how the hemocytometer cell counting process can be automated using an Agilent BioTek cell imaging multimode reader equipped with contrast enhancement technologies. For total cell counts, the cell imager uses automated phase contrast microscopy to count the total number of cells in the field of view, which is approximately 2.9 times larger than the total ruled grid area. This serves to improve the counting statistics relative to counting manually. Furthermore, cell viability can also be determined through the use of automated color imaging of tryptan blue stained cells in the same field of view.

Materials and methods

Materials

Cells: Human neonatal dermal fibroblasts (part number cAP-0008RFP) were purchased from Angio-Proteomie (Boston, MA, USA). The cells were propagated in Advanced DMEM Medium (part number 12491-015) plus Fetal Bovine Serum (FBS), 10% (part number 10437-028) and Pen-Strep-Glutamine, 1x (part number 10378-016) each from Life Technologies (Carlsbad, CA, USA). MCF-7 cells (part number 92020424) were purchased from Cell Biolabs, Inc. (San Diego, CA). The cells were propagated in MEM alpha Medium (part number 12561-056) plus Fetal Bovine Serum (FBS), 10% (part number 10437-028), Pen-Strep, 1x (part number 15070-063), and Human Recombinant Insulin, 10 µg/mL (part number 12585-014) each from Life Technologies. HCT116 cells (part number CCL-247) were purchased from ATCC (Manassas, VA). The cells were propagated in McCoy's 5A (Modified) Medium, HEPES (part number 12330) plus Fetal Bovine Serum (FBS), 10% (part number 10437-028), and Pen-Strep, 1x (part number 15070-063).

Reagents: Trypan Blue, 0.4% (part number 15250-061) was purchased from Life Technologies.

Agilent BioTek Cytation 5 cell imaging reader: The Cytation 5 combines automated digital microscopy and conventional multimode microplate detection providing rich phenotypic cellular information and well-based quantitative data. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C, CO_2/O_2 gas control, and dual injectors for kinetic assays. The phase contrast and color brightfield imaging channels were used to image live and dead cells in areas 1 through 4 of the hemocytometer described in Figure 1.

Agilent BioTek Gen5 data analysis software: Gen5 software controls the operation of the Cytation 5 for both automated digital microscopy and PMT-based microplate reading. Cellular analysis and the addition of subpopulation parameters allow automated total cell, as well as dead cell counting, in addition to live cells/mL calculations.

Methods

Automated hemocytometer phase contrast and color brightfield imaging

Cells were mixed with trypan blue, incubated at room temperature for 5 minutes, and added to the hemocytometer in the same fashion as performing a manual cell count. For the purposes of this application note, 50 µL of resuspended cells were mixed with 30 µL of medium and 20 µL of trypan blue. The hemocytometer was then placed into an adapter which enables use with the Cytation 5. A user prompt asks for the dilution factor when the cells were added to the trypan. If no dilution was performed, a value of “1” is entered.

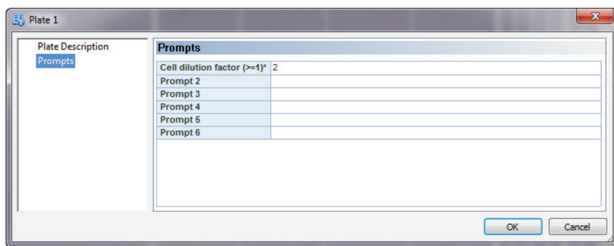

Figure 2. Agilent BioTek Gen5 cell dilution user prompt. The prompt appears before the experiment is begun. A value of “2” is entered to indicate that the cells were diluted 1:2 when mixed with trypan blue.

Automated digital brightfield imaging provides insufficient contrast for accurate cell couting, so automated phase contrast and color brightfield imaging channels were used, along with a 4x objective. A customized image montage read step was incorporated which ensures that the four 16-square grid regions from each side of the hemocytometer are imaged, matching the typical process used in manual cell counting with a hemocytometer.

Live/dead cell calculation using Agilent BioTek Gen5 data analysis software

Cellular analysis was performed using Gen5 software on the 4x phase contrast and color brightfield images captured. Live cells were counted using phase contrast images, while trypan blue stained dead cells were counted using color brightfield images. Tables 1 and 2 describe the parameters used to perform the two cell counts.

Table 1. 4x phase contrast image cellular analysis parameters.

Phase Contrast Detection Channel Cellular Analysis Parameters	
Threshold	10,000 RFU
Minimum Object Size	10 µm
Maximum Object Size	30 µm
Bright Objects on a Dark Background	Checked
Split Touching Objects	Checked
Advanced Options	
Evaluate Background On	5% of lowest pixels
Image Smoothing Strength	0
Background Flattening Size	Auto
Subpopulation Analysis	
Circularity	>0.2
Mean Phase Contrast Signal	>15,000

Table 2. 4x color brightfield image cellular analysis parameters.

Color Brightfield Detection Channel Cellular Analysis Parameters	
Threshold	5,500 RFU
Minimum Object Size	10 µm
Maximum Object Size	35 µm
Bright Objects on a Dark Background	Unchecked
Split Touching Objects	Checked
Advanced Options	
Evaluate Background On	5% of lowest pixels
Image Smoothing Strength	0
Background Flattening Size	Auto
Subpopulation Analysis	
Circularity	>0.5
Mean Red Signal	<12,000

The number of live cells counted from all four 4x phase contrast images is converted into the number of cells which would on average have been counted from the four 16-square grids included in the image. As the area of a 4x image is 2,874,661 µm^2 (Figure 3), and the area of one 16-square grid is 1,000,000 µm^2, the live cell count value is divided by 2.9 to keep the results consistent with what would be reported from a manual count. Additional calculations are then performed to determine the live cells/mL within the cell solution.

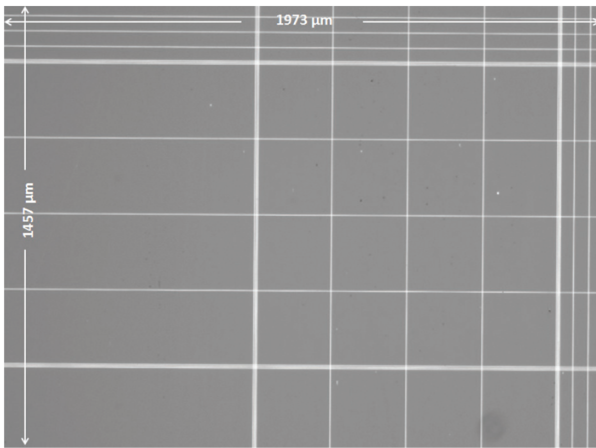

Figure 3. 4x brightfield image of hemocytometer 16-square grid and surrounding area.

The % cell viability is also determined using the following formula:

$$\% \text{ Cell Viability} = \frac{\text{Total Phase Contrast 4x Image Live Cell Count}}{\text{Total Color Brightfield 4x Image Dead Cell Count} + \text{Total Phase Contrast 4x Image Live Cell Count}} \times 100$$

The live cells/mL and % cell viability values are calculated automatically by the Gen5 data analysis software.

Results and discussion

Live cell determination using phase contrast imaging

Live MCF-7 cells appear as structures with a mostly round morphology when viewed on a hemocytometer, due to the fact that they are added as a suspension and have not attached to the bottom surface. Being more compact, the cells cause a relatively uniform change in phase, allowing them to appear as bright white objects on the dark background (Figure 4A). The lower threshold value in the Agilent BioTek Gen5 cellular analysis tool can be set at an appropriate value to correctly identify cellular structures, while ignoring other areas of the image, including trypan blue stained dead cells. Minimum and maximum object sizes can also be optimized to ensure that single cells are identified (Figure 4B). However, dead cell remnants and other noncellular debris can also be added to the counting area. These items typically do not have the same level of signal, and are not as circular as live cells. Therefore two additional subpopulation parameters are applied to the objects that are primarily identified (Table 1). Items that do not meet the circularity and minimum mean phase contrast signal requirements are then eliminated from the final live cell determination (Figure 4C).

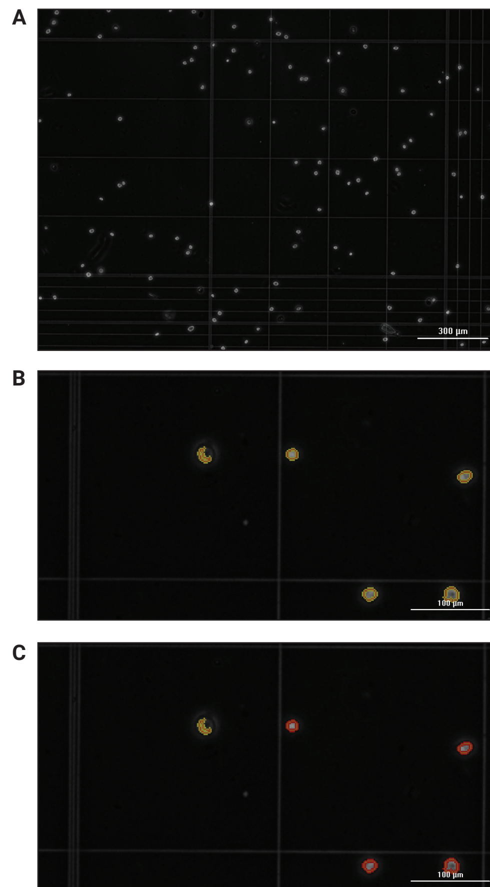

Figure 4. Determination of live MCF-7 cell numbers. (A) 4x phase contrast image of hemocytometer containing MCF-7 cells. (B) Total object count using primary cellular analysis parameters. (C) Live cell count through incorporated subpopulation criteria. Included objects highlighted in red.

The total live cell number per image will be greater than that counted from the 16-square grid alone, due to the larger area used for counting defined by the 4x field of view. This can provide a more accurate cell count due to systematic error, such as when cells are pushed out of the grid area during pipetting of the cell suspension, creating an uneven dispersal of cells. Furthermore, counting more cells provides a statistical advantage reducing random error in the cell count. Finally, the automated procedure is an objective measure of cell count and not a subjective assessment by a

technician. Considering these attributes, the automated cell counting procedure will often times provide a more accurate cell count relative to manual counting. Normalization to cells counted per grid, automatically performed by Gen5, creates a mathematically accurate final live cells/mL value.

Cell count numbers generated by Gen5 using the parameters listed in Table 1 were validated by performing a manual cell count (Table 3).

Table 3. Comparison of manual and Gen5 MCF-7 cell counts. Gen5 cells counted per four 4x images normalized to cells counted per four 16-square grids by dividing by 2.9.

	Manual Cell Count	Gen5 Cell Count
Cells counted per four 4x images		479
Cells counted per four 16-square grids	162	165.2
Cell counted per one 16-square grid	40.5	41.3
Cells per grid x dillution factor	81	82.6
Cells per mL of cell culture	8.1×10^5	8.26×10^5
% Difference between count methods	1.9%	

A difference of 1.9%, or approximately 3 cells per four 16-square grids, was seen between the manual cell count and that produced using Cytation 5 and Gen5, demonstrating the legitimacy of the automated method.

Dead cell determination using color brightfield imaging

Color brightfield imaging is used to identify dead cells within the counting regions. Trypan blue stained dead cells, which appeared as dark areas when using the phase contrast imaging channel, appear as easily identified blue objects with the incorporation of color brightfield (Figure 5).

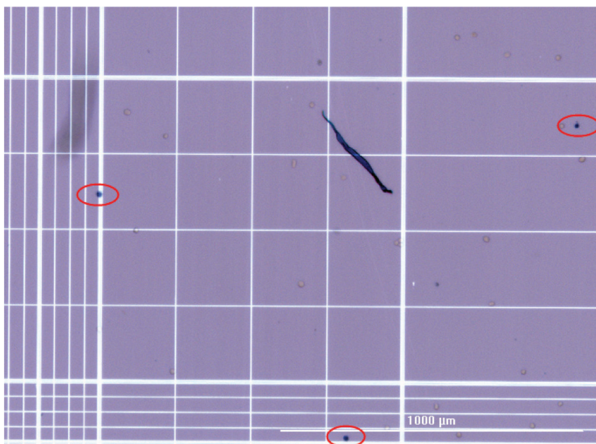

Figure 5. Color brightfield image of live and dead MCF-7 cells. Trypan blue stained dead cells identified by red oval.

The final color brightfield image represents overlaid images taken using red, green, and blue LED excitation. The dark colored trypan blue stained cells show the greatest change in signal when using the red channel (Figure 6A). An inverted threshold measurement is used, such that dark objects are identified. Minimum and maximum object size values once again ensure that single objects are identified. Noncellular objects can again appear within an image, and can occasionally be identified using the primary cellular analysis parameters. In addition, the lines etched into the hemocytometer also yield a bright signal when using color brightfield. This can lead portions of the image containing closely etched lines to also be identified due to the sharp changes in signal within a small area (Figure 6B). The circularity of cells, along with the lower mean signal seen from dead cells, once again eliminates nontarget objects (Figure 6C).

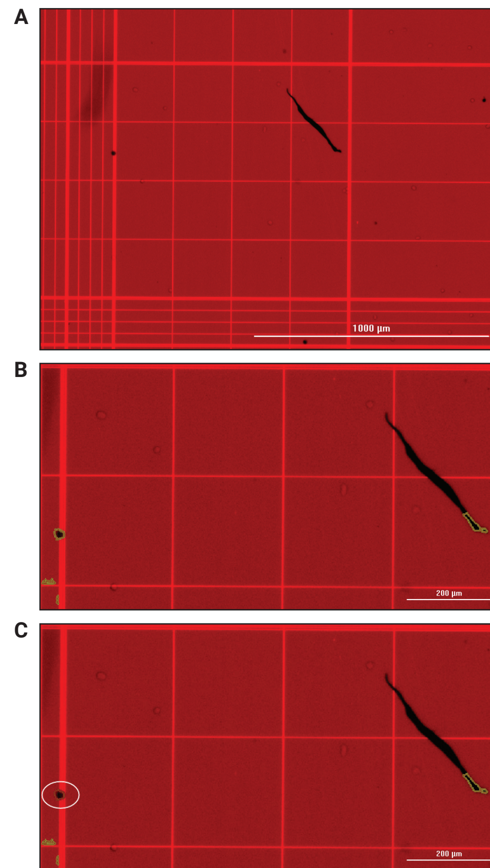

Figure 6. Dead MCF-7 cell determination using red color brightfield excitation. (A) 4x red color brightfield image. (B) Total object count using primary cellular analysis parameters. (C) Objects meeting additional subpopulation criteria. Included object highlighted by white oval.

The final dead cell count, coupled with the phase contrast live cell count, can then be used to calculate a % cell viability value to determine the viability of the cell culture.

Cell count optimization for nontypical cell types

While many cell types have similar sizes and morphologies, not every cell model will appear the same when viewed on a hemocytometer. This will require modifications to the cellular analysis parameters to ensure that proper counts are executed. HCT116 cells have a typical cell diameter less than 10 µm when viewed using phase contrast imaging (Figure 7A). Through an adjustment of minimum and maximum object size to 5 µm and 20 µm, respectively, individual cell diameters can be identified. The cells also commonly appear as small clumps of cells. By raising the threshold signal, the small changes in signal between cells can be used to place object masks around single cells, and provide a more accurate cell count (Figure 7B).

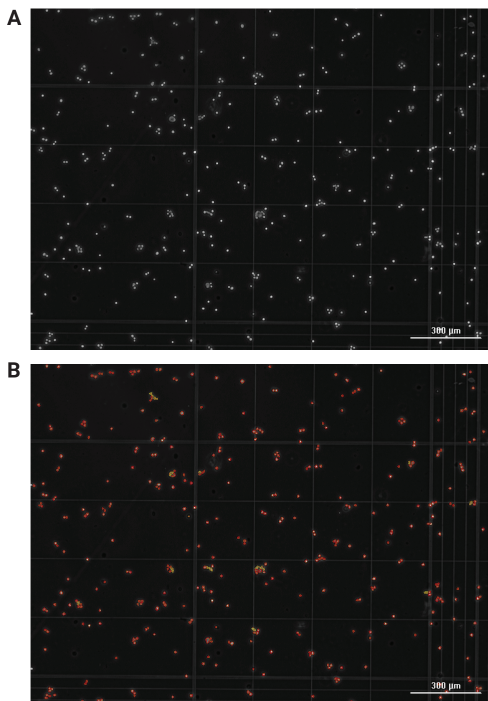

Figure 7. HCT116 imaging and live cell analysis. (A) 4x phase contrast image of HCT116 cells. (B) Object masks drawn around cells using modified primary cellular analysis parameters. Objects highlighted in red included in final live cell count.

A comparison of cells counted manually versus using the Cytation 5 and Gen5 was once again performed (Table 4). Results further validate that the automated cell counting method provides accurate data across a variety of cell types.

Table 4. Comparison of manual and Gen5 HCT116 cell counts. Gen5 cells counted per four 4x images normalized to cells counted per four 16-square grids by dividing by 2.9.

	Manual Cell Count	Gen5 Cell Count
Cells counted per four 4x images		1687
Cells counted per four 16-square grids	573	581.7
Cell counted per one 16-square grid	143.25	145.4
Cells per grid x dillution factor	286.5	290.9
Cells per mL of cell culture	2.87×10^5	2.91×10^5
% Difference between count methods	1.5%	

Primary fibroblasts are a larger cell type with a noncircular, elongated cellular morphology. Therefore the use of a higher degree of circularity as a sub-population delineator may eliminate actual dead cells from the final count. By lowering the circularity level from >0.5 to >0.1, in addition to maintaining the mean signal cutoff used previously, a proper dead cell count can once again be determined.

Conclusion

The use of a hemocytometer and trypan blue to perform live and dead cell counts is a simple, yet highly important beginning to many cell-based experiments. Knowledge of cellular concentration, as well as viability within a cell suspension is critical for proper seeding and data generation. While the procedure is easy to perform manually when examining a single or low number of cultures, the process soon becomes cumbersome when a count is required for large culture numbers, or if counting is performed on a routine basis. Manual counting is also subject to the biases of the individuals performing the procedure, thus actual cells added to a slide or microplate may vary from experiment to experiment. Through the incorporation of phase contrast and color brightfield imaging using the Agilent BioTek Cytation 5, hemocytometer-based live/dead cell counting using trypan blue can be completed in an automated fashion that frees up laboratory personnel to perform other more important tasks. Cell counts performed with Agilent BioTek Gen5 data analysis software and a procedure optimized for individual cell types also ensures that consistent numbers are incorporated with each experiment, reducing both systematic and random error in counting.

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