Application Note

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Monitoring pH in Long-Term Proliferation Assays

Using the Agilent BioTek Cytation 5 cell imaging multimode reader to monitor cell culture status concurrently with imaging

Author

Paul Held, PhD
Agilent Technologies, Inc.

Abstract

Media formulations for the propagation of *in vitro* tissue culture often contain the pH Indicator dye phenol red. It has been previously shown that phenol red absorbance can be used to determine pH in cell cultures. This application note describes the use of the Agilent BioTek Cytation 5 cell imaging multimode reader to monitor culture media pH via absorbance measurements, during multiday imaged-based cell proliferation assays.

Introduction

Characterizing cell proliferation is a crucial aspect of biological research and therapeutic drug development. Most current cell proliferation assays rely on indirect biochemical metrics that are limited by artifacts or imaging-based end-point measures. For example, DNA synthesis can be assessed though the incorporation of thymidine analogs such as BrdU, EdU, and IdU. These analogs are then detected with specific antibodies or chemistries. Cellular metabolism can be assessed with tetrazolium salts that are converted to colored compounds by the action of cellular enzymes. Several different proliferation marker proteins, such as PCNA, Ki67, and MCM-2 can be detected by immunofluorescence. While these technologies can provide a snapshot of cellular growth, they cannot necessary provide information regarding long-term proliferation.

Long-term cellular *in vitro* proliferation assays have been shown to be informative in testing antiproliferative agents.[1]

These assays take place over days in tissue culture media formulations intended to mimic nutrients normally supplied *in vivo*.

Most mammalian tissues exist at a near neutral pH. Human arterial blood is maintained at 7.4 (7.35 to 7.45) by way of a bicarbonate buffer system regulated through normal body respiration. Deviations from the normal range induce the body to increase or decrease lung activity in order to alter CO_2 expiration.[2] Not surprisingly *in vitro* cultivation of cells and tissues prosper at the same pH levels. Cultures are maintained at physiological pH primarily with a bicarbonate-carbonic acid buffer system as well. Bicarbonate-CO_2 systems use a matched concentration of dissolved bicarbonate with artificial levels of carbon dioxide gas. Carbon dioxide dissolves into the media forming carbonic acid as it reacts with water. Carbonic acid and bicarbonate also interact to form an equilibrium that is able to maintain pH at physiological levels.

Regardless of the buffering agent used, tissue culture media is often supplemented with phenol red dye. While phenol red has been described as a weak estrogen under some conditions[3], it is mostly an inert compound added to *in vitro* culture media as a visual pH indicator. While concentrations vary with different media formulations, when present it is typically in the 5 to 15 mg/L range.

Waste products produced by dying cells or overgrowth of contaminants will cause a decrease in pH, leading to a change in indicator color. For example, contamination of a culture of relatively slowly dividing mammalian cells can be quickly overgrown by bacteria, resulting in the acidification of the medium, and the indicator turning yellow. Mammalian cell waste products themselves will slowly decrease the pH, gradually turning the solution orange and then yellow. This color change is an indication that even in the absence of contamination, the medium needs replacing.

Live cell imaging experiments focus entirely on the biology at hand. Whether the images are brightfield, phase contrast or fluorescent, the background milieu of the media is not observed. This application note describes the use of the Cytation 5 cell imaging multimode reader to monitor cell culture pH status while concurrently capturing digital microscopic images. This uses the unique capabilities of the reader to measure well absorbance in conjunction with cellular imaging.

Figure 1. Corning cell culture flasks with phenol red containing media.

Materials and methods

DMEM, fetal bovine serum (FBS), and penicillin-streptomycin-glutamine were purchased from Life Technologies. Clear-bottom, black-sided 96-well microplates (3904) were provided by Corning.

Live cell experiments

HCT116-GFP colon cancer cells expressing a nuclear H2B-GFP chimera fluorescent protein were cultured in Advanced DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO_2. Cultures were routinely trypsinized (0.05% trypsin-EDTA) at 80% confluence. For experiments, cells were plated into Corning 3904 black-sided clear-bottom 96-well microplates. Long-term growth measurements were performed using an Agilent BioTek BioSpa 8 automated incubator connected with an Agilent BioTek Cytation 5 cell imaging multimode reader. The BioSpa 8 automated incubator system controls reader scheduling and maintains cells in a humidified controlled environment (37 °C, 5% CO_2) in between imaging and plate absorbance measurements. As required, the BioSpa 8 transports a microplate to the Cytation 5 for imaging and absorbance measurements and returns it to the incubator afterward.

HCT116-GFP cells were seeded at either 500 or 2,000 cells per well. Cells were allowed to attach overnight before the initiation of growth measurements. HCT116-GFP cells were imaged using the Cytation 5 cell imaging multimode reader configured with a GFP light cube. The imager maintains 37 °C and 5% CO_2 within the read chamber, and uses a combination of LED light sources in conjunction with bandpass filters and dichroic mirrors to provide appropriate wavelength light. The GFP light cube consisting of a 469/35 excitation filter and a 525/39 emission filter for imaging cells expressing GFP. Montaged (2 × 2) digital images were made using a 4x objective and stitched into a single file using Agilent BioTek Gen5 microplate reader and imager software. Primary mask analysis of the captured digital images were used to determine the number of cells. Nuclei are identified as fluorescent objects between 5 to 100 µm in size and having fluorescence more than a threshold of 1,500. Immediately after imaging, the well absorbance at 560 nm was measured using the UV-Vis monochromator module of the Cytation 5.

Results and discussion

Live HCT116 cells expressing GFP in their nuclei are easily counted using image analysis. The green fluorescence identifies each cell without the need for a nucleic acid binding stain that could affect cell growth. When HCT116-GFP cells are seeded at low density and monitored over the course of five days, the 560 absorbance was used to calculate pH in each well by interpolating a previously determined pH calibration curve. The pH in these cultures decreased approximately 0.15 over the course of five days, despite a 50-fold increase in the number of cells counted (Figure 2).

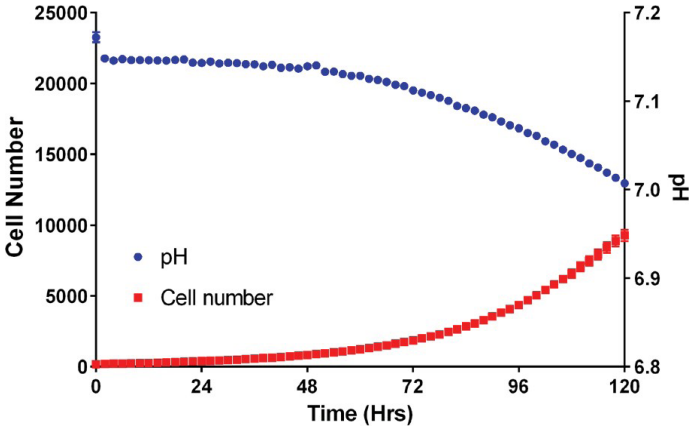

Figure 2. Change in HCT116 cell count and pH over time in nonconfluent cultures. HCT116 cells were seeded at a density of 500 cells per well. After 24 hours to allow for attachment, image-based and absorbance analysis were performed on 96-well plate cultures every two hours for five days and the results plotted. pH was determined by interpolating data from a previously generated calibration curve. Data represent the mean and SEM for 96 determinations at each data point.

In these experiments, cells were seeded at a density such that at the end of five days they have not reached confluence (Figure 3). In this state, the cells are not contact inhibited and can freely divide. The only limitation being media nutrients and media pH status.

This is corroborated with a comparison of the cell number to the 415/560 absorbance ratio. This ratio is a function of the color of the media, which has been shown to be inversely related to its pH.[4] As seen in Figure 4, the cell number increases with time in these cell cultures. Under these conditions, cells are able to grow and divide unimpeded, with time the determined cell number increases. Individual well cell numbers may vary due to variations in the physical location of the cells, as well as differences in initial cell number. Because the stitched image used for cell counting only encompasses the center portion of the well, cells attached around the perimeter of the well would not necessarily be counted.

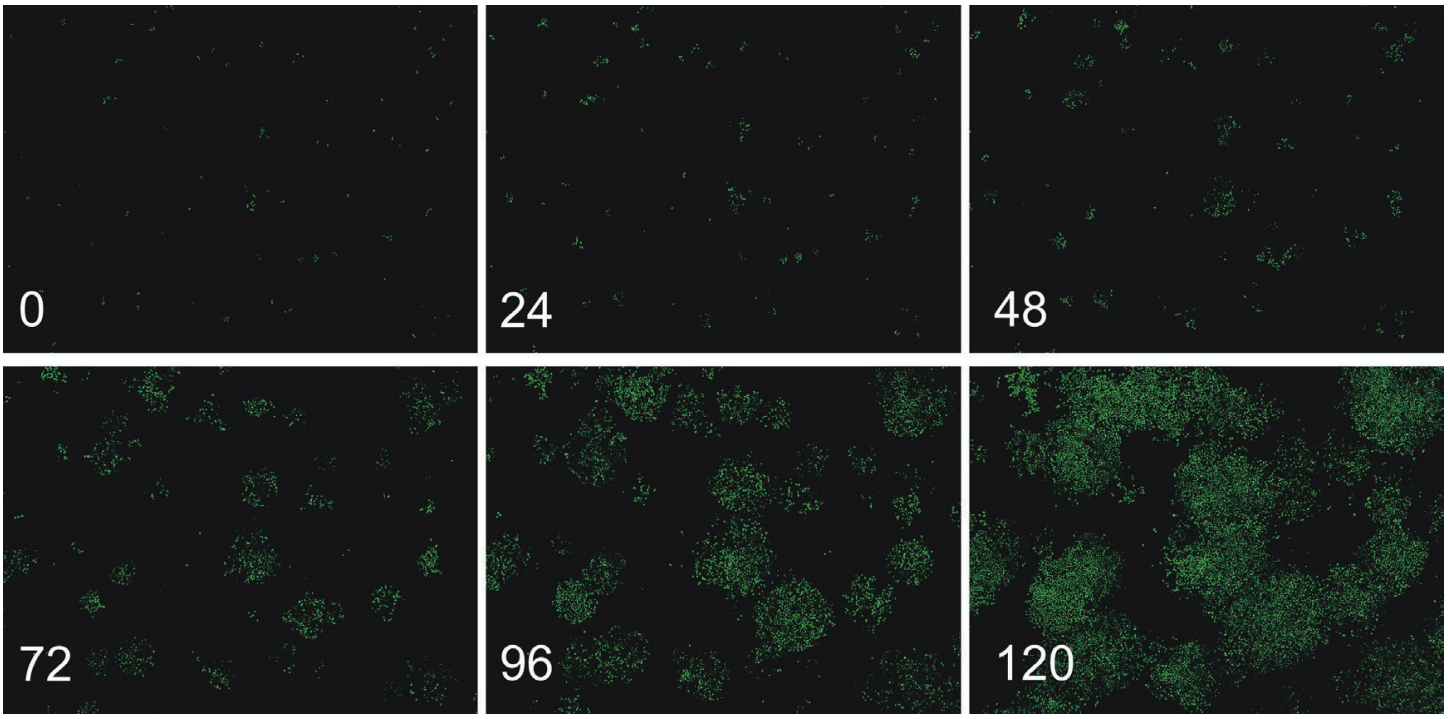

Figure 3. Montage images of HCT116-GFP cells in culture over five days. HCT116-GFP cells were seeded at 500 cells per well into a 96-well plate and allowed to attach for 24 hours. Montage images (2 × 2) were made with a 4x objective every 24 hours using a GFP LED cube.

Wells that receive slightly more or slightly fewer cells with the initial seeding would expect to have different cell counts at later time intervals. Despite the variability in cell count, cell numbers increase in a linear fashion over time. With time and increases in cell number, wells have a lower pH.

When HCT116-GFP cells are seeded at higher density, pH change becomes a greater issue and cell number increase eventually stalls (Figure 5). When cells are seeded at 2,000 cells per well, the increased number of cells causes the pH to drop approximately two-fold more than the lower seeding density. This drop in pH continues the length of the experiment despite the cell number stalling after approximately 80 hours. At higher cell densities, cells continue to respire, despite the cessation of cell number increase, resulting in a decrease in the pH.

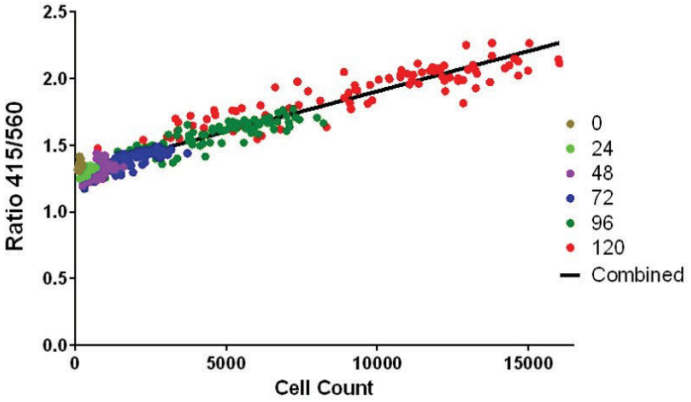

Figure 4. Relationship between media coloration and cell number at low cell density seeding. Cell counts for each well of a 96-well plate cultured over a five-day period plotted against the 415/560 absorbance ratio. Each data point represents an individual well.

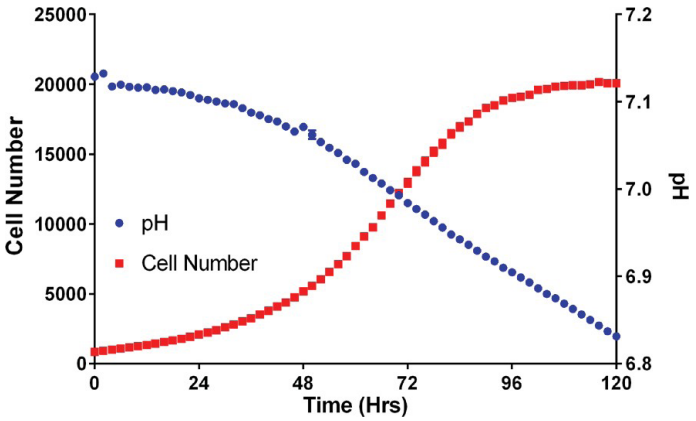

Figure 5. Change in HCT116 cell count and pH over time in confluent cultures. HCT116 cells were seeded at a density of 2,000 cells per well. After 24 hours to allow for attachment, image-based and absorbance analysis performed on 96-well plate cultures every two hours for five days. pH was determined by interpolating the 415/560 ratio values with a previously generated calibration curve. Data represent the mean and SEM for 96 determinations at each data point.

The cellular confluence can be seen in Figure 6, where there is little increase in the fluorescence observed between hour 96 and hour 120.

The inhibition of growth as a result of cellular confluence in long-term cultures can be identified with cell counting. As observed in Figure 7, cell number, as determined from image-based counting of nuclei, no longer increase with time as cells become confluent. The cell number after four or five days does not increase appreciably. However, the pH of the media present does decrease as observed by an increase in the 415/560 absorbance ratio. Despite little to no increase in cell numbers, waste products continue to be excreted and nutrients are consumed by the cells present.

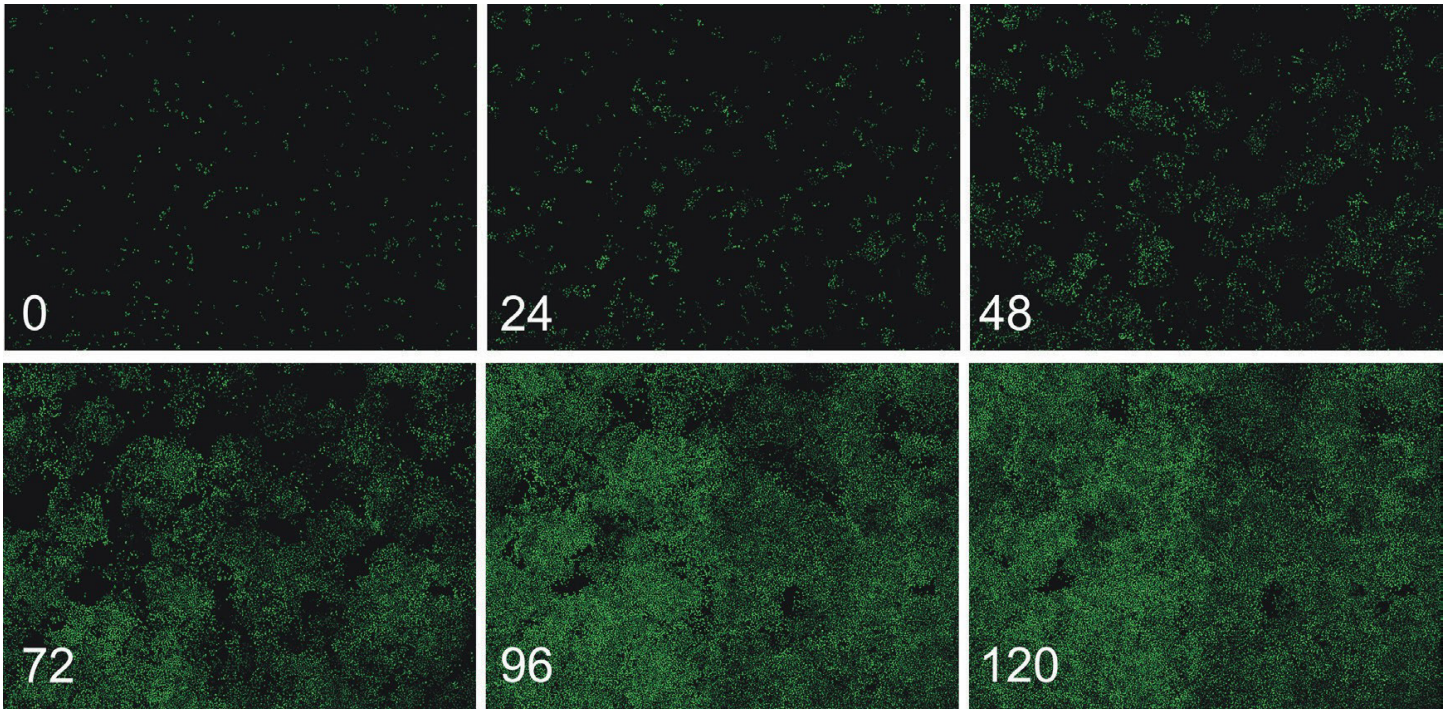

Figure 6. Montage images of HCT116-GFP cells in culture over five days. HCT116-GFP cells were seeded at 2,000 cells per well into a 96-well plate and allowed to attach for 24 hours. Montage-images (2 × 2) were made with a 4x objective every 24 hours using a GFP LED cube.

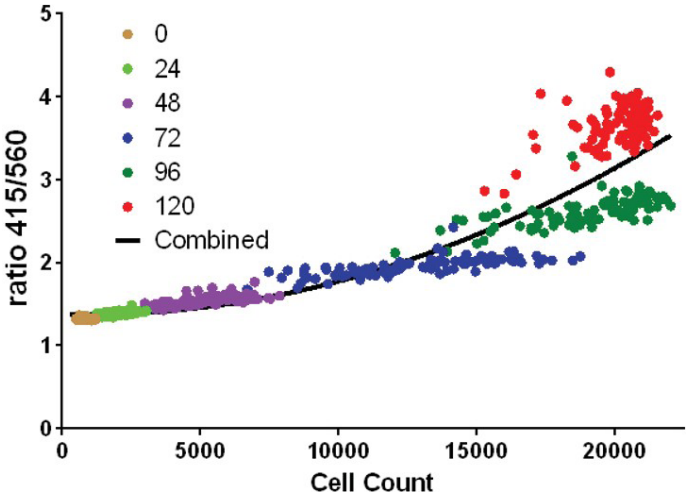

Figure 7. Relationship between media coloration and cell number at high cell density seeding. Cell counts for each well of a 96-well plate cultured over a five-day period plotted against the 415/560 absorbance ratio. Each data point represents an individual well.

Conclusion

These data demonstrate that the Agilent BioTek Cytation 5 cell imaging multimode reader is capable of monitoring cell culture pH in live cell experiments while concurrently imaging. Cell cultures normally become acidic due to an increase in cell numbers and cellular respiration, resulting in a yellowing in color of media formulations containing phenol red. While the change in pH for short-term experiments is often negligible, with long-term live cell experiments increasing cell numbers and the longer duration can overwhelm the buffering capacity of the media formulation. The ability to monitor changes in culture pH this in real time can allow the researcher to have confidence in the observed experimental results or abort experiments that have deleterious pH conditions.

Monitoring cell numbers or cell confluency over time is commonly used to test the efficacy of antineoplastic agents in preventing cell growth. The use of phenol red absorbance to monitor media pH insures that reported inhibition is the result of the test compound rather than poor tissue culture media status.

The Cytation 5 is an ideal platform to monitor phenol red absorbance change with live cell imaging experiments. The reader is unique in that it has the capability of both the absorbance measurements using a dedicated UV-Vis monochromator and microscopic imaging using a 6-position objective turret and LED light cubes. The rapid speed of the absorbance reading adds only seconds to a full 96-well imaging step, but can provide effective information regarding cell culture status. Agilent BioTek Gen5 microplate reader and imager software, besides controlling reader function, can be used to calculate pH from previously established pH calibration curves. This unique combination allows continual real-time monitoring of long-term live cell culture experiments.

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