Application Note

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Using Phenol Red to Assess pH in Tissue Culture Media

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

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Abstract

Media formulations for the propagation of *in vitro* tissue culture often contain the pH indicator dye phenol red. This dye has long been used to quickly assess the status of bulk cultures being propagated for subsequent experimental analysis. However, once an actual experiment has begun, the state of phenol red in the media is typically no longer used, particularly with image-based proliferation assays. This application note describes the use of the Agilent BioTek Cytation 5 cell imaging multimode reader to monitor pH, through absorbance measurements, in tissue culture media.

Introduction

There are numerous formulations for *in vitro* cell propagation. Depending on the intended cell type different mixtures of salts, amino acids, vitamins, buffers, carbohydrates, fatty acids, and supplements such as serum are used. Many of these defined mixtures also contain phenol red. Phenol red has been used as a pH indicator dye in tissue culture media for decades (Figure 1). While its presence is not critical for maintaining cell cultures, it is often used as a quick means for researchers to check on culture stocks.

Figure 1. Typical tissue culture media with phenol red.

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 buffers 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.[1] Not surprisingly, *in vitro* cultivation of cells and tissues prosper at the same pH levels. Cultures are maintained at physiological pH primarily by two different buffer systems. 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.

In addition to bicarbonate systems, the most commonly used alternative is HEPES buffer, which was first described by Good, *et al.* (1966).[2] This acts as a zwitterion and has proven superior to conventional buffers in comparative biological assays with cell-free preparations. It has many properties that make it ideal as a buffer to tissue culture media, principally in that it does not require an enriched atmosphere to maintain the correct pH. Its presence as a buffer agent does not completely eliminate the need for bicarbonate in media formulations.

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[4], it is a generally 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/mL range.

Phenol red, also known as phenolsulfonphthalein, is a pH indicator dye that exhibits a gradual transition from yellow to red over a pH range of 6.2 to 8.2 (Figure 2). Above 8.2 the dye turns a bright fuchsia color. In solution at very low pH phenol red is colorless and exists as a zwitterion, containing both a negatively charged sulfate group and a positively charged ketone group. With an increase in pH (pKa = 1.2) the excess proton in the ketone group is lost, resulting in a yellow color. Further increases in pH (pKa = 7.7) cause a loss of the hydrogen from the hydroxyl group resulting in a red color. The ratio of these three moieties enables phenol red to be used as a pH indicator dye.[3]

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 media needs replacing.

Most cellular experiments focus entirely on the biology at hand. Whether the results are fluorescent, luminescent, or image-based, the background milieu of the media is ignored. This application note describes the use of the Cytation 5 cell imaging multimode reader to monitor cell culture pH status using absorbance-based measurements.

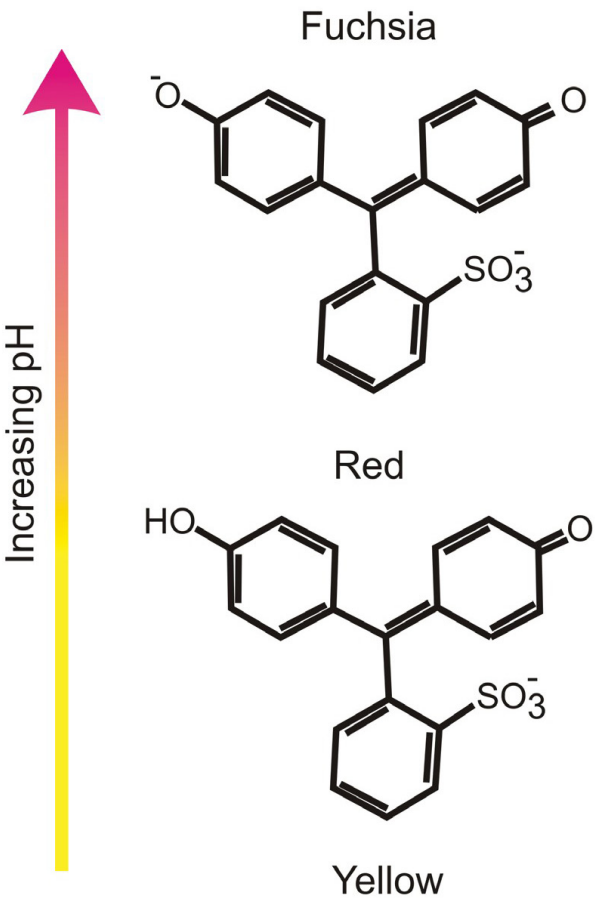

Figure 2. Structure of phenol red and different pH levels. With increasing pH phenol red changes from a zwitterion to an anion structure and eventually a di-anion. In doing so, the color of the compound changes from yellow to red to fuchsia.

Materials and methods

DMEM, RPMI 1640, and McCoys 5A were purchased from Life Technologies (Carlsbad, CA). Phenol red powder (part number P-3532) was from Sigma-Aldrich (St. Louis, MO). Spectral analysis was determined in 1 nm increments using the Agilent BioTek Cytation 5 cell imaging multimode reader and a Hellma quartz 96-well microplate. Spectra were determined in 1 nm increments using a Cytation 5.

For pH studies, complete tissue culture media containing 10% fetal bovine serum (800 µL) was diluted with a series of 100 mM buffers (400 µL) at different pH levels. For pH levels from four to eight phosphate buffer was used, while borate buffer was used at pH levels above 8.0. Buffer stocks were prepared previously and the pH determined using an Orion 3 Star pH meter.

Live cell experiments

NIH3T3 fibroblasts and 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. Humidified growth measurements were performed using Cytation 5 connected with Agilent BioTek BioSpa 8 automated incubator. The BioSpa 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 automated incubator transports a microplate to the Cytation 5 for imaging and absorbance measurements and returns it to the incubator afterward. Nonhumidified experiments were performed directly in Cytation 5 supplied with 5% CO_2 or not as indicated. The plate was maintained at 37 °C with a 2 °C gradient top to bottom to prevent condensation from forming on the top lid. In some experiments, a Breath Easy gas-permeable membrane was used to cover the wells.

Cells were seeded at a density of 4,000 cells per well and allowed 24 hours for attachment and used the following day cells for testing. The effect of carbon dioxide gas loss was tested using absorbance measurements every 30 minutes for 12 hours. Comparisons were made between plates that were maintained in a 5% CO_2 environment with plates that did not. To test the effect of contamination, cells were inoculated with either yeast (*S. cerevisiea*) or bacteria (*E. coli*) diluted in media. Absorbance measurements were performed on 96-well plate cultures every hour for eight hours using Cytation 5. Between absorbance measurements, plates were situated in the BioSpa 8, which maintained cells at 37 °C in a humidified 5% CO_2 atmosphere. pH levels were determined by interpolating a previously generated calibration curve with the 560 nm absorbance values.

Results and discussion

The absorbance spectra of DMEM media and phenol red dissolved in PBS were determined and compared. As observed in Figure 3, complete media has a large peak in absorbance in the UV range and two lesser peaks centered on 415 and 560 nm. These lesser peaks correspond to peaks seen with phenol red only solution. As the concentration of phenol red solution was equivalent to the reported recipe of the media formulation, the near match in the shape and height of the peaks centered on 415 and 560 nm suggests that these peaks represent phenol red absorbance in media. The large UV-peak is most likely due to the large amount of protein, flavonoids, vitamins, and nucleosides present in cell culture media formulations.

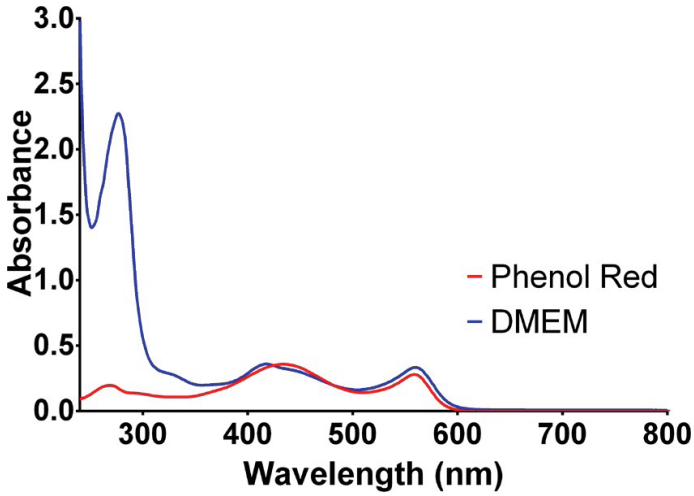

Figure 3. Comparison of phenol red and compete DMEM media absorbance spectra. The absorbance spectra of phenol red (15 mg/L) dissolved in PBS (pH 7.4) and complete DMEM containing 10% FBS were determined. Data represent the mean of three spectral curves after background subtraction.

The pH of complete media was varied from 4.5 to 9.0 using phosphate buffer. At high pH the media has a visible fuchsia color that changes to red then yellow as the pH deceases. This color change is reflected in the changes in the wavelength peaks at 560 and 415 nm. As shown in Figure 4, the absorbance peak at 560 nm is pronounced at pH 9.0, but almost disappears when the pH is decreased to 4.5. A smaller peak is observed at 415 nm with acidic pH levels that diminishes with an increase in pH.

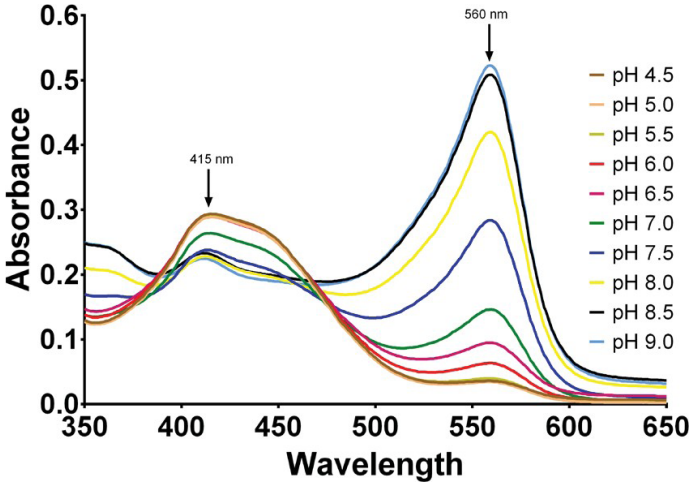

Figure 4. Absorbance spectra of complete DMEM culture media at different pH levels. Wavelengths with changing peak values with pH are indicated with arrows. Data represent the average of three spectral curves for each pH.

Closer examination of the two absorbance peaks in media with and without phenol red reveals an influence from the media constituents to the absorbance at 415 nm (Figure 5), whereas the more substantial peak at 560 nm seems unaffected by media components. This suggests that using the 560 nm peak would be more advantageous for direct calculation of pH. Using a 415:560 ratiometric analysis will provide a greater dynamic change, but results in less accurate pH determination.

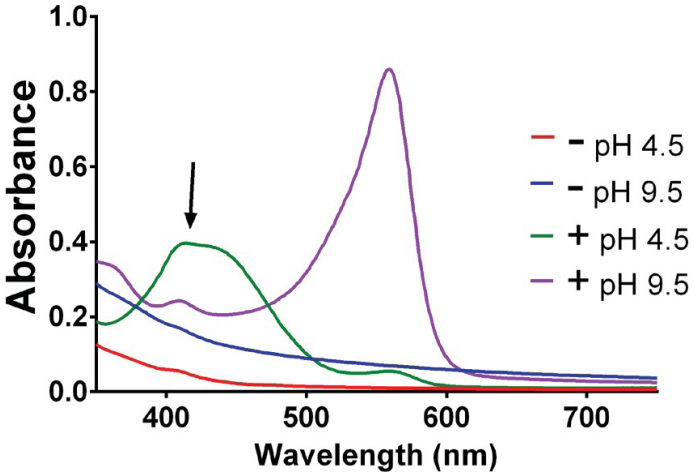

Figure 5. Absorbance spectra of DMEM culture media with and without phenol red. Wavelength with absorbance peak in the presence (+) or absence (-) of phenol red is indicated with an arrow. Data represent the average of two spectral curves for each.

The values of the 560 nm absorbance peak at different pH levels can be plotted and used as a calibration curve for subsequent long-term live cell assays. As shown in Figure 6, the plot demonstrates a linear increase of absorbance from approximately pH 6.5 (~ 0.1 OD) to 10 (~ 1.1 OD).

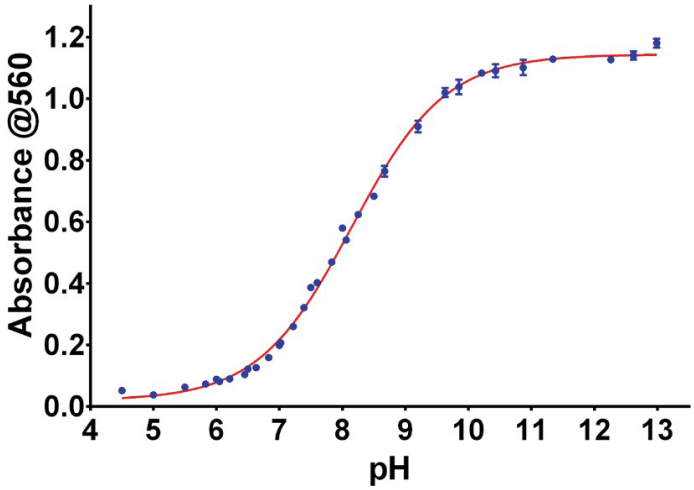

Figure 6. Absorbance at 560 nm for complete media at various pH levels. The absorbance of complete DMEM media was measured at 560 nm at various pH levels and plotted as a function of pH. Data represent the mean and standard deviation of four data points.

Despite the inaccuracies of the 415 nm measurement, the ratio of the 415 and 560 nm absorbance values provides extended coverage in the acidic region of pH where the 560 nm measurement loses sensitivity. As demonstrated in Figure 7, the linear portion of the plot extends from pH 5 (ratio ~ 8) to pH 7.5 (ratio ~ 1).

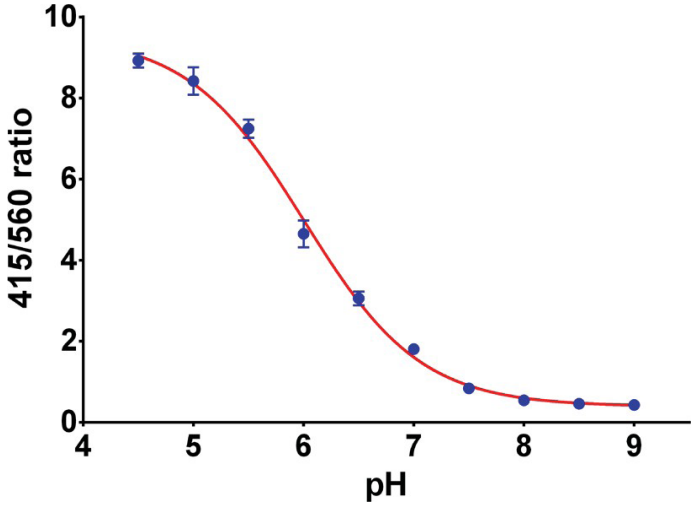

Figure 7. Ratio of 415 and 560 nm absorbance values for complete DMEM media at various pH levels. The absorbance of complete DMEM media was measured at 415 and 560 nm at various pH levels and the ratio of the two values plotted as a function of pH. Data represent the mean and standard deviation of three data points.

In order to effectively be used as a means to determine pH in real time, it is necessary to be able to correct for any background absorbance resulting from the plate itself. Typically, this is accomplished by either dedicating specific blank wells on an experimental plate or using a wavelength not effected experimentally and subtracting its value from the results as the first calculation.

Figure 8 demonstrates that using the absorbance at 750 nm is a viable option for background subtraction. Spectral data indicate that phenol red does not have significant absorbance at that wavelength (data not shown). The resultant values using this method differ only slightly from dedicated blank-well subtraction at very high pH. This method of correction has the added benefit of correcting for specific well-to-well variances, whereas defined blanks can only accommodate an average plate background.

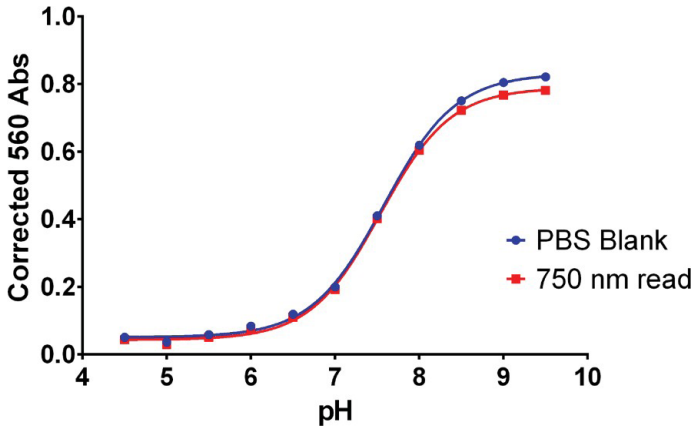

Figure 8. Comparison of background subtraction methods. The absorbance of complete DMEM media was measured at 560 nm at various pH levels using either dedicated blank wells with PBS buffer or the absorbance at 750 nm to subtract background absorbance. Data represent the mean and standard deviation of eight data points.

Absorbance data at 560 nm obeys Beer's law regarding pathlength, as shown in Figure 9. Different sample volumes show linearity concerning volume added to wells of a microplate at all pH levels tested. Not surprisingly, slope of absorbance for the highest pH was the greatest.

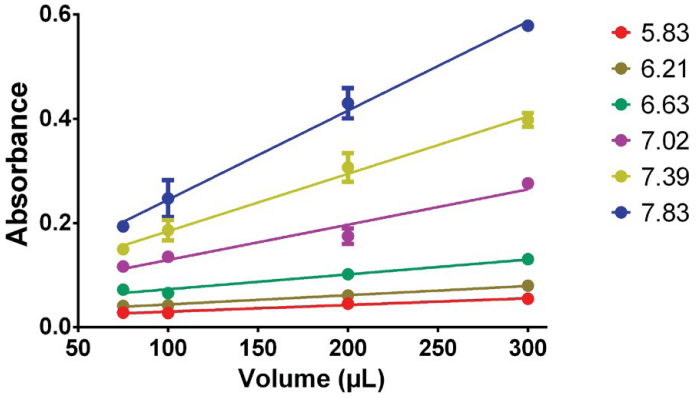

Figure 9. Effect of pH on linearity of 560 nm absorbance with different fluid volumes in each well. Different volumes of DMEM-phosphate buffer mixture were aliquoted and the absorbance at 560 nm determined. Data represent the mean and standard deviation of duplicate data points.

Blanked absorbance analysis of phenol red in media can be performed using any media formulation that contains the dye. When the 560 nm absorbance at different pH levels for DMEM, McCoy's 5A and RPMI 1640 were examined, all three had marked increases with reduced pH. The degree of change is directly proportional to the amount of phenol red in the media. RPMI 1640, which contains 5 mg/L phenol red had a 560 nm absorbance 0.342 at a pH of 9.85, while DMEM, which contains 15 mg/L returned a value of 1.038. McCoy's 5A media, which has a concentration of 10 mg/L was intermediate regarding its response and phenol red free DMEM had virtually no absorbance at 560 nm. All three phenol red containing media formulations had no further increases in 560 nm absorbance above 10.5 (Figure 10).

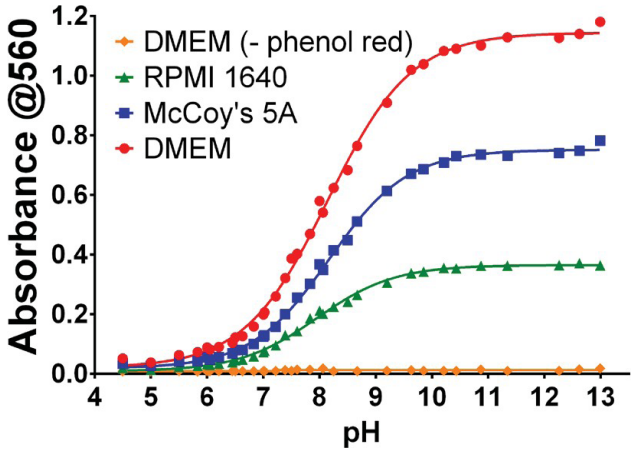

Figure 10. Absorbance at 560 nm for different media formulations. Different media formulations were treated to alter their pH and equal volumes (200 µL) were aliquoted into a Corning 3904 microplate and the absorbance at 560 nm determined. The absorbance values were plotted against pH. Data represent the mean and standard deviation of duplicates.

The pH status of live cells can be monitored using phenol red absorbance. A common problem with live cell experiments is the interruption of carbon dioxide gas supply to the experimental incubator. Because the pH of the tissue culture media is based on an equilibrium between dissolved CO_2 and bicarbonate ion, the outgassing of dissolved CO_2 from the media quickly results in an increase in pH. This is manifested in an increase in absorbance at 560 nm. As shown in Figure 11, DMEM, McCoys 5A, and RPMI 1640 media increase in absorbance over time as dissolved CO_2 outgasses when the carbon dioxide gas is no longer present in the surrounding environment. The raw increase in value is proportional to the amount of phenol red present in the formulation. DMEM, which contains the most phenol red dye had the greatest change, but the relative fold increase for the different media is the same (approximately 2x).

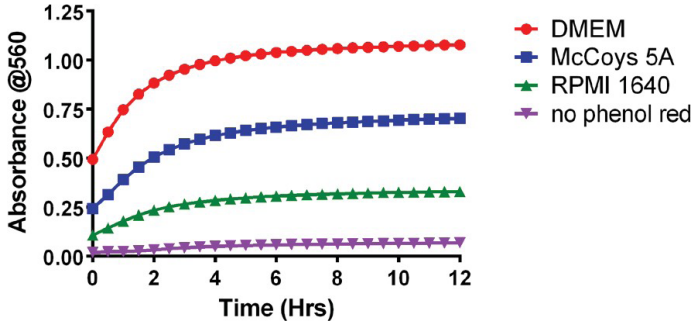

Figure 11. Change in absorbance after cessation of CO_2 supply with different media formulations. Deferent media formulations were aliquoted (200 µL) into wells of a microplate and equilibrated in a CO_2 incubator at 37 °C for four hours. The microplate was then placed in microplate reader and the absorbance at 560 nm measured every 30 minutes at 37 °C without CO_2. Data points represent the mean of 32 determinations.

The extent of pH increase is by interpolating data from calibration curves. As shown in Figure 12, tissue culture media rapidly increases in pH by approximately 2 pH units in the absence of carbon dioxide gas over time. Media formulations are developed for specific CO_2 concentrations. Because these formulations are designed to maintain physiological pH in the presence of 5% CO_2 gas levels, the pH changes for these formulations are similar.

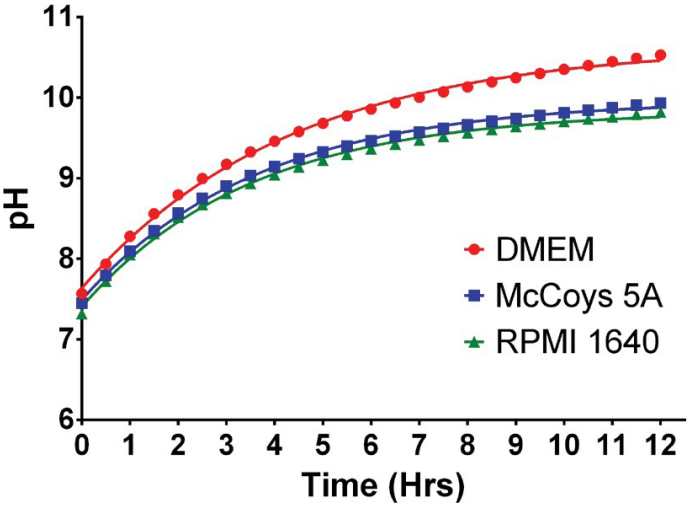

Figure 12. Change in pH levels with DMEM after loss of CO_2 supply. DMED, McCoy's 5A, and RPMI 1640 media were aliquoted (200 µL) into wells of a microplate and equilibrated in a CO_2 incubator at 37 °C for four hours. The microplate was then placed in microplate reader and the absorbance at 560 nm measured every 30 minutes at 37 °C without CO_2. pH values were determined by interpolating the 560 nm absorbance values with a previously generated calibration curve. Data points represent the mean of 32 determinations.

Similar results are observed when live cells are present in a microplate that has been subjected to a loss of carbon dioxide gas. As observed in Figure 13, cultures placed in an environment without carbon dioxide quickly outgas dissolved CO_2 and the pH rises. This rapid rate of pH increase can be lessened by the use of a gas permeable membrane. While these membranes are primarily intended to reduce evaporative loss in a dry environment, also serve to slow outgassing. Plates maintained in either a dry or humidified CO_2 environment maintain their pH.

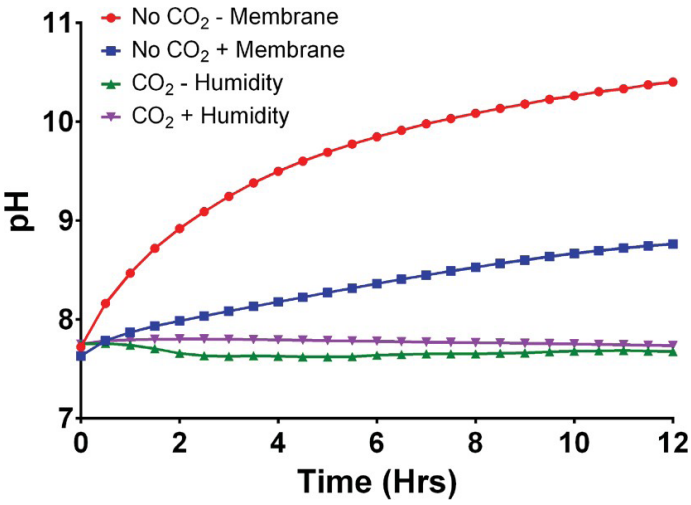

Figure 13. Change in pH levels with DMEM after loss of CO_2 supply. Change in pH in live cell cultures with and without CO_2 loss over time. NIH3T3 cells were allowed to attach overnight in a humidified 5% CO_2 incubator maintained at 37 °C. The following day separate microplates were then placed in microplate reader and the absorbance at 560 nm measured every 30 minutes at 37 °C with or without CO_2. pH values were determined by interpolating the 560 nm absorbance values with a previously generated calibration curve. Data points represent the mean of 96 determinations.

Changes in biological activity within the well will also result in measurable changes in pH. Microbial contamination of mammalian tissue culture often results in a rapid growth of the microbe, which finds favorable growth conditions. Cultures that have been contaminated with microbes will have a rapid decrease in pH due to the high metabolic activity present. As shown in Figure 14, when mammalian tissue cultures are inoculated with either bacteria or yeast a significant and rapid drop in pH, as measured by 560 nm absorbance, can be observed.

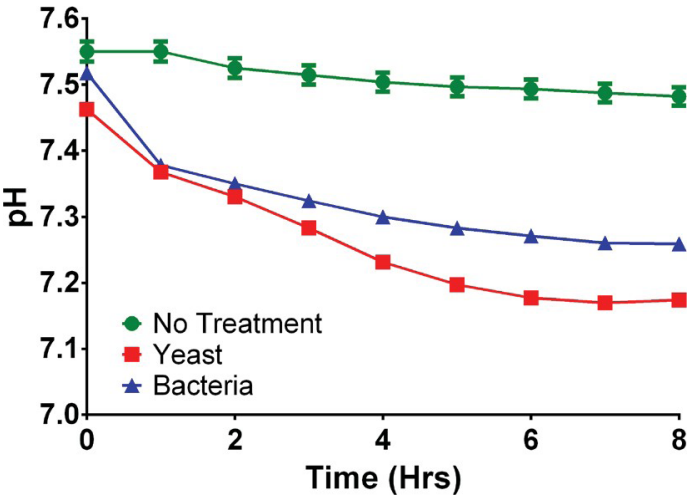

Figure 14. Change in pH with culture contamination. NIH3T3 cells were seeded at a density of 4,000 cells per well. After 24 hours to allow for attachment, cells were inoculated with either yeast or bacterial. Absorbance measurements were performed on 96-well plate cultures every hour for eight hours 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.

These data demonstrate that the Agilent BioTek Cytation 5 cell imaging multimode reader is capable of monitoring cell culture pH in live cell experiments. 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 in real time can allow the researcher to have confidence in the observed experimental results or abort experiments that have deleterious pH conditions.

The 560 nm peak was found to be the most reliable for pH determinations. Changes in absorbance at this wavelength can be used to monitor cell cultures with regard to media pH. Determinations using this wavelength obey Beer's law concerning pathlength and as such can be highly quantitative.

The response of the 560 nm peaks is particularly sensitive to upwards changes, usually the result of loss of CO_2 in the incubation chamber. An increase in pH due to lack of CO_2 exposure would be observed as an increase in 560 nm absorbance, which can increase five-fold with a pH change from seven to nine with DMEM media.

Alternatively, the ratio of the 415 and 560 nm peaks can also be used as a marker for pH change. This analysis is more qualitative than direct 560 nm absorbance, but demonstrates an extended range to lower pH values compared to the 560 nm measurement. This allows one to monitor both cell overgrowth and bacterial contamination. While it is less effective in discerning an increase in pH, usually resulting from a cessation of CO_2 supply, this phenomenon is usually due to mechanical failure rather than a change in true biology.

The use of a second wavelength to either correct for plate absorbance or provide a ratiometric determination has the added benefit in that both will correct for condensation or bubbles in the well that may form over time. The addition of liquid reagents has the potential to produce a small air bubble as the result of surface tension. Likewise, the transfer of a plate to and from the reader has the potential to result in condensation of the under surface of the plate lid. Both phenomena result in an increase in measured absorbance unrelated to true changes in the phenol red absorbance at 560 nm. These increases are consistent across the spectrum and as such, ratiometric analysis or background subtraction will be corrective.

Tissue culture media formulations vary in phenol red concentration. With changes in pH, formulations with higher concentrations exhibit a more pronounced change in 560 nm absorbance as compared to those with lower concentrations, which tends to make them more sensitive to pH change. However, the fold change in the 415:560 ratio is greater in formulations with low phenol red relative to high concentration formulations and the change is most pronounced with a decrease in pH. This makes the ratiometric method desirable as a means to roughly assess cultures and identify contamination. The response of different media formulations will be different depending on the formulation. For example, a 415:560 ratio exceeding three with DMEM would be indicative of an acidic environment, while the same ratio in McCoy's 5A media would require a ratio of approximately eight to indicate the same pH change. Regardless of the media formulation, one should expect a gradual change in pH with time. Even live nondividing cells are still respiring while not increasing in cell number. A rapid color change would be indicative of some sort of microbial contamination.

Conclusion

The Agilent BioTek Cytation 5 cell imaging multimode reader 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 six-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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