Application Note

Cancer Biology

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High-Throughput Fluorescent Colony Formation Assay

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Abstract

The colony formation assay evaluates the proliferative capacity of a single cell. For applications such as cancer drug screening, it is important to distinguish cells that retain this proliferative capacity from those that do not. Conventional analysis of this assay involves manually scoring and quantifying colonies in each well of a multiwell format by eye, limiting its throughput capabilities. Using the fluorescent properties of Crystal Violet, this application note presents a high-throughput method for conducting a colony formation assay in a 96-well microplate using fluorescence microscopy with the wide field of view of the Agilent BioTek Cytation 5 cell imaging multimode reader.

Introduction

Shortly after the first human cells, originating from Henrietta Lacks' biopsied cervical cancer tissue, were successfully maintained *in vitro*[1], it became apparent that a method was needed to culture and isolate clonal populations of mammalian cells. This had never been done with mammalian cells, but such a method would allow scientists to measure the effects of radiation, drugs, and a host of other biological stressors.[2] In 1955, Theodore Puck and Phillip Marcus at the University of Colorado developed the colony formation assay using these "immortal" cells, which gave rise to first clonal population of mammalian cells.[3]

The colony formation assay, or clonogenic assay, is an essential method for cancer research, allowing drug screens and radiation dosing to be conducted.[4-7] The assay is carried out by seeding cells at a low enough density such that individual cells can propagate to a sufficient colony area without impeding on a neighboring colony.[8] At a set time point, adherent colonies are fixed then stained with Crystal Violet colorimetric dye, which allows visual inspection of the culture vessel and quantification of the number of colonies that expanded (Figure 1). A major drawback of this assay is that scoring and quantifying colonies is typically done in a laborious fashion – a trained technician must manually view each well through a low-magnification stereoscope and count each well. This analytical approach hinders the ability to carry out this assay in a high-throughput fashion using microplates with a higher well density than 12- or 24-well plates (Figure 2).

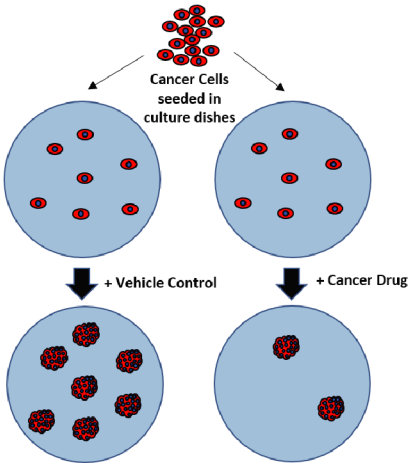

Figure 1. Schematic representation of the colony formation assay. Dissociated cells in suspension are seeded onto tissue culture wells at a low enough density to enable single cells to proliferate into clonal populations. The potency of antiproliferative compounds can be assessed based on the number of surviving colonies relative to control expressed as the number of colonies formed per well.

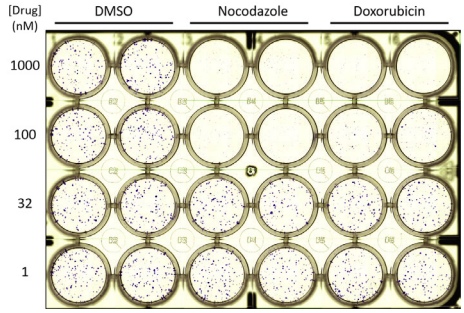

Figure 2. Drug titration using Crystal Violet colorimetric dye in a 24-well plate. The colony formation assay is typically carried out in a multiwell format. Single cells are seeded in each well at low density in the presence of various concentrations of compounds or vehicle control. After a set growth period, colonies are fixed and stained with Crystal Violet, then the number of colonies is scored by visual inspection.

This application note presents an automated high-throughput method for conducting a colony formation assay in a 96-well microplate using fluorescence microscopy. The fluorescent properties of Crystal Violet are used to define the colony area, while Hoechst 33342 is used to quantify the number of cells within the colony. With the wide field of view (WFOV) capabilities of the Cytation 5, and the analytical power of Agilent BioTek Gen5 multimode reader and imager software, Augmented Microscopy enables an automated workflow to both capture whole well images, then identify, quantify, and characterize colonies on a large scale. This approach enables a more robust statistical sample set to be collected, both in terms of replicates, as well as a broader range of drug dosing. This fluorescence-based method is a powerful application of a classic assay, with a streamlined analytical process.

Materials and methods

Reagents

Nocodazole 10 mM in DMSO, Doxorubicin 50 mM in DMSO, and Nigericin 50 mM in EtOH (part numbers 1228, 2252, and 4312, respectively) were purchased from Tocris (Bristol, United Kingdom). PBS was made from tablets (Sigma P4417) dissolved in 1 tab/200 mL deionized H_2O (dH_2O). Four percent paraformaldehyde was prepared from powder (Sigma P6148) by heating to 60 °C in PBS with constant stirring for 1 hour or until completely dissolved and solution was clear, then clarified by passing through a 0.45 µm filter. Hoechst 33342 solution, 20 mM (part number 62249) was purchased from Thermo Fisher Scientific (Waltham, MA), then further diluted to a 10 mM stock with dH_2O. Crystal Violet (CV) (V5265) was purchased from Sigma-Aldrich (St. Louis, MO) as a 25 mM (1% w/v) aqueous solution.

Crystal Violet fluorescence spectra

The fluorescent properties of CV were determined using an Agilent BioTek Synergy Neo2 hybrid multimode reader equipped with monochromators (top position). The peak excitation wavelength of CV was determined by establishing a 300 to 700 nm absorption spectra of 100 µM CV in 50% glycerol using a UV-transparent flat-bottom 96-well microplate (Costar 3635).

The peak excitation wavelength (595 nm) was then used to determine the peak emission wavelength (635 nm) by establishing a 580 to 700 nm emission spectra using a black opaque 96-well microplate (Costar 3915). These spectral properties match best with an Agilent BioTek Texas Red filter cube (Ex: 586/16 and Em: 647/57) (part number 1225002).

Cell culture

Caco2 colorectal adinocarcinoma cells (ATCC HTB-37) were grown at 37 °C in Advanced Dulbecco's modified eagle's medium (Gibco part number 12491) with 10% FBS (Gibco part number 10437) and 1x penicillin/streptomycin/L-glutamine (Gibco part number 10378).

Colony formation assay

For colony formation assays, cells were seeded in 96-well flat clear-bottom black microplates (Costar part number 3904) at a density of 50 cells/well, and proportionally scaled up for 48- and 24-well plates (Costar 3548 and 3524, respectively) based on growth area. First, cells cultured in T75 flasks were passaged with TrypLE (Gibco part number 12605) then transferred to a 15 mL conical and pelleted by centrifugation at 300 ×g for five minutes, followed by resuspension in 10 mL of fresh media. Cell concentration was determined with a hemacytometer. Cells were diluted to 2.5×10^2 cells/mL and seeded at 200 µL/well, giving a density of 50 cells/well. To ensure even distribution of cells across the well bottom by avoiding convection currents from rapidly warming media, plates containing freshly seeded cells were incubated for 30 minutes on a clean countertop at 25 °C before returning to a 37 °C incubator. Colony expansion of single cells were allowed to progress for 6 days, then washed 2x with PBS and fixed with 4% paraformaldehyde in PBS.

Drug treatment

Nocodazole and Doxorubicin (Tocris) were diluted from stock to 10 µM, or 10x of the highest treatment concentration (1,000 nM), in Advanced DMEM. 10x half-log dilutions were prepared from this 10x stock, then 20 µL of this was added to a final volume of 200 µL in 96-well plates containing seeded cells with following 1x final concentrations: 1,000, 320, 100, 32, 10, 3.2, and 1 nM. Samples were set up in quadruplicates with equivalent volumes of DMSO used as a vehicle control.

Crystal Violet staining

1% Crystal Violet (25 mM) was diluted to a working concentration of 250 µM in PBS containing 10 µM Hoechst 33342 nuclear stain. A 100 µL amount of this was added to each well of a 96-well plate (500 µL for 24- and 48-well plates) containing fixed colonies and incubated for 30 minutes at room temperature. The dye was aspirated, and the wells were washed 3x with PBS, then PBS was added back to wells. In this case, the majority of CV will diffuse out of the cell and into solution, which requires additional wash steps to remove CV that has diffused out of the colonies.

Cell imaging

All images were captured using a Cytation 5 equipped with a wide-field-of-view (WFOV) camera, 4x Plan Fluor objective (part number 1220519) and the following filter cubes: Laser Autofocus (part number 1225010), DAPI (Ex/Em 377/447; part number 1225007), and Texas Red (Ex/Em 586/647; part number 1225002). To generate whole-well images of a 96-well microplate at 4x magnification, 2 × 2 montages with 10% overlap were captured, then stitched with the DAPI channel as the reference channel. Laser autofocus was set to the DAPI channel as a reference, and in-focus Texas Red and brightfield channel focal offsets were set accordingly.

Image analysis

Prior to montage stitching, preprocessing individual images of the montage was carried out to enhance the signal contrast of nuclei (DAPI) and colonies (Texas Red). Threshold masking was optimized for whole colonies to be identified as individual objects using Crystal Violet fluorescence in the Texas Red channel as the primary mask, while cells/colony was derived by nuclei count within each colony using the spot counting module as a secondary mask. A complete description of image preprocessing, stitching, and cellular analysis steps is presented in Table 1.

Table 1. Image preprocessing, stitching, and cellular analysis parameters.

Image Preprocessing/Transformation	
Image Set: DAPI 377,447	
Background	Dark
Rolling Ball Diameter	20 µm
Image Smoothing Strength	1 cycle of 3 × 3 average filter
Image Set: Texas Red 586,647	
Background	Dark
Rolling Ball Diameter	1,000 µm
Image Smoothing Strength	10 cycles of 3 × 3 average filter
Stitching	
Registration Channel	Tsf[Texas Red 586,647]
Fusion Method	Linear blend
Crop Borders	Checked
Downsize Image	50%
Cellular Analysis Parameters	
Detection Channel	Stitched[Tsf[Texas Red 586,647]]
Primary Mask and Count	
Threshold	
Value	7,000
Background	Dark
Split Touching Objects	Unchecked
Fill Holes in Masks	Checked
Advanced Detection Options	
Primary Mask	Expand threshold mask by 25 µm
Object Selection	
Minimum Object Size	100 µm
Maximum Object Size	10,000 µm
Include Primary Edge Objects	Unchecked
Analyze Entire Image	Checked
Secondary Mask: Stitched[Tsf[DAPI 377,447]]	
Background	Dark
Measure Within a Primary Mask	Checked: Use primary mask
Measure Within a Secondary Mask	Unchecked
Counting Spots*	10 to 50 µm
* Spot counting module license required.	
Calculated Metrics	
Cell (Colony) Count	
Object (Colony) Area	
Object (Colony) Spot Count (DAPI)	
Subpopulation Analysis	
Object Metric	SpotCount[Stitched[Tsf[DAPI 377,447]]]
Condition	≥32 nuclei/colony
Select Objects Where:	All conditions are met
Calculate:	Cell (colony) count

Results and discussion

Fluorescent properties of Crystal Violet

Crystal Violet has long been used as a colorimetric dye for visualizing colonies (Figure 3A), however the dye also possesses fluorescent properties. In solution (50% glycerol), the peak excitation and emission wavelengths of Crystal Violet are 595 and 635 nm, respectively (Figure 3B). Crystal Violet is readily membrane permeable in dead or fixed cells, however once excess dye is removed and replaced with Crystal Violet-free buffer, the vast majority of dye diffuses out of the cell which renders colonies translucent (Figure 3C). Because Crystal Violet exhibits both DNA and protein binding capacity[9,10], a sufficient amount of Crystal Violet dye is retained to enable fluorescent imaging using a Texas Red filter cube (Figure 3D).

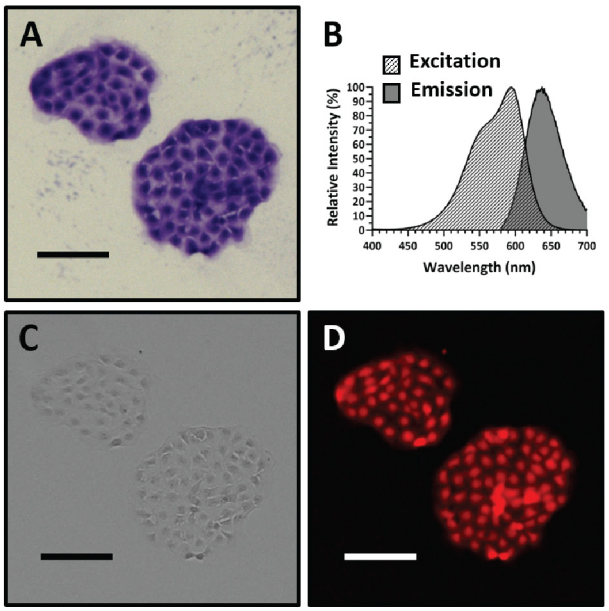

Figure 3. Fluorescent properties of Crystal Violet. (A) Crystal Violet diffuses across the plasma membrane of fixed cells and provides significant colony contrast with brightfield microscopy. (B) Crystal Violet possesses fluorescent properties with a peak excitation and emission of 595 nm and 635 nm, respectively, which is compatible with the Texas Red filter cube (Ex/Em of 586/647 nm). (C) Crystal Violet readily diffuses out of cells when kept in PBS, however (D) its protein- and DNA-binding capacity retains enough dye to enable sufficient signal for fluorescent imaging. Scale bar = 200 µm.

Automated colony screening using fluorescent imaging

Caco2 colonies grown in 96-well microplates were imaged on a Cytation 5 with WFOV. A 2 × 2 montage encompassing the entire well was captured, then a preprocessing background reduction step was applied, followed by a stitching step (Figure 4A). A primary mask in the Texas Red channel was set to define the area of colonies (Figure 4B), while the Spot Counting module within a DAPI secondary mask was used to quantify the number of cells within colonies (Figure 4C and D).

The colony formation assay evaluates the proliferative capacity of a single cell. For applications such as cancer drug screening, it is important to distinguish cells that retain this proliferative capacity from those that do not. Therefore, an important criterion that qualifies a cluster of cells as a colony is the presence of 50+ cells. This criterion is rooted in the application of Crystal Violet colorimetric dye being used to visualize and quantify colonies, then scoring colonies manually by eye. Colonies containing less than 50 cells were deemed difficult to reliably observe by eye.[3] Additionally, the presence of at least 50 cells ensure that multiple rounds of viable cell division have occurred. In an application where microscopic imaging takes the place of manually scoring by eye, there is no longer a limitation on the detection size. Additionally, it can be reasoned that the presence of at least 32 cells indicates that five rounds of divisions have occurred, and sets the criterion for qualifying colonies accordingly (Figure 4E). A complete description of image preprocessing, stitching, and cellular analysis steps is presented in Table 1.

Using the colony formation assay to establish EC_{50} values for anticancer compounds

The power of a high-throughput fluorescence method lies in its ability to increase the sample size of a colony formation assay, as well as automating subsequent analysis (Figure 5). To demonstrate the power of this format, the EC_{50} of two common anticancer compounds was determined using the colony formation assay. Nocodazole is an antimitotic drug that binds to b-tubulin, disrupting microtubule polymerization and ultimately arresting the cell cycle at the G2/M transition.[11,12] Doxorubicin is an antitumor drug that disrupts cell division by intercalating DNA, inhibiting the progression of topoisomerase II, ultimately leading to an inhibition of macromolecular biosynthesis.[13-15] To establish an EC_{50} for these two drugs, Caco2 cells were seeded into 96-well microplates (50 cells/well) in the presence of decreasing half-log concentrations of Nocodazole, Doxorubicin, or DMSO (vehicle control) with each concentration done in quadruplicates. After 6 days in culture, colonies were fixed, stained with Crystal Violet, and imaged on a Cytation 5 WFOV. A subpopulation cellular analysis step was applied to consider only colonies within each well that reached 32 cells/colony cutoff. The mean of qualifying colonies at

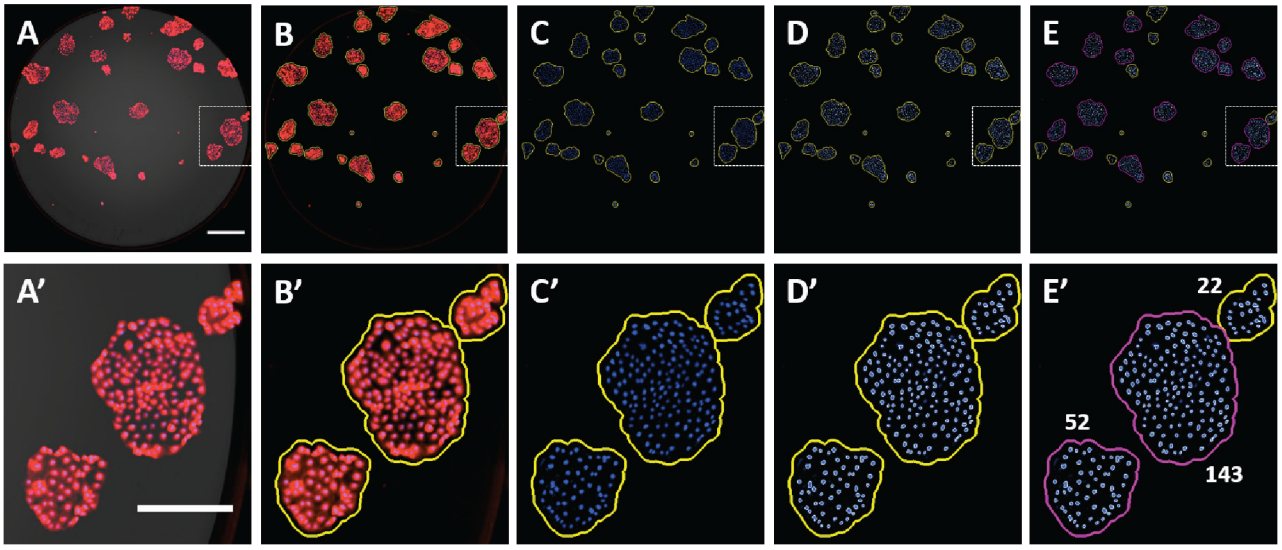

Figure 4. Automated fluorescent colony identification and characterization using the Agilent BioTek Cytation 5 cell imaging multimode reader WFOV and Agilent BioTek Gen5 multimode reader and imager software. A' to E' are insets of the indicated region in A to E, respectively. (A) Whole wells of a 96-well microplate are captured with a 2 × 2 montage that undergoes a background reduction step and stitching. (B) Colony Identification is possible using the fluorescent signal of Crystal Violet as the primary mask in the Cellular Analysis data reduction step. (C) Nuclei are visualized with Hoechst 33342 staining; (D) the nuclei are identified as a secondary mask within each colony using the Spot Counting module, allowing the number of nuclei/colony to be quantified. (E) A subpopulation analysis is set to apply a cut-off for colony size based on number of nuclei. The number of nuclei detected are indicated. Purple = colonies with >32 nuclei/cells. Scale bar = 1 mm (A to E) and 0.5 mm (A' to E'). See Materials and Methods for full data reduction parameters.

each drug concentration was then plotted as a function of the log drug concentration and the EC_{50} was determined by fitting a dose-response curve. With this analysis, the EC_{50}s of Nocodazole and Doxorubicin was determined to be 25.4 nM and 20 nM, respectively.

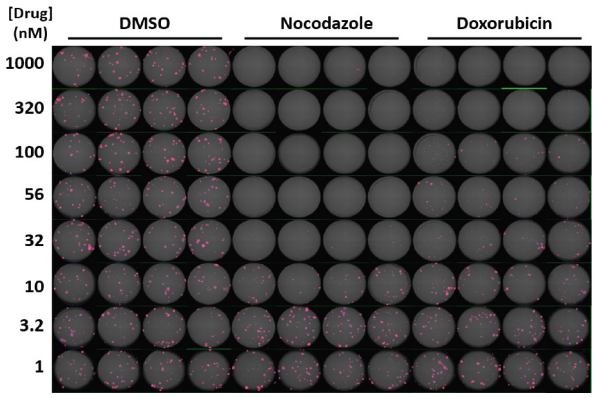

Figure 5. Colony formation assay in a high-throughput format using fluorescence microscopy. Limitations associated with the low-throughput format and manual analysis of the colony formation assay can be overcome with the Agilent BioTek Augmented Microscopy workflow using an Agilent BioTek Cytation 5 cell imaging multimode reader WFOV to image a 96-well microplate complemeted by the analytical power of Agilent BioTek Gen5 multimode reader and imager software, which enables increased sample replicates and concentrations, as well as automated analysis and quantification.

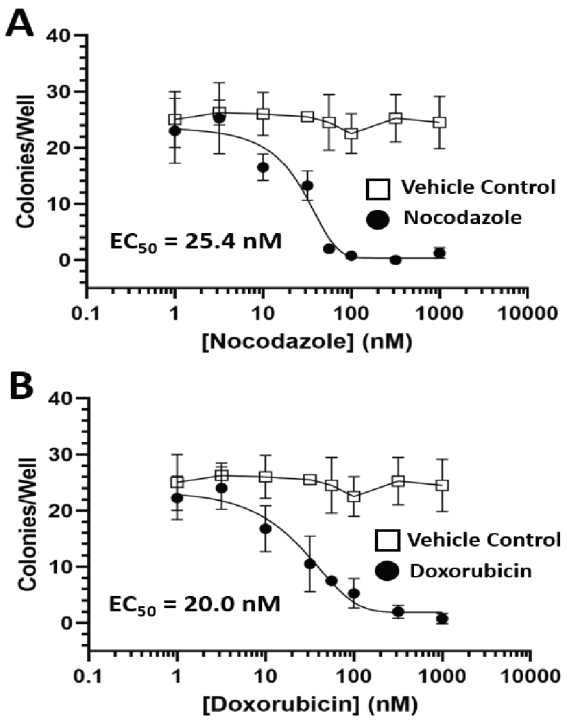

Figure 6. EC_{50} determination of anticancer drugs with the colony forming assay using high-throughput fluorescent analysis. Increased sample replicates and expanded drug dilutions of a 96-well microplate, coupled with the automated workflow of Agilent BioTek Gen5 multimode reader and imager software enable robust datasets to be generated. Colony forming assay conducted with a dilution series produce an estimated EC_{50} for Nocodazole (A) and Doxorubicin (B) of 25.4 nM and 20.0 nM, respectively.

Conclusion

The Agilent BioTek Cytation 5 cell imaging multimode reader with wide field of view capabilities enables automated whole-well imaging and analysis of 96-well microplates and is ideal for large scale screening of potential cancer therapeutics. Crystal Violet is a colorimetric dye that contains fluorescent properties, which can be used to fluorescently image colonies culture in a 96-well microplate, enabling high-throughput applications. This application note couples the imaging power of Cytation 5 WFOV with a fluorescence-based adaptation of the colony formation assay, which permits statistically robust data acquisition that is crucial for drug development applications such as cancer therapeutics.

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