High Throughput Anchorage-Independent Cell Colony Growth Assay with the IsoCyte™-HTS Platform

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Abstract

The advantages of anchorage-independent cell colony growth assays for drug development are well known. Monitoring proliferation of cells in colony form is more biologically relevant than as single cells and formation of 3-dimensional (3D) colonies in semi-solid media better represents *in vivo* environments. There are many challenges associated with performing this assay in high-throughput fashion with microscope based systems because of the need to tile multiple regions to get sufficient field-of-view and the need to stack multiple z-heights to adequately sample thick media. Here we report on the use of the IsoCyte™-HTS for automated anchorage-independent growth assay. The IsoCyte™ is a laser scanning cytometer that scans whole well areas in a single pass and has a large depth of field making it well-suited for colony characterization in thick media. Colony assays using human lung carcinoma A549 cells in agarose were prepared in 96-well plates, incubated overnight, and treated with a dilution series of staurosporine. The cultures were grown for 14 days and then stained with epicocconone (a non-toxic live-cell dye), incubated at room temperature for 2.5h, and analyzed using the IsoCyte™. Fluorescence images were acquired using 488nm excitation, detection with a 600LP filter, and a sampling of 5 μm giving an inspection cycle time of <5 minutes/plate. Cell colonies were analyzed automatically for size, number, and total colony area. The results demonstrated that A549 colony formation was inhibited in a concentration dependent manner by staurosporine. The use of epicocconone with the IsoCyte™ allows a true "mix-and-read" cell colony growth assay ideal for a high-throughput automation environment. The IsoCyte™-HTS platform, integrated with a Twister II™ plate handler, enables a high degree of process control and automated data processing important for therapeutic discovery and development environments.

Materials and Methods

Cells and Culture Conditions. Human lung carcinoma cells, A549 (ATCC, The American Type Culture Collection catalog CCL-185) were cultured in RPMI-1640 medium supplemented with 10% Fetal bovine serum + 25 mM Hepes + 2 mM Glutamax-1 + 1 mM sodium pyruvate + 1 mM non-essential amino acids + 100 units/ml penicillin, 100 μg/ml streptomycin. Cells were passaged prior to achieving confluency. All culture media and supplements were purchased from Invitrogen.

Assay Plate Preparation. In a sterilized glass bottom 96-well tissue culture plate (MatriCal), a 100 μl base layer of 0.8% (w/v) agarose was prepared by adding sterile 1.5% agarose solution to complete RPMI-1640 medium. Agarose was obtained from Chemicon's cell transformation assay kit (catalog #ECM570). A549 cells that had attained 80% confluency in a T-75 tissue culture flask were washed twice in pre-warmed phosphate buffered saline, lifted by adding 5 mls of TrypLE™ Select Animal-Origin-Free Trypsin-Like Enzyme solution (Invitrogen), and incubated for 5 min at 37°C. The cell suspension was removed and centrifuged for 5 min at 500 X g. The cells were resuspended in 5 ml cell culture medium and the number of cells was determined. A working cell suspension of 1.5 X 10^4 cells/ml was prepared in a mixture of 0.3% agarose solution and complete RMPI-1640 medium. Cell suspension (100 μl containing 1000 cells per well) was added to the top of the base layer and allowed to solidify for 1 hour at 37°C. 100 μl of RPMI-1640 media was added to wells and the plate was incubated overnight at 37°C and 5% CO2. The following day, 100 μl of 4X dilutions of staurosporine (Calbiochem, catalog #569397) in media were added to appropriate wells in replicates of five.

Colony Identification. The cultures were microscopically monitored for colony formation. Day 14 cultures were stained with 2.4 μM LavaCell™ (Epicoconnone; Active Motif Cat # 15004) by removing growth media and adding 100 μl of dye in HBSS. Plates were incubated for 1-2 hours and analyzed using the IsoCyte™.

Detection of Colonies by IsoCyte™. The IsoCyte™ laser scanning platform was setup for 2 channel acquisition with 600 long pass filters in detectors channels 3 and 4 for LavaCell™ emission. Image acquisition was done at 5 x 5 micron sampling with a total scan and analysis time of 4 minutes. A schematic of the IsoCyte™ optical setup that provides highly sensitive binocular detection with a large depth of field is shown in **Figure 1**. Whole well image results and the analysis process are shown in **Figure 2**.

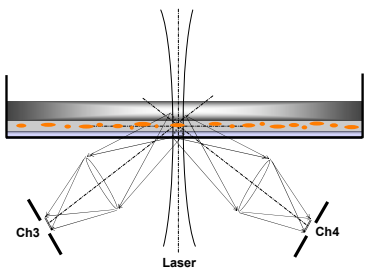


Figure 1. Schematic diagram of the IsoCyte™ optical setup showing laser excitation path and two detection channels in a binocular arrangement. The laser has a large depth of field (>150 μm) and the collection heads have a 400 μm confined detection region allowing the system to image cell colonies over whole plates without need of focus adjustment or acquisition of multiple z-heights.

IsoCyte™ Binocular Imaging of Thick Samples

(US Patent 7,141,378; patents pending)

Cell Colony Protocol

Add 100 μl base agar (0.8%) in growth medium

↓

Add 100 μl cell suspension in growth medium containing 0.3% agarose

↓ Solidify @ 37° C for 1 hour

Add 100 μl of growth medium

↓ Incubate @ 37° C and 5% CO_2 overnight

Add 100 μl of 4x concentrations of staurosporine in growth medium

↓ Incubate @ 37° C and 5% CO_2 14 days

Remove growth medium and add 100 μl of 2.4 μM LavaCell™ dye in HBSS

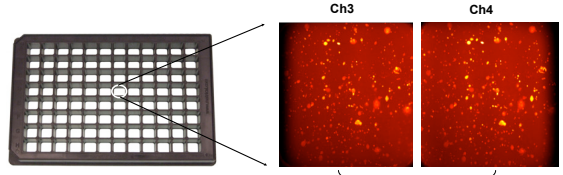
↓ Incubate @ room temperature 1-2 hours

Image and analyze colonies by IsoCyte™

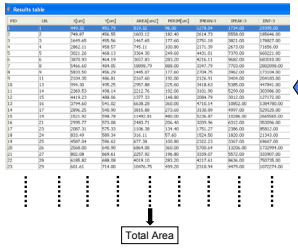
Figure 2. Outline of cell colony protocol and illustration of image acquisition and automatic colony quantification. The samples were placed as shown and the outer rows of the 96-well MatriCal plate were filled with media only for evaporation control. The location of the glass bottom of the MatriCal 96-well plate allowed better optical access and the square wells resulted in improved image quality and reduced shadowing. The selected well (white circle) is from the column treated with 0.1 nM staurosporine. The images were acquired at 5 x 5 micron sampling and an automated image analysis process was implemented. Because of the shadowing observed from imaging colonies in thick media (see loss of image quality on left well wall for Ch3 and right well wall for Ch4 images), the Ch3 and Ch4 images were added and the resulting composite image was used for object thresholding. The objects were filtered using area filters and the classified object data was saved as a list file (.csv) enumerating object-by-object data for each well.

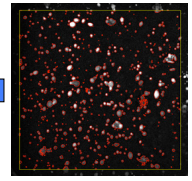
Results and Discussion

The colony assays using human lung carcinoma A549 cells was prepared using a dilution series of staurosporine from 1x10^{-6} to 1x10^{3} nM concentrations. The cultures were grown for 14 days and then stained with LavaCell™ (epicocconone) as described and analyzed using the IsoCyte™. Fluorescence images for 0.01, 0.1, 1, and 10 nM staurosporine concentrations are shown in **Figure 3**. A clear change in cell colony size and morphology is observed. Cell colonies were analyzed for size and the total colony area was calculated for each well. A plot of total colony area versus staurosporine concentration is shown in **Figure 4**. The results demonstrated that A549 colony formation was inhibited in a concentration dependent manner by staurosporine. The calculated IC50 is 0.62 nM.



Tabulated Results for Each Colony





Composite Image and identified objects (red outline)

Figure 2

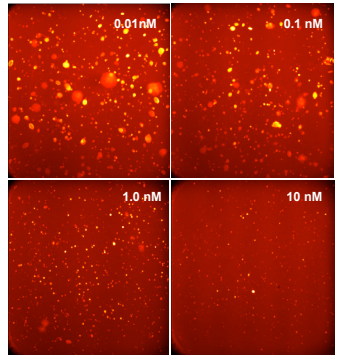


Figure 3. Whole well images of wells treated with the indicated concentrations of staurosporine and stained with LavaCell™.

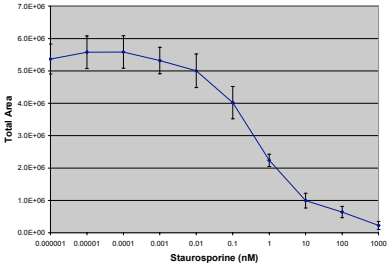


Figure 4. Staurosporine dose response curve as determined by total cell colony area. Measurements were done in replicates (n=6) and the error bars represent ± one standard deviation of the measurements.

Image Analysis Process

1. Acquire whole well images on IsoCyte in two channels (binocular view)
2. Add Images from two channels (Ch3 + Ch4)
3. Threshold on composite image
4. Use filters (area, intensity, etc.) to classify objects for analysis
5. Determine total cell colony area (sum of area measurements from all classified objects)
6. Image analysis occurs concurrently with scanning and the results can be viewed immediately afterward
7. Other colony parameters also available for analysis (e.g., colony size distribution)

Summary

- The IsoCyte™ allows a rapid image acquisition and robust image analysis process for quantitation of cell colony growth
  - Whole well images of LavaCell™ stained colonies detected in 2 channels
  - Composite image used for image thresholding to define objects
  - 96-well plate is scanned with concurrent analysis in 4 minutes or < 5 min cycle using an integrated plate handler (Twister II) for walk-away operation
- An automation ready protocol has been established for cell colony growth assay
  - Robust assay assembly and cell colony growth was achieved with MatriCal 96-well plate
  - Use of LavaCell™ achieved a one step colony staining process
  - Provides whole well, colony-by-colony information for quantitation
- Further optimization of protocols and development of novel 3-D assays are in progress