Technical Overview

Cell Analysis

Agilent

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Whole-Well Cell Proliferation Imaging with the Agilent InteroCyte Shadow-Free Imaging Microplate

Introduction

In order to determine the effect of a test molecule on target cells, it is common to perform cell proliferation assays. This is true during the drug discovery process for assessment of negative toxic side effects, or during general cellular research. While capturing a single image from the middle of each well and counting the included cells may suffice for certain applications, due to uneven cell distribution, it is common to image the entire well to achieve the most accurate cell count.

As many cell proliferation assays are still performed with cells plated in a 2D manner, microplates containing a tissue culture treatment and a cylinder-shaped well are typically incorporated into the workflow. In addition, it has become increasingly desirable to carry out such assay procedures using label-free methods to ensure that changes in proliferation rates are truly from a test molecule, and not from the addition of a fluorescent probe to the cells. This poses a particular problem when using a microplate with cylindrical wells due to what is traditionally known as the meniscus effect. The meniscus of a liquid forms when that liquid comes into contact with the sides of a microplate well. The tissue culture treatment causes the surface to be hydrophilic. Therefore, the molecules of the liquid have a stronger attraction to the surface than to themselves, causing concave menisci to form at the inner edge of the well (Figure 1).

Figure 1. Microplate well demonstrating liquid meniscus effect.

When performing label-free imaging—where white light is passed from a source above the well, through the liquid to the sample, and then down to the camera for quantification—any curvature of the light causes a dark shadow to form. Because of this phenomenon, cells within these areas of the well are poorly illuminated and almost impossible to properly quantify. Software-based image-processing algorithms can be applied to "even out" the illumination of the image from the center to the edge of the well. However, more often than not, the level of processing that is required makes any contrast between the cells and background indeterminable near the inner edge of the well.

To provide researchers with the solution they require to perform robust cell proliferation assays, we developed the 96-well Agilent InteroCyte shadow-free imaging (SFI) microplate. The unique well design simply eliminates the shadow caused by the liquid meniscus from the imaged area, with volumes commonly used in 96-well format (Figure 2).

Figure 2. Agilent InteroCyte SFI microplate well.

No image processing is necessary. The plate also conforms to ANSI/SLAS plate standards for length and width, allowing it to be used not only with Agilent imaging systems, such as the BioTek Cytation and Lionheart systems, and the xCELLigence RTCA eSight, but also with any microscopy system.

Experimental

Materials

Cells and media

HeLa cells, engineered to express a red fluorescent protein (RFP) such that the signal could be captured by the Texas Red imaging cube, were used for this evaluation. The cells were cultured in Eagle's minimum essential medium (ATCC, part number 30-2003), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep), according to the supplier's instructions.

Instrumentation

Images were captured using the wide field of view (WFOV) camera on the Agilent BioTek Lionheart FX automated microscope. An Agilent 4x plan extended apochromat air objective (part number 1220573) was used to capture all images. The high-contrast brightfield (HCBF) imaging module was used to capture label-free images, while the fluorescence imaging module, along with the Agilent 590 nm LED cube (part number 1225002) and Texas Red filter cube (part number 1225102), were used to capture fluorescent images.

Software

Agilent BioTek Gen5 Image Prime software was used for image capture and analysis.

Methods

Cell seeding for meniscus shadow-free imaging

Cells were seeded in a volume of 250 µL at a density of 2,000 cells/well into 16 wells of three InteroCyte SFI microplates (part number 204973-100), as well as three competitive 96-well clear microplates containing cylindrical wells (control plates). A volume of ≥ 250 µL is required for shadow-free imaging. The cells were allowed to settle to the bottom of the plates for one hour in a biosafety cabinet at room temperature before being placed into a 37 °C / 5% CO_2 incubator. Also, 50% media exchanges were performed on a 24-hour basis.

Imaging and analysis

A two-row by two-column 4x image montage, using the objective and imaging modes previously described, was incorporated to image the entire well for each plate type. Images were captured using the default autofocus method every 24 hours over a total 72-hour time period to track cell proliferation. A second set of high-contrast brightfield images was also captured by decreasing the focal height to a point where maximum contrast between cell and background brightness was achieved.[1] Following image capture, each image montage was stitched together. The total number of cells imaged, either using label-free methods or via fluorescence, was then quantified.

Results and discussion

Results

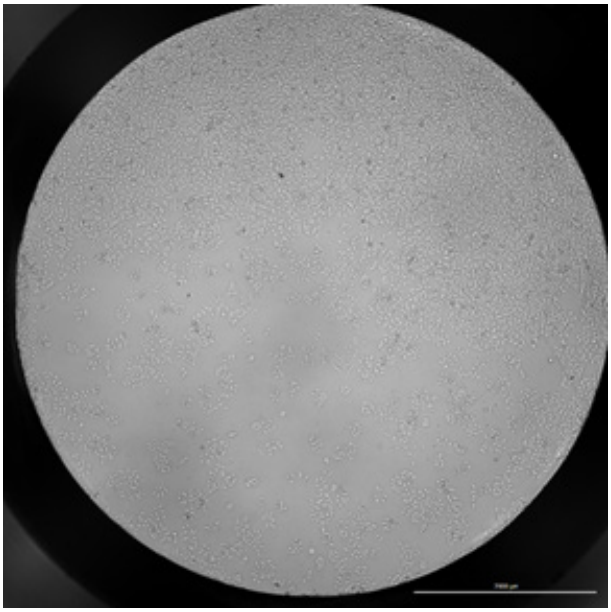
When comparing the 4x HCBF image montage (Figure 3A) captured using the InteroCyte SFI microplate, it can be easily seen that the well of the InteroCyte SFI microplate is evenly illuminated from the middle to the outside of the well. RFP-expressing cells, visualized in the Texas Red channel (Figure 3B), are also clearly visualized in the HCBF channel no matter their placement within the well, but particularly at the well edge.

In comparison, in the HCBF image montage captured from a control well plate (Figure 3C), a liquid meniscus shadow can be clearly seen around the outside of the well. The shadow, in essence, hides the cells at the outer edge of the well, which can be visualized in the Texas Red channel.

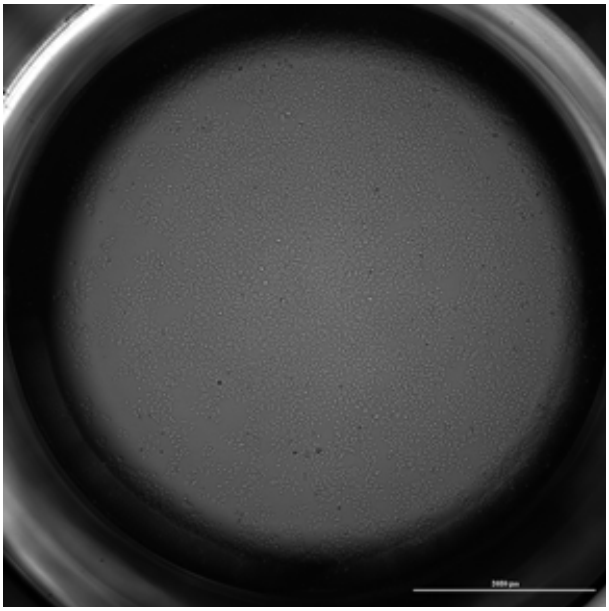
Quantification of the meniscus shadow effect

When performing cellular analysis of captured images, using equivalent analysis criteria, quantification of the effect the meniscus shadow has on data quality can be clearly seen (Figure 4).

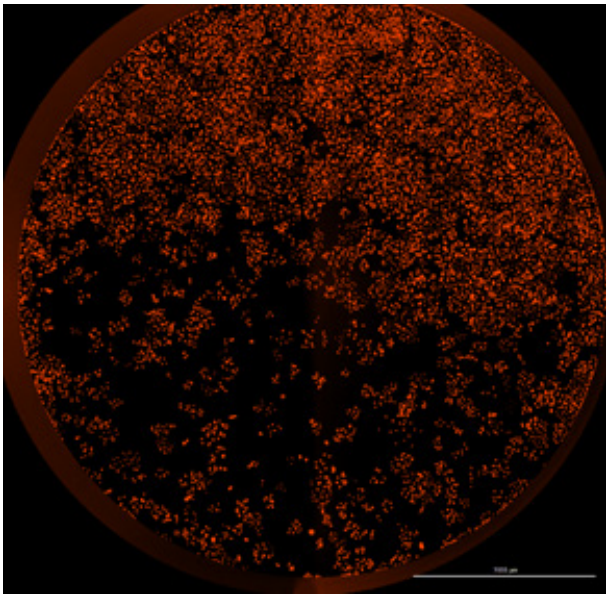
A.



C.



B.



D.

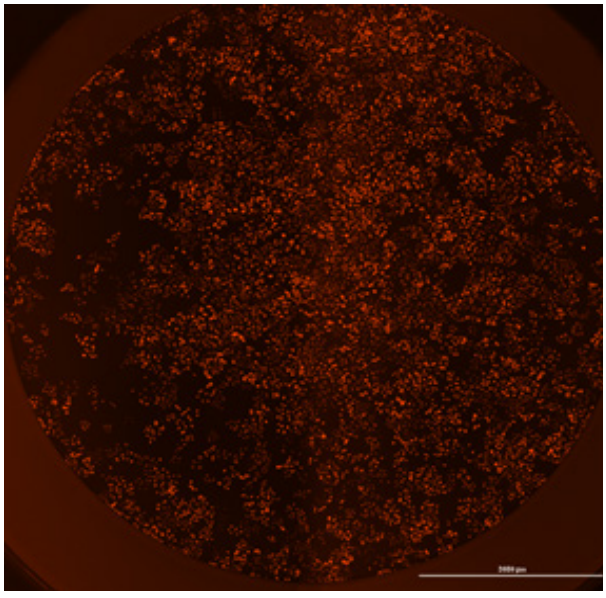


Figure 3. A 4x image montage captured from the well of an Agilent InteroCyte SFI microplate (A and B) and the well of a control plate (C and D). Images were captured using HCBF imaging (A and C), as well as the Texas Red fluorescence channel (B and D).

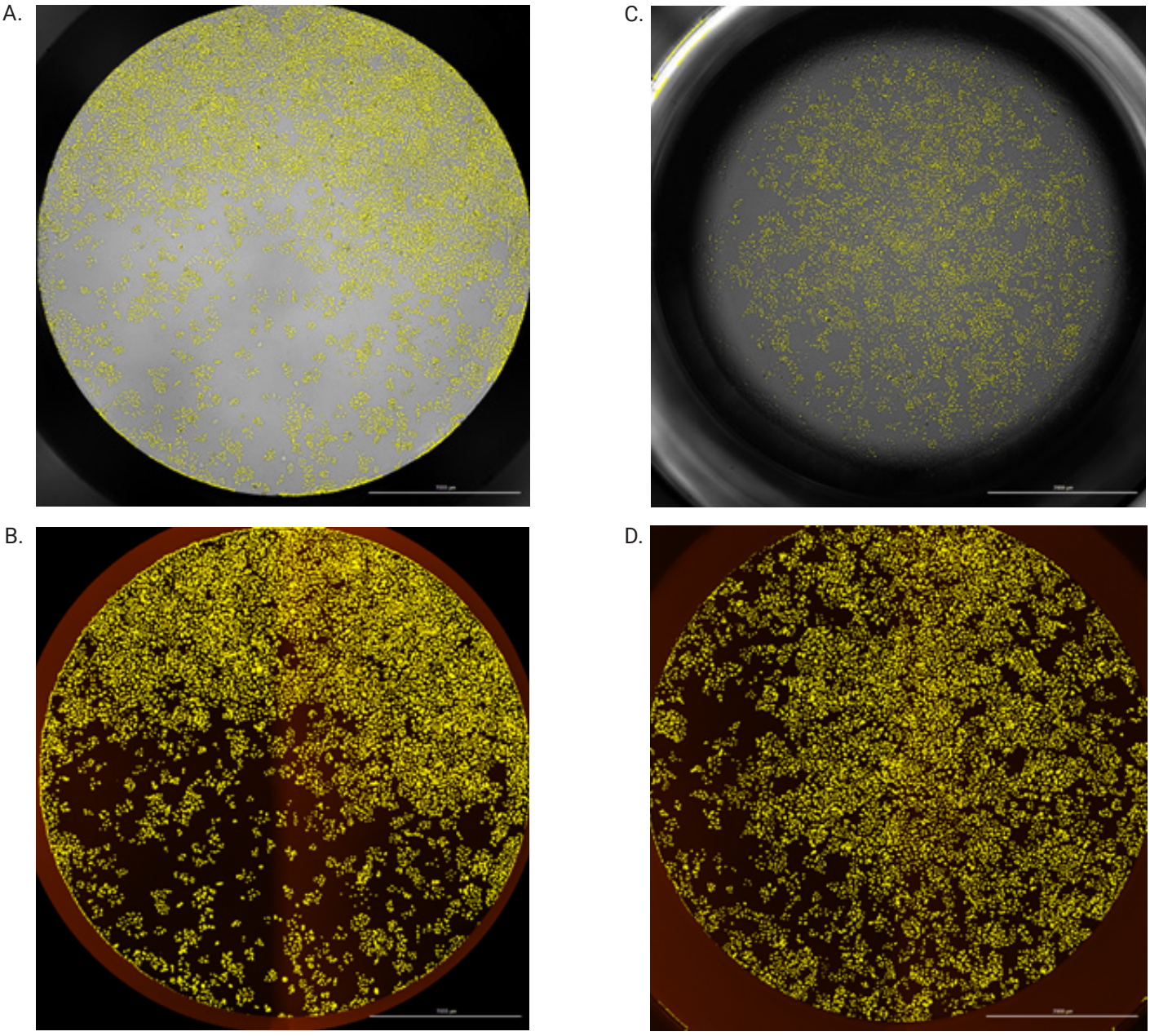


Figure 4. Object masks placed following cellular analysis of a 4x image montage captured at the 72-hour time point, from the well of an Agilent InteroCyte SFI microplate (A and B) and the well of a control plate (C and D). Analysis was performed on HCBF images (A and C), as well as Texas Red fluorescent images (B and D).

The cells counted within the representative InteroCyte SFI microplate well when using the HCBF channel (13,225) is 99.6% of the cells counted from the Texas Red channel (13,280). By comparison, the cells counted from the representative control plate well when using the HCBF channel (8,711) is only 85.8% of the cells counted from the Texas Red channel (10,156).

Quantification of the meniscus shadow effect on cell proliferation data

Cell counts, calculated from test plates included in the cell proliferation study, were then plotted over time. The graphs in Figures 5A to C show that an equivalent number of cells are consistently counted from HCBF and Texas Red images captured from the InteroCyte SFI microplates. The graphs in Figures 5D to F, however, demonstrate a difference in cell

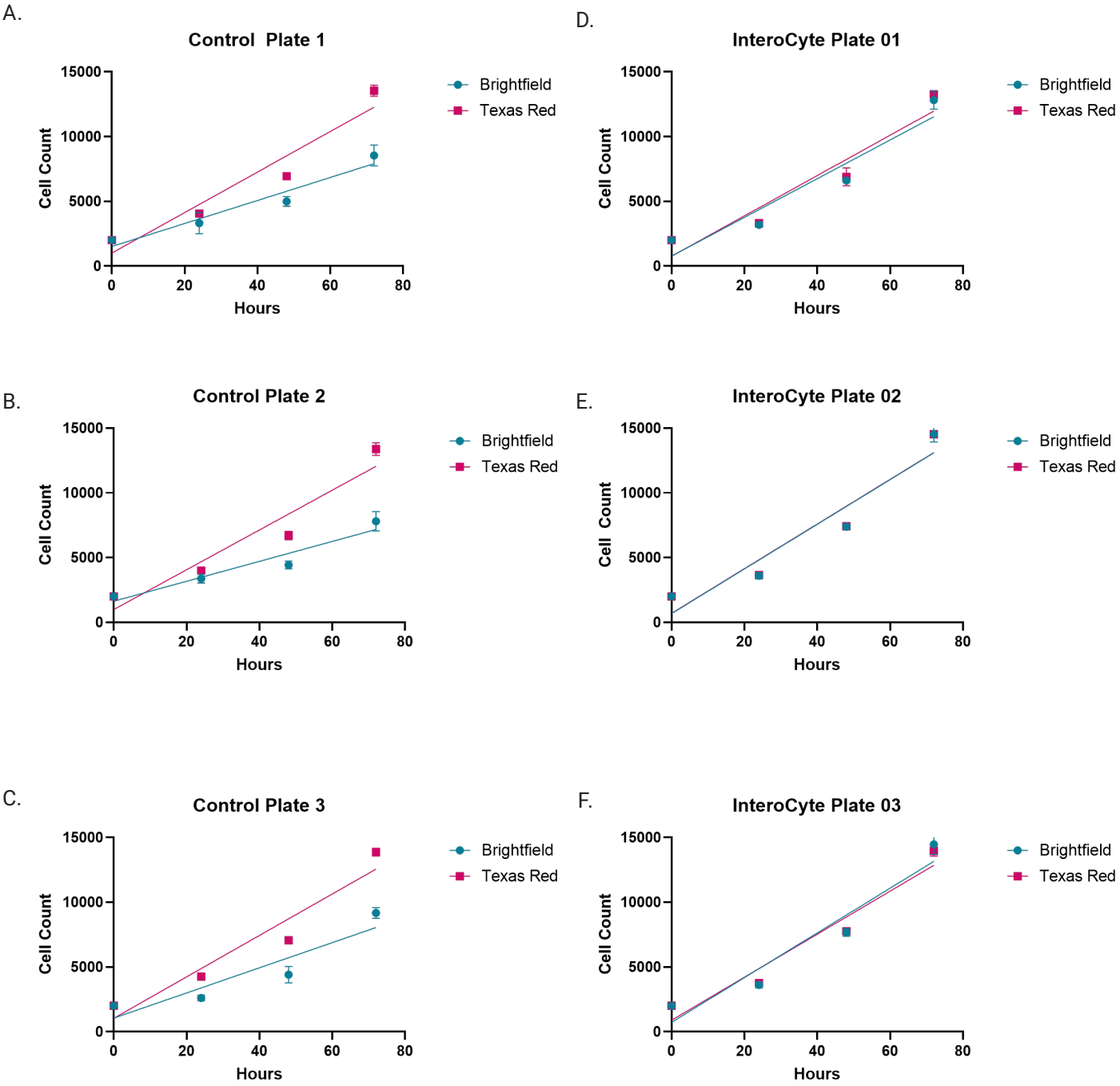


Figure 5. The 72-hour cell proliferation curves, plotted from HCBF or Texas Red images, using either Agilent InteroCyte SFI microplates (A to C) or control plates (D to F).

counts from the control plates. When imaged in the HCBF channel, cells at the edge of the well remain uncounted across the course of the study. As these cells continue to proliferate, the inaccuracy of the cell count continues to increase.

A statistical analysis of the difference between the slope of the generated HCBF and Texas Red cell proliferation curves, equivalent to an analysis of covariance, was then carried out using GraphPad Prism (Table 1).[2]

Table 1. Curve slopes generated from HCBF and Texas Red cell counts for three control plates and three Agilent InteroCyte SFI microplate test plates. The *p*-values are also reported from slope comparison tests carried out using GraphPad Prism.

Plate	HCBF Curve Slope	Texas Red Fluorescence Curve Slope	*p*-value
Control 1	88.84	156.4	<0.0001**
Control 2	76.95	153.6	<0.0001**
Control 3	97.14	160.0	<0.0001**
Test 1	149.4	155.7	0.4771
Test 2	172.4	172.3	0.9933
Test 3	172.6	166.1	0.4381

The *p*-values from the comparison of cell proliferation curves generated with control plates are all less than 0.0001. According to established criteria, the difference between the two curves is extremely significant, as their *p*-values are less than 0.001. The *p*-values calculated from each InteroCyte SFI microplate, on the other hand, are all greater than 0.4 and, according to criteria, are not significantly different when the *p*-value is greater than 0.05.

Discussion

The image in Figure 3C, captured from a control plate containing a columnar well shape, demonstrates the problem seen using traditional microplates. The liquid meniscus remains within the imaged area, causing a shadow that impedes proper illumination of the well edge. The effect decreases cell counts (Figures 4C and D) and negatively affects cell proliferation curves (Figures 5E to H, and Table 1). This can lead to false assumptions being made about the growth rate of test cell types, or the effect that a test molecule has on target cells.

By comparison, the image from Figure 3A demonstrates how the unique well geometry of the InteroCyte SFI microplate provides an equally illuminated full-well image. Cells at the edge of the well can be seen and counted as easily using the HCBF channel (Figure 4A) as in the Texas Red channel (Figure 4B). This allows researchers to have complete confidence that cell counts and cell proliferation curves (Figure A to D, and Table 1) captured using label-free methods are accurate and repeatable.

Conclusion

The Agilent InteroCyte shadow-free imaging microplates provide a robust method to achieve shadow-free, whole-well images. The unique well design eliminates the effects created by the liquid meniscus from the imaged well, at volumes commonly used with cell-based assay workflows. The result creates accurate, trustworthy data when performing label-free cell analysis assays, such as cell proliferation and toxicity assessments. Non-whole-well, label-free imaging with volumes less than 250 µL, in addition to fluorescence imaging, is also possible using the InteroCyte SFI microplate. Other traditional types of imaging applications are also possible with the InteroCyte SFI microplate using volumes less than 250 µL, such as label-free imaging of a portion of each well, in addition to fluorescence imaging. The combination makes the InteroCyte SFI microplate a highly versatile consumable that can be integrated into multiple assay workflows performed in modern research laboratories.

References

1. High Contrast Brightfield, https://www.agilent.com/cs/library/technicaloverviews/public/high-contrast-brightfield-5994-3444EN-agilent.pdf. Accessed 12 June 2025.
2. GraphPad. "GraphPad Prism 10 Curve Fitting Guide – Comparing Sloped and Intercepts." Home-GraphPad, https://www.graphpad.com/guides/prism/latest/curve-fitting/reg_comparingslopesandintercepts.htm. Accessed 12 June 2025.

Products used in this application

Agilent products

Agilent Interocyte Shadow-Free Imaging Microplates

www.agilent.com/lifesciences

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