

Trends in Cell-based Assays

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Cell-based Assays using Microplates

Cells are the smallest unit of life and thus are often used as a model system to study the biology of an organism. Over the last five years or so, there have been two growing trends in the use of cell-based assays. One is to increase the biological relevance of assays, such that in-vivo conditions in humans are more closely resembled; and the other is a return to classical pharmacology methods using phenotypic assays to measure functional responses of cells to stimuli.

The interest in providing greater biological relevance to cell-based assays has included using

- cell lines with endogenous expression of protein(s) of interest
- freshly isolated primary cells from the organism of interest
- assays with live cells and kinetic readouts
- assays that use 3-D cell culture which provide a tissue-like structure

Each of these advances has made assay development more difficult as endogenous expression levels of proteins of interest are typically low, compromising assay robustness. Primary cells are difficult to culture and undergo senescence after a few passages. Live cell assays require non-toxic reagents to be used and tissue culture conditions should be maintained (37 °C/5% CO₂) for long kinetic readouts. And finally, 3-D cell culture requires completely different cell culture techniques.

Target-based drug discovery leans heavily on the validation of the chosen drug target for disease intervention. Poor efficacy remains one of the most common reasons for attrition in drug discovery, suggesting that drug target validation requires more effort and in many disease states, multiple targets must be addressed for satisfactory efficacy. Thus many efforts are returning to classical pharmacology methods [1]. Prior to the rise of target-based drug discovery, these methods were centered on functional activity: a classic example is the use of organ baths, where compounds could be tested for a physiological response. These classical pharmacological assays required no preconceived knowledge of drug target or mechanism of action, which on one hand make it more difficult to develop assays relative to target-based methods; yet on the other, complex disease biology associated with multiple proteins acting in concert can be assayed for using these functional readouts. Phenotypic assays conducted today use the same notion of monitoring phenotype, but typically the vessel of choice is microplates for cells displayed either in a monolayer adhered to microplates or in 3-D structures.

Microplate Readers for Cell-based Assays

As the cellular model associated with cell-based assays has become progressively more complex, microplate readers need to adapt to accommodate these advances. These include the addition of highly sensitive detection modes, such as time-resolved fluorescence to better develop robust assays with the endogenous expression of proteins of interest, whether in cell lines or primary cells. Microplate readers are now available with environment control within the detection chamber such that tissue culture conditions can be maintained (37 °C/5% CO₂) for long kinetic readouts. Microplate readers can be used with 3-D cell cultures, such as those that use scaffolds like hydrogels or extra cellular matrix, but there can be issues with sensitivity if spheroids are employed as their relative size compared to the microplate well is small. As the PMT-based optics of microplate readers have been designed to capture as much light as possible from the microplate well, assays involving live cell spheroids capture mostly background rather than signal. Imaging of the microplate well can circumvent these issues as the field of view can be easily varied by use of different microscope objectives to localize the spheroid and thus improve signal to background ratio. This has been demonstrated with a number of microplate readers that are actually automated digital fluorescence microscopes that image cells in microplate wells [2-4].

The microplate reader is a workhorse for cell-based assays that are target-based. Commercial reagent vendors offer myriad kits and reagents for quantifying protein expression in [5] and secretion from cells [6], receptor binding [7], and cellular signaling [8]. One of the most common assay formats are immunoassays using a variety of labels. Microplate readers can also be used for phenotypic assays, such as measuring the production of reactive oxygen species [9], but imaging of cells in the microplate well using automated digital fluorescence microscopes remains a highly popular detection method for phenotypic assays as cells can be treated with a broad palette of fluorescent probes to enable the analysis of multiple cellular phenotypes [10].

Measuring a cell phenotype can also provide more accurate results relative to surrogate markers typical of microplate readers. This was evident in a study of antiproliferative agents, where an image-based assay that counted cells and determined cell cycle phase distribution was proved more accurate than two metabolism-based cell viability/proliferation assay formats. It was found that depending on compound mechanisms of action, the metabolism-based surrogate assays were frequently prone to significant underestimation of compound potency and efficacy [11].

Combining Automated Fluorescence Microscopy with a Microplate Reader

It is unequivocal that cell-based assays conducted in microplates have evolved over the last decade and there is no doubt that they will continue to do so. From the previous discussion, it appears there is high utility for combining traditional PMT-based optics typical of microplate readers with automated fluorescence microscopy typical of HCS readers. This will allow for the broadest range of cell-based assay applications to be conducted in a single laboratory instrument. The PMT-based optics of the microplate reader provides highly sensitive assays reflecting a cumulative signal from the cell population in the well; whereas CCD-based imaging allows for sub-population analysis of the cells in the well and the visualization and quantitative assessment of phenotypes using one of more fluorescent probes.

This is now possible with the Cytation™ 3 Cell Imaging Multi-Mode Reader. Cytation 3 combines automated digital microscopy and conventional microplate detection. It has been designed to provide suitable performance for all of the latest types of and trends in cell-based assays, including

- assays using endogenous expression of targets and substrate in either cell lines or primary cells
- live cell kinetic assays that extend out to days
- assays conducted in 3D formats including both scaffold and spheroid technologies
- image-based phenotypic assays

Figure 1 below illustrates the three separate optical paths that are provided with a Cytation 3 as modules. The upper portion of the instrument houses conventional PMT-based microplate reader optics, although both monochromator- and spectral filter-based optical paths can be chosen in the same instrument. The lower portion of the instrument contains CCD-based microscopy using either brightfield or fluorescence.

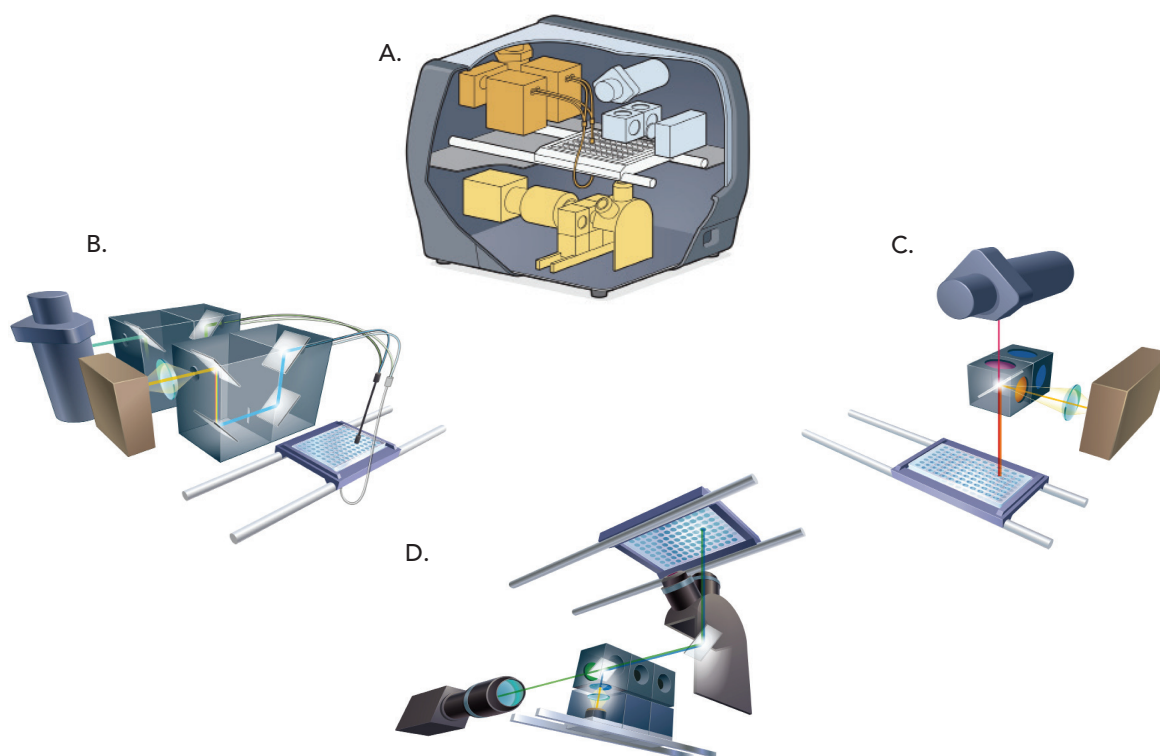


Figure 1. Schematic of Cytation 3 Cell Imaging Multi-Mode Reader illustrating the three separate optical paths. **A:** Configuration of the three separate optical paths within Cytation 3; **B:** PMT-based detection using monochromator wavelength dispersion; **C:** PMT-based detection using spectral filter cubes for wavelength isolation; **D:** CCD-based microscopy using LEDs, spectral filter cubes and microscope objectives ranging from 2x – 60x.

Applications using Cytation™ 3 Cell Imaging Multi-Mode Reader

A number of applications that take advantage of both PMT- and CCD-based optics within the Cytation 3 will be described. Many of these applications utilize the latest trends in and types of cell-based assays as described previously.

Live Cell Assays using Endogeneous Expression of Target Proteins

It has been described in the literature that IL-6 secretion is a putative biomarker for late stage ovarian cancer [12] and poor prognosis. In a previously published application note, we used an HTRF sandwich immunoassay to quantify EGF-induced IL-6 secretion from an endothelial ovarian cancer cell line, SKOV-3 [13]. This assay was then used to investigate the inhibition of IL-6 secretion by a series of both small molecule and monoclonal antibodies that inhibit both at the level of EGF's receptor (EGFR) and at various points in the activation pathway. A two-plate protocol was used where an aliquot of the cell supernatant was transferred to a shallow well 384-well plate for IL-6 quantification using HTRF and high sensitivity PMT-based spectral filter cube optics. This transfer served to miniaturize the assay, conserving expensive reagents; but also allowing for a phenotypic LIVE/DEAD assay to be conducted on the remaining cells in the original assay plate using CCD-based fluorescence microscopy. This assay is comprised of a blue emission cell-membrane permeable probe that binds to nuclear DNA and serves as a LIVE cell indicator; and a red emission cell membrane impermeant probe that only binds to nuclear DNA if the cell membrane is compromised, as in necrosis or apoptosis. Figure 2 below demonstrates this workflow.

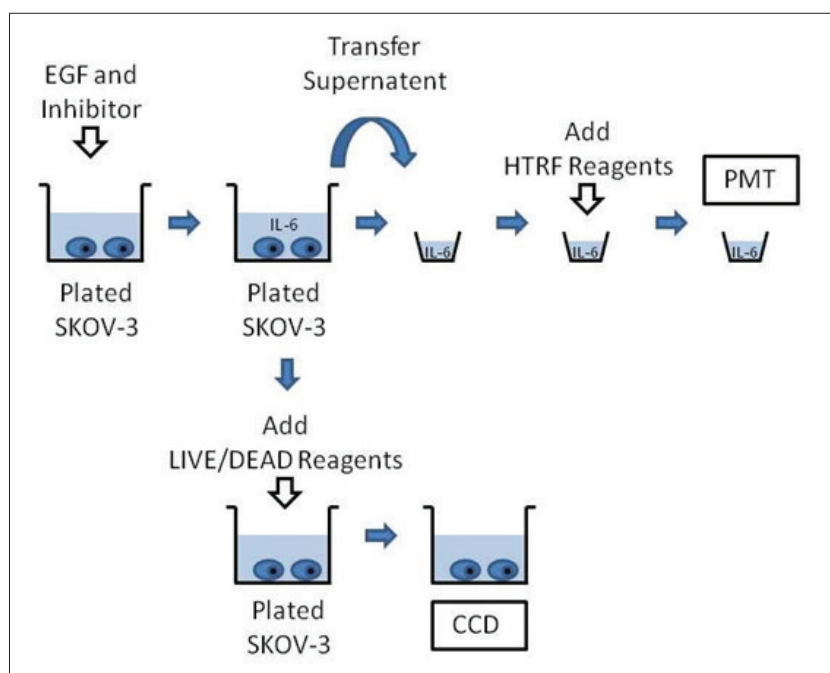


Figure 2. SKOV-3 cells plated in a 96-well microplate were induced/inhibited to secrete IL-6. After a suitable incubation time, 16 mL of supernatant was transferred to a shallow well 384-well microplate and IL-6 quantified using high sensitivity PMT-based spectral filter cube optics. In addition, the remaining SKOV-3 cells in the 96-well microplate were tested for cell viability using LIVE/DEAD fluorescence probes and CCD-based fluorescence microscopy.

One small molecule inhibitor used demonstrated significantly higher potency in full dose response than other inhibitors tested. The wells of an IC_{50} and a high dose concentration were imaged and a sub-population analysis performed on the cells in the field of view to allow for computation of the percentage of cells that have had their plasma membrane disrupted and are considered dead. Figure 3 below demonstrates that AG1478, an EGFR inhibitor, is toxic to the SKOV-3 cells at high dose and contributes to its apparent inhibition potency.

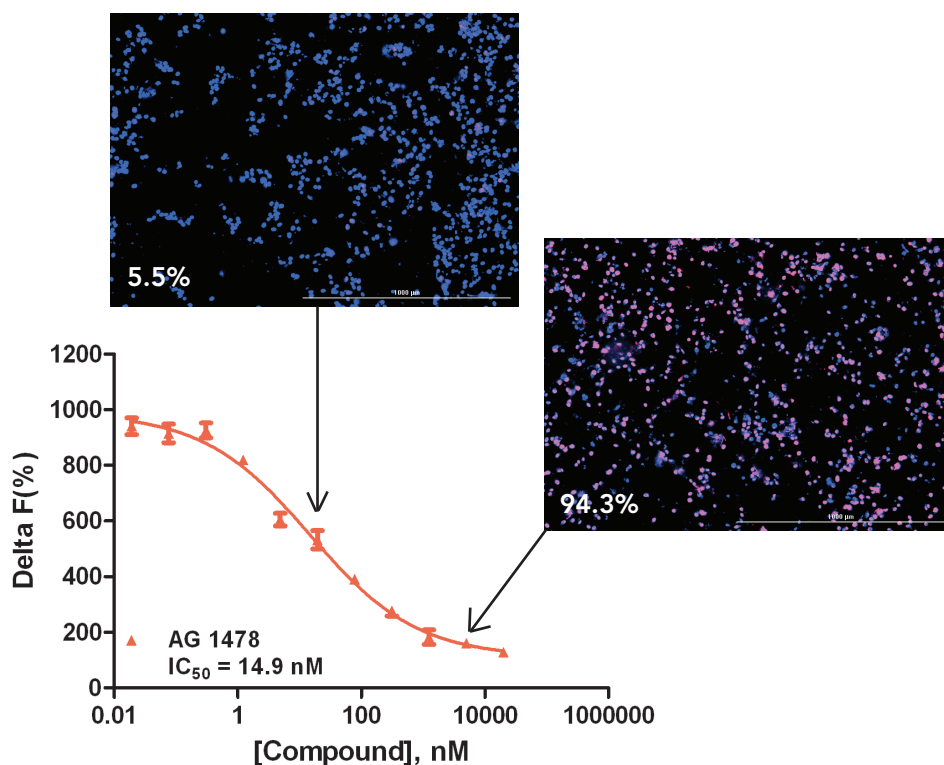


Figure 3. Full dose response curve for AG 1478, an EGFR inhibitor, obtained using PMT-based optics; with superimposed images of cells at IC_{50} and a high dose using CCD-based fluorescence microscopy. A 4x microscope objective was used to obtain a field of view that images about 1000 cells in the microplate well. At the IC_{50} concentration, only about 5% of cells show any loss of viability; yet at ~ 10M, almost 95% of cells show loss of cell membrane integrity.

Phenotypic Assays

Probing cellular phenotypes associated with disease or disease intervention is a return to classical pharmacology practices. We recently developed assays for monitoring oxidative stress and hypoxia in immortalized keratinocytes where the cellular phenotypes were chemically induced using $CoCl_2$ [14]. The fluorogenic probe that monitors oxidative stress is a fluorescein derivative which generates green fluorescence intensity proportional to reactive oxygen species (ROS) production; a red fluorogenic probe monitored nitroreductase activity and was used as a measure of hypoxia. Conventional PMT-based microplate readers can be used to assess these phenotypes, but the background inherent to these fluorogenic probes can be high.

CCD-based automated digital fluorescence microscopy allows for cell subpopulation analysis which can assess the fluorescence confined to the cell itself through identifying cells in the field of view through nuclear staining (i.e. DAPI, Hoechst dyes). This serves to dramatically improve signal to background ratios (S/B) for phenotypic assays. This can be illustrated using the oxidative stress phenotype associated with ROS production in the chemically induced keratinocytes. Using Gen5 Image+ software provided with Cytation 3, the average green fluorescence associated with the oxidative stress phenotype from all the cells in the field of view can be determined using a mask created through DAPI staining. This method measures ROS production in the environs of the nucleus of each cell in the field of view, excluding bulk solution background fluorescence. This method can be improved upon by extending the area of the mask through Gen5 Image+ analysis parameter adjustments to the green channel such that portions of the cytosol can be assessed for the green fluorescence associated with ROS production.

Each of these CCD-based image analysis methods reduces background and provides significantly greater S/B relative to PMT-based optics (Figure 4).

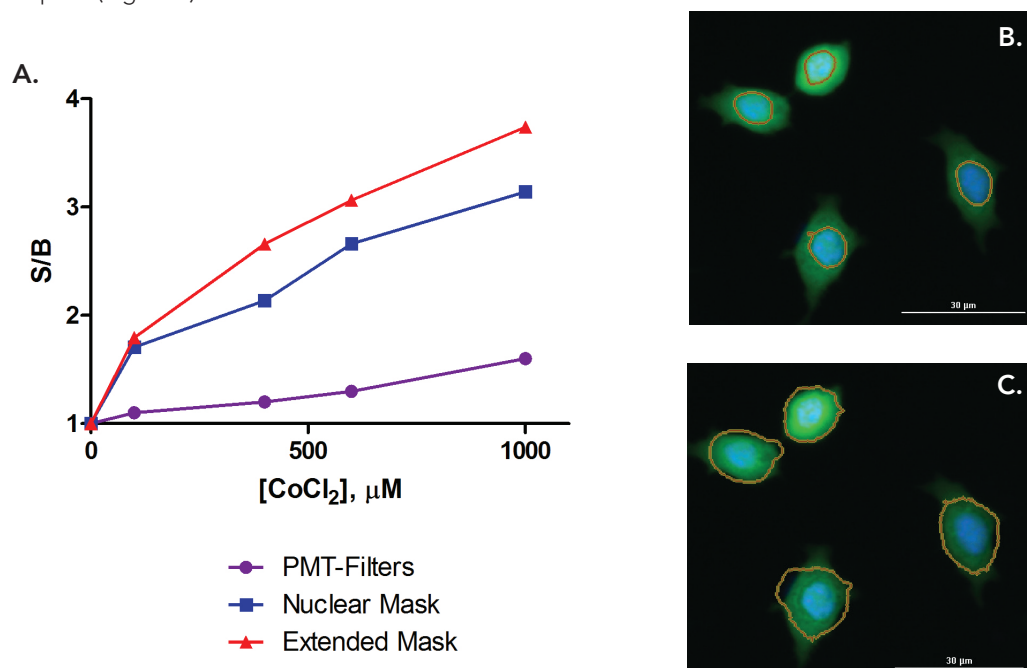


Figure 4. A: Comparison of assay performance quantifying ROS production associated with an oxidative stress phenotype as measured with PMT-based optics and spectral filters (purple circles); CCD-based microscopy with DAPI-based nuclear mask (blue squares); and CCD-based microscopy with an extended nuclear mask that captures a portion of cytosolic ROS production (red triangles); **B:** Zoomed 20x image of keratinocytes with DAPI-stained nuclei and a mask associated with nuclear staining used to quantify ROS production in the green channel (Threshold: 10,000, Image Smoothing Strength: 0); **C:** Zoomed 20x image of keratinocytes with DAPI-stained nuclei and an extended mask used to quantify ROS production (Threshold: 500, Image Smoothing Strength: 1). In each image, the yellow perimeter defines the area over which green signal associated with ROS production was quantified.

Live Cell Phenotypic Assays using Spheroid-based 3D Cell Culture

This improved relative assay performance between PMT- and CCD-based optics can be even more dramatic if 3D cell culture methods, such as spheroids, are used in live cell assays. Spheroids are cell aggregates comprised of about 10^4 to 10^5 cells which can be grown in either suspended drops of media from special microplates or in microplates with ultra low absorbance coatings. The spheroids are typically < 0.5 mm in diameter. When compared to the dimensions of a typical 96 well plate, the relative area a 200 μm ID spheroid would cover of the well bottom is $\sim 0.1\%$. PMT-based optics in microplate readers are designed to maximize capture of signal intensity emitting from the bulk solution in the well. When the signal is emitted from a spheroid, these optics have the disadvantage in collecting mostly background signal.

Conversely, microscopy has the advantage of being able to control the field of view through microscope objective selection. The Cytation 3's CCD-based optics and a 4x microscope objective limits the field of view to an area of about a tenth of the microplate well bottom. Thus the spheroid occupies $>1\%$ of this field of view. This improves with the use of a 10x objective, where the field of view is reduced to an area that is of the same scale as the 200 μm ID spheroid where it now encompasses $>10\%$ of the field of view. Background rejection can be further improved upon by using Gen5™ Image+ s/w to create a fluorescence mask of the same area as the spheroid.

This is demonstrated in a phenotypic assay for hypoxia using a red fluorogenic probe that measures nitroreductase activity and a 200 μm ID spheroid comprised of about 2,000 cells. Figure 5 shows the images of four time points in the hypoxia time course illustrating the growth of the hypoxic signal using the 10x microscope objective.

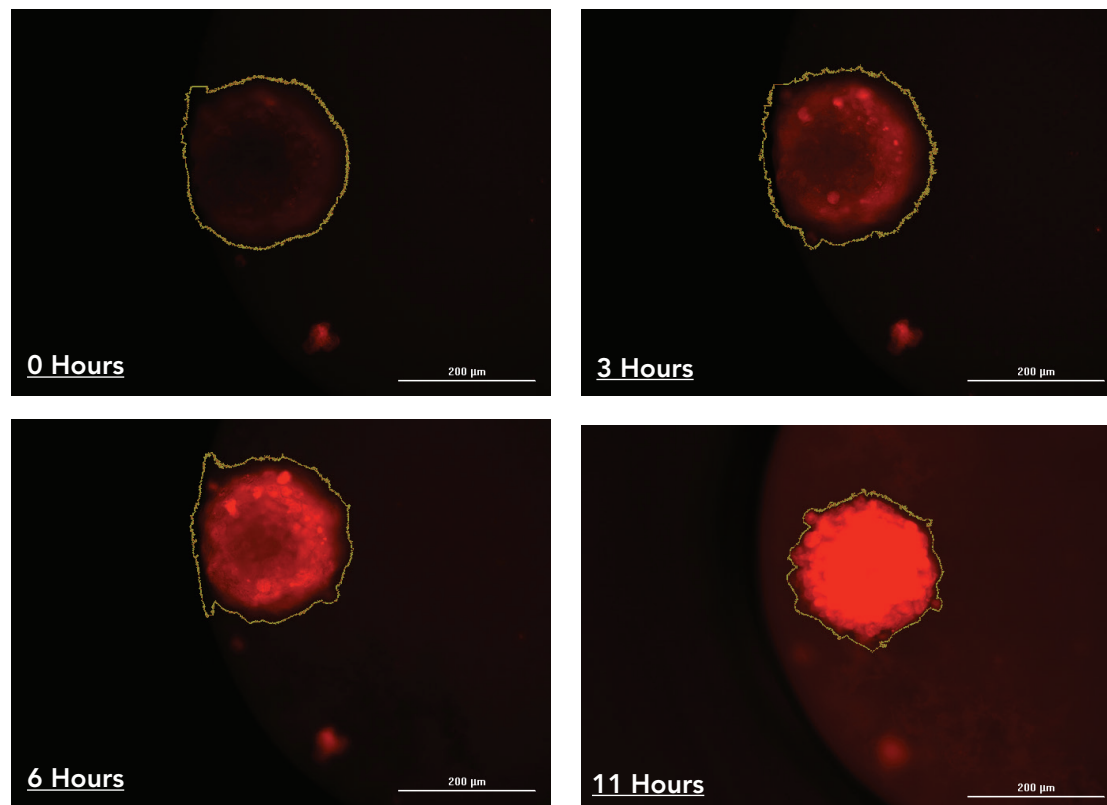


Figure 5. Time course study of the generation of hypoxic conditions in a liver microtissue spheroid imaged using a 10x microscope objective. The yellow perimeter seen around the spheroid image is the mask created by Gen5™ software. Signal collection can be confined to the CCD pixels within this mask for optimization of S/B.

Figure 6 demonstrates the relative assay performance using PMT-based monochromator optics and digital fluorescence microscopy with the Cytation™ 3 using 4x, 10x and the 10x microscope objectives with a red fluorescence mask around the spheroid. It is evident that the best assay performance is available with the method that rejects most background: the 10x microscope objective with red fluorescence mask.

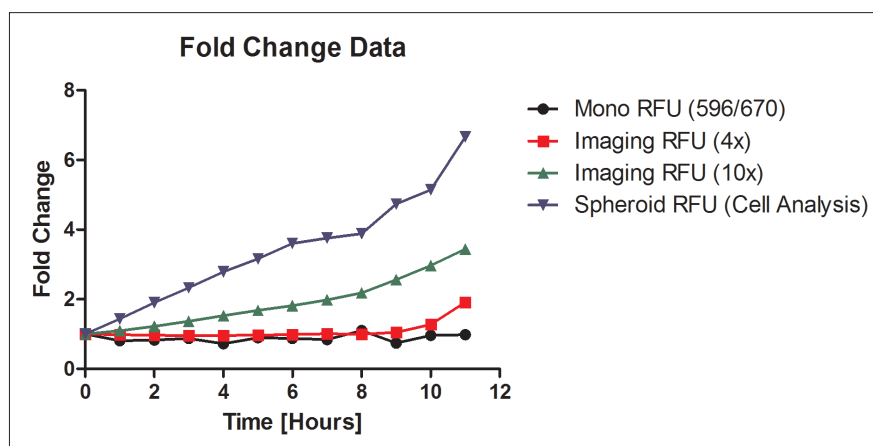


Figure 6. Assay Performance for live cell spheroid-based hypoxia assay using PMT- and CCD-based optics.

Assay Development for Spheroid Formation using Spheroid-based 3D Cell Culture

Automated digital microscopy is also useful in the laboratory for methods development. As an example, the Cytation 3 has been used for monitoring cell aggregation in spheroid formation using the Perfecta3D® hanging drop plate technology. These plates allow researchers to create their own spheroids of varying size by adjusting cell density addition to the hanging drop plates. The standard protocol calls for the addition of 40 L of cells and media to a well of the Perfecta3D® plate for each spheroid desired. This volume is sufficient to allow for a drop of media and cells to form suspended below the plate. As there is no plasticware for the cells to attach to, over time cell aggregation occurs forming spheroids. Spheroid formation is highly dependent on the cell/cell line used. Co-cultures are often used as well so methods development is usually required to optimize spheroid formation using various relative amounts of the cells used, additives and other conditions. The hanging drop plate assembly contains a series of reservoirs to maintain humidity levels within the plate contained between lid and tray. This ensures that little to no evaporation of media occurs during spheroid formation.

The Cytation™ 3 can be used to monitor spheroid formation [15]. The Cytation 3 uses a low magnification, long working distance microscope objective, such as a Zeiss 2.5x to image spheroid formation through the tray (Figure 7).

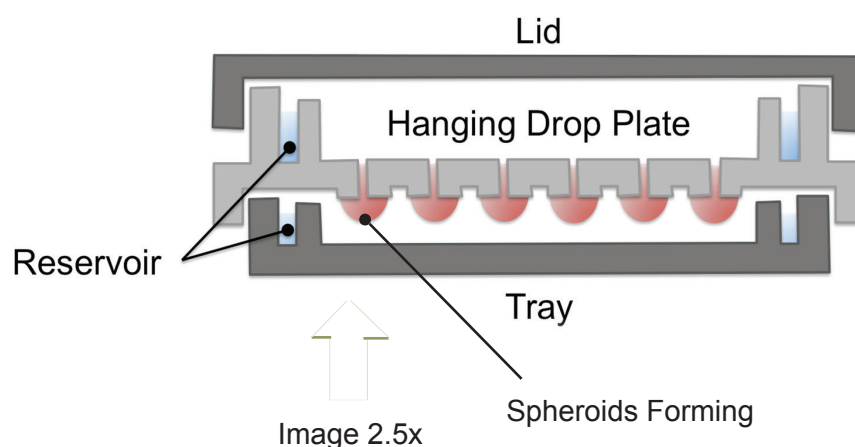


Figure 7. Imaging spheroid formation in suspended drops of media and cells in the Perfecta3D® Hanging Drop Plate.

Spheroid formation can be monitored over several days using the gas controller option which can maintain CO₂ incubator conditions within the detection chamber. Figure 8 demonstrates the aggregation of 5,000 cells of the breast cancer cell line MCF-7 over the period of 4 days using bright field microscopy.

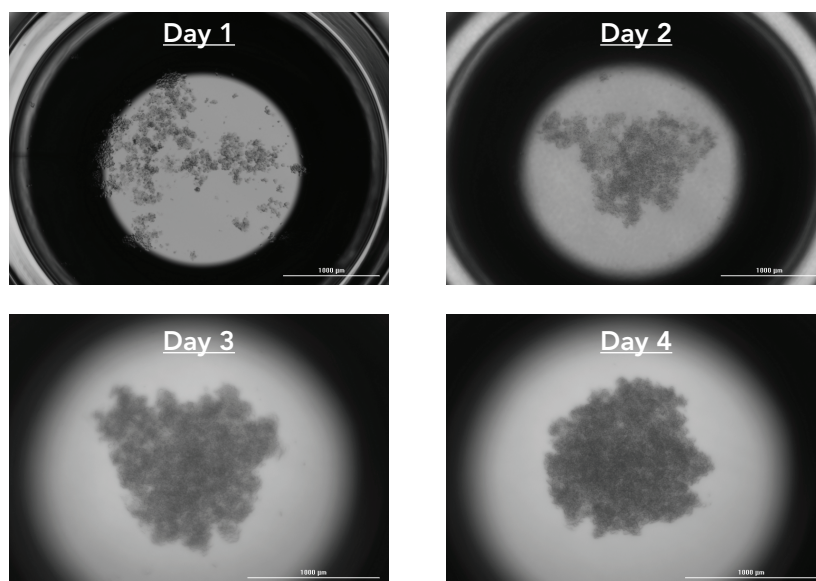


Figure 8. MCF-7 cells aggregating into spheroids in hanging drops from a Perfecta3D® plate using a Zeiss 2.5x microscope objective.

"Hit Picking" in High Content Analysis

Phenotypic assays often involve automated digital fluorescence microscopy of cells in microplates due to the ability to capture multi-parametric data and spatial information of cellular components, which has been termed "High Content Analysis" or HCA. These HCA instruments have been designed to image each well of a microplate using a number of fluorescence channels as rapidly as possible. They tend to be very expensive and reserved for industrial end-users or core labs which have sufficient capital budgets.

A key advantage of combining PMT-based optics and CCD-based microscopy in a single instrument is "hit-picking" of phenotypic assays. For assays involving a fluorescence intensity change, the PMT-based optics can be used to rapidly scan the whole plate and only return to wells for imaging provided the fluorescence intensity changes cross a threshold level, in essence a "hit." This has been demonstrated in an experiment to identify potential inhibitors of cellular hypoxia [14]. Immortalized keratinocytes were cultured in the presence of cobalt chloride (CoCl_2), a known inducer of hypoxia-like responses, along with a REDOX compound library made up of several known antioxidants. A red fluorogenic probe was used that provides a measure of hypoxia based on nitroreductase activity. Prior to reading the assayed microplates, a single Cytation 3 hit pick protocol was created. The protocol used the results from a monochromator-based read of the signal from the hypoxia dye to initiate imaging of wells whose RFU values were greater than or equal to 50% inhibition of the hypoxic condition, as assessed from controls.

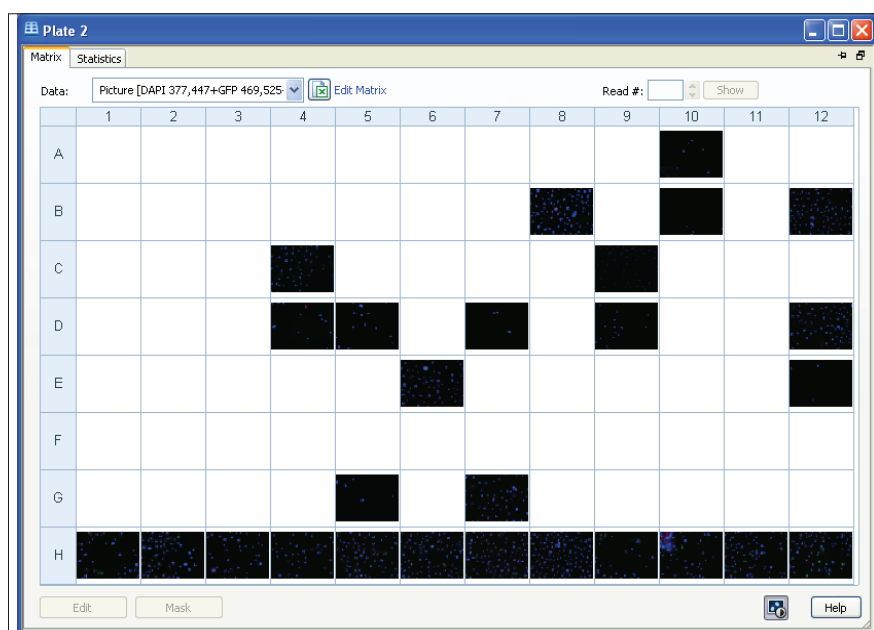


Figure 9. Selected wells for imaging using "Hit Pick" protocol criteria.

Per Figure 9, a total of 27 wells were imaged using the protocol, including 15 "hit-picked" wells and 12 control wells in row H. The time to capture three images (3 separate fluorescence channels) from these wells is 3 minutes and 22 seconds. When compared to the total time required to capture three images from all 96 wells, which is 12 minutes, this represents a 3.5 fold savings in time, as well as required storage space. If one considers that the typical hit rate for HCA screening is in the order of 1-5%, a screening campaign can be accelerated using PMT-based "hit picking" by a higher margin, closer to an order of magnitude while requiring only about 1-5% of the data storage space.

Conclusions

The Cytation 3 enables many of the latest trends in cell-based assays as demonstrated by the applications described herein. These include assays of ever greater physiological relevance that make for challenging conditions for adequate assay performance. Having the three available optical paths involving both PMT-based detection and CCD-based imaging provides great flexibility in selecting the most suitable readout for the assay desired. In addition, the two forms of detection can be used together to reduce phenotypic screening times and data storage requirements. Finally, the microscopy module can be used for routine laboratory practices such as cell counting and determination of transfection efficiency; and for methods development such as monitoring spheroid formation in hanging drop plates.

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