

# Improving Fluorescence-Based Assay Performance Using Automated Digital Microscopy



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## Introduction

Fluorescence remains one of the most popular detection modes used in cell-based assays primarily due to the wide array of fluorophores available. These can be grouped into three main families of fluorophore:

- Reactive dyes that can be used to make fluorescent conjugates of biomolecules (i.e. antibodies) that can be used to probe cellular processes
- Fluorogenic probes that upon binding undergo significant changes in fluorescence quantum yield
- Photoproteins like the family of GFP variants that can be transfected into cells as reporter systems or fusion proteins.

One of the main limitations of fluorescence detection is background fluorescence, either from autofluorescence from endogenous cellular material, impurities from reagents added to the assay, unbound fluorescent conjugates or fluorogenic probes, or unreacted photoprotein. Autofluorescence and background from impurities can be reduced by the use of red-shifted fluorophores; unbound fluorescent conjugates can be removed through gentle wash cycles that allow cells to remain adhered to plasticware; but for homogeneous assays in microplates that rely on simple mix and read workflows, background from unbound probe or unreacted photoprotein can be a problem that limits assay performance.

Here we demonstrate three unique ways in which the incorporation of imaging, in addition to cellular analysis, allow for a more accurate analysis of the event taking place within the microplate well, and improve assay performance.

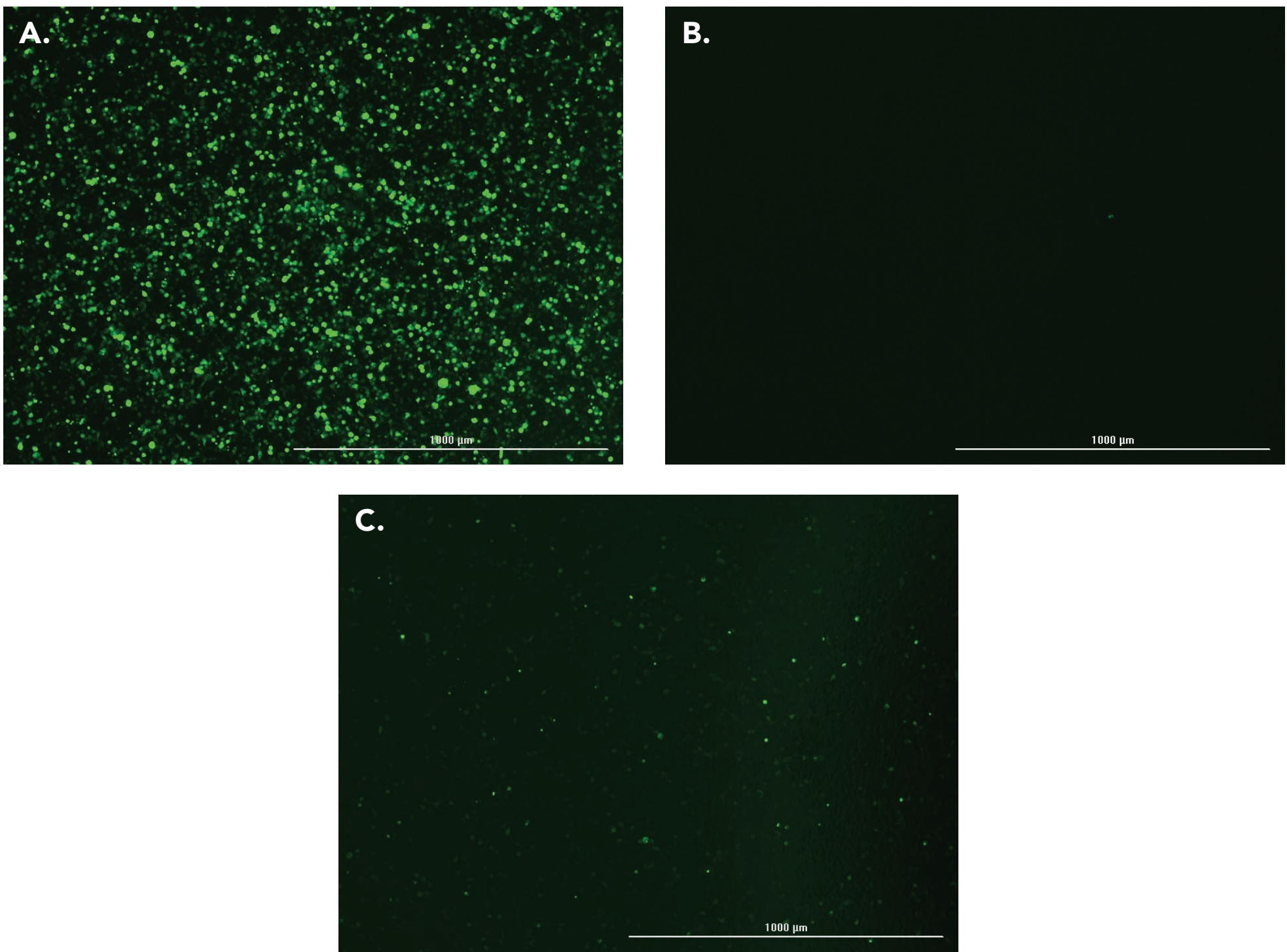
## BioTek Instrumentation and Analysis Software

**Cytation™3 Cell Imaging Multi-Mode Microplate Reader.** Cytation 3 combines automated digital microscopy and conventional multi-mode microplate detection providing rich phenotypic cellular information and well-based quantitative data. With special emphasis on live-cell assays, Cytation 3 features temperature control to 45 °C (37 ± 0.5°C), CO<sub>2</sub>/O<sub>2</sub> gas control and dual injectors for kinetic assays. The instrument was used to image cells and spheroids using multiple unique fluorescence imaging cubes.

**Gen5™3 Data Analysis Software.** Gen5 software controls the operation of the Cytation 3 for both automated digital microscopy and PMT-based microplate reading. Image acquisition is completely automated from sample translation, focusing and exposure control. Image analysis allows for cell counting and sub-population analysis suitable for phenotypic assays.

## Transfection Efficiency Determination

The efficiency of transfection of mammalian cells with exogenous DNA plasmids is often monitored with genetically encoded fluorescent protein markers such as GFP. Efficiency can be determined using either a PMT-based whole well measurement or a CCD-based image. In both cases the efficiency of the experimental sample is the ratio of the sample to the positive control after the negative control has been subtracted.



**Figure 1. GFP Protein Transfection Efficiency Conformational Imaging.** Images captured using GFP imaging filter cube and 4x magnification. (A) Positive control containing stably expressing GFP cells. (B), Negative control containing non-GFP expressing cells. (C) Test well containing transfected cells expressing GFP protein.

	Well RFU	Cell Count
Negative Control	19556	1
Positive Control	45029	2668
Transfected Cells Control	24118	104
Transfection Efficiency (%)	17.9%	3.9%

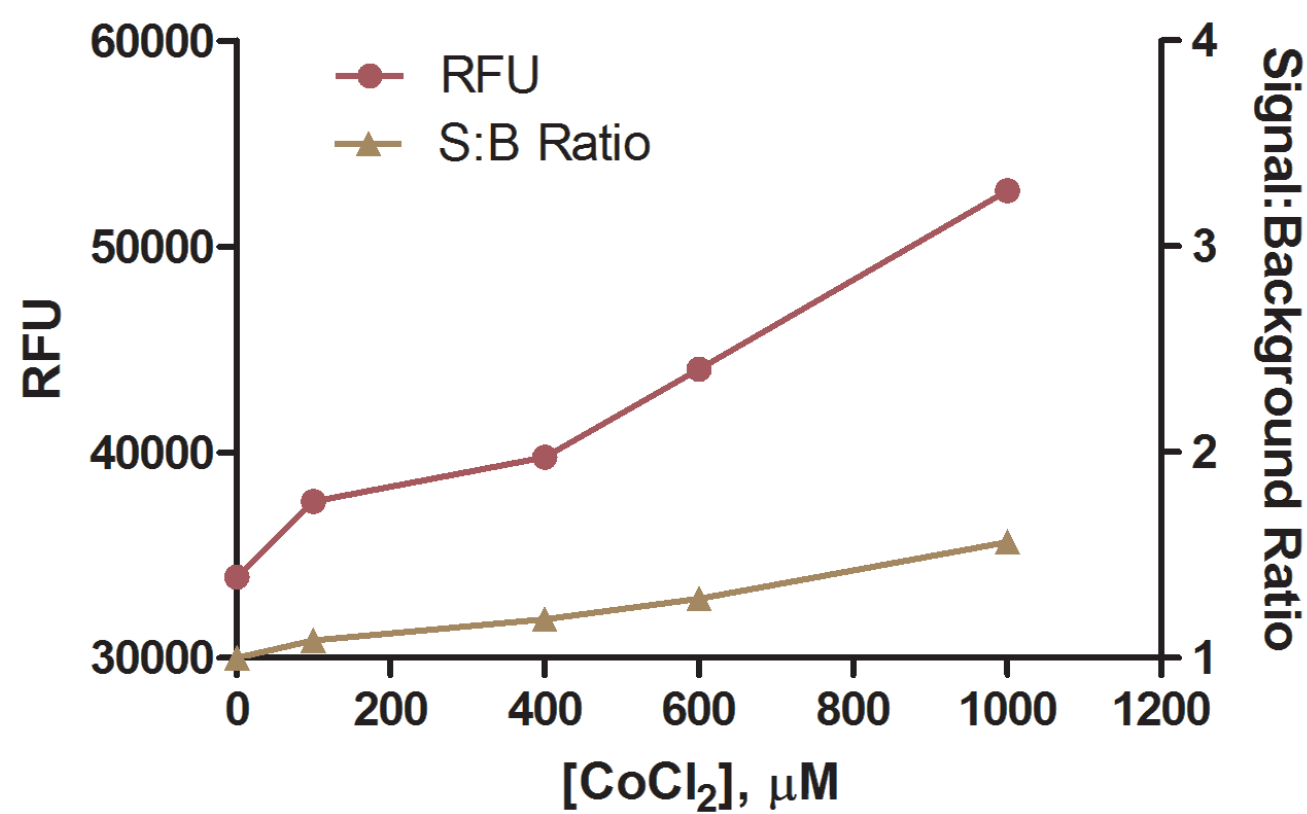
**Table 1. PMT- and Imaging-Based Transfection Efficiency Results.** Fluorescence values captured from whole well PMT-based microplate reads, in addition to cell counts performed with 4x images captured of positive, negative, and test wells.

Whole well fluorescence measurements result in a calculated 17.9% efficiency based on the blanked ratio of the experimental sample to the positive control. CCD-based image analysis cell counting identified 104 positive cells in the experimental well, while the positive well had a total of 2668 and 1 cell in the negative control resulting in an efficiency of 3.9%. Background issues with PMT-based measurements can limit effectiveness of this method. Autofluorescence from the microplate, cell media and untransfected cells is captured and recorded. CCD-based imaging along with image analysis capability allows for cell segmentation. Threshold fluorescence levels, which are used to identify true cells, can be applied to count discreet cells only, while ignoring background fluorescence, thus providing greater accuracy in determining transfection efficiency.

## Phenotypic Assay Analysis in Cells

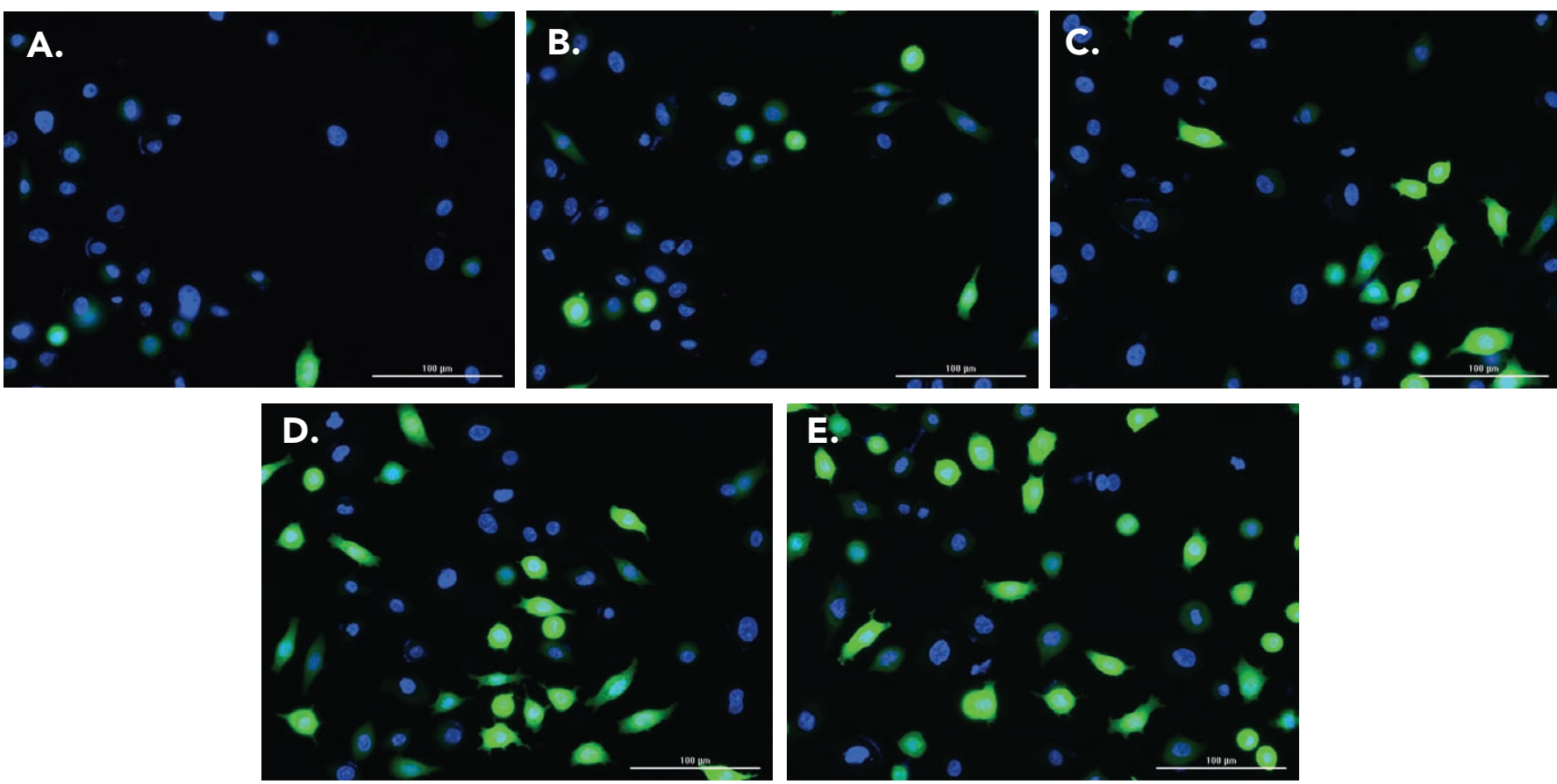
Fluorescence-based assays have been created to track a variety of different phenotypic responses following environmental or molecular-based challenges to a cell. While live cell assays have gained in popularity, many are still added following treatment, incubated with the cells, and then unincorporated dye is washed away using buffer. For many years the standard has been to perform this process and then use a microplate reader to detect the fluorescence from each well.

The Oxidative Stress Detection Reagent used in the following application represents such an assay. Primary keratinocytes were treated with CoCl<sub>2</sub> to induce stress, reagent was added and washed, followed by microplate reading.



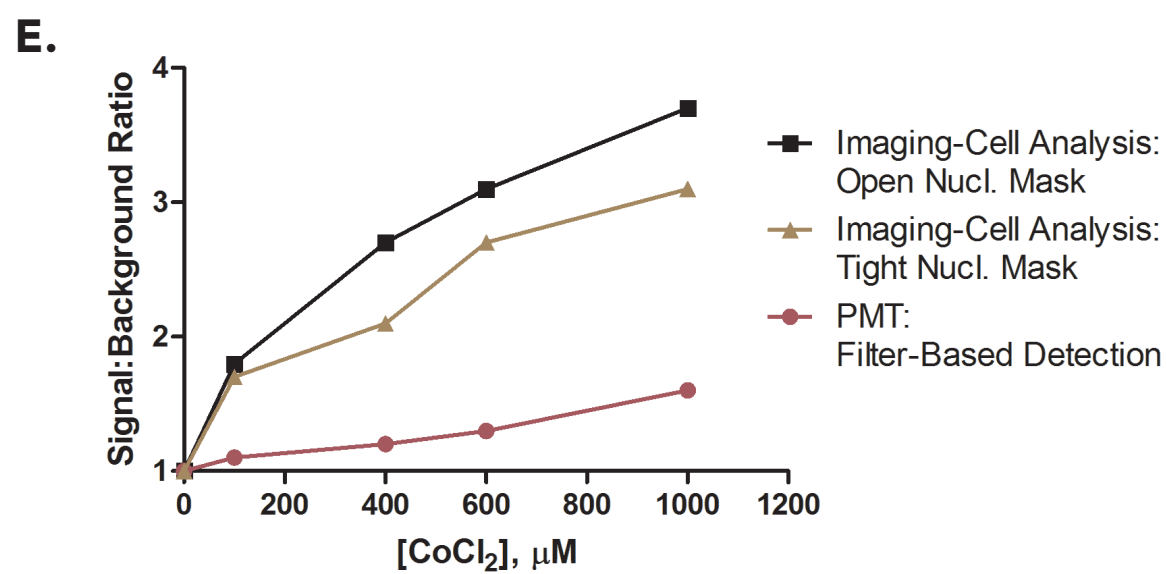
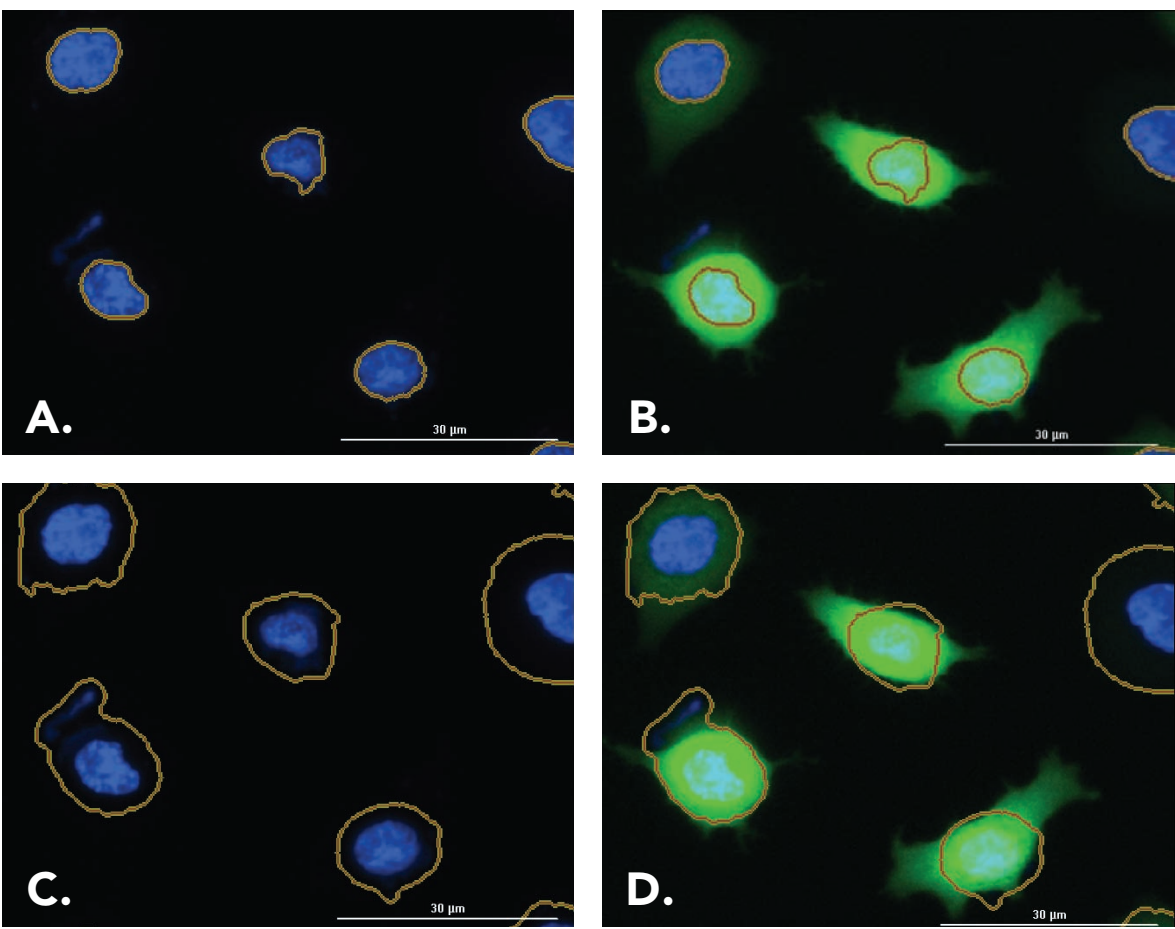
**Figure 2. PMT-Based Fluorescent Signal Detection Results.** Fluorescent signal captured from Oxidative Stress Reagent, part of Cyto-ID® Hypoxia/Oxidative Stress Detection Kit (Enzo Life Sciences, Farmingdale, NY). Raw fluorescence units (left Y-axis) and signal:background ratio (right Y-axis) exhibited.

Results from the PMT-based analysis illustrate that while a change in fluorescent signal is detected, the inherent background fluorescence from the reagent creates an almost undistinguishable change in signal: background ratio across all concentrations of CoCl<sub>2</sub> tested. Therefore a method is required which can eliminate background fluorescence from experimental analysis. Incorporation of digital microscopy, combined with cellular analysis provides such a means. As such, cellular imaging was also performed with each experimental well.



**Figure 3. Cellular Images of Fluorescently Stained Keratinocytes.** 20x overlaid images of keratinocytes treated with (A.) 0, (B.) 100, (C.) 400, (D.) 600, or (E.) 1000 µM CoCl<sub>2</sub>. Hoechst 33342 stained nuclei and Oxidative Stress Reagent visualized using DAPI and GFP imaging filter cubes, respectively.

Cellular analysis was then completed using the Hoechst 33342 stained nuclei captured with the DAPI channel (Figure 4A). The Gen5 data analysis software also reports fluorescent signals from other imaging channels detected within the object masks drawn using the primary channel (Figure 4B). As seen in Figure 3, the signal from the Oxidative Stress Reagent is found not only in the nucleus, but in all parts of the cell. Accordingly, a second analysis was performed using a lowered fluorescent threshold in the DAPI channel. The result is an “opened” mask drawn around the nucleus, and a greater incorporation of the fluorescent signal from the Oxidative Stress Reagent (Figure 4C and D).



**Figure 4. Cellular Analysis of Oxidative Stress Reagent Signal.** (A.) “Tight”, and (C.) “Open” nuclear masks drawn using fluorescence thresholds in the DAPI channel of 10,000 and 500 RFU, respectively. (E.) Signal:Background ratios generated with Oxidative Stress Reagent RFU values captured using PMT-Based detection, as well as from the signal incorporated using (B.) “Tight” and (D.) “Open” nuclear masks.

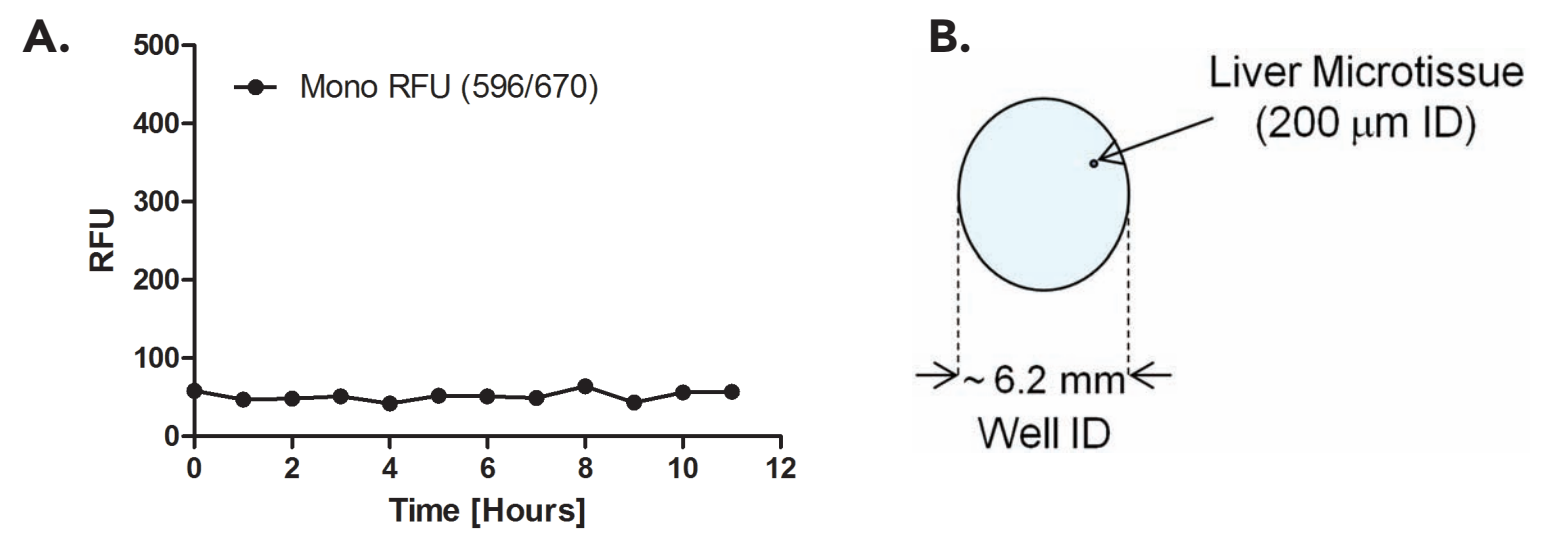
The results in Figure 4E illustrate how cellular analysis, allowing for the incorporation of signal emanating solely from inside a cell, can lower background fluorescence; allowing for larger, more easily detected phenotypic changes. Inclusion of lower threshold cutoff values further enhances the detectable change and helps create the most robust experimental analysis procedure.

## Live Cell Assay Analysis using a 3D Spheroid Model

The evolution into live cell formats provides valuable data that may be missed in a single time point, lytic assay. A downfall of this and other assay types is that cells grown on solid, flat substrates do not display the same morphology, and may not behave in the same manner, as those grown *in vivo* (Zang et al., 2012).

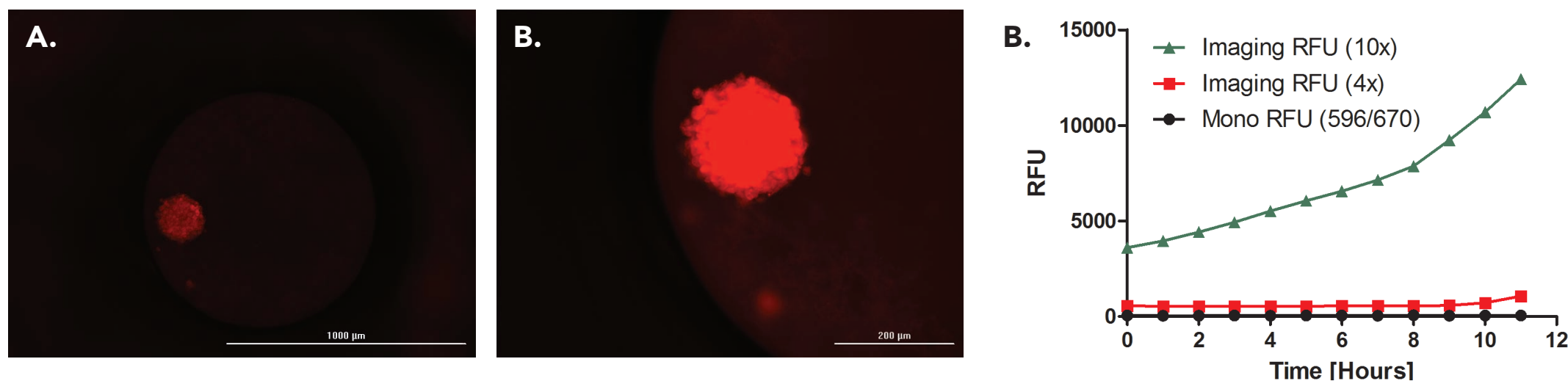
Unlike *ex vivo* 2D cultured cells, those grown *in vivo* interact with nearby cells and the extracellular matrix (ECM) to form complex communication networks that control a number of cellular processes (Bissel et al., 2002). New 3D culture methods encourage cells to aggregate into clusters, thereby forming vital communication networks, and more closely mimicking *in vivo* structures.

Here a live cell detection reagent was added to a 3D liver microtissue model, the spheroid exposed to a low oxygen environment for a total of 11 hours, and analysis of hypoxia induction carried out kinetically during that timeframe.



**Figure 5. Monochromator-Based Hypoxia Induction Analysis.** (A.) RFU values from monochromator-based microplate reads (Ex: 596 nm/Em: 670 nm). (B.) Diagram of relative spheroid size compared to size of normal 96-well microplate well.

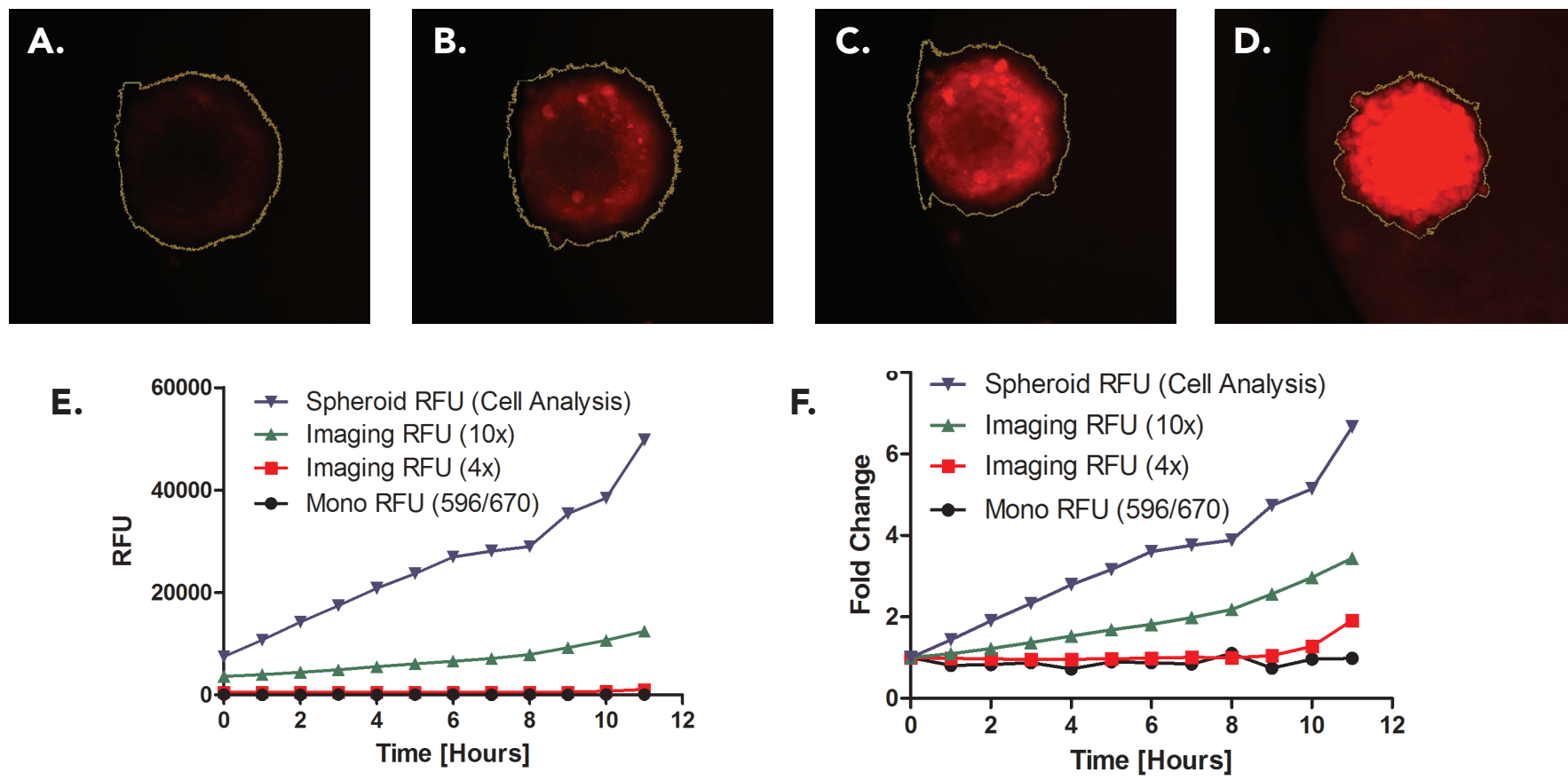
Figure 5A demonstrates that no significant change was seen in fluorescence values captured with monochromator-based detection over the 11 hour time period. The reason being that the area of a liver microtissue is approximately 1000X smaller than the area of the well of a 96-well microliter plate (Figure 5B). PMT-based optics are designed to capture as much light from the well as possible, thus for live cell microtissue analysis, these optics collect largely background as evident from the temporal response in Figure 5A. Cellular imaging, which provides a method to more closely focus solely on the spheroid, can overcome this problem.



**Figure 6. Analysis of Hypoxia Induction using Microscopy and Image Analysis.** Images captured of spheroid-based signal from Hypoxia Red Detection Reagent following 11-hour incubation period, using (A.) 4x, and (B.) 10x magnification. (C.) Graph of average fluorescent signal from 4x and 10x images, compared to signal measured using PMT-based detection.

From the mean fluorescence values calculated (Figure 6C) using 4x (Figure 6A) and 10x magnification (Figure 6B), it is evident that the combination of LED excitation and CCD-based quantification, as well as being able to focus on a smaller portion of the well containing the sample of interest, creates a more robust analysis of the change in signal from the hypoxia reagent. This is especially true when focusing on the results from the 10x image analysis, where a steady increase in fluorescent signal is seen at each time point.

Using Gen5, a Cellular Analysis was also performed using the 10x images captured. By setting appropriate parameters, the software is able to define a mask around the liver microtissue (Figure 7A-D), which allows signal quantification from the object as a whole.



**Figure 7. Cellular Analysis of Kinetic 10x Images.** Object masks drawn around spheroids following (A.) 0, (B.) 3, (C.) 6, and (D.) 11 hours of incubation. Raw fluorescence values (E.) and fold change calculated from captured signal (F.) plotted for each timepoint during kinetic analysis

When performing a fold change analysis using the fluorescence values calculated with all methods of signal quantification (Figure 7F), it can be observed that the cellular analysis method creates the largest change in fluorescent signal over time. Using this method, only the fluorescence within the drawn mask is calculated, while all other areas of the image consisting of background signal are ignored. Therefore cellular analysis represents the most robust manner in which to evaluate the results from this type of experiment.

## Conclusions

- PMT-based detection is optimal for assays where signal is emitted from bulk solution
  - Optics designed to collect as much light from the microplate well as possible
  - Good choice for cell-based assays incorporating a lysis step
- CCD-based detection is optimal for assays where signal is restricted to cells, especially for live cell applications
  - Magnification restricts field of view reducing background signal collection
  - Cellular Analysis capabilities of Gen5 restricts signal collection to cells and cellular structures such as spheroids
  - Together, imaging and Cellular Analysis can significantly improve signal: background ratios, and robustness for live cell, phenotypic assays