Meeting Ultra-High Throughput Imaging Challenges: • Low Resolution • 1536 Well Plates • Homogeneous Assays

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Introduction

Cell based assays using high content imaging are often favored for drug screening or toxicity testing due to their biological relevance and the ability to investigate multiple parameters even in heterogeneous populations. However, defining a streamlined, yet flexible, workflow for complex cell-based assays can be challenging. This presentation will demonstrate solutions for some of those challenges, such as scaling up to 1536-well plates, obtaining useful imaging data from homogeneous (no-wash) assays, and shortening time to results with a multi-colour cell toxicity assay.

Screening in 1536 well plates can often be time consuming, but with the large field-of-view enabled with the ImageXpress® Micro XLS System and improvements in MetaXpress® 5 High Content Image Acquisition and Analysis software, whole well images of an entire plate can be acquired at 3 wavelengths in 15 minutes and analysed using either the standard analysis software or accelerated with MetaXpress® PowerCore™ parallel processing software. This experiment demonstrates equivalent results in an antibody binding assay using beads, whether utilising standard imaging methods or a new ultra-high throughput workflow.

Obtaining useful data from homogeneous assay images can be difficult due to high background fluorescence in the media. We successfully transferred a homogeneous assay, traditionally used to identify hybridomas that are secreting monoclonal antibodies into the supernatant, onto two different imaging platforms. This robust assay can measure antibody in the low ng/mL range in 384 or 1536-well plates.

Identifying toxic compounds and instantly calculating IC_{50} estimates is possible by combining a new multi-colour cell toxicity kit with low magnification imaging. These experiments demonstrate the rapid image acquisition of the SpectraMax® MiniMax™ 300 Imaging Cytometer and simultaneous analysis with SoftMax Pro® software for easy generation of tables and graphs to dramatically shorten time to result in common toxicity assays. Results are compared to those obtained with ImageXpress Micro XLS High Content Imaging system.

Materials & Methods

Assays

- Microplates – 384 well black-walled (Corning P/N 3712); 1536 well black-walled (Greiner P/N788092)
- EarlyTox™ Cell Integrity Kit (Molecular Devices,) using HeLa cells
- Homogeneous Antibody Assay:
  - Microspheres – 7.4 µm colorless Goat anti-Mouse IgG coated (Spherotech P/N MPFc-60-5) and 3.5 µm Blue Goat anti-Human IgG coated (Spherotech P/N HPBFc-30-5)
  - Cells – non-adherent CHO cells
  - Analyte – hybridoma supernatant (in cell-based assay), human IgG (Jackson ImmunoResearch P/N 009-000-003), mouse IgG (Jackson ImmunoResearch P/N 015-000-0003)
  - Anti-species secondary antibodies labeled with AlexaFluor 488 or Texas Red

High Content Image Acquisition & Analysis

- ImageXpress® Micro XLS High Content System
- SpectraMax i3 Multi-Mode Detection Platform with MiniMax 300 Imaging Cytometer
- Images were analysed using either user-customized or standard algorithms from MetaXpress® or SoftMax Pro Software including the modules:
  - Cell Scoring – identify cells or area of cells within a well that are positive for 1, 2, or up to 7 wavelengths
  - Live Dead – classify cells as live or dead based on 2 separate cell markers

Reducing time-to-result in 1536 well plates

Many fluorescent bead or cell-based assays can be scaled down to a few microliters of volume, amenable to running in a 1536 well plate. Whole well images can be acquired in a 1536 well plate using 10X magnification but 4 wells at one time can be acquired using 4X magnification. This assay compares the amount of time required to achieve relevant results in an assay read with two different objectives and the data output when analysed with a standard cell scoring software module in MetaXpress software.

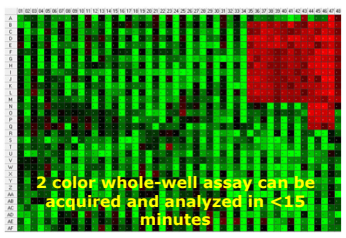
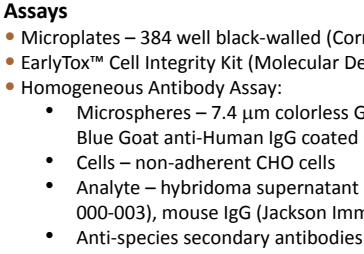
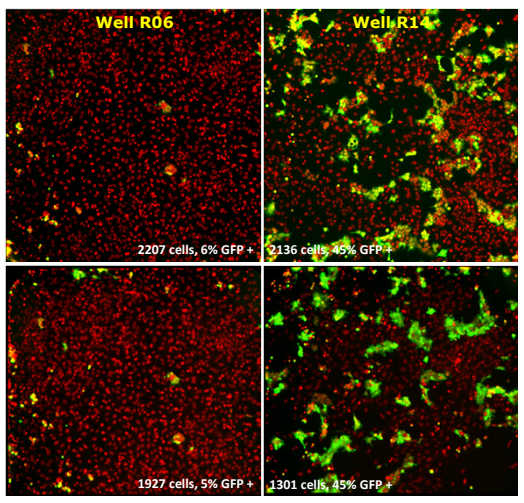


Figure 1. **Above.** Heat map of analyzed 1536 well plate showing most hits in top right quadrant of the plate. **Right**. Whole well images acquired with a 10X objective (Top) and 4X objective (bottom) zoomed to display cells in each image as similar in size. Total nuclei stained with DRAQ5 (red) were identified and the percentage of the subpopulation of cells expressing GFP (green) was calculated. **Bottom Graph.** Mean results and standard deviations from this ultra-high throughput assay were comparable whether the plate required 23 minutes (4X) or 93 minutes (10X) to acquire and analyse.



10X



4X

By acquiring the images at lower magnification, one field of view captures a sufficient number of cells to yield statistically relevant data for many cell-based assays. In addition, the acquisition time is reduced by nearly 4 times. A typical 2-3 wavelength assay read in 1536 wells can be completed in 15-25 minutes depending on staining intensity and required exposure times. In this 1536 well assay, the final well volume was 6 µL, allowing a dramatic decrease in reagent use.

Homogeneous Antibody Detection Assay

Two representative bead-based assay were developed which utilise commercially available polystyrene particles (beads). Two different populations of species-specific coated beads allowed multi-plexing within one well. A combination of secondary antibodies against human or mouse IgG, each labeled with a different fluorophore, could be added to each well. Figure 2 demonstrates how the beads can be distinguished.

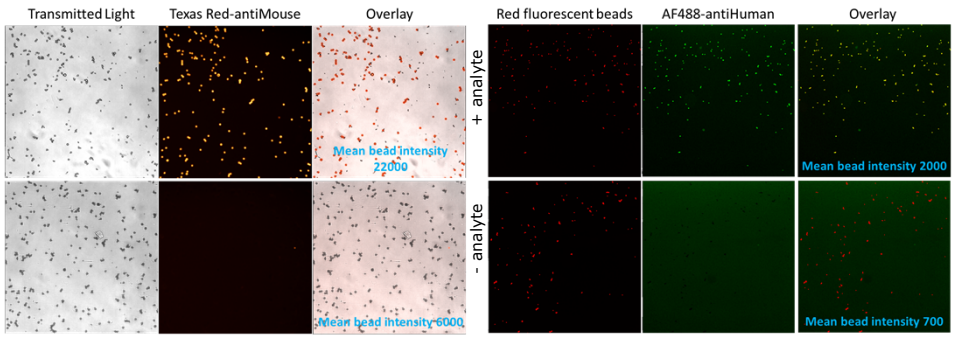


Figure 2. Colorless 7.5 um anti-mouse coated beads (left) and 3.5 um anti-human coated red beads (right) can be individually segmented. The secondary antibody signal associated with the bead surface is used to detect presence (top) or absence (bottom) of analyte in the well. Images shown on left were acquired from a 384 well plate and on right from a 1536 well plate with a 20X PF objective using ImageXpress® Micro XLS system

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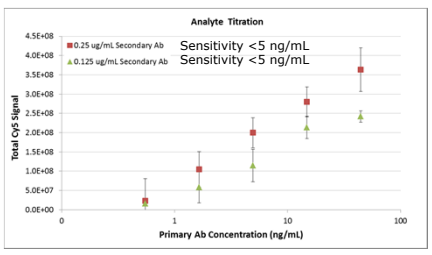
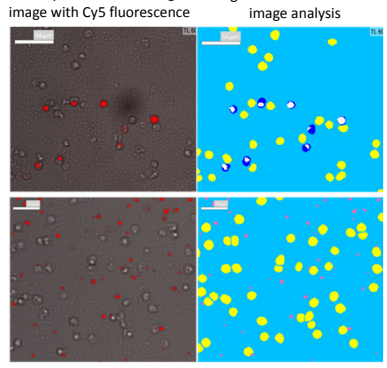
The same homogeneous assay was run on CHO suspension cells. If the cells had a receptor to the analyte, it bound and then was detected with a Cy5 labeled secondary antibody. With the customisable image analysis available in the MetaXpress Imaging software, several parameters were evaluated: total cells as identified in a transmitted light image, number of Cy5-positive cells, total signal from positive cells, number of negative cells, and overall Cy5 intensity throughout the image (to flag wells with many fluorescent aggregates).

Figure 3. **Top Images.** Cells that are positive for antibody binding show up brightly in the Cy5 channel. In the segmentation mask on the right:
Yellow = cells negative for Cy5 **Blue** = cells that are found in transmitted light AND contain Cy5 White = areas within a cell that are also red.
Bottom Images. All cells are negative for antibody binding. Although overall Cy5 signal is high, none is associated with a cell. In the segmentation mask :
Yellow = cells negative for Cy5 Pink = non-cellular debris positive for Cy5

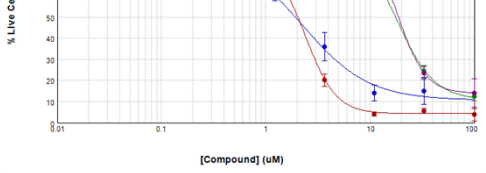




In this optimised cell-based homogeneous assay, plates were stable for many hours and the lower detection limit of the target antibody was 0.5 ng/mL.

Homogeneous Cell Toxicity Assay

The EarlyTox™ Cell Integrity Kit for conducting cytotoxicity assays contains the EarlyTox Live Red Dye and the EarlyTox Dead Green Dye. EarlyTox Live Red Dye is a cell permeable DNA-selective dye whose fluorescence is enhanced upon binding to DNA. EarlyTox Dead Green Dye is a cell impermeable dye for staining DNA in dead, fixed or apoptotic cells. This kit is useful for the rapid quantification of cell viability when used with the SpectraMax MiniMax 300 Imaging Cytometer or with other high content imaging systems. A cocktail of the two dyes is added to treated and untreated cells in equal volume to the media in the well and is ready to read at standard FITC and Cy5 wavelengths after a 60 minute 37°C incubation. No aspirate or wash step is required before or after staining. The results below demonstrate a dose response to compound dilutions in a 384 well plate read with 4X magnification with the SpectraMax MiniMax 300 Imaging Cytometer, or at 10X magnification with the ImageXpress Micro Widefield System.



When using the SpectraMax MiniMax 300 Imaging Cytometer, preconfigured algorithms in the SoftMax Pro software allow on-the-fly analysis of the toxicity assay and instant generation of graphs, complete with curve fitting and IC_{50} calculations.

Figure 4. **Top.** A no-wash live-dead assay measures cytotoxicity in 384 well plates read on a benchtop imaging cytometer (SpectraMax MiniMax 300) or a high content screening system (ImageXpress Micro XLS). Dose curves and data quality were very similar.
Bottom. Compound IC_{50}s are virtually identical whether the plate was imaged at 4X and analyzed using SoftMax Pro software or imaged at 10X and analyzed with MetaXpress High Content Analysis software.

Cytotoxic Compound	MiniMax 300 IC_{50}	ImageXpress Micro IC_{50}	Treatment
Staurosporine	2.4 µM	2.3 µM	Overnight @ 37°C
Mitomycin C	18.1 µM	18.6 µM	Overnight @ 37°C

Summary

- Ultra high throughput assays are possible even with cellular imaging by developing assays in 384 or 1536 well plates that require no wash steps.
- Whole well imaging can be done with the ImageXpress Micro XLS large field-of-view paired with low magnification to obtain a large sample size of cells and improve statistical relevance of results.
- With the new 2 color EarlyTox Cell Integrity assay, meaningful data can be obtained rapidly with low magnification settings using either the SpectraMax MiniMax 300 Imaging Cytometer or the ImageXpress Micro XLS High Content System.

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