High-Throughput Cellular Assays

Using the DropArray™ 'Well-Less' Plate Format with the Formulatrix Tempest

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Efficient high-throughput screening is a complex system of samples, chemistry, automation, and data analysis. Traditional high-throughput systems utilize 6- to 1536-well plates to support the large number of experimental conditions required to produce reliable and statistically significant studies. Current technologies require a substantial investment in samples and reagents and are limited in application based on the type of cells that are being studied.

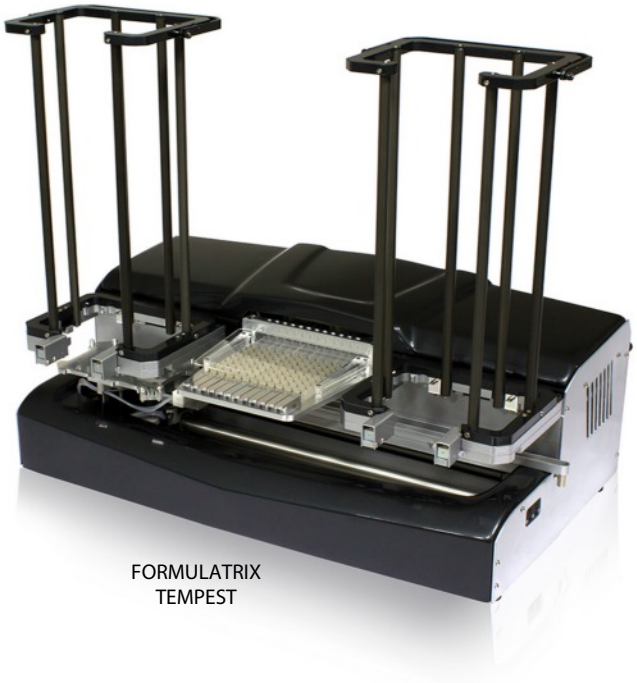
For example, adherent cells are typically required for studies with multi-step processing (e.g. multiple washes), while suspension cells are well-suited for homogenous assays where washing is not needed (e.g. Add, Mix, Read). Here we present a unique system for the automated processing of both adherent and suspension cells by using Curiox Biosystems' DropArray™ Plates in combination with non-contact, low-volume automated liquid dispensing using the Formulatrix Tempest.

About the Curiox Biosystems DropArray™ Microplate

The DropArray™ Microplate achieves "wall-less" drop separation by leveraging hydrophobic and hydrophilic properties on the planar surface of a plate. Hydrophilic islands arranged in a grid pattern on a hydrophobic substrate "focus" dispensed drops and maintain separation between samples. Once drops have been dispensed to the specially coated surface, immiscible Liquid Lid Sealing Fluid is added to the plate to prevent cross-contamination and evaporation. For more information, please see http://www.curiox.com/products.html.

About the Formulatrix Tempest

The Formulatrix Tempest is a non-contact, bulk reagent dispenser based on proprietary modular microfluidic chip technology. It is configurable to simultaneously deliver any volume of up to 12 ingredients through 96 individually controlled channels. The DropArray technology benefits from the X-Y spatial precision and the accurate and non-contact dispensing capabilities of the Tempest liquid dispenser. The Tempest's patented microfluidic valve clusters use positive displacement to dispense discrete volumes of liquid to 96-, 384-, or 1536-well plates with non-recoverable dead volumes as low as 50 µL per ingredient. The optional stackers and barcode reader add additional flexibility to the instrument.



FORMULATRIX
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METHODS AND MATERIALS

I. Cell Lines and Reagents

COS-7 (African Green Monkey Cercopithecus aethiops Fibroblast-like Kidney Cells) and human prostrate epithelial (hPRE) cell lines were purchased from the American Type Culture Collection (ATCC) and maintained in culture according to ATCC recommendations. Cytotoxic T (CD8+ T) cells were purified from human Peripheral Blood Mononuclear Cells (PBMCs) isolated from peripheral blood of random healthy donors via the use of a CD8+ T-cell isolation kit (130-094-156; Miltenyi Biotec).

Antibodies and stains used were as follows: Monoclonal antibody APC Mouse Anti-Human CD8 (555369; BD Biosciences), Hoechst 33342 nucleic acid stain (H-3570; Molecular Probes), Phalloidin-Tetramethylrhodamine B isothiocyanate (P1951; Sigma-Aldrich), ZO-1 Rabbit Polyclonal Antibody (61-7300; Invitrogen) and Alexa Fluor ® 488 Goat Anti-Human IgG (H+L) Antibody (A-11013; Invitrogen).

Anti-CD3/CD19 bispecific antibody was produced by Chinese hamster ovary cells and purified from cell culture supernatant as previously described (1). The endocytic inhibitor Dynasore hydrate (D7693; Sigma-Aldrich) was used for live imaging applications.

The following cell viability assays reagents were used: CellTiter-Glo® Luminescent Cell Viability Assay (G7570; Promega), CellTracker™ Green CMFDA (5-Chloromethylfluorescein Diacetate) (C7025; CMFDA; Molecular Probes), ethidium bromide solution (EB; E1510; Sigma-Aldrich), Acridine Orange hydrochloride hydrate fluorescent stain (AO; 318337; Sigma-Aldrich), and Propidium iodide counterstain (PI; P3566; Molecular Probes).

II. Cell Transfections

COS-7 cells were transfected with complementary DNA (cDNA) by using FuGENE® 6 Transfection Reagent (E2691; Promega) according to the manufacturer's standard protocol at a ratio of 6 µL of Fugene 6 per 1 µg of DNA diluted in OPTI-MEM I

CURIOX DROPARRAY
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(31985-070; Invitrogen). Cells were grown for 48 hours before staining.

III. Cell Viability Assays

Cells were treated for 72 hours with different concentrations of Monomethyl auristatin E (MMAE), a synthetic antineoplastic agent, before performing the specific cell viability assay. For the CellTiter-Glo® assays, 2 µL drops of CellTiter-Glo® were added to the 2 µL drops of cells on the DropArray™ plate. The plate was read on an EnVision 2102 Multilabel Plate Reader (Perkin Elmer) after incubation for five minutes at room temperature.

For fluorescence-based assays, cells were labeled with the CellTracker™ Green CMFDA with the addition of MMAE. The drops were washed to remove dead cells and cellular debris before the plate was imaged with an IN CELL Analyzer 2000 (GE Healthcare Life Sciences) and analyzed by using the IN CELL Developer Toolbox v1.9 image analysis software.

IV. Immunofluorescence Staining

Cells were fixed with 4% paraformaldehyde for 20 minutes, washed with 1X phosphate-buffered saline (PBS) and blocked for 30 minutes with PBS/1% Bovine Serum Albumin (BSA).

The cells were incubated with Fc-tagged bait proteins or human-specific antibodies for 1 hour in PBS/1% BSA, washed with 1X PBS and incubated with appropriate fluorescently-labeled secondary antibodies at room temperature for 45 minutes, then washed with 1X PBS and imaged.

V. Automation Equipment

In addition to the Curiox Biosystems plate washer, the complete automated system to process the Curiox Biosystems DropArray™ plates includes the Overlord3 scheduling software controlling a Formulatrix Tempest, a Dynamic Devices Oasis and a Peak Analysis & Automation KiNEDx robot. This system processes adherent and suspension cells through various immunochemistry, transfections and cell viability assays with reduced reaction volumes while producing robust data.

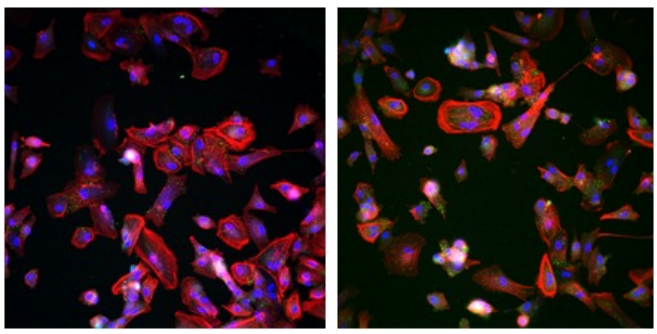

Figure 1. Comparable results staining hRPE cells stained with Hoechst, ZO-1, and Phalloidin and processed in "Well-less" DropArray™ plates and standard 384-well plates. 20X objective, scale bar = 70µm.

RESULTS

Traditional 384-Well Plate vs. DropArray™ Plate

The DropArray™ well-less plate enables low-volume adherent and suspension cell culture in a 384-well format. Incubation oil covering the 2 µL drops of cell culture medium eliminates evaporation and enables long-term cell culture conditions (Figure 1).

Cell Viability Assays Using the DropArray™ Technology with Suspension and Adherent Cells

Several cell viability assays were tested using the Tempest and DropArray™ technology. Reducing the reaction volume to a total of 4 µL (2 µL cells and 2 µL CellTiter-Glo®) instead of 40 µl (20 µL cells and 20 µL CellTiter-Glo®) with standard 384-ul plates resulted in a similar calculated half maximal inhibitory concentration (IC50) compared to fluorescent live cell staining with CellTracker™ Green CMFDA (Figure 2). These results show that the low-volume culture and assay conditions produce similar results

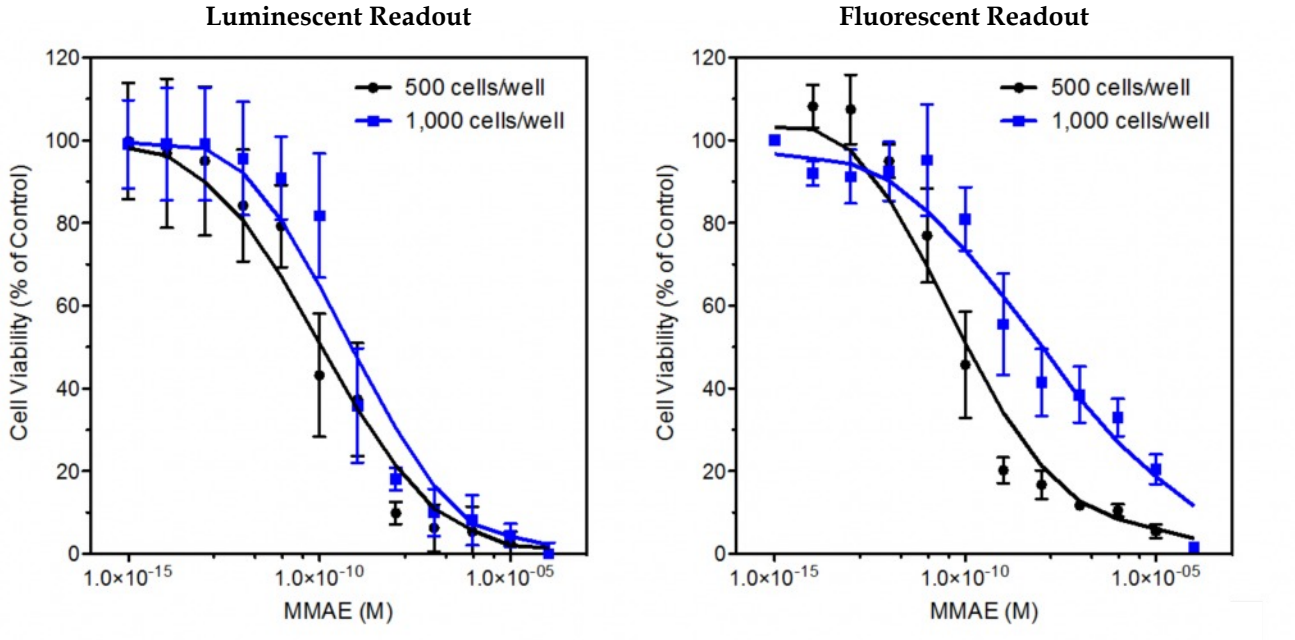

Figure 2. Similar IC_{50} curves produced with washing the cells (fluorescence detection) and without washing the cells (luminescent detection).

compared to traditional plate-based methods requiring larger volumes of reagents.

Cell-Surface Protein-Protein Interaction Investigations via the DropArray™ versus Microtiter Plate

Performance of the DropArray™ plate in a multi-step cell-based assay procedure was analyzed with an expression cloning experiment to investigate protein-protein interactions of IgLON family members. Using optimized washing conditions (2), the DropArray™ technology was compared with conventional 384-well plates using a regular automated washing procedure. COS-7 cells (adherent), Human Embryonic Kidney 293T (HEK-293T) cells (semi-adherent), and HEK-293S cells (suspension-adapted) were transiently transfected with limbic system-associated membrane protein (LSAMP), human neurotrophin (hNT), or opioid binding protein/cell adhesion molecule-like (OPCML) constructs.

Cells expressing LSAMP, hNT, or OPCML were then incubated with bait protein. NEGR1-hFc binding was detected by using Alexa Fluor ® 488 Goat Anti-Human IgG (H+L) Antibody. A 5-fold increase in mean intensity in response to the specific binding of NEGR1-hFc to LSAMP, hNT or OPCML was observed compared with the non-specific binding in non-transfected cells on either plate format (Figure 3B).

NEGR1-Fc binding to LSAMP-, hNT-, or OPCML-transfected HEK293T or S cells was not detected in the 384-well plate because these semi-adherent and suspension-adapted cells were removed during the washing procedure (Figure 3B). However, processing cells with the DropArray™ plates and the optimized

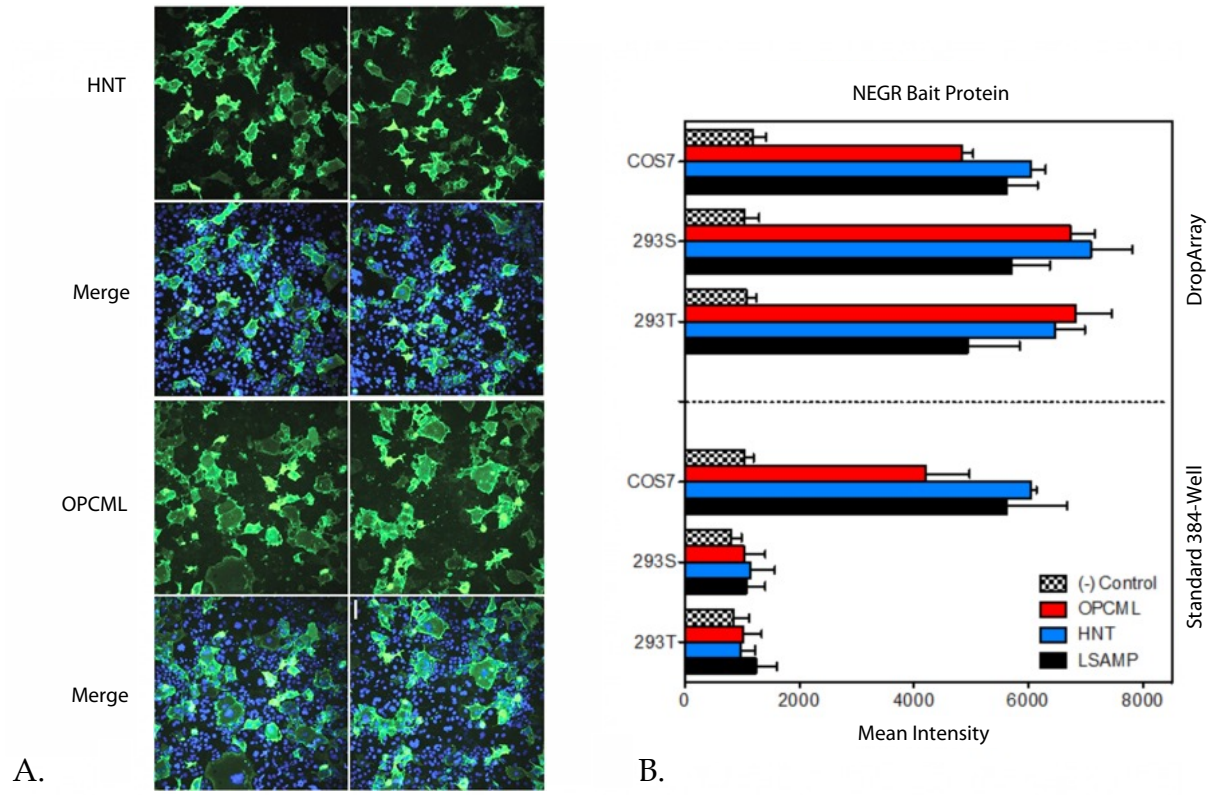

Figure 3. Cell-based extracellular protein-protein interactions. (A) COS-7 cells transfected with hNT or OPCML expression constructs were fixed and incubated with NEGR1-hFc bait protein. The cells were washed and stained with anti-human AF488 (green) and scanned with an IN Cell Analyzer 2000. The same reagents were used for transfection and staining in a standard 384-well microtiter plate and a DropArray™ plate for a direct comparison. Similar cell density was measured in both plate formats by using Hoechst staining (blue) of the nuclei. Scale bar = 70 µm. (B) The mean intensity of the green channel from the acquired images was quantified. Similar intensity values were observed when NEGR1-hFc binds to the three different cell types expressing the three different expression constructs of the NEGR1 binding partners. Similar values were generated from the adherent cell line (COS7) cultured in standard 384-well format but the weakly adherent (293T) and suspension adapted (293S) cell lines were removed during the washing procedure in the standard 384-well plate format.

washing conditions resulted in NEGR1-Fc binding to LSAMP-, HNThNT-, or OPCML-transfected HEK-293T or HEK-293S cells similar the COS-7 cells.

Volume Difference per 40 Plate Run of 384-Well Plates versus DropArray™ Plates

The DropArray™ plate and Tempest liquid dispenser system resulted in a 10-fold reduction for most reagents used in the transfection experiments (Table 1), enabling a significant reduction in reagent volumes and a substantial cost savings per sample. The reduced cost per sample makes the assays less expensive to complete and increases accessibility based on the total cost for a given study.

	Standard 384-well system	DropArray™-Tempest system
Transfection Reagent	1980 µL	198 µL
Bait Protein	2.2 mg	220 µg
Detection Antibody	1.0 mL	100 µL
Nuclear Stain	800 mL	80 µL
Cell Viability Reagent	7680 mL	768 µL

Table 1. Reduced reagent usage with the DropArray™/Tempest system. Using the DropArray™ plates with the Tempest liquid dispenser reduced the required transfection reagents by 90%.

Conclusions

The challenge of high-throughput screening is to efficiently gather sufficient reliable data with the least cost and effort. Here we present novel instrumentation and labware which reduces the cost and work required to process adherent and non-adherent cultured cells with homogenous assays, immuno-fluorescent assays, and transfections. The data gathered by utilizing the Formulatrix Tempest and the Curiox Biosystems' DropArray™ Plates compares favorably to similar data obtained using standard 384-well plates, but with substantial reagent and therefore cost savings. The integrated automation platform includes the Overlord3 scheduling software controlling a Formulatrix Tempest, a Dynamic Devices Oasis and a PAA KiNEDx robot. This platform provides a robust and reliable system to perform cell-based assays with adherent, semi-adherent and suspension cells with a substantial reduction in reagents while maintaining the quality of results obtained with traditional high-throughput screening plate formats.

References

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2. Quiñones G, Moore T, Nicholes K, Lee H, Kim S, Sun L, Jeon NL, & Stephan JP. (2012), "Application of a new wall-less plate technology to complex multi-step cell-based investigations using suspension cells." *Blood*. **121**:e25-e33.