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Use of the CellCard™ System for Analyzing Multiple Cell Types in Parallel

Oren Beske, Daniel Bassoni, and Simon Goldbard

Summary

The CellCard™ system enables the analysis of multiple cell types within a single microtiter well. In doing so, the CellCard system not only determines the effect of an experimental condition on a cell type of interest, but also the relative selectivity of that response across nine other cell types. In addition, this approach of cellular multiplexing is a means of miniaturization without the necessity of microfluidic devices. The standard 96-well plate generates ten 96-well plates of data (or, the equivalent of a 960-well plate). Taken together, the CellCard technology enables multiple cell types to be assayed within a single microtiter well allowing for the simultaneous determination of cellular activity and compound selectivity. This chapter will describe a method by which multiple cell types can be simultaneously assayed for biological parameters of interest.

Key Words: Cell-based assay; CellCard carrier; selectivity.

1. Introduction

The introduction and adoption of "high content" assays has spurred a desire for technologies and assays that provide increasing amounts of biologically relevant data from a single assay reaction, typically from a microtiter well *(1–3)*. Automated high-resolution microscopy combined with sophisticated image analysis algorithms have gone a long way in providing the tools enabling this data to be accessible to the drug discovery industry. For example, the ability of cells to reduce a tetrazolium salt (e.g., MTT) has widely been used as a surrogate for cellular health *(4)*. However, there are many phenotypes and/or mechanisms of toxicity that can be missed or underestimated by this type of an assay. An ability to quantitatively assess the multiple cellular responses associated with toxicity enables the measurement of unique and/or subtle phenotypes. One high content approach in measuring cellular toxicity is to stain cells with a nuclear dye, a mitochondrial dye, and a lysosomal dye. When imaged and quantified these dyes can provide information regarding cell number, nuclear condensation and/or fragmentation, mitochondrial mass, mitochondrial function, lysosomal mass, and lysosomal pH and other relevant parameters. Clearly this approach provides a more comprehensive understanding of the nature of the cellular response to a potential toxin.

This assay strategy has enabled drug discovery scientists to better understand the effects of a potential therapeutic on cells *(5–8)*. Indeed, the main goal of these assay strategies and technologies is to make the drug discovery process more efficient (and informative) thereby reduce the large failure rate currently being experienced with investigational new drugs. It has been postulated that a reason for this high failure rate is the lack of compound selectivity. This selectivity can be viewed as cross reactivity with similar targets, unrelated targets, or other cell types. These

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off-target effects can lead to a lack of efficacy and/or undesired side effects. The CellCard™ System *(9–11)* was developed to provide a means by which the selectivity of an experimental condition (e.g., treatment with a small molecule) can be robustly and routinely determined by assaying multiple targets and/or cell types within a single microtiter well.

The CellCard System is a microparticle-based technology optimized for cell-based assays *(8)*. These particles, termed CellCard carriers, are designed to incorporate a colored barcode that allows different classes of carriers to be uniquely identified. Therefore, when different cell types are associated (e.g., grown on) with the CellCard carriers, the cell types become uniquely identifiable by virtue of the carrier code. Once the assay is performed and read with the CellCard reader, the cellular data is extracted, associated with the code, and returned to the researcher. It then follows that if multiple cell types are associated with uniquely coded carriers, then the data retrieved will provide insight into the cell type selectivity of the treatment. In addition, because the data is obtained from a single well the cellular data is unequivocally comparable. The selectivity observed is not subject to common sources of assay variation that is experienced with sequential approaches to selectivity determination. When multiple cell types are assayed sequentially, well-to-well or, more importantly, week-to-week variation in reagent stability and/or cellular physiology can lead to a false representation of the selectivity observed. However, obtaining these data points from a single reaction in a single microtiter well will remove these sources of variation.

In this chapter, we describe how the CellCard System can be used to profile the cell type selectivity of antiproliferative compounds. This is illustrated with an example in which 10 cell types were treated with antiproliferative compounds in an eight-point dose–response of in triplicate. A simple fluorescent assay designed to quantify the confluence of the cells (a marker of general cellular toxicity) was performed. In doing so, 40 triplicate dose–response curves were generated in a single 96-well microtiter plate.

2. Materials

1. *CellCard System*: the CellCard System (Vitra Bioscience Inc., Mountain View, CA) is a multi-component, commercially available platform. The system consists of tissue culture consumables, CellCard Carriers, CellCard Dispenser, CellCard Reader, and associated software.
2. *Cells*: all cells were provided by a development partner under confidentiality and are herein anonymized. The cells were grown adherently in 10-cm dishes using standard tissue culture techniques. Briefly, the cells were split using trypsin-EDTA (Invitrogen, Carlsbad, CA) and maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), and an antibiotic/antimycotic (Invitrogen) in a humidified 5% CO_2 incubator at 37°C.
3. *Assay reagents*: the cells were stained with 2 µg/mL Hoechst (Invitrogen) in phenol red free, serum free RPMI. The potentially tested anticancer therapeutics was provided by a development partner under confidentiality and is herein anonymized.

3. Methods

3.1. Experiment Design and Plate Layout

A core software component of the CellCard system is the Experiment Manager application. This application provides an interface to design and manage all aspects of the multiplexed experiment to be performed. It stores information (i.e., fluorochromes, analysis parameters, number of plates, and so on) about the assay being run in order to appropriately configure the CellCard Dispenser and Reader. In addition, the software allows the user to log cellular and compound information (i.e., lot number, concentration), that are useful for downstream data analysis can also be managed with this software tool.

3.2. CellCard Preparation

Preparation of a CellCard CellPlex experiment requires standard tissue culture steps in which cells are seeded into wells previously loaded with CellCard carriers (*see* **Note 1**). The carriers

are laid onto CellCard ladles, preinserted into wells of standard six-well tissue culture plates, which are designed to facilitate the subsequent mixing of CellCard carrier codes.

Users of the System must first determine an appropriate number of six well plates and CellCard ladles needed for an experiment during the initial experiment design. The Experiment Manager software contains a "ladle calculator" function to determine the total number of ladles, thus determining the number of CellCard carriers that will be required for the experiment. By inference, the number of ladles needed for the experiment will determine the amount of CellCard carriers that will be needed; carriers are packaged in vials containing a measured amount of one class (code) of carriers that will be transferred to a ladle. However, as a rule of thumb, 10 vials of CellCard carriers and 10 CellCard ladles are required for each 96-well assay plate. Therefore, if 10 cell types are to be assayed, a single vial of 10 separate carrier codes is sufficient; if five cell types are used in the CellPlex experiment, then two vials each of five carrier codes are required per 96-well plate.

Once the CellCard carrier and ladle requirements are established, the user is ready to begin the experiment. The disposable CellCard ladles are first placed in the wells of dry six-well tissue culture plates. Then 2.5 mL of complete media is added to each ladle-containing well. The ladles are now ready for the deposition of the carriers.

One vial of carriers will be deposited onto each ladle (*see* **Note 2**). It is impractical to transfer dry carriers to the ladles. Therefore, carrier transfer is facilitated by adding 1 mL of the complete media to the CellCard carrier vials. A sterile, disposable transfer pipet is used to aspirate the carriers out of the vial and then deposit them onto the ladles by simply touching the tip of the transfer pipette to the media above the ladles. Slight positive pressure and capillary action will result in the majority of the carriers falling out of the pipet and onto the ladle. Gentle "washing" of media through the pipet will remove carriers remaining in the pipette tip. The carriers are dispersed into a monolayer on the ladles by mounting the six-well plates on the CellCard Disperser and shaking for 45 s at 110 rpm. Dispersing the carriers is a critical step to ensure that a maximum number of carriers will be exposed to the cells that will be added to the wells.

3.3. Tissue Culture

The cell lines to be multiplexed are maintained using standard tissue culture protocols in the appropriate media. Cells are removed from plates; pellet in conical centrifuge tubes, resuspended and accurately counted before plating them on the CellCard carriers. Typically, $1–5 \times 10^5$ cells are seeded into the wells containing the carriers. The actual number is dependent on cell doubling time, total incubation time of cells on carriers, and desired final cell confluence. A range of seeding densities should be tested to ensure the proper confluence of cells for the particular assay that is to be performed. The cells should be allowed to adhere (under standard incubation conditions) for an appropriate amount of time to ensure robust adhesion to the particles. The rate of adhesion and cell spreading is cell-type specific and can range from 5 to 18 h. We recommend allowing the cells to adhere, spread, and initiate their cell cycle during an overnight incubation.

3.4. CellCard Carrier Mixing and Dispensing Into 96-Well Format

After the cells have adhered to the carriers, they are ready for mixing (*see* **Note 3**). This is a simple process that is started by attaching a purpose-built funnel to a standard 15-mL conical tube. The funnel-tube apparatus is filled with approx 55 mL media and the carriers are transferred to the funnel by simply gripping the peg-shaped handles of the CellCard ladles with forceps, lifting the ladles out of their wells, submerging the ladles in the funnel and allowing the CellCard carriers to fall off the ladle into the conical tube. Up to 40 ladles (or vials) worth of carriers, enough for four 96-well assay plates can be transferred to one conical tube.

After all carriers are transferred to the 15 mL tube, the excess media from the funnel is aspirated leaving the 15 mL tube full to its brim. The funnel is detached from the tube and capped. The carriers are then thoroughly mixed with a single 360° inversion of the conical tube. After mixing, the media is then aspirated from the tube down to the top of the stack of carriers to remove cells that might have been loosened from the carriers by the mixing process. The tube is refilled with fresh complete media to the 6 mL mark. The CellCard carriers are now ready for dispensing into assay plates.

CellCard carrier dispensing is accomplished by a liquid handling robot, called the CellCard Dispenser, designed to transfer CellCard carriers to 96-well assay plates. The Dispenser includes custom built pipet tips that are specially designed to aspirate a metered amount of carriers to be deposited into the 96-well assay plates. A dispense run is set up by mounting the 15-mL tube containing the CellCard carriers and the appropriate number of 96-well plates onto the Dispenser. A 50-mL tube containing a reservoir of media is also mounted on the Dispenser. The start cycle of the Dispenser requires the user to adjust the starting height of the dispensing tips through the software controller interface. From there, the Dispenser will automatically transfer carriers from the 15-mL conical tube to the assay plates. On completion of carrier dispensing, the carriers are then dispersed into a monolayer via the CellCard Disperser.

3.5. Compound Addition

Excess media is removed from each assay well with a wand aspirator or a plate washer to ensure that the same residual volume is present in all wells. We recommend leaving 50 µL of residual media in each well to minimize disturbance of the CellCard carriers while adding compound. The compound to be added is made up at 2X (twice the desired final) concentration, using the appropriate media, and an equal volume (50 µL in this case) of the 2X solution to each well to create a 1X final assay concentration. After the compounds have been added to each of the wells return the plates to the cell culture incubator for the appropriate incubation time. This incubation time is assay dependent and can range from 30 min for a signaling (i.e., nuclear translocation) assay to multiple days for a proliferation assay.

3.6. Assay Staining Protocols

In general, only minor alterations in standard assay protocols needs to be made when using the CellCard System. Generally, staining reagent concentrations and incubation times will be the same for assays run with or without CellCard carriers. For the Calcein AM/Hoechst assay presented here, the assay plate wells are first aspirated to 30 µL. The staining solution is made by mixing 4.5 µL of Calcein AM (4 m*M* stock) and 8 µL Hoechst (1 mg/mL stock) in 8 mL phenol red-free RPMI 1640. Add 75 µL of the staining solution to each assay well. Incubate the plate for 30 min at 37°C, 5% CO_2. At the completion of incubation, aspirate the wells to 50 µL. Top off the wells with approx 300 µL phenol red-free RPMI 1640 (*see* **Note 4**).

3.7. CellCard Reading

Before scanning the plates with the CellCard Reader, the appropriate plate description file (generated in the Experiment Manager application) must be loaded. This will configure the Reader to address the appropriate wells and acquire images with the appropriate assay specific fluorochromes.

Some preparation of the assay plates will also be necessary. The nature of the carrier recognition algorithm in the Image Analyzer software necessitates a noncolored solution in the assay wells. Any colored solutions must be washed out of the wells with solution such as 1X PBS (for fixed assays) or phenol red-free media (for live assays). In order to eliminate any shadowing within the bright field images, which can affect carrier code recognition, the wells must be completely topped off with the appropriate solution such that any meniscus is eliminated from each well.

After the microtiter plate has been placed in the Reader (either manually or through a robot) the image acquisition parameters must be set. The parameters for acquiring the bright field images are automatically set by the reader without user intervention. This will ensure that the colored bands of the CellCard codes will have the hues required by the Image Analysis software for robust code recognition. The parameters for fluorescent image acquisition are adjusted by the user. A detailed protocol for this is provided in the User's Guide. Briefly, exposure, gain, and offset parameters are set to produce high signal, low background images that can be robustly processed by the System's image analysis algorithms to provide the biological data. Once the acquisition parameters are set, the plate is scanned by the Reader and the images are saved to an appropriate file directory designated during the design of the experiment in Experiment Manager. Acquisition parameters are saved by the Reader software to alleviate any need to set exposure parameters for the remaining assay plates of the experiment.

3.8. Image Analysis and Data Visualization

To extract the cell type specific data, the images acquired by the CellCard Reader must be processed by a series of image analysis algorithms. The software will read in assay specific default image analysis parameters, including minimum pixel intensity thresholds, before proceeding with the analysis. The Image Analyzer software will allow the user to examine the effect of the threshold parameters on the analysis of the images. If necessary, the user can adjust these thresholds to ensure that the image analysis algorithms accurately extract biologically relevant data apparent in the images. We recommend that this threshold analysis be run on positive and/or negative control wells, with known and/or expected biological responses, before analyzing the entire experiment. The CellCard System User's Guide provides a detailed description of how to set proper image analysis parameters. Once appropriate analysis parameters are set, the image analysis of the entire experiment, typically a set of plates with the same assay, can be performed. The Image Analyzer will automatically analyze every well of every plate in the experiment and generate a fully annotated data table.

The data tables generated by the Image Analyzer software are saved as tab-delimited flat files. The files contain data generated from a variety of measures, including measures of stained area, spots, intensity of stains, and the like. These data files can then be opened and visualized in the Vitra Bioscience Data Analyzer software or other standard data visualization applications (i.e., Spotfire). The flat file structure also allows the data to be parsed and uploaded into an enterprise database for further analysis and storage.

3.9. Example Data

In this example 10 cell types were maintained using standard tissue culture protocols and seeded onto CellCard carriers as described earlier. The cells were then exposed to a dose–response of a small molecule compound, a known toxin and potential anti-cancer therapeutic, for 2 h. The compound was subsequently removed. The wells were then washed and fresh media (without the compound) was added to the wells for a 48 h chase period. At the end of this period the cells were stained with the nuclear dye and subsequently scanned and analyzed using the CellCard System (**Fig. 1**).

Compound A is known to be toxic and, therefore, was predicted to induce a dose-dependent decrease in the signal generated by the nuclear dye. Indeed that was the case. As shown in **Fig. 3B**, the relative number of cells decreased as the dose of the compound was increased. Interestingly, the 10 cell types partitioned into three classes of response profiles. The class-1 response is described as a gradual dose–response curve wherein cells begin to respond to the compound at a relatively low dose. This response gradually increases as a function of dose but does not reach a maximal response within the dose range tested (no right side plateau). The classes-2 and -3 responses, characterizing three and five cell types, respectively, result in sigmoid shaped curves

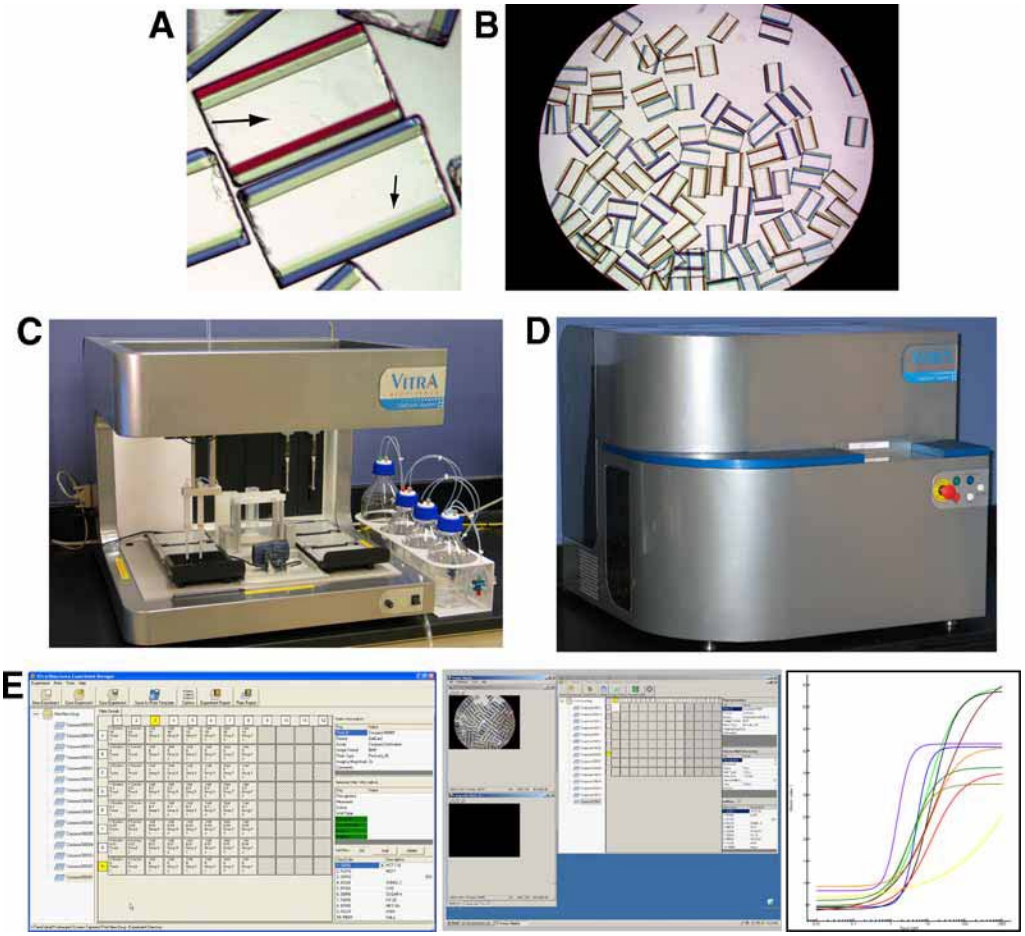

Fig. 1. System overview. **(A)** Image of CellCard carriers showing the coding bands (small arrow) and optically clear data read-out (big arrow) sections. **(B)** An image of a single well from a 96-well microtiter showing approx 100 CellCard carriers dispersed throughout the well. **(C)** The CellCard dispenser. It is designed to transfer approx 100 CellCards from a 15-mL conical tube into the wells of the microtiter assay plate. **(D)** The CellCard Reader. A CCD camera based reading device that acquires all the requisite bright field and fluorescent images to both decode the carrier codes as well as extract biological data. **(E)** A subset of the CellCard System suite of software. This figure shows screen shots from the Experiment Manager, CellCard Reader, and Data Analyzer software applications.

with plateaus on the low high dose ranges connected by a steep transition area. That is, at low doses there is no response of these cells to the compound, but, once a critical concentration is used, it does not require a large dose increase of the compound to elicit a maximal response (the high dose plateau). Although the mechanisms behind these different responses are not understood, by using the CellCard System multiple sources of assay artifact can be removed as potential explanations. For example, if the 10 cell types were assayed sequentially over a period of days to weeks, compound stability issues with multiple freeze-thaw cycles may result in the different cellular responses observed. By implementing the CellCard System, each of the cell

Fig. 2. *(Opposite page)* Schematic of the CellPlex assay workflow. CellCard carriers are first placed into 6-well format and cell types seeded onto the carriers. After mixing the carriers and attached cells they are dispensed into the 96-well microtiter plate, subjected to the experimental treatment of interest, and stained for the biological parameters of interest. Finally, the plates are imaged, analyzed, and the data plotted.

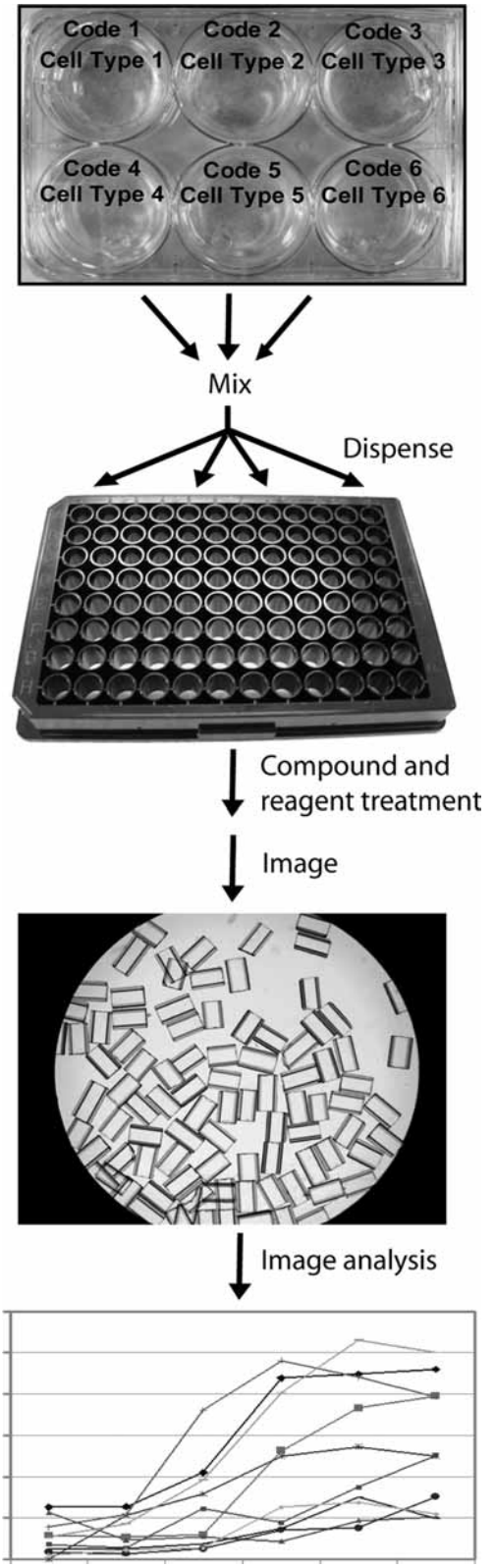
Code 1
Cell Type 1
Code 2
Cell Type 2
Code 3
Cell Type 3
Code 4
Cell Type 4
Code 5
Cell Type 5
Code 6
Cell Type 6
Mix
Dispense
Compound and
reagent treatment
Image
Image analysis

Fig. 2.

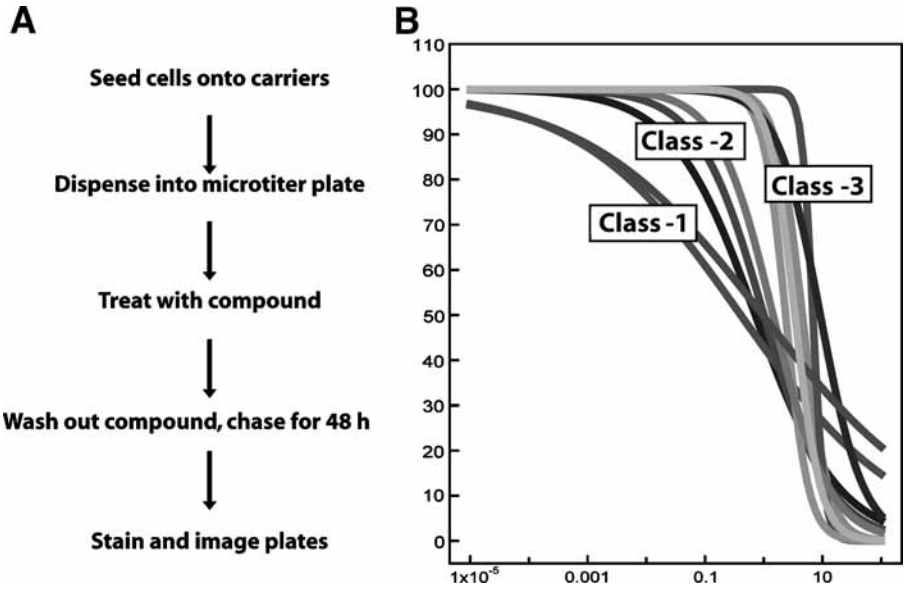

Fig. 3. Case study data example. **(A)** Outline of the protocol used. Ten cell types were introduced into the CellPlex experiment as described in the methods. They were treated with compound for 2 h, washed, then incubated in for another 48 h with standard growth media. **(B)** Dose–response of 10 cell types to a cytotoxic compound. These 10 cell types tended to separate into three distinct classes. The class-1 response in left shifted and shallow. The classes-2 and -3 responses are much more steep with the class-2 response being more potent.

Table 1
A Table Showing Examples of Cell Types Used and Assays Performed on the Cellcard System

Example cell types assayed on CellCard carriers	Example assays performed on CellCard carriers
A549, A431, MCF7, COLO205, SKMEL28, SW620, OVCAR3, OVCAR5, HCT116, HT29, T47D, LOVOS, HEK-293, CHO, HELA, COS, primary HUVEC, primary preadipocytes, primary renal tubule, and others	• β-lactamase reporter gene • Proliferation—cell count, BrdU incorporation, mitotic index • Toxicity—TUNEL, Caspase-3 activation, propidium iodide, C alcein-AM • Adipocyte differentiation—lipid accumulation and others

types were assayed simultaneously within a single well. Therefore, even if there were assay errors introduced by reagent stability issues, these cell types, in this assay, on this day, experienced identical assay conditions. The relative responses between the cell types within the well is unequivocal; at the low doses in which the class-1 group of cells show a slight but significant response and the classes-2 and -3 cells show no response, this result must be cell-type dependent.

The CellCard System provides a means by which ten unique cellular conditions can be assayed simultaneously. These conditions are typically represented by 10 different cell types (**Table 1**) but could also represent seeding densities, extra-cellular matrix coatings, and the like. When performing CellPlex assays, significant miniaturization is realized within the 96-well plate. That is, when assaying 10 cell types, the plate effectively becomes a 960-well plate. Finally, the number of cells analyzed per data point is significantly reduced. Moving forward, we have been exploring assays in which it is desirable to increase the number of data points derived

from rare and/or valuable cells (i.e., human primary cells) ***(12)***. We believe that this will result in a very powerful application of the CellCard System wherein patient samples could be assayed along side a clinical trial ***(13,14)*** leading to the development of in-vitro surrogate markers of compound efficacy and potential theranostic indicators.

4. Notes

1. The determination of which cell lines to be used in a CellPlex assay should follow a few guidelines. First of all, because the assay will be performed simultaneously across multiple cell types, the kinetics of the response to be measured should be similar. For example, if measuring activity across ten G protein-coupled receptors (GPCRs) simultaneously using a reporter gene assay, one should choose GPCRs with similar activation kinetics to be included in the same well ***(10)***. An incubation time of 3 h could be chosen because the primary receptor for which the compound was identified showed a robust response within that time. However, if the maximal reporter gene expression for some of the cell lines in the CellPlexed experiment is 6 h, a lack of activity on that cell line would be interpreted as compound inactivity although the compound could be active albeit with slower kinetics. Similarly, when assessing experimental effects on the cell cycle the cells to be included in the same well should have similar doubling times. This will ensure that within the experimental incubation time, on average, the cell types will have experienced comparable cell cycle passage.
2. It is recommended only to dispense a single vial of carriers onto each ladle. However, with large experiments, it is advantageous to limit the total number of ladles and six-well plates that need to be handled. When placing more than a single vial of carriers onto a single ladle the probability of the carriers overlapping increases. If the carriers are overlapped when the cells are seeded, those on the underside will not be available for the cells to adhere to. When taken through to the assay output, for assay measures that depend on the overall cellular density, the data from CellCard carriers that were never seeded with cells often are identified as outliers. For those ratiometric assay measures that are not dependent on cellular density, it is worth noting that CellPlex assays derive data from very few cells (about 500/data point) and any process that decreases the size of the cellular population to be analyzed is more likely to increase the variance of the assay.
3. The generation of the array of cell types to be assayed requires that the cells (and CellCard carriers) be mixed. If this step is omitted, the carriers will be layered in the conical tube from which the dispensing into the 96-microtiter plate performed. Therefore, the last code put into the conical tube (the resulting top layer) will be the only carrier type dispensed in to the first few wells and the first code transferred to the conical tube (the bottom layer) will be the only code in the last few wells. Assuming that the experiment contains ten cell types, nine of these 10 cell types will be absent from a majority of the wells. The mixing procedure is simple requiring a single inversion of the conical tube (*see* **Subheading 3.4.**). If insufficient mixing is suspected, it is recommended that a second inversion of the tube be performed.
4. All cell washes must be done as gently as possible to limit CellCard carrier agitation or loss. In addition, wells should not be aspirated dry as carriers might be aspirated into the aspiration device resulting in a potential clog. The carriers also have a tendency to float when liquid is reintroduced to a well that has been aspirated dry. We recommend leaving 50 µL in each well between wash steps to minimize carrier disturbance or loss. For postwash aspiration steps, we recommend leaving more than 20 µL in each well; live assays (e.g., cells are not fixed) may require more than 30 µL. A 96-well plate washers can be used routinely, provided that they dispense gently enough to minimize carrier agitation; we recommend using a Tecan PW384 or a BioTek ELX405 Select CW for this purpose.

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