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Optimizing the Integration of Immunoreagents and Fluorescent Probes for Multiplexed High Content Screening Assays

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Summary

Immunoreagents formed the basis of early fixed end point high content screening (HCS) assays and their use in HCS applications in drug discovery will continue to increase. One important application of immunoreagents is their incorporation into multiplexed HCS assays in which multiple physiological features are simultaneously measured and related in the same cells. However, creating multiplexed HCS assays that incorporate multiple immunoreagents presents issues such as reagent compatibility, spectral signal overlap, and reproducibility that must be addressed. Here, an example multiplexed fixed end point HCS assay is used to guide potential assay developers on how to optimize complex, yet cellular information rich, multiplexed HCS assays although avoiding some common pitfalls.

Key Words: Cell cycle; fluorescence-based immunoassays; high-throughput screening; microtubule cytoskeleton; systems cell biology; tumor suppressors.

1. Introduction

Fluorescent immunoreagents have become important tools for fixed end point high content screening (HCS) because of their exquisite specificity and sensitivity. Furthermore, fluorescent immunoreagents can also be combined with additional immunoreagents as well as other fluorescent physiological indicators and biosensors in the same cells to produce multiplexed HCS assays.

With the commercial availability of thousands of immunoreagents and fluorescent probes, large numbers of multiplexed fixed end point HCS assays are possible. However, the compatibility of reagents when integrated into a single assay can be problematic. Furthermore, multiplexed HCS assays involving the use of several immunoreagents have an inherent complexity that demands strict attention to detail to achieve a validated assay for HCS. Nevertheless, the systems cell biology knowledge gained from multiplexed fixed end point HCS assays more than compensates for the extra assay development effort required.

In the drug discovery process, simultaneous measurement of the effects of potential lead compounds on multiple cellular pathways has become a valuable tool, especially when applied early on in the discovery process to create systems cell biology knowledge *(1)*. Thus, the following multiplexed HCS assay, which involves the use of six immunoreagents plus a fluorescent DNA-binding probe provides information on how libraries of compounds affect cell cycle regulation, the degradation of DNA and changes in nuclear morphology associated with apoptosis, the stability of the microtubule cytoskeleton, the activation of the tumor suppressor protein p53, and the

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activity of a signaling kinase associated with chromatin structure. A brief description of how to design and perform a four-color multiplexed HCS assay is presented. In addition, several reagent issues unique to multiplexed HCS assay development are also considered and addressed.

2. Materials

1. The cell permeabilization reagent Triton X-100, the DNA-binding fluorescent probe Hoechst 33342, mouse anti-α-tubulin antibody (no. 6), dimethyl sulfoxide (DMSO), 5-fluorouracil, and vinblastine are from Sigma Chemical Company (St. Louis, MO).
2. Cell culture medium, antibiotics, fetal bovine serum, trypsin, 37% formaldehyde, and Hank's balanced salt solution (HBSS), and 384-well microplates (Falcon, cat. no. 3962) are obtained from Fisher Scientific (Pittsburgh, PA).
3. Sheep antihuman p53 pantropic polyclonal antibody (cat. no. PC35) is purchased from Calbiochem (La Jolla, CA) (*see* **Note 1**).
4. Rabbit antiphospho histone H3 antibody (cat. no. 70) is obtained from Upstate, Inc. (Charlottesville, VA).
5. The fluorescently labeled secondary antibodies, which include fluorescein isothiocyanate (FITC)-labeled donkey antimouse IgG (cat. no. 715-095-150), Cy3-labeled donkey antirabbit IgG (cat. no. 65-152), and donkey antisheep IgG (cat. no. 713-175-147) are from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) (*see* **Note 2**).

3. Methods

1. For multiplexed HCS assays, many primary cell types and established cell lines have been used. In this example, A549 lung carcinoma cells (CCL-195; ATCC, Manassas, VA) were used. The cells were cultured in Ham's F12 medium plus 10% fetal bovine serum and penicillin/streptomycin. For this type of multiplexed HCS assay, it was important to use actively growing and dividing cells. The most reproducible way to obtain these cells was to allow them to grow in a culture vessel for not more than 36 h. We often use a "shuffling" procedure in which cells are trypsinized and plated into large culture vessels (e.g., T-150 or T-175 flasks) on the day before transferring them to microplates.
2. For the multiplexed HCS assay, cells were trypsinized from flasks and plated at a density of 7500–8000 cells per well (40 μL) in 384-well microplates that were coated with collagen I using an automated liquid handling system (Biomek® 2000; Beckman-Coulter, Inc., Fullerton, CA). This alternative to precoated microplates was preferred for two main reasons: (1) economic and (2) manually coating microplates provided cell morphologies much more amenable to high-content imaging than did the precoated microplates. Furthermore, it was useful and relatively easy to prepare rat tail collagen I solutions using established procedures *(2)*.
3. Cells were exposed to drugs within 2–8 h of plating. Concentrated stocks of all drugs were diluted into solutions of HBSS plus 10% fetal bovine serum and added to the microplates (10 μL per well). For this assay, which is optimized to measure the effects of compounds on the regulation of the cell cycle, cells were incubated in the presence of compounds for 20–24 h (*see* **Note 3**).
4. After incubation with compounds, the solution was removed from the microplates by shaking them out and immediately replaced with a solution of HBSS containing 4% formaldehyde to fix the cells.
5. After incubation at room temperature for 30 min, the solution was removed from each well and replaced with HBSS (100 μL/well). At this point, microplates could be sealed and stored at 4°C overnight.
6. After removing the HBSS from each well, 0.5% (w/w) Triton X-100 in HBSS was added (10 μL/well) and the plate incubated for 5 min at room temperature to detergent extract a fraction of the soluble cellular components including destabilized tubulin.
7. The wells were washed with HBSS (100 μL/well) followed by the addition of a primary antibody solution containing mouse anti-α-tubulin (1/3000), rabbit antiphospho histone H3 (1/500), and sheep antihuman p53 (1/400) in HBSS (10 μL/well) (*see* **Notes 4** and **5**).
8. After a 1 h incubation at room temperature, the microplate wells were washed with HBSS as in **step 7** followed by the addition of a secondary antibody solution containing FITC-labeled donkey antimouse (1/300), Cy3-labeled donkey antirabbit (1/300), and Cy5-labeled donkey antisheep (1/300) antibodies diluted in HBSS containing 10 μg/mL Hoechst 33342 (10 μL/well).
9. After a 1 h incubation at room temperature, the microplate wells were washed as above and HBSS was added (100 μL/well) before sealing the microplates. Labeled microplates could be stored at 4°C for up to 2 wk before high content analysis.

10. HCS of microplates prepared using the above method is platform independent provided that the HCS reader employed has the capability to image all four of the fluorescent labels. HCS was routinely performed with a V3.1 ArrayScan® HCS Reader (Cellomics, Inc., Pittsburgh, PA). In this example, the instrument was used to scan multiple optical fields, each with multiplexed fluorescence, within a subset of the wells of a 384-well microplate. Typically, 1000 cells per well were measured and was usually accomplished by scanning three to four fields per well. However, significant effects induced by compounds could also be measured by scanning only one field per well, which typically provided 250–300 cell measurements. Thus, a trade-off exists between the sample scanning rate and the confidence with, which cellular responses can be measured.
11. Methods for the detailed interpretation of multiplexed HCS assay data have been previously described *(3–7)*. For this example, which is focused on immunoreagent optimization, some representative multiplexed HCS assay images of cells that were either untreated or were treated with two drugs, vinblastine and 5-fluorouracil are provided **(Fig. 1)**. These images show that the signals from each of the fluorescent labels were well separated and balanced such that crosstalk between the fluorescence channels was minimal. Visual inspection of the images from this assay revealed the profiling capabilities of the multiplexing approach, but the resulting conclusions only a fraction of the information that was extracted using imaging algorithms. For example, 100 n*M* vinblastine induced nuclear condensation, microtubule destabilization (note the loss of microtubule structure), and increased histone H3 phosphorylation, whereas 10 μ*M* 5-fluorouracil had little visually detectable effect on nuclear morphology and microtubule stability. However, it is easy to discern that 5-fluorouracil produced a considerable activation of p53 as well as inhibition of histone H3 phosphorylation to levels below that of cells treated with DMSO. Therefore, this brief example shows how multiplexed immunoreagents have the potential to generate large amounts of systems cell biology knowledge when coupled with the appropriate bioinformatics tools *(7,8)*.

4. Notes

1. Storage of primary antibody solutions in a liquid form at –20°C provided the most flexibility in developing and executing HCS assays. Thus, primary antibody stock solutions as received from the manufacturer were routinely diluted 1:2 with glycerol, if not done so by the manufacturer, and stored at –20°C in the liquid form until use.
2. Secondary antibodies optimized for multicolor labeling is used for multiplexed HCS assays are highly recommended. Fluorescently labeled secondary antibody solutions were stored in the dark at 4°C as recommended by the manufacturer.
3. In designing multiplexed fixed end point HCS assays, careful attention must be paid to building an acceptable suite of immunoreagents. In the example we showed here, a primary–secondary antibody labeling approach was used that relied on three primary antibody reagents raised in three types of animals. In general, choosing compatible primary immunoreagents is the most difficult part of multiplexed fixed end point HCS assay development. However, the increasing commercial availability of fluorescently labeled primary antibodies and other fluorescent physiological indicators and biosensors is reducing the complexity of multiplexed HCS assay design.
4. Once the primary antibodies have been chosen, the obvious controls in which cells are labeled with individual primary–secondary antibody pairs must be performed. It is important during this step to balance the fluorescence signals from each primary–secondary antibody pair. Balancing the signals can be accomplished by varying the dilution strengths of each of the primary antibodies as well as the secondary antibodies, if necessary. The fluorescence signal from a single primary–secondary antibody pair is deemed too strong if it overflows into one or more of the other fluorescence channels. In the example presented here, a combination of the quality of the antitubulin antibody and the high-intracellular concentration of tubulin dictated that a high dilution of the primary antitubulin antibody relative to the dilutions of the other primary antibodies was necessary.
5. After optimization of the primary–secondary antibody pairs in isolation, mixtures of these reagents can be tested for compatibility. In the procedure presented here, it was possible to incubate the reagents with cells using a mixture of all three primary antibodies followed by incubation with a mixture of all three secondary antibodies. Occasionally, immunoreagent incompatibilities arise and are often solved by doing serial labeling where necessary. Finally, nonimmunoreagents (e.g., Hoechst 33342 in this example, labeled phalloidins, fixable small molecule physiological indicators, and so on) are added to the

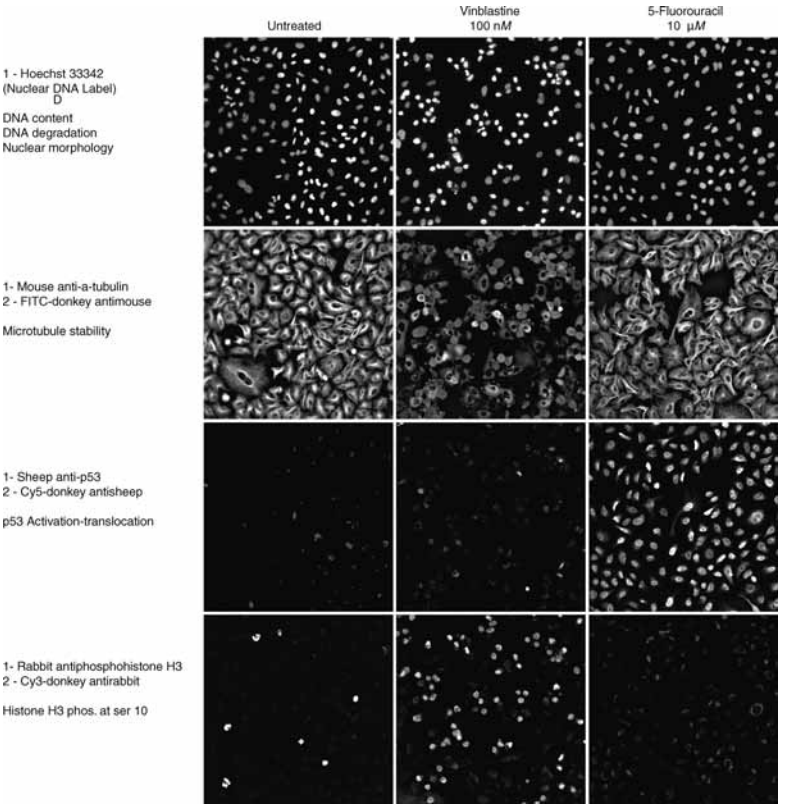

Fig. 1. An example multiplexed HCS assay that incorporates multiple immunoreagents and a physiological indicator probe. A549 human lung tumor cells were treated with the anticancer agents vinblastine and 5-fluorouracil for 24 h and then labeled for multiplexed HCS using the reagents indicated. Example cellular feature measurements are shown in bold next to the images in which the information was extracted using the HCS bioapplication. Thus, visual inspection alone shows the compatibility of the multiplexed reagents and that each drug produced an individual response profile.

labeling protocol where necessary. For example, Hoechst 33342 could be incubated with cells along with the labeled secondary antibodies.

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