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A one step imaging assay to monitor cell cycle state and apoptosis in mammalian cells

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Abstract

High-content screening (HCS; fluorescence microscopy with multiple markers followed by automated image analysis) is gaining its popularity in drug discovery due to the rich information it reveals about drug responses. It is particularly useful in studying anti-mitotic drug responses since mitotic arrest provides an activity biomarker. One conventional way to probe mitotic arrest and downstream apoptosis response is to use mitosis- and apoptosis-specific antibodies in cell-based imaging assays. However, weakly attached cells, especially dead cells, are mostly washed out during antibody labeling steps. Here, we report a rapid and convenient one-step cell-imaging assay that accurately measures cell-cycle state and apoptosis in mammalian cells. Our assay uses three fluorescent dyes to stain living cells, involves no wash, and is fixable after live-cell labeling. Compared to the antibody-based method, our assay is quicker, more cost-effective, and gives more accurate dose-response results.

Keywords

High-content screening; imaging assay; mitosis; apoptosis; dose response; pharmacology

Introduction

Understanding variation in drug response is crucial in cancer pharmacology (Ma and Lu, 2011; Madian et al., 2012). Conceptually, variation in drug sensitivity and selection for resistance can occur at any step in the drug response pathway, from upstream target engagement to downstream signaling activation/inactivation (Tang et al., 2013). One approach to parse out these different mechanisms is to conduct high-content imaging which uses multiplexed readouts to reflect changes relevant to drug responses. For anti-mitotic small-molecule screens, it is important to understand whether drug resistance is due to poor target inhibition or downstream apoptosis resistance.

We developed a cell-based imaging assay for screening anti-mitotic compounds (Tang et al., 2013). Conventionally, antibodies have been favored as imaging markers due to their broad applicability, high specificity and strong signal (Bullen, 2008; Lang et al., 2006; Zanella et al., 2010). However, multiple wash steps in antibody-labeling bear the strong risk of losing

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weakly attached cells, e.g., mitotic arrested cells and apoptotic cells, making accurate quantification of these cell types almost impossible. For this, we developed a high-content assay where living cells were labeled with three fluorescent dyes, followed by fixation, but with no washes or medium changes to minimize cell loss. Compared to the antibody-based assay, our one-step dye-base assay is quick, cost-effective, and gives more accurate quantification of mitotic and apoptotic cells. We also developed a customized image analysis method for automated cell scoring.

Basic Protocol: One-step imaging assay using three fluorescent dyes to accurately detect mitotic, apoptotic, and interphase cells

This assay was performed on 33 cancer cell lines in (Tang et al., 2013).

Materials

Consumables

Cells and Cell culture components (cell culture incubator, serological pipettes, cell culture flasks)

384-well black clear-bottom imaging plates (Corning 3712)

Aluminum plate seals (Corning 6570)

Small-molecule compounds to be screened, diluted in DMSO

Reagents

Growth medium

0.5% Trypsin-EDTA

Phosphate-buffered saline pH 7.4

4x Cocktail of cell-staining reagents, made up in PBS:

1 ug/ml LysoTracker-Red (Invitrogen, cat. No L-7528)

4 ug/ml Hoechst 33342 (Sigma, cat. No. B2261)

2 uM DEVD-NucView488 Caspase-3 substrate (Biotium, Inc., cat. No. 10402)

2% formaldehyde solution diluted in PBS

Instrumentation

Matrix *WellMate* (for liquid dispensing)

(Rudnicki and Johnston, 2009)

Epson Compound Transfer Robot (for compound transfer)

(Rudnicki and Johnston, 2009)

Molecular Devices ImageXpress Micro microscope (for multi-well microplate imaging)

Steps

1. Cells were trypsinized, re-suspended in growth media and dispensed into clear-bottom black 384-well imaging plates (30 μ L/well) at a pre-determined optimal seeding density. Optimal seeding densities of these cell lines were pre-determined so that cells grew to ~80% confluence by 72hrs in the absence of compound treatment. For most cell lines, 2000 to 3000 cells per well were plated. For each timepoint to be monitored, a separate assay plate for each cell line should be produced—thus, if monitoring the assay at 24H, 48H, and 72H, three assay plates for each cell line would be made. Then, at each timepoint, one plate per cell line can be processed (see Step 4).
2. Let cells settle in the plates in cell culture incubator (37°C, 5% CO₂) for 24hrs.
3. Perform a pin transfer (using Epson Compound Transfer Robot, for example at a screening facility) to add 100 nL compounds from the compound plate to each assay plate, with a dilution factor of 300. Typically, compound stocks are in DMSO solvent in a 384-well source plate. Compounds in the stock plate might be plated in a dilution series (as in Tang et al. 2013) so that dose response curves can be produced from the assay data.
4. At the end point (e.g. 24, 48, and 72hrs after the compound transfer), perform the following:
 - a. Dispense 10 μ L of the 4x cocktail of cell staining reagents (4 μ g/mL Hoechst 33342, 2 μ M NucView488, and 4 μ M LysoTracker-Red in PBS) into each well (so that the final concentration of Hoechst 33342 is 1 μ g/mL, NucView488 is 500nM, and LysoTracker-Red is 1 μ M) using a Matrix *WellMate* plate filler.
 - b. Incubate the plates in a cell culture incubator (37°C, 5% CO₂) for 1.5 hrs.
 - c. Dispense 40 μ L of pre-warmed 2% formaldehyde in PBS (37°C) into each well (final concentration 1%), using a Matrix *WellMate*. Plates are spun in a table-top centrifuge at 1000 rpm, while cells are being fixed, for a total of 20 min at room temperature.
 - d. Seal the plates using aluminum plate seals (Corning 6570).
 - e. Image the plates (best if imaged within the same day) using an ImageXpress Micro (Molecular Devices) with 10x Plan Fluor objective lens, and suitable filters: DAPI (Excitation 377/50; Emission 447/60), FITC (Excitation 482/35; Emission 536/40), and Texas Red (Excitation 562/40; Emission 624/40). Four sites should be imaged toward the center in each well. Typically, ~2000 cells are expected to be imaged in the four sites 24hrs after DMSO control, and ~500 cells are imaged in the four sites 24hrs after Staurosporine treatment.

Analyzing the data

5. Automated scoring of mitotic, apoptotic and interphase cells can be done with a customized image analysis tool written in MATLAB, available through GitHub (https://github.com/xietiao/Tang_et_al_LINCS_cell_scoring). This algorithm is freely available and customizable as desired.

6. A detailed description of the image analysis algorithm can be found in (Tang et al., 2013). In brief, nuclei are scored in the DAPI channel and used to “segment” the image to identify individual cells. In the Texas Red channel, bright, rounded cells (a subset of the cells stained by LysoTracker-Red) are mitotic cells. In the FITC channel bright spots correspond to NucView staining of apoptotic cells. Finally, late-stage dead cells were identified as those that had a “blurry” DAPI morphology and no NucView or LysoTracker Red staining.

7. In reporting results from the assay, five different parameters were tracked: A. Cell Count (the total number of cells that stained with Hoechst 33342 and scored in the DAPI channel), B. # Interphase Cells (the total number of cells less the number of Apoptotic cells, Dead cells, and Mitotic cells), C. #Apoptotic Cells (the number of cells that stain with NucView), D. # Dead cells (the number of late-stage dead cells described in step 6), and E. # Mitotic cells (the cells that stain brightly with LysoTracker Red and that have a rounded morphology).

8. Since we are interested in studying how drug responses (in our case, mitotic index that is the percentage of mitotic cells in total cell count; and apoptotic index that is the percentage of apoptotic and late-stage dead cells in total cell count) change over different drug exposures (or drug doses), we first plot (drug response) ~ (drug dose) then fit the data points to generate dose-response curves.

Different models can be used to fit dose-response curves. In our case, we choose to fit using Prism 6 software, and the following 3-parameter nonlinear regression model is used:

$$Y = E_{min} + (E_{max} - E_{min}) / (1 + 10^{((LogEC_{50} - X))})$$

Y: Drug response, mitotic index or apoptotic index in this case;

X: log of the drug concentration (in μ M)

E_{min} : baseline response in absence of drug

E_{max} : maximum achievable response

EC_{50} : concentration that produces half maximal effect

Detailed step-by-step procedures in fitting and analyzing dose-response curves in Prism 6 can be found in User's Manual or at <http://www.graphpad.com/www/graphpad/assets/File/Prism%20-%20Dose-response.pdf>.

Commentary

Background Information

Compared to a single GI_{50} readout (GI_{50} : the concentration of a drug that inhibits the growth of cells by 50%), cell-based HCS is more useful in drug discovery due to the rich information that it reveals in cellular response besides cell proliferation. It's an obvious choice for anti-mitotic drugs, since mitotic arrest provides an activity biomarker. Modulation of different pathways in the drug response can lead to resistance to anti-mitotic drugs. For example, overexpression of a drug efflux pump (i.e., P-glycoprotein) in a cell line would result in less binding of target by drug. Other cell lines might have mutations in apoptosis pathway components that blunt apoptotic responses after mitotic arrest (Letai, 2008; Smyth et al., 1998). Because they provide more information about cell phenotypes after drug treatment than simple GI_{50} measurements, imaging assays can be used to distinguish different mechanisms that might lead to differential sensitivity to anti-mitotic drugs across a panel of cell lines.

Conventionally, antibodies have always been an obvious choice for imaging assays due to their specificity and strong signal. However, in cases where mitotic arrest and apoptotic response are the focus of the study, the antibody method is not optimal since loosely-attached mitotic and apoptotic cells are prone to be lost due to multiple washes in antibody labeling methods. To find a better way to accurately quantify mitotic and apoptotic responses, we tested a dye-based assay where first living cells are labeled with three different fluorescent dyes, then fixed in formaldehyde. Since no wash is involved in the dye-based assay, it gave more accurate quantification of mitotic and apoptotic populations, compared to the antibody method (Tang et al., 2013). Fixing the cells, even if only lightly, makes the assay more stable to any time delays that might occur during the imaging step.

Critical Parameters

The dye-based assay in this article uses LysoTracker-Red at a fairly high concentration (1 μ M) to label the whole cytoplasm although it's more selective in labeling lysosomes at lower concentrations, and uses the round-up morphology when cells enter mitosis as a criterion to score mitotic cells in our automated image analysis. Therefore, this dye-based assay to score mitosis only works with adherent cells but not suspension cells since the latter has round morphology in all cell-cycle states.

For most of cell lines we tested, the staining concentrations of the 3 fluorescent dyes in the Basic Protocol suffice. However, different cell lines have different staining intensities with Hoechst 33342, NucView488 and LysoTracker-Red. In order to obtain accurate image analysis results, it is important to set the correct background signals in these three channels for each cell line assayed. Also, it might be necessary to adjust the dye concentration and incubation time if the image quality of a particular cell line is sub-optimal, ranging from 0.1x – 10x of what was used in the Basic Protocols.

Another important factor that affects image analysis outcome is cell morphology either before or after drug treatment. Some cell lines inherently clump more, which can make

segmentation in the image analysis step difficult, and therefore yielding slightly less accurate quantification of mitotic and apoptotic populations.

To obtain consistent and reproducible drug response results, it is important to have consistent cultured cells including minimizing passage number, seeding cells at a consistent density, and making sure cells grow in logarithmic phase before performing the assay.

Troubleshooting

The 33 cell lines studied in (Tang et al., 2013) double between 18 – 40hrs. For slow-growing cells with a doubling time more than 72 hrs, an assay endpoint of 72 hrs may not be enough to capture the maximum efficacy in drug response, especially for anti-mitotic compounds that affect dividing cells. In other words, for anti-mitotic compounds, cells should be allowed to go through cell cycle at least once before performing this assay.

As mentioned previously, cell morphology affects image analysis results. Some cell lines tend to clump more than others. In these cases, to make sure that cells were singly seeded in the wells at the beginning is important. Several efforts can be taken to achieve more homogenous cell mixture, including increasing trypsinization time, pipetting trypsinized cell mixture for a longer time to break small clumps, and filtering cell mixture through membranes before cell seeding.

Anticipated Results

Our dye-based assay is robust in labeling DNA, cytoplasm, and apoptotic populations across 33 different adherent cell lines studied in (Tang et al., 2013). Depending on the morphology/clumping of a cell line either before or after drug treatment, image analysis results could vary. Among the 33 cell lines we tested in (Tang et al., 2013), ~40% of them exhibit some level of clumping either prior to or post drug treatment. Although the image analysis algorithm we employed was not able to yield accurate single cell segmentations as expected for these clumping lines, it still enabled us to generate reasonable and reproducible dose-response curves for clumpy lines despite their non-ideal growth patterns.

Time Considerations

One investment of time before performing the assay is to determine the optimal seeding density of a cell line. Normally, different seeding densities (ranging from 300 to 6000 cells/well) are tested to determine the one that gives ~80% confluency at the assay endpoint (i.e., 72hrs). This step may take 1-2 weeks.

After cells are seeded into multi-well plates, cells need to re-attach (or settle down) to the plates for 24hrs. Then compounds can be transferred to the assay plate through robotic pin transfer. On the day when assay is performed (either 24, 48, or 72 hrs after compound transfer), it takes a few hours to dispense the 3-dye solution to cells, incubate, dispense fixative (formaldehyde) to cells, then seal all the plates. Depending on the number of assay plates, this total processing time could vary. Typically, processing 16 assay plates, through the fixation and plate sealing step, took 4 hours.

Plates are then imaged on ImageXpress Micro using a 10x objective lens for 3 channels with 4 sites/well. Normally it takes ~1.5 hrs to image 1 assay plate.

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