[16] Development and Implementation of Multiplexed Cell-Based Imaging Assays

By BONNIE J. HOWELL, SEUNGTAEK LEE, and LAURA SEPP-LORENZINO

Abstract

Fluorescence microscopy, image analysis, and automated screening technologies are some of the most powerful tools enabling cell biologists to investigate complex signaling pathways and compound or siRNA effects on cellular function in individual cells. Researchers can now use multiple fluorescent probes to quantify effects on intracellular molecular events, measure phenotypic changes, and provide contextual information about cellular pathways not discernible by traditional single-parameter, end point experiments. This chapter focuses on fluorescent labeling techniques and methods for designing image-based assays, multiplexed readouts, and image analysis routines. Case studies are presented describing the use of cell-based imaging assays for monitoring cell proliferation, cell cycle stage, and apoptosis.

Introduction

One of the most powerful tools enabling cell biologists to monitor physiological processes and interrogate protein function at the cellular and molecular level is the fluorescence microscope. This tool, coupled with sophisticated image analysis and automated screening technologies, provides a unique opportunity to investigate complex signaling pathways and effects of compounds or other agents on cellular function in individual cells (Mitchison, 2005). Through the use of multiple fluorescent probes, researchers can now quantify intracellular molecular events, measure phenotypic changes, and provide contextual information about cellular pathways not discernible by traditional single-parameter, end point experiments. Furthermore, this multiparametric approach can filter out false positives and false negatives quite effortlessly during screening campaigns, resulting in higher-quality leads. This chapter focuses on fluorescent labeling techniques and methods for image-based assay design, including multiplexed readouts and image analysis routines for high-content screening. Case studies are presented describing the use of cell-based imaging assays for monitoring cell cycle stage and apoptosis.

METHODS IN ENZYMOLOGY, VOL. 414 0076-6879/06 $35.00

DOI: 10.1016/S0076-6879(06)14016-1

General Considerations

Several labeling approaches exist for visualizing intracellular components and physiological processes in cells, including incubation of cells with membrane-permeable chemical fluorophores, microinjection of fluorescently labeled proteins, transfection with DNA constructs expressing fluorescent proteins, for example, green fluorescent protein (GFP), and immunostaining with antigen-specific antibodies. The desired labeling approach is often dictated by the biology, hardware accessibility, reagent availability, throughput, and cost. For example, if temporal and spatial information is desired, then time-lapse fluorescence microscopy using nondestructive, cell-permeable chemical fluorophores, fluorescently labeled proteins, or fluorescent fusion proteins is the preferred approach. If kinetic information is not necessary and/or if multiplexing is desired, then immunofluorescence or a combination of labeling approaches is preferred. Advantages and limitations of fluorescent labeling approaches are discussed later and reviewed in Table I.

Fluorescent dye technology is a rapidly evolving approach to labeling cells. Chemical dyes, together with appropriate imaging technologies, allow characterization of cellular architecture and detection of cellular events in the absence of cell perturbation. Increased utilization of chemical fluorophores is driven, in part, by improved brightness and photostability of dyes, wider selection and improved filters to separate colors, low cost, and flexibility to use across cell lines. Shortcomings include possible compound interference and limited choice in wavelength selection. Overall, fluorescent dyes offer the benefit of multiplexing with other probes, do not require sophisticated hardware capabilities, and are widely available through several vendors.

A second and more conventional approach to studying cellular processes and protein behavior is indirect immunofluorescence. Traditionally, this method has been performed on slides with complex, laborious protocols. However, with today's advanced liquid-handling technologies, immunofluorescence assays have become a popular tool for high-throughput screening, target validation, and lead optimization. Advantages of fixed-cell immunofluorescence studies include stable fluorescent signal for convenient imaging, flexibility in cell line choice, and large availability of antigen-specific antibodies. However, the greatest advantage is the ability to multiplex probes and proteins to produce multiparametric, high-content information in one simple assay. Historically, multiplexing has been limited to three or four probes; however, advances in multispectral imaging equipment and quantum dot technologies offer wide spectral resolution and new promises in this area. Immunofluorescence approaches have the drawbacks of possible fixation and permeabilization artifacts, lack of kinetic information, and laborious assay development.

TABLE I
CELLULAR MARKERS FOR USE WITH AUTOMATED FLUORESCENCE MICROSCOPY

Technology	Biology	Application
DAPI, Hoechst 33342, DRAQ5	Nucleic acid stain/marker; DAPI can be used for fixed cell assays, whereas Hoechst and DRAQ5 can be used for both live and fixed cell assays	Nuclear size and intensity, cell cycle profile, endoreduplication, apoptosis, cytotoxity, morphology
Propidium iodide	Only taken up by dead cells or cells with compromised cell membrane. Binds DNA	Cell viability
Calcein AM	Nonfluorescent membrane permeant cellular marker cleaved by intracellular esterase to fluorescent form in living cells	Cell viability
Ethidium homodimer	Cell-impermeant marker; only taken up by dead cells or cells with compromised cell membrane. Binds DNA	Cell toxicity
7-AAD	Nucleic acid stain taken up by dead or apoptotic cells	Cell cycle and apoptosis analysis
Cyclin B1-GFP, cyclin B1 antibody	Cyclin B1 marker	Identifies G1/S, G2, mitotic cells
PhosphoHistone H3 antibody	G2/mitosis marker	Identifies G2 and mitotic cells
MitoTracker	Mitochondrial membrane potential sensing dyes	Measures disruption of active mitochondria for apoptosis analysis
Annexin V	Binds to phosphatidyl serine	Early stage apoptotic marker
Cleaved caspase 3 antibody	Binds to active caspase 3	Midstage apoptotic marker
Cleaved PARP antibody	Binds to active PARP	Detects late-stage apoptosis
TUNEL	Detects DNA strain breaks	Detects late-stage apoptosis

The field of cellular imaging has exploded even more within the last decade with recent advances in molecular cloning, optical imaging, and the discovery of fluorescent proteins. Fluorescent proteins, for example, GFP, provide a nondestructive method for studying dynamic processes *in vivo* and offer several advantages over conventional immunofluorescence. These labeling

technologies allow quantification of cellular events and protein behavior in real time, are often less expensive, and provide flexibility in cell line choice. Additionally, the fluorescence from fluorescent proteins, for example, GFP, remains after fixation of cells with paraformaldehyde and therefore this approach can also be combined with conventional immunofluorescence or membrane-permeable dyes. Disadvantages include lengthy assay development periods for establishing transfection conditions or making stable cell lines, need for specialized equipment for kinetic studies, and concern that the fluorescent tag may alter protein folding or function.

Because assay design and image quality lay the foundation for distinguishing between on-target vs off-target effects, careful consideration should be given when choosing cell lines, reagents, image acquisition technologies, and image analysis routines. Lee and Howell (2006) provide detailed discussion regarding assay design and choice of hardware/software. This chapter describes basic protocols for visualizing cell cycle and apoptosis events using automated fluorescence microscopy and outlines caveats to bear in mind when choosing between different fluorescent labeling approaches.

Monitoring Cell Cycle Progression

One of the most fundamental processes occurring in eukaryotic cells is the cell division process in which cells duplicate their contents and distribute them equally to two daughter cells. The cell cycle is regulated both temporally and spatially and is divided into four discrete phases: G1, S, G2, and mitosis (M). Historically, flow cytometry has been the gold standard for measuring DNA content and determining cell cycle stage. With this technology, cells are first incubated with a fluorescent DNA-binding dye and then passed through a detector one cell at a time. Because G2/M cells display exactly twice the DNA content of G1 cells, and S cells have an intermediate DNA content, flow cytometry can be used to generate distribution plots of cell number and DNA content and quickly access fractions of cells in different phases of the cell cycle. This approach can be applied to automated fluorescence microscopy (Fig. 1); however, microscopy and image analysis offer additional measurements compared to flow cytometry. With the same DNA stain, information such as nuclear size and DNA fragmentation (measuring irregular staining and shape of the nucleus) can be obtained along with the cell cycle profile.

An alternative approach to using DNA-binding dyes is quantification of protein expression and/or posttranslational modifications of cell cycle-regulated proteins. One example is cyclin proteins, as these undergo a cycle of synthesis and degradation during cell cycle progression. Here we show the use of cyclin B1 to distinguish among G2, M, and G1/S phases of

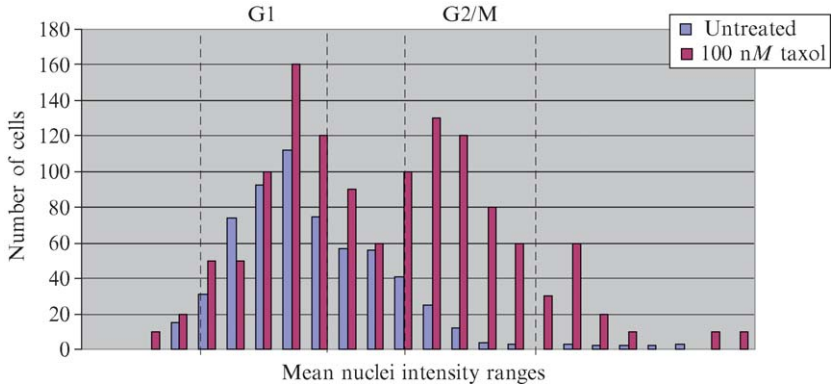


FIG. 1. Cell cycle analysis by quantification of nuclear stain. HCT116 cells were left untreated or treated with 100 n*M* paclitaxel for 24 h, fixed with paraformaldehyde, and stained with DAPI. Nuclear intensity was quantified using the object intensity algorithm from GE Healthcare and cells were binned according to the intensities and cell numbers. Paclitaxel treatment (red bars) resulted in increased G2/M population compared to untreated cells (blue bars).

the cell cycle. Using cell cycle analysis algorithms from GE Healthcare, one can quantify the localization and intensity of cyclin B1 in individual cells and classify cells into distinct phases of the cell cycle (http://www.mdyn.com/aptrix/upp01077.nsf/Content/ProductsTree; Fig. 2). This approach is multiplexed easily with other probes to interrogate cell cycle dependence of key processes or pathways and can be run either as an end point, immunofluorescence study using antibodies to cyclin B1, or as a real-time kinetic study using a fluorescent fusion protein, for example, GFP.

Posttranslational protein modifications also have a major role in driving cell cycle regulation, as well as many other biological processes, and therefore serve as excellent markers of cellular events. For cell cycle analysis of G2/M cells, we chose antibodies that recognize phosphorylated histone H3. Histones are among the numerous DNA-binding proteins that control DNA condensation/decondensation during cell cycle progression, and phosphorylation of histone H3 has been tightly correlated with chromosome condensation during mitosis (Prigent and Dimitrov, 2003). Therefore, antibodies to phosphorylated histone H3 can be used to identify cells in G2 and M phases of the cell cycle, and this can be multiplexed with other probes for correlating cell cycle dependency with drug efficacy or toxicity. The following sections describe various methods for identifying cell cycle stage using fluorescence microscopy.

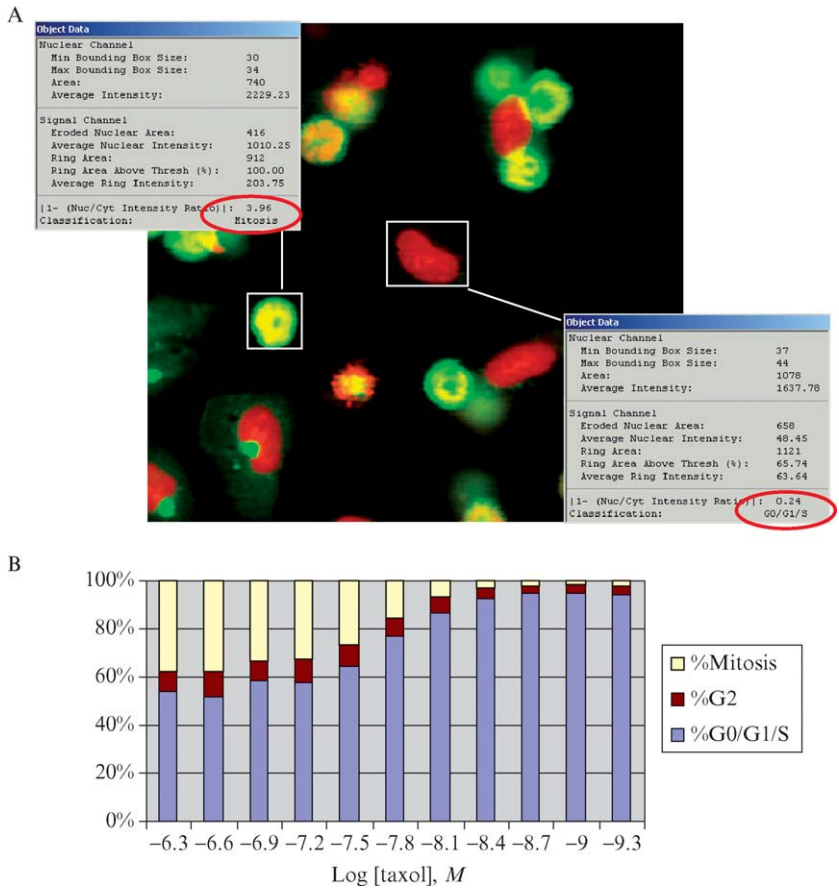


FIG. 2. Cell cycle analysis using cyclin B1. U2OS cells expressing cyclin-B1-GFP were treated with paclitaxel at various concentrations, and cell cycle stage was determined using the cell cycle phase module by GE Healthcare. This algorithm classifies individual cells based on intensity and localization of cyclin B (A) and can then be used to generate a histogram of cell cycle response for the entire well (B).

Protocols for Cell Cycle Analysis

Cell Culture

Seed cells onto 96-well microtiter plates in 100 μl media and incubate at least 12 h, preferably 24 h, to allow them to adhere. For drug treatments, add 100 μl of a 2$\times$ stock of the preferred drug, or add directly from

dimethyl sulfoxide stock. For inducing mitotic arrest, paclitaxel (Taxol) can be used as a positive control at a final concentration of 0.1 to 1 μM. For cells with a doubling time <25 h, cells should be incubated with drug for ~18–24 h to see maximal mitotic arrest.

Determining Cell Cycle Distribution by Quantification of Nuclei Intensity

1. For live cell assays, stain nuclei by adding 1 to 10 μg/ml Hoechst 33342 (final concentration; Invitrogen) and incubate 30 to 60 min before imaging.
2. For fixed cell assays, remove media, fix for 15 min with 2% paraformaldehyde/phosphate-buffered saline (PBS), rinse once with PBS, and then add 100 μl PBS containing 1 to 5 μM DAPI (Invitrogen) or 1 to 10 μg/ml Hoechst 33342 (Invitrogen). Incubate 15 min and read on a fluorescence microscope.
3. Quantify nuclear intensity and display as histogram of binned intensities

Note: Some high-content screening (HCS) companies, for example, Cellomics (http://www.cellomics.com), offer analysis modules that specifically bin cells into one of the four cell cycle stages based on DNA staining intensity (similar to flow cytometry applications).

Cyclin-B1-GFP Live Cell Assay. The cyclin-B1-GFP assay described here was developed by GE Healthcare (http://www.mdyn.com/aptrix/upp01077.nsf/Content/ProductsTree). For this assay, the N-terminal region of cyclin B1 containing the localization and destruction domain is fused to GFP under control of the cyclin B1 promoter and stably expressed in U2OS cells. The assay can be performed as a kinetic or an end point assay, and the cells can be imaged while still alive or after paraformaldehyde fixation.

Cells are grown per manufacturer's recommendations, seeded in 96-well microtiter plates at 8000 cells per well in 100 μl of growth medium, and incubated for 24 h at 37°, 5% CO_2/air prior to drug treatment. Phenol red-free media should be used to reduce background autofluorescence.

1. Treat cells with 2× drug in 100 μl phenol red-free media.
2. For kinetic studies, incubate for desired period, add live-cell nuclear stain (e.g., Hoechst 33342 or DRAQ5), and image at defined time points. Be sure platform is equipped with appropriate environmental controls.
3. For end-point studies, incubate for desired period, remove media, and add 4% paraformaldehye (final concentration) in PBS. Incubate at room temperature for 30 min, remove fixative, rinse twice with PBS, and add 100 μl nuclear stain (e.g., Hoechst 33342, DRAQ5, or DAPI) in PBS.

4. The cell cycle stage can be quantified using the cell cycle phase module from GE Healthcare or custom algorithms that quantify fluorescence intensity and localization of cyclin B1-GFP.
5. For the GE Healthcare software module, results are reported as percentage of cells in G0/G1/S, G2, prophase, and mitosis.

Cyclin B1 Immunofluorescence Assay. Cell lines are cultured according to vendor recommended growth conditions.

1. Plate cells at appropriate seeding density (e.g., 8000–10,000 cells/well in 96-well microtiter plate), incubate overnight to allow adherence, and drug treat as described earlier.
2. Incubate for desired time periods.
3. Remove media by gentle aspiration, leaving ~15 μl in the well so as not to disturb the bottom layer of cells.
4. Add 100 μl fresh 4% paraformaldehyde in PBS and incubate 15 min (perform this operation in a hood).
5. Remove paraformaldehyde, leaving ~15 μl.
6. Rinse three times with 200 μl PBS, leaving ~15 μl after each rinse.
7. Permeabilize, block, and immunostain cells in TBS supplemented with 0.1% (v/v) Triton X-100, 5% bovine serum albumin (BSA), and 1 to 2 μg/ml FITC-conjugated anticyclin B1 antibodies (Santa Cruz) for 90 min at 37°, 4 h at room temperature, or overnight at 4° (high backgrounds can be reduced by adding 0.1% Triton X-100 to the rinse step and lengthening its duration).
8. Remove staining solution and add 150 μl PBS supplemented with DNA stain, such as 1 to 10 μg/ml Hoechst 33342 (Invitrogen) or 1–5 μM DAPI (Invitrogen).
9. Image on a fluorescence microscope.
10. The cell cycle stage can be quantified using the cell cycle phase module from GE Healthcare, as described earlier.

Phosphohistone H3 Immunfluorescence Assay for Detecting Late G2 and Mitotic Populations. Cells are cultured according to vendor-recommended conditions.

1. Plate cells at appropriate seeding density (e.g., 8000–10,000 cells/well in 96-well microtiter plate), incubate overnight to allow adherence, and drug treat as described earlier. Incubate for desired time periods.
2. Remove media by gentle aspiration, leaving ~15 μl in the well so as not to disturb the bottom layer of cells.
3. Add 100 μl fresh 4% paraformaldehyde in PBS and incubate 15 min (perform this operation in a hood).

4. Remove paraformaldehyde, leaving ~15 μl.
5. Rinse three times with 200 μl PBS, leaving ~15 μl after each rinse.
6. Permeabilize, block, and immunostain cells in TBS supplemented with 0.1% (v/v) Triton X-100, 5% BSA, and 1:50–1:100 dilution of fluorophores-conjugated antiphosphohistone H3 antibodies (Cell Signaling) for 60 min at 37°, 2 h at room temperature, or overnight (high backgrounds can be reduced by adding 0.1% Triton X-100 to the rinse step and lengthening its duration).
7. Remove staining solution and add 150 μl PBS supplemented with DNA stain, such as 1 to 10 μg/ml Hoechst 33342 (Invitrogen) or 1 to 5 μM DAPI (Invitrogen).
8. Image on a fluorescence microscope.
9. The cell cycle stage can be quantified by thresholding for cells with phosphoHistone H3 positive signal colocalizing with nuclear stain. The mitotic index is reported as number of thresholded objects divided by number of total objects $\times$ 100.

Visualizing Apoptosis by Fluorescence Microscopy

Apoptosis is a well-regulated form of cell death initiated by a wide range of physiological and pathological conditions (Vermes *et al.*, 1995). It is unique from other forms of cell death, such as necrosis, autophagy, and mitotic catastrophe, and can be characterized by a sequence of biochemical and morphological events beginning with cell surface changes and caspase activation and ending with DNA fragmentation and cell death/rupture. The time interval between reception of an apoptotic signal and execution of downstream signaling events may vary between cell lines and between apoptotic stimuli, as well as the duration of the stage (window for detection). Therefore, it is essential to designs experiments that carefully monitor apoptotic stage of interest.

Several tools exist for detecting apoptosis by fluorescence microscopy. For early apoptosis detection, researchers can monitor loss of cell membrane integrity using dyes that are impermeant to intact membranes but selectively pass through the plasma membrane of apoptotic cells (Darzynkiewicz *et al.*, 1997). Second, dyes that detect changes in mitochondrial membrane potential can also be used (Darzynkiewicz *et al.*, 1997). Often, annexin V conjugates or antibodies to phosphatidyl-serine (PS) are used to detect the apoptosis-initiated translocation of PS from the inner surface of the cell membrane to the external surface (Vermes *et al.*, 1995). Finally, antibodies to activated caspases can be used, as described in detail later, or fluorescent inhibitors to caspase, which are nonfluorescent until they enter cells and bind activated caspases (Villa *et al.*, 1997). Many of these reagents are used to detect early to midstage apoptotic events; however, late-stage apoptosis can be visualized

using antibodies to cleaved PARP or TUNEL labeling to detect DNA strand breaks (Villa *et al.*, 1997). The following sections describe approaches for apoptosis induction using antibodies to cleaved caspase 3 or cleaved PARP and the TUNEL method for detecting DNA breaks.

Apoptosis Detection Using Antibodies to Cleaved Caspase 3

Cells are cultured according to vendor-recommended conditions.

1. Plate cells at appropriate seeding density (e.g., 8000–10,000 cells/well in a 96-well microtiter plate), incubate overnight to allow adherence, drug treat, and incubate for desired time period.
2. Remove media by gentle aspiration, leaving ~15 μl in the well so as not to disturb the bottom layer of cells.
3. Add 100 μl fresh 4% paraformaldehyde in PBS and incubate 15 min (perform this operation in a hood).
4. Fix cells for 10 min at room temperature in 4% paraformaldehyde/PBS solution.
5. Remove formaldehyde, leaving a small amount (~20 μl).
6. Rinse three times with PBS (150 μl per rinse). Leave a small volume between rinse steps when using a plate washer (~20 μl).
7. Block with 100 μl of 2% FBS in PBST (PBS supplemented with 0.1% Triton X-100) for 30 min at room temperature. Rock gently (optional).
8. Remove blocking solution.
9. Add 1:100 dilution of FITC-conjugated rabbit anticaspase-3 antibody (Cell Signaling Technologies) in PBST + 2% FBS.
10. Incubate overnight at 4° in dark.
11. Remove staining solution and add 200 μl PBST per well. Repeat twice, leaving 10-min incubations between rinses.
12. Add 100 μl DAPI or 1–10 μg/ml Hoechst 33342 in PBS.
13. Incubate at room temperature for 15 to 30 min.
14. Image plates.

Note: To minimize cell loss, we suggest spinning plates at 300*g* for 5 min between wash steps and before imaging.

Apoptosis Detection Using Antibodies to Cleaved PARP

Cells are cultured according to vendor-recommended conditions.

1. Plate cells at appropriate seeding density (8000–10,000 cells/well in a 96-well microtiter plate), incubate overnight to allow adherence, drug treat, and incubate for desired time period.
2. Remove media by gentle aspiration, leaving ~15 μl in the well so as not to disturb the bottom layer of cells.
3. Add 100 μl fresh 4% paraformaldehyde in PBS and incubate 15 min (perform this operation in a hood).

4. Fix cells for 10 min at room temperature in 4% paraformaldehyde/PBS solution.
5. Remove formaldehyde, leaving a small amount (~20 μl).
6. Rinse three times with PBS (150 μl per rinse). We use a plate washer for this and leave a small volume in the wells between rinse steps (~20 μl).
7. Block in 100 μl of 1% BSA/TBST (TBS supplemented with 0.1% Triton X-100) for 30 min at room temperature.
8. Remove blocking solution and rinse once with 150 μl PBS, leaving 20 μl.
9. Add 100 μl of staining solution (1–10 μg/ml DAPI or Hoechst 33342, 1–5 μg/ml FITC-conjugated PARP antibody in 1% BSA/TBST). The antibody is from BD Biosciences.
10. Incubate for 90 min at room temperature.
11. Wash plate twice with PBST (PBS supplemented with 0.1% Triton X-100), leaving a small volume between rinses.
12. Add 100 μl PBS and image plates.

Note: To minimize cell loss, we suggest spinning plates at 300*g* for 5 min between wash steps and before imaging.

TUNEL Method for Apoptosis Detection

This assay uses reagents obtained from the Invitrogen Apo-BrdU TUNEL labeling kit, and the protocol has been modified for multiplexing with cell cycle probes and for preventing loss of apoptotic cells during high-content screening.

Reagent Preparation

DNA labeling mix (50 μl per sample): 5 μl TdT reaction buffer (green cap), 0.38 μl TdT enzyme (yellow cap), 4 μl Brd-UTP (violet cap), and 40.63 μl of distilled water. Keep on ice when using or at 4° for up to 24 h.

Anti-BrdU staining mix (50 μl per sample): 2.5 μl Alexa Fluor 488 anti-BrdU (orange cap), 47.5 μl rinsing buffer (red cap), and 1 to 10 μg/ml DAPI or Hoechst 33342. Keep on ice when using or at 4° for up to 24 h. Protect from light.

Protocol. Cells are cultured according to vendor-recommended conditions.

1. Plate cells at appropriate seeding density (e.g., 8000–10,000 cells/well in a 96-well microtiter plate), incubate overnight to allow adherence, drug treat, and incubate for desired time period.

2. Remove media by gentle aspiration, leaving ~15 μl in the well so as not to disturb the bottom layer of cells.
3. Add 100 μl fresh 4% paraformaldehyde in PBS and incubate 15 min (perform this operation in a hood).
4. Fix cells for 10 min at room temperature in 4% paraformaldehyde/PBS solution.
5. Remove formaldehyde, leaving a small amount (~20 μl).
6. Rinse three times with PBS (150 μl per rinse) or with wash buffer provided in kit. We use a plate washer for this and leave a small volume in the wells between rinse steps (~20 μl).
7. Spin plate(s) at 300*g* for 10 min, remove buffer, leaving 20 μl, and add 50 μl of DNA labeling reaction per well.
8. Incubate 1 hl at 37° (shake every 15 min or place on rocker if available).
9. Add 150 μl rinsing buffer (red cap) from kit or PBS.
10. Centrifuge plates at 300*g* for 10 min, remove buffer, leaving 20 μl, and add 100 μl anti-BrdU staining solution.
11. Incubate 30 min at room temperature in the dark.
12. Add 150 μl rinse buffer (from kit) or PBS, spin at 300 *g* for 10 min, and remove buffer, leaving a small amount. Repeat.
13. Resuspend in 125 μl PBS and image plate.

An example image is shown in Fig. 3.

Detecting Morphological Changes Associated with Apoptosis

An alternative approach to detecting apoptotic cells other than using an apoptosis-specific marker is to quantify the morphological hallmarks associated with late-stage apoptosis, such as DNA fragmentation. To apply this approach, cells are first stained with a nuclear marker (e.g., DAPI, Hoechst, DRAQ5) and then ''granularity'' or ''spot detection'' algorithms are used to quantify the punctate appearance of nuclei. The following methods are used for visualizing apoptosis in cells by fluorescence microscopy.

1. Cells are cultured according to vendor specifications, plated in 96-well microtiter plates, drug treated, and incubated for desired time periods.
2. Nuclear dye, for example, 1 to 5 μM DAPI, Hoechst, or DRAQ5 in PBS, is added to wells and is incubated for 15 to 60 min before imaging.
3. Spot detecting algorithms are used to identify fragmented nuclei and to calculate apoptotic index. Examples are seen in Fig. 4.

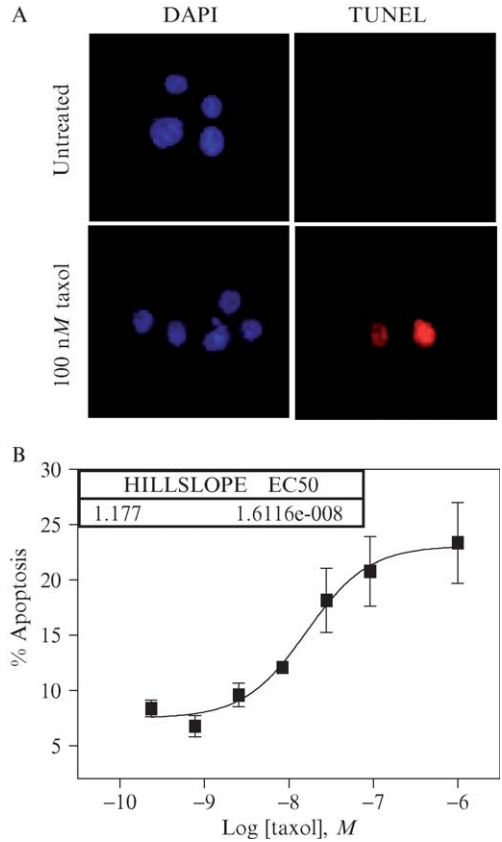
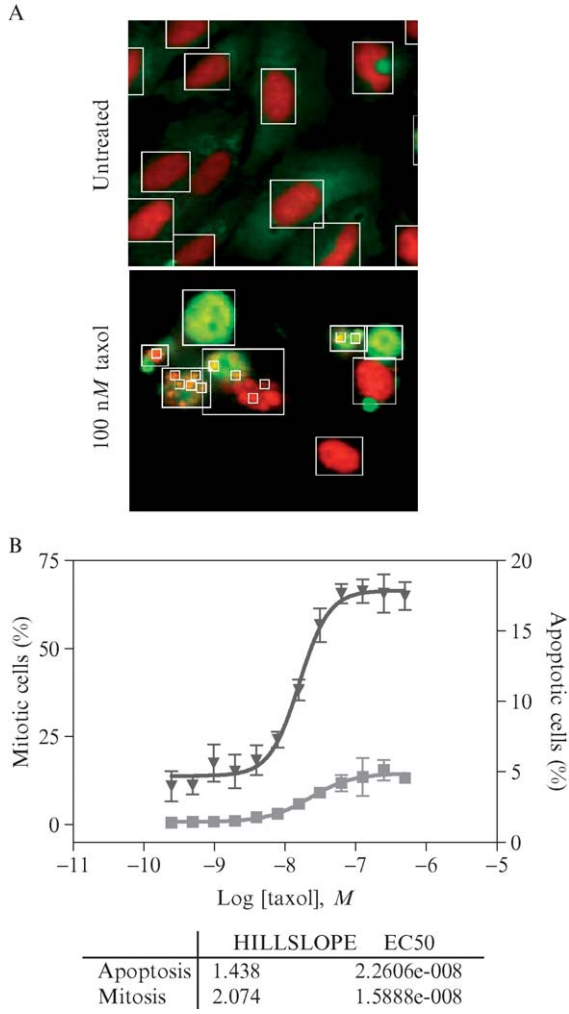


FIG. 3. Apoptosis detection using by TUNEL. HCT116 cells were left untreated or treated with paclitaxel for 48 h, fixed with paraformaldehyde, and labeled for DNA strand breaks via the TUNEL method. Paclitaxel treatment results in positive TUNEL staining (A) and an increase in the total number of apoptotic cells (B).

Multiplexing Cell Cycle and Apoptosis Assays

Many fluorescent probes and HCS assays are multiplexed easily. For example, nuclear stains are commonly used to identify individual cells in HCS assays; however, these stains can also be used to classify cell cycle stage, quantify apoptotic events, detect endoreduplication, and measure cytotoxicity. An example can be seen in Fig. 4 for cell cycle/apoptosis analysis in which the nuclear stain is also used to detect fragmented nuclei. A second approach is to combine antibodies, as described later in the



	HILLSLOPE	EC50
Apoptosis	1.438	2.2606e-008
Mitosis	2.074	1.5888e-008

FIG. 4. Apoptosis detection using morphological quantification. U2OS cells expressing cyclin-B1-GFP were treated with paclitaxel at various concentrations and cell cycle stage and apoptosis effects. Paclitaxel treatment resulted in increased DNA fragmentation (A), a morphological hallmark characteristic of late stage apoptosis, which was quantified using the granularity algorithm by GE Healthcare (B). Cell cycle quantification was performed using the cell cycle phase module (B), as described in Fig. 2.

phosphoHistone H3/TUNEL assay for cell cycle and apoptosis detection. Immunofluorescence markers are multiplexed easily but care should be taken to use antibodies from different species or primary-conjugated antibodies to avoid cross-reactivity. A major challenge to this approach is ensuring that the fixative used adequately preserves the antigen epitope and maintains antibody recognition. Advances in antibody-labeling technologies, such as the zenon-labeling approach from Invitrogen (http://www.Invitrogen.com), offer scientists a unique opportunity to use primary antibodies derived from the same species, rapidly label them with fluorophores of their choice, and avoid the use of secondary detection reagents. This allows versatility in assay design and minimizes cross-reactivity.

Multiplexing of PhosphoHistone H3/TUNEL

This assay uses reagents obtained from the Invitrogen Apo-BrdU TUNEL labeling kit, and the protocol has been modified for multiplexing with cell cycle probes and for preventing loss of apoptotic cells during high-content screening.

Reagent Preparation

DNA labeling mix (50 μl per sample): 5 μl TdT reaction buffer (green cap), 0.38 μl TdT enzyme (yellow cap), 4 μl Brd-UTP (violet cap), and 40.63 μl of distilled water. Keep on ice when using or at 4° for up to 24 h

Anti-BrdU staining mix (50 μl per sample): 2.5 μl Alexa Fluor 488 anti-BrdU (orange cap), 47.5 μl rinsing buffer (red cap), and 1–10 μg/ml DAPI or Hoechst 33342. Keep on ice when using or at 4° for up to 24 h. Protect from light.

Protocol. Cells are cultured according to vendor-recommended conditions.

1. Plate cells at appropriate seeding density (e.g., 8000–10,000 cells/well in a 96-well microtiter plate), incubate overnight to allow adherence, drug treat, and incubate for desired time period.
2. Remove media by gentle aspiration, leaving ~15 μl in the well so as not to disturb the bottom layer of cells.
3. Add 100 μl fresh 4% paraformaldehyde in PBS and incubate 15 min (perform this operation in a hood).
4. Fix cells for 10 min at room temperature in 4% paraformaldehyde/PBS solution.
5. Remove formaldehyde, leaving a small amount (~20 μl).

6. Rinse three times with PBS (150 μl per rinse) or with wash buffer provided in kit. We use a plate washer for this and leave a small volume in the wells between rinse steps ($\sim$20 μl).
7. Spin plate(s) at 300*g* for 10 min, remove buffer, leaving 20 μl, and add 50 μl of DNA labeling reaction per well.
8. Incubate 1 h at 37° (shake every 15 min or place on rocker if available).
9. Add 150 μl rinsing buffer (red cap) from kit or PBS.
10. Centrifuge plates at 300*g* for 10 min, remove buffer, leaving 20 μl, and add 100 μl anti-BrdU staining solution supplemented with 1:50 to 1:100 dilution of fluorophore-conjugated antiphosphohistone H3 antibodies (Cell Signaling) and DNA stain (e.g., 1–10 μg/ml Hoechst 33342 or 1–5 μM DAPI).
11. Incubate 30 min at room temperature in the dark.
12. Add 150 μl rinse buffer (from kit) or PBS, spin at 300*g* for 10 min, and remove buffer, leaving small amount. Repeat.
13. Resuspend in 125 μl PBS and image plate.

Conclusions

Multiple approaches exist for fluorescent labeling of intracellular components in cells, including immunostaining with antibodies, using dyes or stains specific to cellular components or events, and fluorescent proteins for live-cell imaging. Whatever the need, there is a plethora of choices in fluorescent probes, imaging platforms, automation, and image analysis modules that offer researchers unique opportunities to interrogate complex signaling pathways, measure phenotypic changes, and exploit mechanistic effects of agents on cellular function in individual cells. For successful screening campaigns, care should be given when choosing cellular probes (e.g., dyes, fluorescent proteins, and antigen-specific, noncross-reactive antibodies), fixatives, buffers, imaging hardware, analysis software, and visualization tools. Nevertheless, with these resources in hand, automated fluorescence microscopy and high-content screening have the potential to reduce target and lead identification time and accelerate the drug discovery process.

Acknowledgments

The authors acknowledge Mike Cerra, Brandi Dickinson, Frank Traynor, and Shawn Gauby for their assistance with tissue culture and immunostaining.

References

Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T., and Traganos, F. (1997). Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). *Cytometry.* **27,** 1–20.

Lee, S., and Howell, B. J. (2006). High-content screening: Emerging hardware and software technologies. *Methods Enzymol.* **414**(this volume).

Mitchison, T. J. (2005). Small-molecule screening and profiling by using automated microscopy. *Chembiochem.* **6,** 33–39.

Prigent, C., and Dimitrov, S. (2003). Phosphorylation of serine 10 in histone H3, what for? *J. Cell Sci.* **116,** 3677–3685.

Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutellingsperger, C. (1995). A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Methods.* **184,** 39–51.

Villa, P., Kaufmann, S. H., and Earnshaw, W. C. (1997). Caspases and caspase inhibitors. *Trends Biochem. Sci.* **22,** 388–393.

[17] High-Throughput Screening for Modulators of Stem Cell Differentiation

By PAUL J. BUSHWAY and MARK MERCOLA

Abstract

Realizing the potential of stem cell biology requires the modulation of self-renewal and differentiation, both of which are incompletely understood. This chapter describes methods for the design, development, and implementation of cell-based screens of small molecules, genes and expressed proteins for modulation of stem and progenitor cell fate. These include the engineering of embryonic and other stem cells with gene promoter–reporter protein constructs and their application in automated screening. We discuss considerations of promoter reporter selection, assay development and implementation, and image acquisition, analysis, and data handling. Such black-box screens are useful for the identification of probes of developmental processes and should provide tools that will identify druggable targets for biochemical assays.

Introduction

Stem cells combine the potential for self-renewal with the ability to give rise to one or more differentiated cell types, and the ability to manipulate these cells offers unprecedented opportunities to increase our understanding of developmental biology and to provide a renewable source of cells to

METHODS IN ENZYMOLOGY, VOL. 414 0076-6879/06 $35.00

DOI: 10.1016/S0076-6879(06)14017-3