Screening and Development of Antibodies for Use in Cell-Based Assays Using Immunofluorescence Microscopy

Application Note

Contents

Introduction

High-content imaging is rapidly becoming a mainstay in pharmaceutical and life science research laboratories.[1] The power of imaging resides in its ability to measure not only fluorescence intensity changes within cells, but also proximal and temporal relationships and morphological features. In recent years, the availability of a new generation of automated imaging platforms has created a unique opportunity to develop novel cell-based assays.[2,3,4] The abundant use of antibodies in immunofluorescence applications has advanced high-content microscopy. These reagents have enabled high-resolution localization of important molecular targets in individual cells. For example, antibodies can be used to detect specific stages in apoptosis, in the activation of signaling cascades, the organization or reorganization of cellular structures, and identification of stages in the cell cycle.

However, many of the antibodies currently available have not been specifically tested for use in automated imaging applications. In addition, the potential value of high-content screening relies on the multiplexing of cellular information. However, multiplexing is often limited when using standard unlabeled primary antibodies with labeled secondary antibodies due to species cross-reactivity. To facilitate the use of antibodies for imaging applications, BD Biosciences embarked on a screening process using automated imaging to evaluate a large library of monoclonal antibodies for utility in cellular imaging. The library of antibodies included specificities that recognize proteins involved in cell signaling, cell cycle, apoptosis, and cancer.

Antibodies were tested using cell lines and methods that are relevant to high-content screening applications on automated imaging platforms. Specific criteria regarding signal to noise, subcellular localization, and other important parameters were used to qualify reagents. Two distinct BD™ Bioimaging Certified reagent product lines have resulted from this antibody screen. The first is a continually expanding collection of more than 200 unlabeled primary antibodies that have been shown to have general utility in cellular imaging applications. A subset of these reagents has been developed into primary conjugated antibodies to truly enable high-content screening using antibody multiplexing. These reagents, combined with an automated imaging platform, can enable rapid development of novel cell based assays.

Methods

Screening of Purified Antibodies

Antibodies from the BD Biosciences library were screened for utility in cellular imaging applications. The antibodies recognized proteins in different areas of cell biology that included cell signaling, apoptosis, cell cycle, and cancer. For ubiquitously expressed proteins, the antibodies were screened on three different cell lines routinely used in high-content imaging applications. HeLa (human cervical carcinoma), A549 (human lung carcinoma), or U-2 OS (human osteosarcoma) cells were seeded at approximately 10,000 cells per well in 96-well clear bottom imaging tissue culture plates optimized for automated imaging applications. Eighteen hours later, cells were either treated with an appropriate agonist/antagonist and then fixed and permeabilized, or if not treated, immediately fixed and permeabilized by the following two methods:

Fixation

1. Remove the culture medium from wells.
2. Add 100 µL of freshly prepared 3.7% formaldehyde, diluted in 1X phosphate buffered saline (PBS) and pre-warmed to 37°C, to each well.
3. Incubate at room temperature for 10 minutes.
4. Remove the fixation solution and drain the residual liquid by inverting the plate briefly onto absorbent paper.
5. Wash twice by adding 100 µL of 1X PBS to each well.
6. Proceed to either the Triton™ X-100 or Methanol permeabilization steps described as follows.

Triton X-100 Permeabilization

1. Remove the 1X PBS and add 100 µL of a 0.1% Triton X-100 solution diluted in 1X PBS to each well.
2. Incubate at room temperature for 5 minutes.
3. Remove the permeabilization solution and drain the residual liquid.
4. Wash twice with 1X PBS.
5. Proceed to the Blocking step.

Methanol Permeabilization

1. Remove the 1X PBS and add 100 µL of a chilled 90% methanol (-20°C) solution diluted in water to each well.
2. Incubate at room temperature for 5 minutes.
3. Remove the permeabilization solution and drain the residual liquid.
4. Wash twice with 1X PBS.
5. Proceed to the Blocking step.

Blocking

1. Remove the 1X PBS and add 100 µL of 1X PBS supplemented with 3% fetal bovine serum (FBS) to each well.
2. Incubate at room temperature for 30 minutes.
3. Proceed to the Primary Antibody Staining step.

Primary Antibody Staining

1. Remove the blocking solution and add 50 µL of primary antibody diluted in blocking solution to the appropriate concentration to each well.
2. Incubate at room temperature for 1 hour.
3. Remove the primary antibody solution.
4. Wash three times with 1X PBS.
5. Proceed with the Secondary Antibody Staining.

Secondary Antibody Staining

All antibodies were diluted in blocking solution to 0.05 mg/mL. Several different second step reagents were used, including FITC goat anti-mouse IgG (Cat. No. 554001), APC goat anti-mouse IgG (Cat. No. 550826), Alexa Fluor® 555 goat anti-mouse IgG, and Alexa Fluor® 488 goat anti-mouse IgG.

1. Remove the 1X PBS and add 50 µL of diluted secondary antibody to each well.
2. Incubate at room temperature for 1 hour in the dark.
3. Remove the secondary antibody solution.
4. Wash three times with 1X PBS.
5. Add 100 µL of 1X PBS containing 2 µg/mL of Hoechst 33342 to each well.
6. Cover the plate and let stand protected from light for 15 to 30 minutes.
7. Image plates or store at 4°C in the dark prior to imaging.

Screening of Directly Conjugated Antibodies

The same procedure as outlined previously with one alteration was followed. The conjugated antibodies were used in the primary antibody staining step. Unless otherwise stated, all data shown used the conjugated antibodies at their predetermined optimal concentration. After conjugated-antibody binding, cells were washed, Hoechst dye was added, and the samples were imaged.

Modified Staining Procedure for Cleaved (Active) Caspase-3

1. Do not remove the medium from wells. Fix cells by adding an equal volume of 7.4% pre-warmed formaldehyde (3.7% final concentration) to the medium and incubate for 1 hour at room temperature.
2. Remove the formaldehyde solution and drain the residual liquid.
3. Wash once with 1X PBS.
4. Add 100 µL of 90% methanol (-20°C) to each well and incubate for 10 minutes at -20°C.
5. Remove the methanol and drain the residual liquid.
6. Wash twice with 1X PBS.
7. Add 100 µL of 1X PBS containing 5% goat serum and 0.3% Triton X-100 to each well, and incubate for 1 hour at room temperature.
8. Add 50 µL of purified anti-cleaved caspase-3 (Cat. No. 559565), diluted to 1.25 µg/mL in blocking buffer, to each well and incubate overnight at 4°C.
9. Wash three times with 100 µL per well of 1X PBS.
10. Add 50 µL of Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody to each well.
11. Incubate at room temperature for 1 hour in the dark.

12. Remove the secondary antibody solution.
13. Wash three times with 1X PBS.
14. Add 100 µL of 1X PBS containing 2 µg/mL of Hoechst 33342 to each well as described previously, and image.

Differentiation and Staining Procedures for PC12 Cells

1. Coat the wells of a 96-well clear bottom imaging tissue culture plate with type 1 rat tail collagen (Cat. No. 354236).
2. Seed PC12 cells in DMEM with 10% FBS, 5% horse serum, and 1% pen/strep, at approximately 15,000 cells per well.
3. After 24 hours, replace the medium with differentiation medium (DMEM with 0.1% FBS, 0.05% horse serum) containing two-fold dilutions of nerve growth factor (NGF) ranging from 400 to 6.25 ng/mL along with untreated control. Replenish the medium every third day for ten days.
4. On day 10, do not remove the medium from wells. Fix cells by adding a one-third volume of 11.1% pre-warmed formaldehyde (3.7% final concentration) to the medium and incubate for 20 minutes at room temperature.
5. Remove the fixation solution.
6. Wash twice with 1X PBS.
7. Proceed with the Triton X-100 permeabilization step outlined previously.
8. Wash once with 1X PBS.
9. Cells were then stained using either the primary/conjugated secondary or the conjugated primary antibody staining procedures outlined previously.

Staining Procedure for Paraffin Embedded Tissue Sections

1. Deparaffinize sections in two changes of xylene followed by two changes in 100% alcohol and two washes in 1X PBS.
2. Submerge sections in BD™ Retrievagen A (Cat. No. 550524) and heat in a decloaking chamber (pressure cooker, Biocare Medical) to 121°F for 10 minutes (approximate pressure of 17 psi).
3. Allow to cool slowly in the decloaking chamber to room temperature.
4. Wash twice with 1X PBS.
5. Stain simultaneously with Alexa Fluor® 488 anti-BrdU (Cat. No. 558613), Alexa Fluor® 647 anti–β-tubulin (Cat. No. 558606), and Alexa Fluor® 555 anti-actin (Cat. No. 558657) for 2 hours at room temperature in the dark.
6. Wash twice with 1X PBS and incubate with 1X PBS containing 2 µg/mL of Hoechst for 30 minutes in the dark.
7. Wash once with 1X PBS.
8. Seal sections under a cover slip using Aqua-Mount (Fisher Scientific) and image.

Imaging and Analysis

Cells were imaged using a 20x (0.75 NA) objective. Image and data analysis was performed. Cell populations were analyzed for nuclear and/or cytoplasmic fluorescence intensity.

Results and Discussion

Identification of BD Bioimaging Certified Primary Antibodies

All antibodies were originally screened at 1 µg per well. All second-step reagents were used at 0.25 µg per well. Typically, 47 antibodies were screened in duplicate on a 96-well cellular imaging plate with two wells serving as negative controls. Therefore, to encompass the three cell lines and the two permeabilization methods, six 96-well plates were required for each set of 47 antibodies (Figure 1). In anticipation of a broad range of fluorescence intensities, three exposures of each well were taken, 30, 100, and 300 milliseconds (ms). If the staining intensity for a given antibody was saturating at the lowest exposure time, a titration series of the purified antibody was performed to determine the optimal concentration.

Many of the antibodies screened recognized ubiquitously expressed proteins that were not expected to show an altered phenotype upon cell activation. For these proteins, if the subcellular location was correct (based on the literature) and the signal was at least two fold over background, the antibody was classified as a BD Bioimaging Certified reagent (Figure 2). However, some antibodies recognized proteins that were expected to redistribute upon cell stimulation, or they were expected to recognize phosphospecific or cleaved epitopes. For these antibodies, testing in an appropriate cell model system was required. Two such examples, one for active caspase-3 and the other for phospho-STATs 1 and 3, are shown in the following sections.

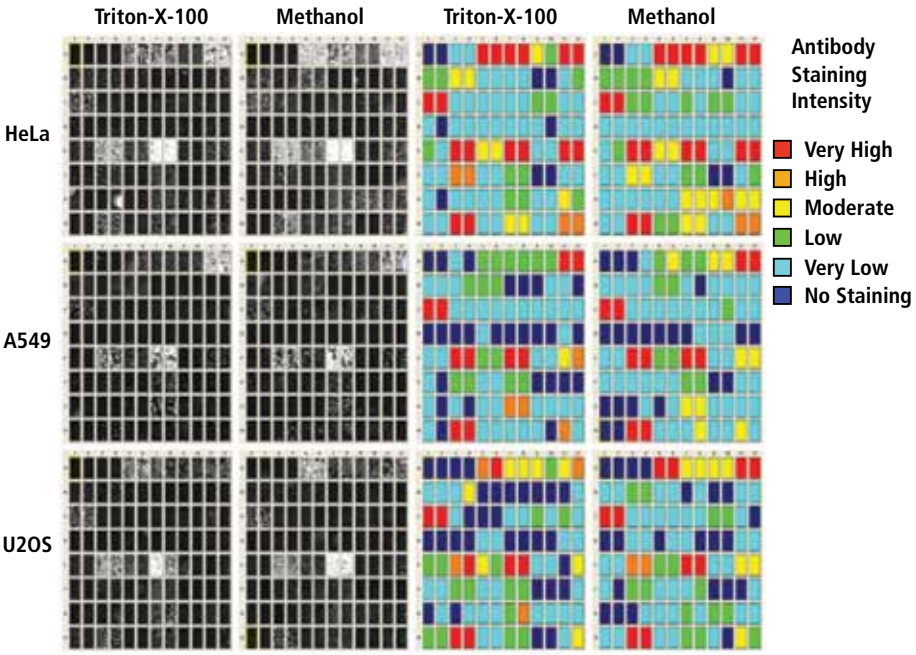

Figure 1. Purified antibody screen using FITC goat anti-mouse IgG second-step reagent.

Representative data from a 47-antibody screen (100-ms exposure) is shown in the two left columns. The cell lines and fix/perm procedures used are indicated. Data classification software was used to determine the level of antibody staining intensity over negative controls, based on user defined intensity thresholds. The individual wells were then scored and classified into six groups ranging from very high staining intensity (red) to no staining (blue) for both the nuclear and cytoplasmic regions. Heat maps of the corresponding cytoplasmic staining intensities are shown in the two right columns.

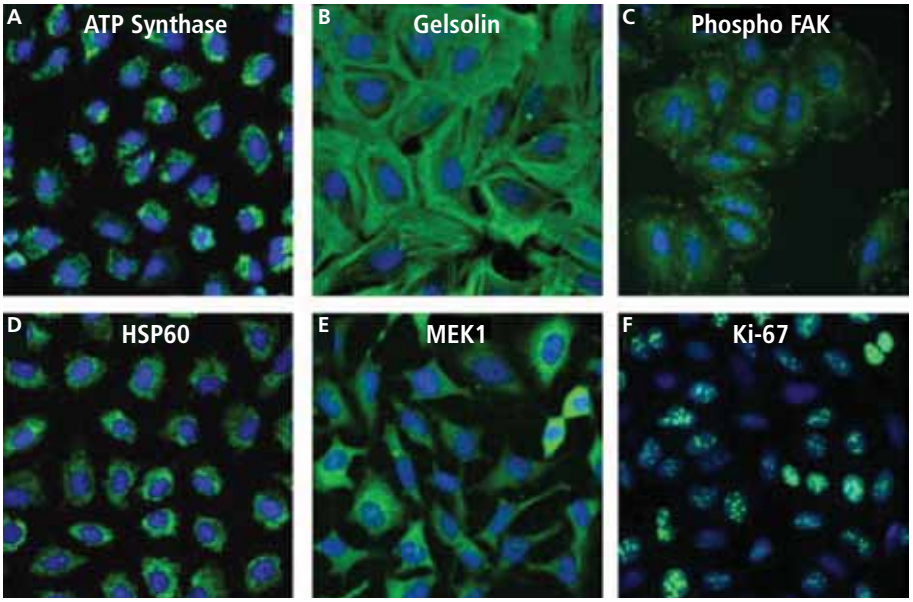

Figure 2. Staining patterns of purified antibodies identified as BD Bioimaging Certified reagents

All images were acquired with a 20x (0.75 NA) objective. Hoechst 33342 staining is pseudocolored blue and antibody-specific staining is pseudocolored green. Images A and B show A549 cells stained with purified anti-ATP Synthase (0.5 µg per well, Cat. No. 612518) and anti-Gelsolin (0.25 µg per well, Cat. No. 610412), respectively. Cells were prepared using the methanol permeabilization method, and the second-step reagent was FITC goat anti-mouse IgG. Images C and D show A549 cells stained with purified anti-phospho-FAK (Y397, 1 µg per well, Cat. No. 611806) and anti-HSP60 (0.06 µg per well, Cat. No. 611562), respectively. Cells were prepared using the Triton X-100 permeabilization method, and the second-step reagent was Alexa Fluor® 488 goat anti-mouse IgG. Images E and F show HeLa cells stained with purified anti-MEK1 (0.5 µg per well, Cat. No. 610121) and anti-Ki-67 (0.5 µg per well, Cat. No. 556003), respectively. Cells were prepared using the methanol permeabilization method, and the second-step reagent was FITC goat anti-mouse IgG for image E and Alexa Fluor® 555 goat anti-mouse IgG for image F.

Detection of Cleaved (Active) Caspase-3

The caspase family of cysteine proteases plays a key role in apoptosis and inflammation.[5] Caspase-3 is a key protease activated during the early stages of apoptosis. Like other members of the caspase family, it is synthesized as an inactive proenzyme that is processed in cells undergoing apoptosis.

Active caspase-3 proteolytically cleaves and activates other caspases, as well as other relevant targets in the cytoplasm and in the nucleus.[6] Anti-cleaved caspase-3 (Cat. No. 559565) was used to detect active caspase-3 in HeLa cells that were treated with increasing concentrations of staurosporine to induce apoptosis (Figure 3). As the concentration of staurosporine increased so too did the percentage of cells staining positive for active caspase-3, as seen by the increase in cells pseudocolored green in panels B and C of Figure 3. This monoclonal antibody therefore provided a means to detect early apoptotic events in a population of cells by image analysis. The validated reagent provides consistent data from which dose-response curves and EC_{50} values can be calculated. Our data supported findings by Jessel et al (2002), who report an EC_{50} of 200 nM for staurosporine.[7]

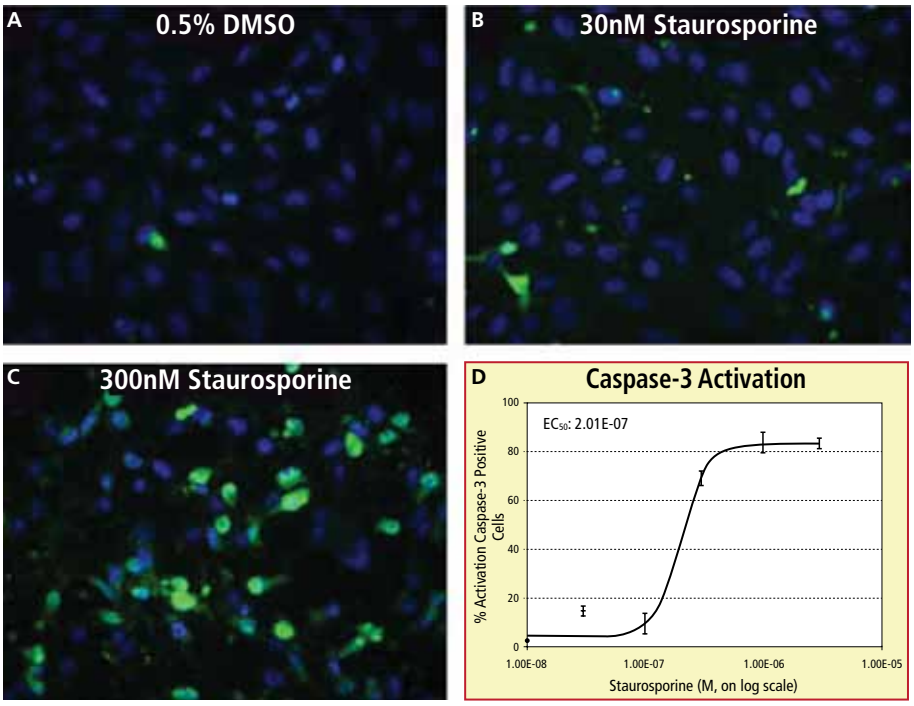

Figure 3. Dose response of caspase-3 activation.

HeLa cells were plated as described in the Methods section. Eighteen hours later, cells were exposed to increasing concentrations of staurosporine for 4 hours. After the treatment, cells were stained with anti-active caspase-3 antibody, as described in the Methods section, and imaged with a 20x (0.75 NA) objective. Merged images are shown, nuclei are pseudocolored blue, and active caspase-3 staining is pseudocolored green. Panel A shows cells that were treated with vehicle alone (control), panel B shows cells treated with 30 nM of staurosporine, and panel C shows cells treated with 300 nM of staurosporine. For a more complete analysis, the active caspase-3 fluorescence intensity values were analyzed. Control cell intensities were used to set a threshold for the treated cells, and a dose-response curve for the titration series plotting the percentage of cells above the threshold was generated (panel D). Data points from n = 2 wells are shown as the mean ±SEM.

Detection of Activated STAT1 and STAT3

STAT proteins function as both cytoplasmic signal transducers and as nuclear activators of transcription. STATs respond to a wide array of stimuli including cytokines and growth factors.[8,9] In response to interferon alpha (IFN-α) treatment, STAT1 and STAT3 become tyrosine phosphorylated, form homodimers, and migrate to the nucleus where they subsequently direct the transcription of IFN-α responsive genes. Anti-phospho STAT1 (pY701, Cat. No. 612233) and anti-phospho STAT3 (pY705, Cat. No. 612357) were used to detect increased phosphorylation of these STAT proteins in A549 cells that were treated with increasing concentrations of IFN-α (Figure 4). Panels A, B, and C show phospho-STAT1 staining with increasing concentrations of IFN-α. Panel A shows a low level of constitutive phospho-STAT1 staining, while B and C, with 2.7 ng/mL and 100 ng/mL of cytokine, respectively, show increased nuclear staining intensity. This suggests that the percentage of STAT1 molecules activated increased with IFN-α concentration. The same was true for phospho-STAT3 staining as shown in panels D, E, and F. Both monoclonal antibodies were therefore able to detect increased phosphorylation of the STAT proteins in response to IFN-α in a dose-dependent manner.

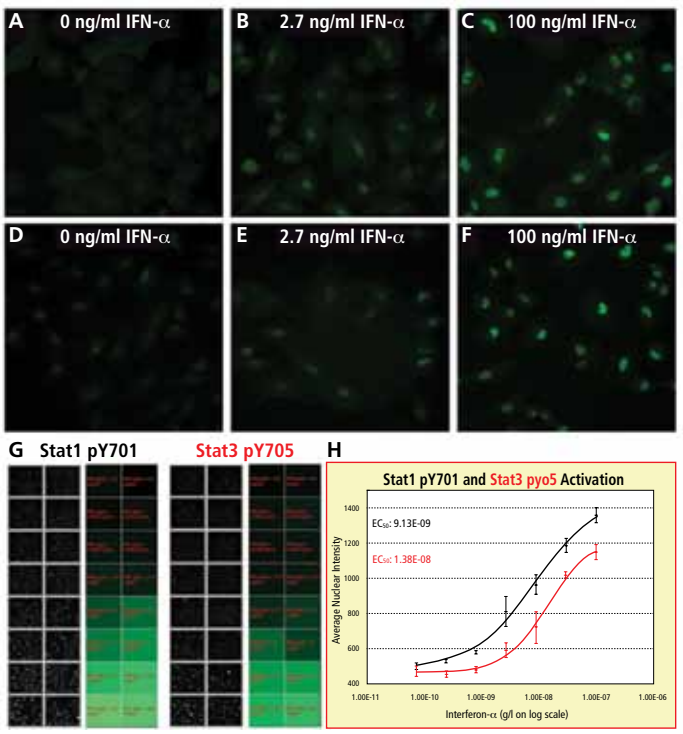

Figure 4. Activation of STAT1 and STAT3 in A549 cells.

A549 cells were plated as described in the Methods section. After overnight incubation, cells were treated with increasing concentrations of IFN-α (Sigma) or medium for 30 minutes. After the stimulation, cells were fixed and permeabilized using the methanol protocol. Mouse anti-phospho-STAT1 and -STAT3 antibodies (0.5 µg per well) were used to detect the specific phosphorylation events. The second-step reagent used was Alexa Fluor® 488 goat anti-mouse IgG. Images were captured using a 20x (0.75 NA) objective, and phosphospecific staining in images A through F is pseudocolored green. Fluorescence intensity values were used to generate dose-response "heat" maps based on nuclear staining intensity for the two phospho-STAT antibodies (G). For each antibody, the black and white thumbnail images of the green channel displayed in the left panels (G) show the increase in fluorescence intensity with increasing IFN-α doses (0 ng/mL at the top up to 100 ng/mL at the bottom). The average nuclear intensity per well is expressed using a software defined color scale in which bright green denotes higher average nuclear intensities and dark green denotes lower intensities. Finally, EC_{50} curves were generated that were also based on average nuclear intensities for the two responses (H). Data points from n = 2 wells are shown as the mean ±SEM.

Development of Primary Conjugated Antibodies

Primary conjugated antibodies, labeled with dyes suitable for high-content imaging platforms, greatly simplify the development and implementation of multiplexed assays. In addition, the use of primary conjugated antibodies eliminates the secondary antibody staining step, thereby greatly reducing the number of wash steps. Once purified antibodies were qualified as BD Bioimaging Certified reagents for use in high-content analysis, as well as other imaging applications, a subset of antibodies was selected for development as primary conjugated antibodies. To facilitate multiplexing, the selected antibodies were conjugated to multiple dyes (Alexa Fluor® dyes 488, 555, and 647) and then tested using the same methods used to screen the primary purified antibodies.

The fluorophore:antibody (F/P) ratio used for conjugation can dramatically affect the signal to noise (S/N) ratio. In addition, the reactivity of a conjugated antibody can vary depending on the dye that it is conjugated to. This makes it essential to empirically determine the optimal F/P ratio for each particular antibody/dye combination. Therefore, to ensure development of optimal high-content imaging reagents, each dye was conjugated to the antibody at three different F/P ratios, high, moderate, and low. During initial testing, conjugated antibody staining was compared to that of the purified with a second-step reagent and was analyzed for correct subcellular location and S/N ratio. Based on these initial results, the best F/P ratio was chosen for further development. Purified anti–β-tubulin, mouse monoclonal 5H1 (Cat. No. 556321) is an example of a purified clone that passed the criteria for a BD Bioimaging Certified reagent and was subsequently chosen for conjugation to Alexa Fluor® dyes at three different F/P ratios for each dye.

The nine conjugates were then tested to see if they stained appropriately. Different trends were observed for the different dyes (Figure 5). For the Alexa Fluor® 647 conjugates, the lowest F/P ratio gave the brightest signal while the highest F/P ratio gave the lowest signal. For the Alexa Fluor® 555 conjugates, the lowest F/P ratio gave an intermediate signal, the middle F/P ratio gave the highest signal, and the highest F/P ratio gave the lowest signal. The Alexa Fluor® 488 conjugates gave the lowest signal at the lowest F/P ratio and as the F/P ratio increased, there was a concomitant increase in signal intensity (data not shown). Therefore, for this particular antibody, each Alexa Fluor® dye had a different optimal F/P ratio. Testing of other conjugated antibodies showed that the optimal F/P ratio also differed for each and that there was no consistent trend for any of the three dyes tested.

The use of directly conjugated antibodies dramatically reduces the number of wash steps needed during plate processing. This attribute can be particularly advantageous when performing assays on fragile cell types such as differentiated neurons, which are prone to breakage due to pipetting sheer force. Figure 6 shows PC12 cells differentiated with increasing concentrations of nerve growth factor (NGF). At higher NGF concentrations (from 50 ng/mL upward), very extensive neural networks are formed. These more complex networks are especially prone to aspiration/dispensing sheer force and were negatively impacted when a two step antibody staining protocol was used to detect the neurites. Neural networks were preserved when a primary conjugate was used for detection.

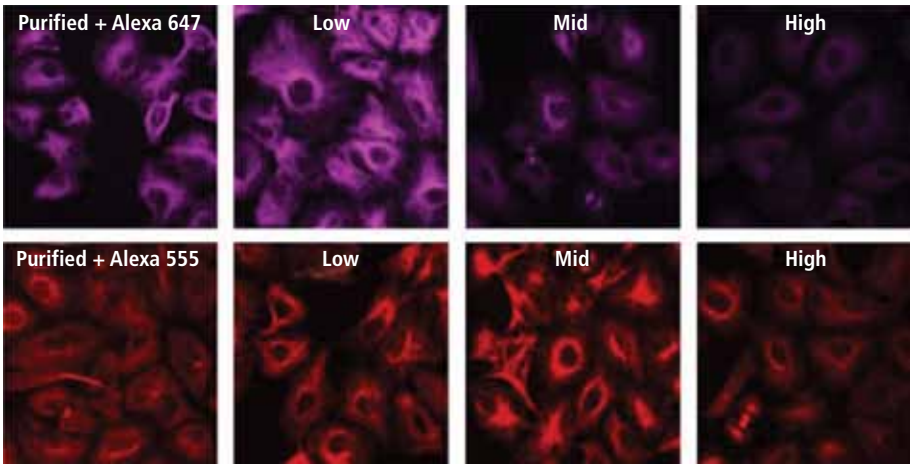

Figure 5. Determining the optimal F/P ratio for Alexa Fluor® conjugated anti–β-tubulin.

A549 cells were plated as described in the Methods section. After overnight incubation, cells were fixed and permeabilized using the methanol procedure. Cells were then stained with the unlabeled purified antibody/labeled secondary antibody as a control, or the primary conjugates at the three different F/P ratios. The purified and conjugated primary antibodies all were used at the same concentration, and images were captured using a 20x (0.75 NA) objective. The β-tubulin–specific stain is pseudocolored magenta for the Alexa Fluor® 647 conjugates and red for the Alexa Fluor® 555 conjugates. The Alexa Fluor® dyes and F/P ratios tested are shown in the figure.

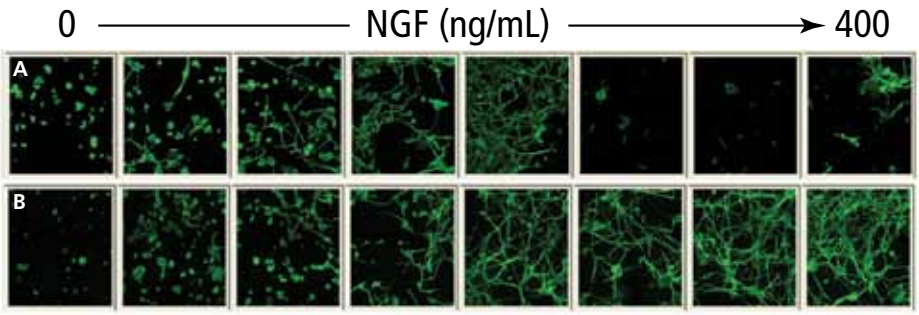

Figure 6. Comparison of the conventional two step staining procedure to the one step procedure using a primary conjugated antibody.

PC12 cells were differentiated into neurons as described in the Methods section. Panel A shows cells stained using purified anti–β-tubulin and Alexa Fluor® 488 conjugated goat anti-mouse IgM (Invitrogen, A21042) second-step reagent, while panel B shows cells stained with Alexa Fluor® 488 conjugated anti–β-tubulin (Cat. No. 558605). Images were captured using a 20x (0.75 NA) objective, and β-tubulin staining is pseudocolored green.

Example of a Multiplexed Assay

Using conjugated primary antibodies for imaging provides users the flexibility to mix and match specificities and obtain multiple data points per well. To demonstrate this, Alexa Fluor® 647 anti-β-tubulin (Cat. No. 558606) and Alexa Fluor® 555 anti-phospho-Histone H3 (S28, Cat. No. 558613) were used to monitor the effects of colcemid on microtubules and the phases of the cell cycle in U-2 OS cells. Microtubules, which are composed of tubulin, are part of the eukaryotic cytoskeleton and are essential for cell division, since they form the mitotic spindle, which in turn directs chromosome segregation.

Colcemid disrupts spindle formation by inhibiting microtubule polymerization. Without spindles, chromosomal segregation cannot occur and consequently, cells accumulate in M phase. The DNA binding protein Histone H3 is specifically phosphorylated at serine 28 during the early stages of mitosis and is often used

as a marker for M-phase cells. Therefore, disruption of microtubules and the accumulation of cells in M phase were monitored using anti–β-tubulin[10] and anti-phospho-Histone H3 (S28)[11] conjugated antibodies, respectively (Figure 7). The β-tubulin specific signal intensity decreased as the concentration of colcemid increased, indicating a decline in microtubules (analysis shown in panel D). Conversely, for phospho-Histone H3, as the concentration of colcemid increased, the percentage of cells positive for phospho-Histone H3 increased, indicating an increased percentage of cells blocked in M phase (analysis shown in panel E).

To determine if there was indeed a correlation between microtubule disruption and accumulation in M phase, the dose-response curves for anti-β-tubulin and phospho-Histone H3 were compared (shown in panel F). At low doses of colcemid, the percentage of cells positive for β-tubulin is close to 100% and the percentage of cells in M phase is below 5%. However, as the colcemid concentration increases, the percentage of cells positive for β-tubulin decreases while the percentage of cells in M phase increases. At colcemid concentrations above 15 ng/mL, the percentage of cells positive for β-tubulin approaches background levels and the percentage of cells accumulating in M phase reaches its maximum (40% to 50%), indicating the dependence of the cell cycle on microtubules.

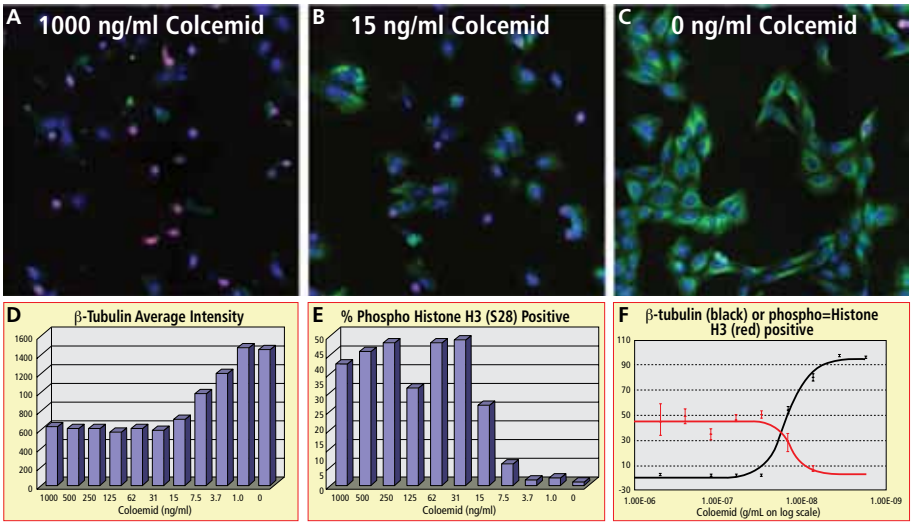

Figure 7. Effects of colcemid on microtubules and cell cycle.

U-2 OS cells were plated as described in the Methods section. After overnight culture, cells were treated with increasing concentrations of colcemid or medium (control) for four hours and then fixed and permeabilized using the methanol procedure. Cells were then stained with Alexa Fluor® 647 anti–β-tubulin and Alexa Fluor® 555 anti–phospho-Histone H3 simultaneously, washed, and stained with Hoechst 33342 before imaging. Images were captured using a 20x (0.75 NA) objective. Hoechst staining is pseudocolored blue, β-tubulin staining is pseudocolored green, and phospho-Histone H3 staining is pseudocolored red. Colocalization of the pseudocolored phospho-Histone H3 and Hoechst stains is seen as purple in the images. Cells in panel A were exposed to 1,000 ng/mL of colcemid, those in panel B to 15 ng/mL of colcemid, and those in panel C were controls. The data in panels D, E, and F was generated. For β-tubulin (panels D and F), the cytoplasmic intensity was measured using an 8-pixel ring dilated out from the nuclear mask (identified by Hoechst staining). For phospho-Histone H3 (panel E), the data is reported as the percentage of cells positive above a user-defined threshold that was based on cells negative for the phospho-Histone H3 stain. In Panel F, the phospho-Histone H3 data (red) was generated as for panel E, and for β-tubulin (black), the percentage of cells positive was calculated using signal intensity above a user-defined threshold based on β-tubulin negative cells (high drug treatment). Bar graphs are shown as the mean from n = 2 wells. Dose-response values are shown as the mean from n = 2 wells ±SEM.

In Situ Detection Using Primary Conjugates

In-situ staining applications enable the study of defined cells within the micro-environment of a tissue. Using directly conjugated antibodies, multiple targets within a tissue sample can be simultaneously detected with relative ease. In the example shown in Figure 8, mouse gut was used as a target tissue to identify proliferating cells using BrdU. BrdU, a thymidine analog, is incorporated into the DNA of cells progressing through S phase and is identified using an anti-BrdU antibody. Therefore, control mice were compared to mice that had received an intra-peritoneal injection of 1 mg of BrdU 24 hours prior to sacrifice. Paraffin embedded gut sections were then simultaneously stained with Alexa Fluor® 488 anti-BrdU (Cat. No. 558599), Alexa Fluor® 647 anti–β-tubulin, and Alexa Fluor® 555 anti-actin (Cat. No. 558622). The anti-BrdU antibody identified proliferating cells only in BrdU-treated mice (Figure 8). In addition, the anti-actin antibody identified the smooth muscle of the small intestine while the anti–β-tubulin antibody identified β-tubulin in all cells, but the stain was particularly useful in the identification of the columnar cells.

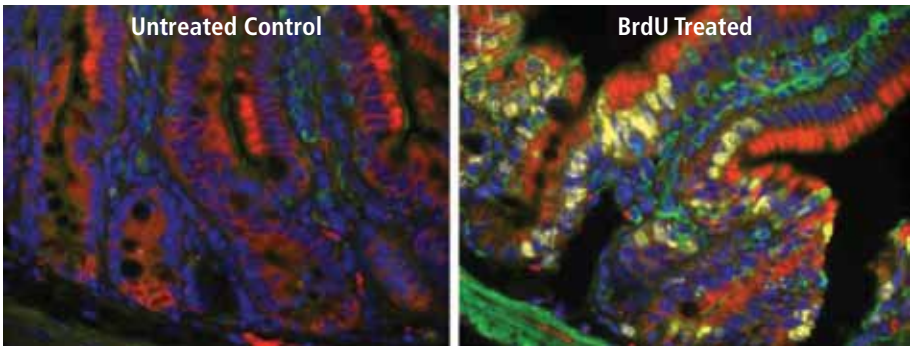

Figure 8. Immuno staining of paraffin embedded mouse gut sections with primary conjugated antibodies.

Gastrointestinal sections were stained as described in the Methods section. Images were captured as a 2 x 2 montage in confocal mode using a 40x (0.9 NA) objective. Five z sections separated by 0.8 microns were acquired and collapsed for display purposes. Hoechst staining is pseudocolored blue, actin staining green, β-tubulin staining red, and BrdU staining yellow.

Summary

BD Biosciences is working to eliminate the need for researchers having to source multiple antibodies to find reagents that work well in imaging applications. We have identified BD Bioimaging Certified monoclonal antibodies that recognize important proteins in the areas of signal transduction, cell cycle, apoptosis, and cancer. Each of these reagents must meet the following criteria to qualify as a BD Bioimaging Certified antibody. The antibody must exceed a signal intensity threshold over background in commonly used cell lines, work with widely used fixation and permeabilization methods, localize to the appropriate region within a cell, and show an appropriate response (when applicable) following stimulation. Developing this line of reagents using monoclonal versus polyclonal antibodies also ensures consistent results by reducing lot-to-lot variability. To date, we have identified several hundred BD Bioimaging Certified antibodies, and will continue to identify additional reagents in major research application areas, including neurobiology and stem cells.

In this application note we have demonstrated the utility of these reagents to detect specific cellular responses such as induction of apoptosis (for example, anti-active caspase-3–specific antibody), cell signaling events (for example, anti–phospho-STAT antibodies), cell cycle status (for example, anti-BrdU and anti–phospho-Histone H3 antibodies), and subcellular structures (for example, anti– β-tubulin antibody). In addition to the unlabeled BD Bioimaging Certified antibodies, we are also developing BD Bioimaging Certified primary conjugated antibodies that enable true high-content screening. These reagents will be available in several colors, promoting a mix-and-match approach to multiplexing. We anticipate that multiplexing conjugated antibodies will allow researchers to gain 2 to 3 fold more information from a cell than previously was possible. This ultimately will enable the development of novel high-content cell-based assays.

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