ThermoFisher SCIENTIFIC

Western blotting

In Cell Western™ Analysis with iBright™ FL1500 Imaging Systems

Introduction

In cell western (ICW) also commonly known as, in cell ELISA (ICE), cytoblot, or cell-based ELISA, is a simple and rapid immunocytochemistry method for the qualitative and quantitative analysis of protein expression and protein post-translational modifications in cultured cells. This method relies on antibodies whose specificity has been confirmed by western blotting. ICW is a high-throughput assay that involves seeding and treating cells in microplates, followed by fixation, and probing with primary and secondary antibodies. ICW is utilized to:

- Measure the relative amount of protein phosphorylation in a signaling pathway
- Monitor inhibitor or activator activity
- Screen cell lines, antibodies libraries and drug-targets

With the iBright Imaging Systems it is now possible to capture the image and analyze ICW data as well as visually assess cell density and uniformity of cell adherence. Quantitative analysis can be easily performed in the iBA software with preconfigured data formatting

Protocol

This protocol has been optimized for the cell line HCT116 and the specified targets and can be used as a guide to develop protocols for other cell lines and antibodies. HCT116 cells were cultured in DMEM-High Glucose medium with 10% FBS and Penicillin-Streptomycin, and plated into 96 wells plates at a concentration of 20,000 cells per well. The cells were allowed to adhere overnight and reach a confluency of ~90%. Where indicated, cells were serum starved with serum free medium for 16 hours and then treated with serial dilutions of IGF-1 starting at 200 ng/ml, for 15 mins at 37°C. The cells were then fixed with 4% PFA and permeabilized with 1x Triton X-100 detergent. They were then blocked with 1X Blocker™ FL Fluorescent Blocking Buffer prior to incubation with primary and secondary antibodies. The cells were washed 3 times with 1X TBS-T after the primary and secondary antibody incubations. Plates were then covered with tinfoil to prevent photobleaching. Images of the plates were captured on an iBright FL1500 imaging system, upon removal of the tinfoil.

Note: After the cells have been fixed, the plates can be stored at -80°C for long term storage

How to capture an ICW 96 well micro plate image

Note: Before imaging, remove as much liquid as possible from the wells, by inverting, flicking, and blotting the plate on a paper towel.

1. Remove the lid from the plate and place it upside down on the center of the turntable of an iBright Imaging System.
   a. Using the Invitrogen™ iBright™ System Tray Adapter for E-Gel™ 48-/96-well Agarose Gels (A56599) helps ensure the plate is centered and straight on the turntable which helps ensures optimal zoom, improving image quality (Figure 1A).
   b. Additionally, the grid in "**Sample Rotation**" tool helps ensure the plate is vertically and horizontally straight, which in turn facilitates template placement upon analysis. To do this select "**More options**", "**Camera**", "**Sample Rotation**". Check the "**Show grid lines"** box and use the arrows to rotate as needed (Figure 1B).
2. Close the drawer and select "**Fluorescent Blots Mode**", then select the desired dyes.
3. The camera focus may need to be adjusted to account for the height of the plate. To do this select "**More options**", "**Camera**", "**Zoom/Focus**". Once the desired zoom level is achieved, (1.5x zoom helps provide maximum image resolution and facilitates alignment of the plate to the analysis template) select "**Auto focus**" to re-focus the camera on the plate (Figure 2).

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4. Select "**Done**" and capture an image using the "**Smart Exposure™ automatic exposure**" feature for an optimized exposure time.
5. If analysis is desired, export the image from the instrument as a g2i file, and then import into the iBright Analysis (iBA) Software for further analysis and annotation.

Note: For more information on importing images into the iBA software, see the user guide.

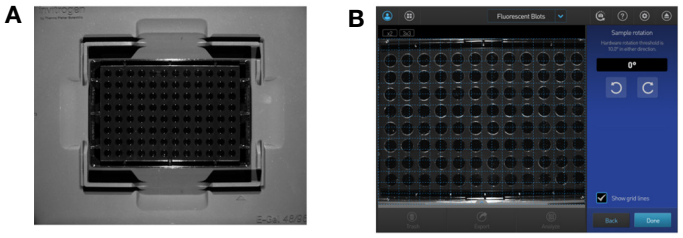

Figure 1. Live view of a 96 well plate on an iBright FL1500 using the Invitrogen™ iBright™ System E-Gel Tray Adaptor, 48/96-well (A) and grid guided sample rotation (B)

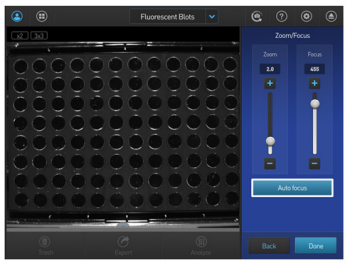

Figure 2. Re-focusing of the camera using the Auto Focus function

Analyzing images in the iBright Analysis Software

Open the desired image file in the "**Image Analysis**" viewport and select the "**Flip**" icon in the "**Adjust Image**" accordion on the right side of the screen (Figure 3A). This will cause a sub-menu to appear under the image. Select the "**Flip Horizontal**" icon to view the data in the appropriate orientation (Figure 3B). Deselect the "**Flip**" icon in the "**Adjust Image**" accordion to save the changes.

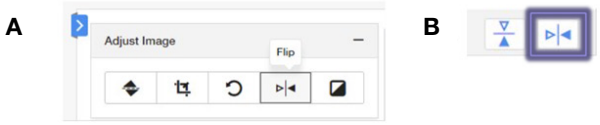

Figure 3. How to enable image Flip in iBA software (A) and selections for Vertical flip or Horizontal flip (B).

Under the "**Analyze Image**" accordion, select "**96-well ICW**" in the "**automatic analysis**" option drop down menu (Figure 4A). Select "**Yes**" in the pop up menu to confirm changes. To adjust template and wells, select and drag corners or handles to resize. Once adjustments are completed, select "**Apply**" to save any changes (Figure 4B). Under the "**Analyze Image accordion**", select the "**Data**" tab to view data in a 96 well plate formatting for exporting and easy analysis (4C). Data for volume, local background corrected volume, and area will be displayed (4D).

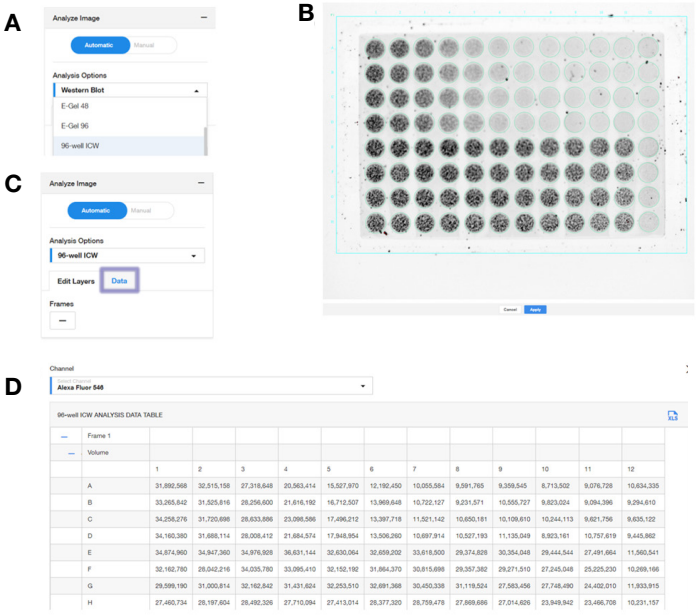

Figure 4. Selecting 96-well ICW template (A) and adjusting the template and wells for accurate analysis (B). Selecting the Data tab (C) will show data displayed in a 96 well plate format (D).

Target activation with treatment of IGF-1

After HCT116 cells were treated with IGF-1, the plates were probed with primary antibodies against AKT, pAKT, pPRAS40, and pPTEN at 1:500 dilutions to determine the phosphorylation response to the treatment (Figure 5). The targets were detected with fluorescently-abeled secondary antibodies at 1:1,000 dilutions. Primary antibody concentrations may require optimization dependent on cell lines and targets used.

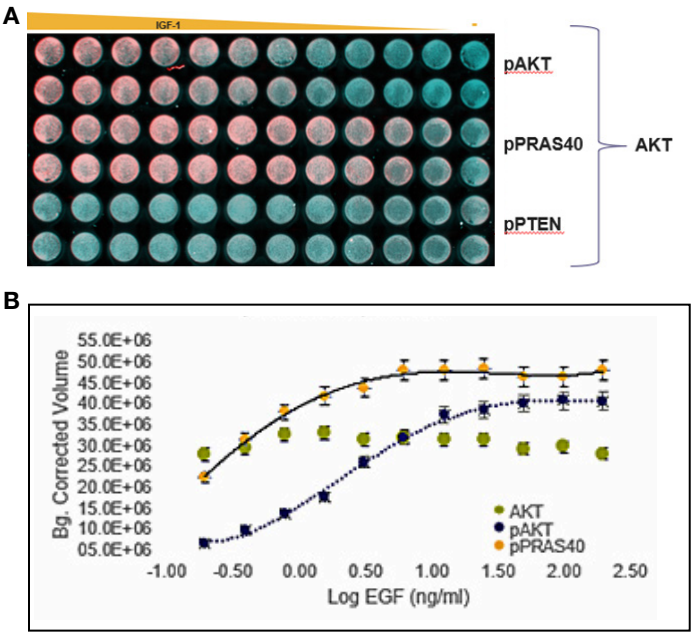

Figure 5. Composite color overlaid image of dose responses of pAKT, pPRAS40, pTEN and AKT in cell line HCT116. Phosphorylated target proteins were imaged on the iBright FL1500 in channel 647 and compared with total AKT expression levels (phosphorylated and non-phosphorylated), imaged in channel 488 (A). pAKT reveals a sigmoidal response to IGF-1, while a saturated response can be observed for pPRAS40 at higher concentrations of IGS-1. (B).

Whole cell stains imaged on the iBright FL1500 Imaging System

HCT116 cell line was plated with a serial density dilution and stained with Qtracker™ 800 and HCS CellMask™ Orange Stain (Figure 6). Qtracker™ 800 uses a custom targeting peptide to deliver Qdot nanocrystals into the cytoplasm of the live cell. It provides an intense, stable fluorescence that is retained during cell fixation. Qtracker is offered in a range of 8 dyes and is an excellent normalization tool for multiplexing protocols.

HCS CellMask™ Orange Stain similarly stains the whole cell by labelling the cytoplasm and nucleus. It can be applied immediately after fixation or in the last step of the multiplexing protocol, if used for normalization. When using whole cell stains for normalization, the optimal concentrations may need to be experimentally determined.

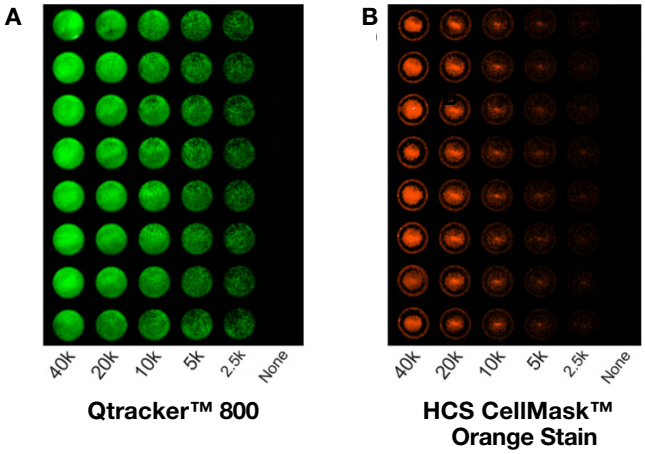

Figure 6: Cell density titer visualized using whole cell stains. Qtracker ™ 800 is a long stoke shift dye (Ex/Em 405-760/800 nm) and must be imaged in universal mode in iBright FL1500 imaging systems (A). A fluorescent custom dye was created with X1 excitation and M5 emission filters selected. HCS CellMask™ Orange Stain is a fluorescent dye (Ex/Em 556/572 nm) that can be imaged with channel 2 (Alexa Fluor 555) on the iBright FL1500 imaging systems (B). Smart exposures were used for both captures to display optimal images.

Conclusions

The iBright Imaging Systems offer a range of extended application support far beyond Western blots and DNA gels. The iBright FL1500, with its five fluorescence channels, support a wide selection of compatible fluorophores used in ICW assays. Additionally, the powerful detector and intuitive analysis software further enhance the instrument's versatility.

Materials

- HCT116 Cells
- Gibco DMEM (Cat.No: 11995040)*
- Gibco HBSS (Cat.No: 14175079)*
- Gibco Penicillin-Streptromycin (Cat.No: 15140122)
- Gibco FBS (Cat.No: A3160401)*
- Thermo Scientific™ 96 Well Black/Clear Bottom Plate, Poly-D-Lysine Coated Surface (Cat.No: 152037)
- Gibco Human IGF-I Recombinant Protein (Cat.No: PHG0071)
- Thermo Scientific Paraformaldehyde, 16% (Cat.No: 043368-9M)
- Blocker™ FL Fluorescent Blocking Buffer (Cat.No: 37565)
- Pierce™ TBS Tween 20 Buffer (Cat.No: 28360)
- Invitrogen Qtracker™ 800 Cell Labeling Kit (Cat.No: Q25071MP
- Invitrogen™ HCS CellMask™ Stains (Cat.No: H32713)
- Phospho-Akt (Ser473)
- Phospho-PRAS40 (Thr246)
- Phospho-PTEN (Ser380)
- Akt (pan)
- Goat anti-Rabbit Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 488 (Cat.No: A32731)
- Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 647 (Cat.No: A-21235)
- Invitrogen iBright FL1500 (A44241)

Troubleshooting

Problem	Possible cause	Solution
Cells are detaching during plating and washing steps of protocol	Dispensing of wash solutions into wells is causing cell detachment	Avoid pipetting directly onto cells. Always pipette down the side of the well and decrease pipette speed if needed.
	Removing wash solutions from wells is causing cell detachment	Gently invert and flick the plate to remove most of the liquid. Then place plate upside down on a paper towel to allow remaining liquid to drain.
	Cells do not have very adherent properties and are not strongly adhered to the plate	Use poly-D-lysine coated plates for cell lines that do not strongly adhere
Weak or no signal	Insufficient amount of primary antibody	Use an antibody titration to determine best antibody concentration. For ICW, primary antibodies may need a strong concentration, as low as 1:500 dilution
	Exposure time is too short	Increase exposure time or use the Smart Exposure feature to determine an optimal exposure time
	Cell density is too low, or cells are not confluent enough	Signal is best detected when cells are allowed to reach ~90% confluency
Background is too high in wells	Plate may be auto fluorescing	Use 96 well black flat well plates to avoid cross talk and auto fluorescent issues
My images are blurry	Camera's focus is not adjusted for the height of the plate	Use the auto focus function found under more options to readjust the camera focus
I am doing a lot of adjustments to my well template during analysis	The plate is not vertically and horizontally straight	Use of the Invitrogen™ iBright™ System Tray Adapter for E-Gel ™ 48-/96-well and grid guided sample rotation will assist in centering and straightening your sample for less manipulation in downstream analysis
I am seeing bleed through in my multiplexing protocols	The appropriate channels were not selected for multiplexing	When multiplexing, choose these channels in the following order to avoid bleed through: 1. Channel 555 2. Channel 647 3. Channel 800 4. Channel 488 *Never use channel 647 & 680 together as their filter sets are overlapping

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