Using Total Cell Staining and Advanced Imaging to Unlock the Full Power of In-Cell Westerns

Introduction

In the dynamic landscape of life sciences research, the need for precise, high-throughput, and versatile methods to study cellular responses has never been greater. The study of cellular responses to various stimuli, including changes in protein expression and post-translational modifications, is pivotal to understanding fundamental biological processes and advancing therapeutic intervention. In-Cell Westerns (ICW) have emerged as a powerful and versatile technique, facilitating the study of cellular behaviors at a molecular level.

The In-Cell Western assay is a fluorescence-based immunodetection technique that combines the principles of immunofluorescence and Western blotting, allowing researchers to probe specific target proteins within whole cells. This innovation has revolutionized the field by bridging the gap between conventional Western blotting, which requires cell lysis and disrupts cellular environments, and live-cell imaging, which often lacks the molecular specificity of immunodetection. In-Cell Westerns enable the quantitative assessment of protein expression and modifications directly within the cell and surrounding cellular environment, preserving critical physiological context.

As In-Cell Westerns continue to advance our understanding of the cellular world, the significance of two crucial components – total cell staining and advanced imaging – becomes increasingly apparent. These elements, when combined, play a pivotal role in unlocking unparalleled insights and ensuring the reliability of data in ICW experiments.

Total cell staining lays the foundation for data accuracy and normalization. Using a total cell stain for normalization corrects for variations in cell number between samples, ensuring that protein expression data reflect true changes in individual cells rather than differences in cell density.

In addition to total cell staining, a high-resolution laser scanner is an indispensable tool to capture the subtleties, dynamics and nuances of cellular responses. However, even with an advanced imaging system, interference between imaging channels can compromise data integrity and accuracy. Using a multi-channel imaging system with little to no cross-talk is crucial to ensure the quality of data by reducing signal interference and cross-talk. The combination of a top-tier multi-channel laser scanner with high-quality, validated reagents enables researchers to differentiate between specific signals effectively, allowing for the capture of even the most subtle variations in protein expression.

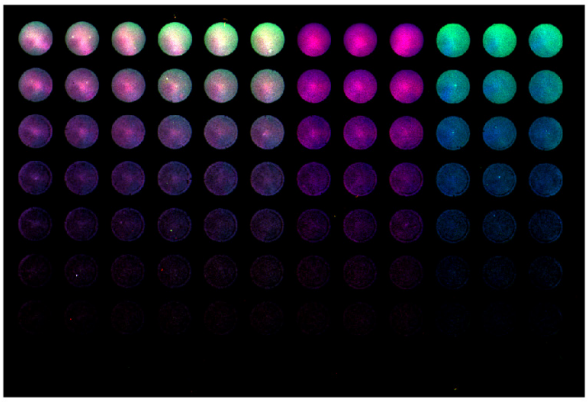

Figure 1. Image of In-Cell Western experiment.

Materials

Step	Material Used	Source	PN
Cell Culture	HeLa Cells	ATCC	CCL-2
	96-well Plates	Greiner	655097
	EMEM Media	ATCC	30-2003
	Fetal Bovine Serum	ATCC	30-2020
	0.25% Trypsin	ATCC	30-2101
	PBS	ATCC	30-2200
	IFN alpha	CST	36000S
In-Cell Western	Methanol	Fisher	A452SK-4
	Rabbit anti-STAT3	CST	4904S
	Mouse anti-pSTAT3	CST	4113S
	AzureCyto In-Cell Western Kit Rb700/Ms800	Azure Biosystems	AC2022
	AzureCyto In-Cell Western Kit Ms700/Rb800	Azure Biosystems	AC2023
Imaging	Azure Sapphire FL Biomolecular Imager	Azure Biosystems	IS4000
	532 Standard Optical Module	Azure Biosystems	IS4002
	685 Standard Optical Module	Azure Biosystems	IS4004
	784 Standard Optical Module	Azure Biosystems	30-2200

Method

Cell Culture

HeLa cells were serially diluted and seeded into a sterile 96-well tissue culture plate at a volume of 115µL per well. The plate was incubated overnight before treating cells in columns 4–12 with IFN-alpha for 15 minutes. Wells were then washed with PBS prior to performing the In-Cell Western.

In-Cell Western

Cells were fixed with 100% methanol for 20 minutes at -20°C then permeabilized by incubating with AzureCyto™ Permeabilization Solution for 5 minutes. Wells were then blocked with AzureCyto Block Solution for one hour at room temperature with shaking. Cells were then probed for STAT3 and/or pSTAT3 according to Table 1 and stained for total cells by co-incubating the primary antibodies with AzureCyto Total Cell Stain overnight at 4°C. Wells were washed with PBS prior to incubation with AzureSpectra 700 and AzureSpectra 800 secondary antibodies for 30 minutes at room temperature. Wells were washed with PBS prior to imaging.

Imaging

Plate was imaged on the Azure Sapphire FL using the 532, 685, and 784 Standard Optical Modules at a resolution of 10µm with the focus set to +3.00mm.

Normalization

To account for variations in cell number and protein content between wells, signals for the proteins of interest in each well were normalized to the signal of the total cell stain for that well. The highest total cell signal intensity was identified for each cell volume of the dilution series. The remaining signal values for each cell volume were then divided by this maximum value to determine a normalization factor for each well. The signals for the proteins of interest in each well were then divided by the corresponding normalization factor to obtain the normalized signal volumes.

Column	Treatment	Probed For
1	Untreated	STAT3 & pSTAT3
2	Untreated	STAT3 & pSTAT3
3	Untreated	STAT3 & pSTAT3
4	IFN-alpha	STAT3 & pSTAT3
5	IFN-alpha	STAT3 & pSTAT3
6	IFN-alpha	STAT3 & pSTAT3
7	IFN-alpha	STAT3
8	IFN-alpha	STAT3
9	IFN-alpha	STAT3
10	IFN-alpha	pSTAT3
11	IFN-alpha	pSTAT3
12	IFN-alpha	pSTAT3

Table 1. Plate columns were treated and probed according to the table above.

Results and Conclusions

The detection of increased expression of pSTAT3 in response to IFN-alpha serves as a compelling illustration of the robust dose and treatment response ICW can capture when combined with total cell staining for normalization. Total cell normalization proved to be instrumental in ensuring the reliability and accuracy of the data by accounting for variabilities in cell volume within each individual well.

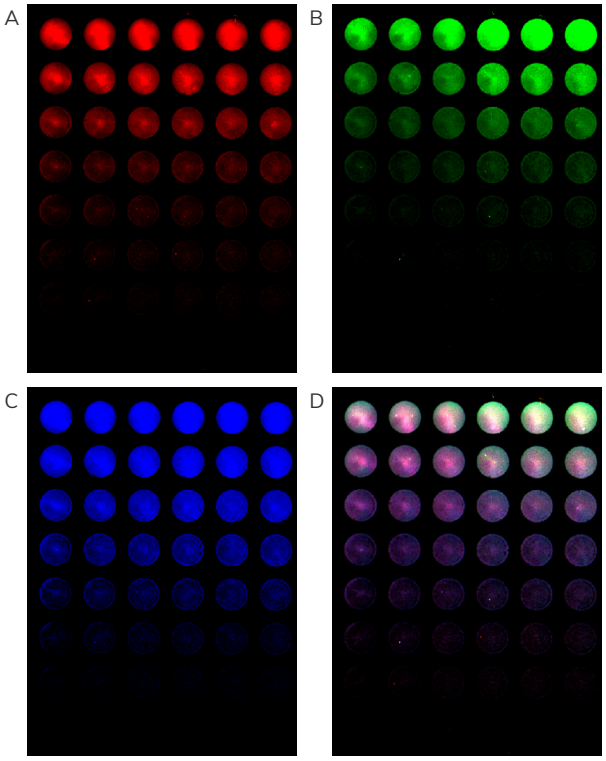

Figure 2. Columns 1–6 illustrate the increased expression of pSTAT3 due to IFN-alpha treatment. A) STAT3 detected with AzureSpectra 700 using the 685 channel of the Azure Sapphire FL (red). B) pSTAT3 detected with AzureSpectra 800 using the 784 channel of the Azure Sapphire FL (green). C) Cell DNA detected with AzureCyto Total Cell Stain using the 532 channel of the Azure Sapphire FL (blue). D) Overlay image of all three channels.

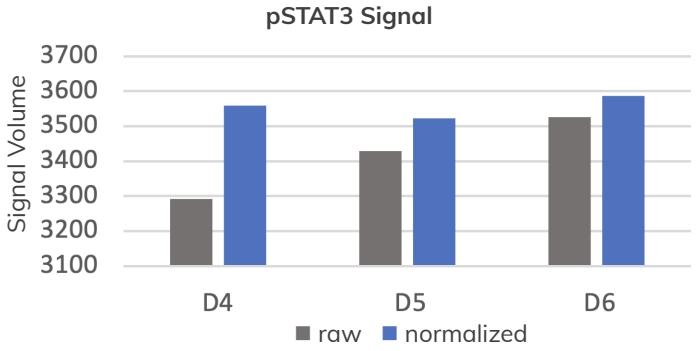

Figure 3. When measuring the pSTAT3 signal (AzureSpectra 800 in green) in wells D4–6, the raw signal volumes initially appear quite different (gray). However, once normalized to the total cell signal in each well, it's apparent the pSTAT3 expression is actually very similar when total cell volume is taken into account.

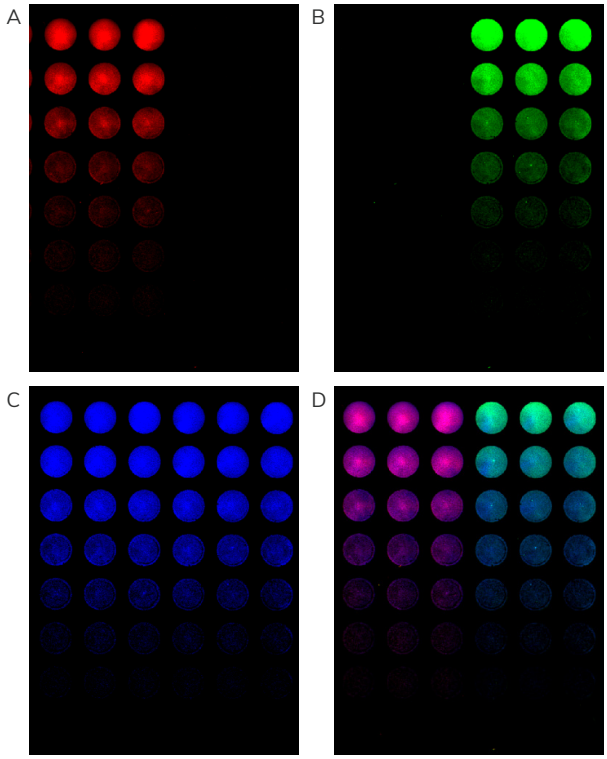

Figure 4. Columns 7–12 were scanned using the Azure Sapphire FL showing high detection sensitivity and low cross-talk between the individual channels. A) STAT3 detected with AzureSpectra 700 using the 685 channel of the Azure Sapphire FL (red). B) pSTAT3 detected with AzureSpectra 800 using the 784 laser of the Azure Sapphire FL (green). C) Cell DNA detected with AzureCyto Total Cell Stain using the 532 channel of the Azure Sapphire FL (blue). D) Overlay image of A and B shows lack of cross-talk between the signal channels.

The absence of cross-talk between individual scanning channels is notable in this In-Cell Western experiment. It instills a high level of confidence in the quality and validity of the acquired signals – the signals detected can be attributed directly to the targeted proteins under investigation. This absence of of cross-talk reinforces the reliability and integrity of the In-Cell Western data.

In this note, we scratched the surface of the capabilities of In-Cell Westerns. The experiment conducted here offers a glimpse into the potential of this technique in the study of intricate cellular responses. In-Cell Westerns are a powerful tool for high throughput drug and treatment screening and for gaining insights into the world of cell signaling and protein dynamics. However, the true potential of ICW shines when coupling robust, validated reagents and total cell staining – providing a comprehensive view of cellular protein expression and allowing for more meticulous and meaningful quantification. Combined with an advanced imaging system with high sensitivity and minimal cross-talk, ICW becomes a dynamic and versatile tool, capturing subtle variations in protein expression and treatment response.

Figure 5. Sapphire FL is a laser scanner designed for multicolor fluorescent imaging, NIR fluorescent imaging, chemiluminescence, phosphor imaging, and more.

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