# Using Single-Cell Westerns to Validate Single-Cell RNA-Seq Data

*Dhananjay Wagh, Ph.D. and John Coller, Ph.D., Stanford Functional Genomics Facility, Stanford University School of Medicine, Palo Alto, CA*
*Jason Stafford, Ph.D. and Kelly Gardner, Ph.D., ProteinSimple, San Jose, CA*

## Introduction

Single-cell gene expression analysis of heterogeneous cell populations such as those contributing to cancer and the immune response has begun to revolutionize our understanding of biology. The increased resolution provided by single-cell technologies allows researchers to identify rare cell subpopulations that play a key role in disease and profile variation in response to treatment across heterogeneous patient samples.

Single-cell RNA sequencing (RNA-Seq) allows researchers to measure transcript levels in thousands of individual cells in a single assay to measure RNA expression heterogeneity. However, cellular mRNA transcript levels do not always directly correlate with levels of functional protein. In fact, mRNA and protein levels can vary significantly due to translational and post-translational regulation. MicroRNAs can modulate gene expression by inhibiting translation of mRNA. Proteins may also be rapidly degraded after they are translated via proteases and the proteasome. Studies have demonstrated that only about 40% of cellular protein levels are directly proportional to mRNA concentration. It is therefore vital to validate any single-cell RNA expression studies with single-cell protein expression data to ensure accurate and complete conclusions about cellular function.

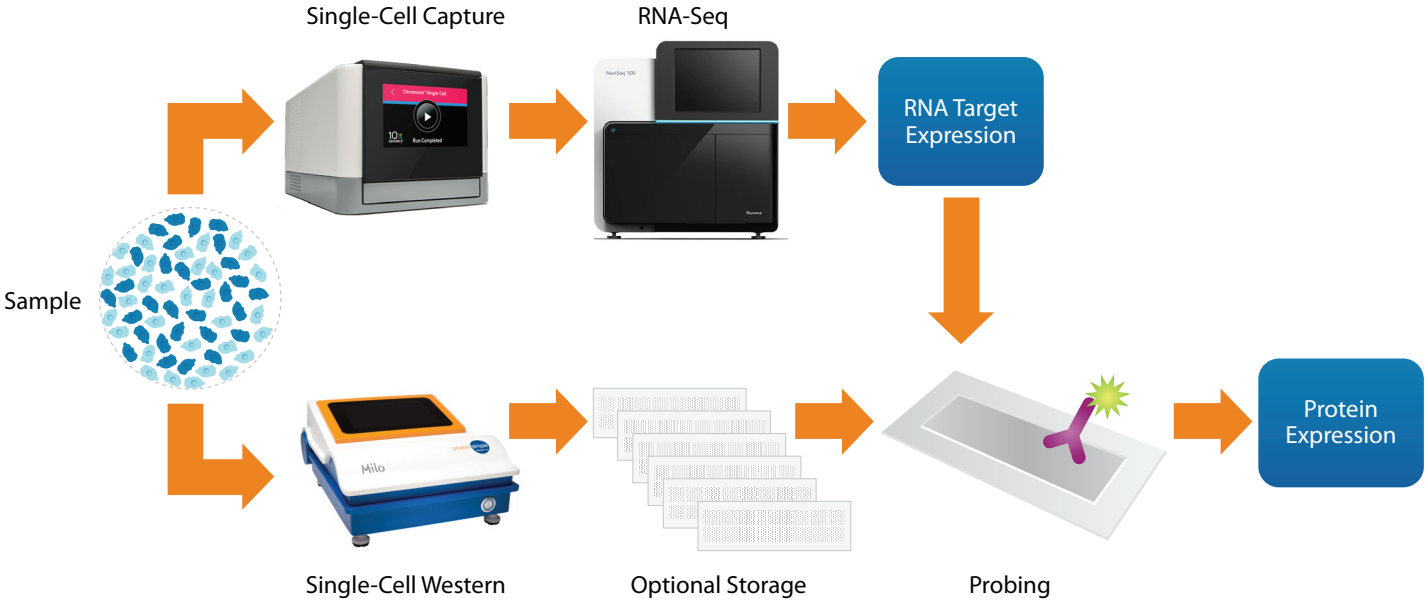



**FIGURE 1.** Workflow for protein validation of single-cell RNA-Seq data.

## Single-Cell Westerns for Protein Expression Heterogeneity Analysis

The Milo Single-Cell Western platform provides scientists with protein validation data for their single-cell RNA-Seq gene expression data (**Figure 1**). Milo users can measure protein expression levels in over 1,000 individual cells per run, and can multiplex with typical assays detecting approximately four proteins per cell simultaneously using a variety of multiplexing approaches.

Regardless of what targets are uncovered in your RNA-Seq run, Milo has the flexibility and versatility to detect them at the protein level. Milo uses commercially-available Western antibodies, giving users access to the broadest set of detection reagents to validate even uncommon targets that emerge from their sequencing runs. Furthermore, users can measure proteins irrespective of where they are located in or on the cell, and can measure transcription factors and protein isoforms which can be challenging to measure by flow cytometry. Single-Cell Westerns can also be used to study post-translational modifications such as phosphorylation that are not revealed by RNA-Seq analysis.

## HIF-1α mRNA and Protein Levels Do Not Always Correlate

Proteins can be rapidly degraded after they are translated, leading to significant differences in the levels of mRNA versus functional protein within a cell. Expression of the transcription factor known as Hypoxia-inducible factor 1-alpha (HIF-1α) is a classic example. HIF-1α mRNA and protein are constitutively expressed in cells. However, under normoxic conditions (when $O_2$ is readily available) HIF-1α is rapidly ubiquinated, targeting it to the proteasome for degradation (**Figure 2**). This process is mediated by an oxygen-dependent prolyl hydroxylase (PHD) and an E3 ubiquitin ligase known as von Hippel-Lindau (VHL) protein.

Under hypoxic conditions (when $O_2$ is scarce), PHD activity is inhibited and HIF-1α escapes proteosomal degradation. HIF-1α heterodimerizes with HIF-1β to form a transcriptionally-active complex that regulates the expression of >60 genes including vascular endothelial growth factor (VEGF) and erythropoetin (EPO), signaling molecules important for increasing $O_2$ delivery to hypoxic

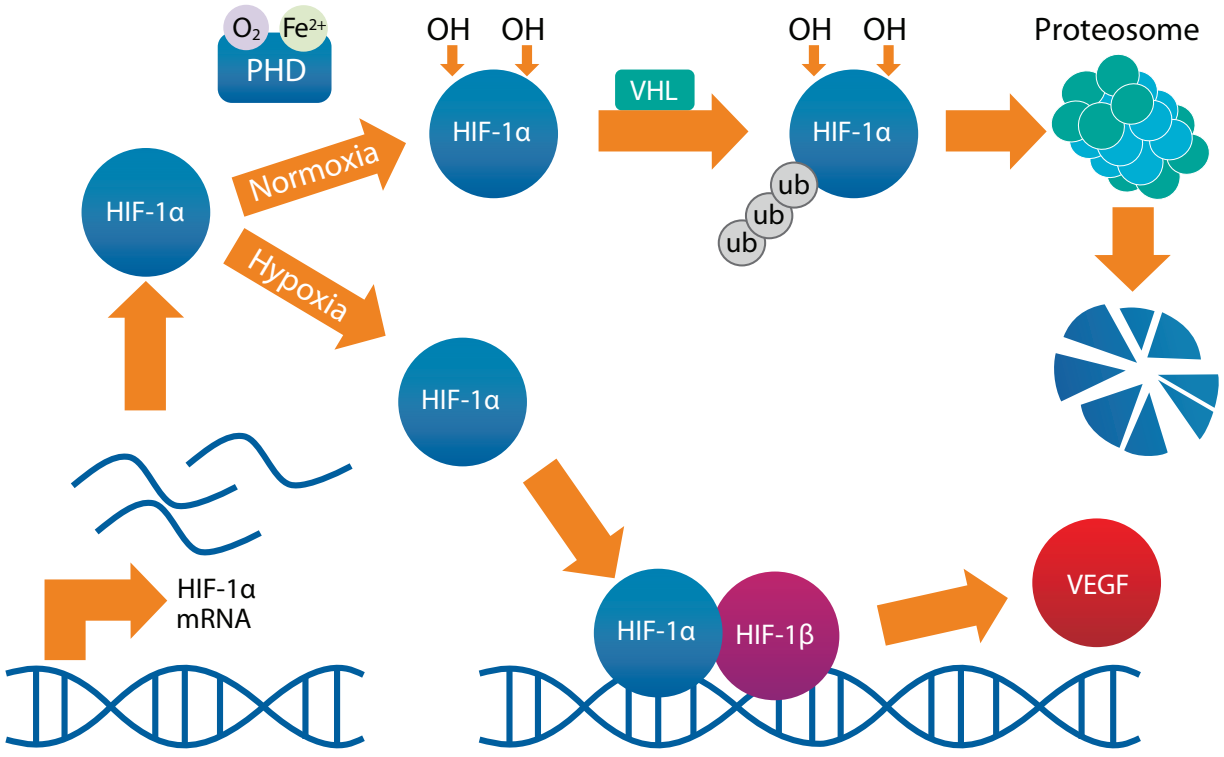



**FIGURE 2. HIF-1α protein regulation**. HIF-1α protein is rapidly degraded when $O_2$ is available (normoxia). When $O_2$ is not available (hypoxia) HIF-1α escapes degradation and acts as a transcription factor.

tissues. As a result, HIF-1α mRNA and protein levels only correlate under hypoxic conditions but differ substantially under normoxic conditions.

## Parallel Single-Cell RNA-Seq and Single-Cell Western Workflow

Highlighting the need to validate gene expression at the protein level, Milo was used in parallel with single-cell RNA-Seq experiments to validate HIF-1α protein levels in a heterogeneous cell population consisting of cells subjected to either hypoxic or normoxic conditions.

HeLa cells were treated for 24 hours with 0.5 mM deferoxamine (DFO), an iron-chelating agent that mimics hypoxia by inhibiting PHD activity. Untreated HeLa cells (normoxic conditions) were used as a control. Manufacturer's guidelines were followed (10x Genomics) for cell preparation. Both treated and untreated cells were dissociated, washed, counted and mixed at a 1:1 proportion.

The mixed sample was then split and analyzed in parallel on the Milo Single-Cell Western system and the Chromium Controller system using the v2 Chromium Single-Cell 3' Solution (10x Genomics) followed by sequencing on a NextSeq 500 (Illumina) (workflow shown in **Figure 1**).

### SINGLE-CELL RNA-SEQ WORKFLOW

One thousand cells (DFO-treated and untreated mix) were targeted for capture using the 10x Chromium Controller and Chromium Single Cell 3' v2 chemistry. The manufacturer's protocol was followed for cDNA amplification, fragmentation and library preparation without any modifications. The library was sequenced on a NextSeq 500 instrument. Data was analyzed using cell ranger (version 1.3.1). A total of 204 cells were captured. About 1.5 million reads per cell were obtained. Normalized expression values for HIF-1α and β-tubulin (BTUB) were extracted from each cell and a scatter plot of HIF-1α vs BTUB was created (**Figure 3**).

### SINGLE-CELL WESTERN WORKFLOW

One thousand cells (DFO-treated and untreated mix) were targeted for capture on an scWest chip using the standard Single-Cell Western workflow. Briefly, 1 mL of a 100,000 cell/mL cell suspension was loaded onto a Large scWest chip and allowed to settle for 10 minutes. Single-cell occupancy was confirmed via brightfield microscopy and the scWest chip was then run on the Milo instrument with the following conditions: 10 sec lysis, 75 sec electrophoresis at 240 V, and 240 sec UV exposure. The chip was then simultaneously probed for 2 hours with a primary antibody cocktail of mouse anti-HIF-1α antibody (BD Biosciences 610958) at 100 µg/mL final concentration and rabbit polyclonal anti-β-tubulin antibody (Abcam ab6046) at a 50 µg/mL final dilution. After washing 3x15 min with Wash Buffer, the chip was then probed for 1 hour in the dark with a secondary antibody cocktail containing donkey anti-mouse IgG Alexa Fluor 647 (Thermo A-31571) and donkey anti-rabbit IgG Alexa Fluor 488 (Thermo A-21206), each at a final concentration of 100 µg/mL. The chip was then washed and imaged on an Axon 4400a microarray scanner (Molecular Devices). Images were analyzed using Scout Software (ProteinSimple) to quantify peak areas for HIF-1α and β-tubulin in each single-cell lysate.

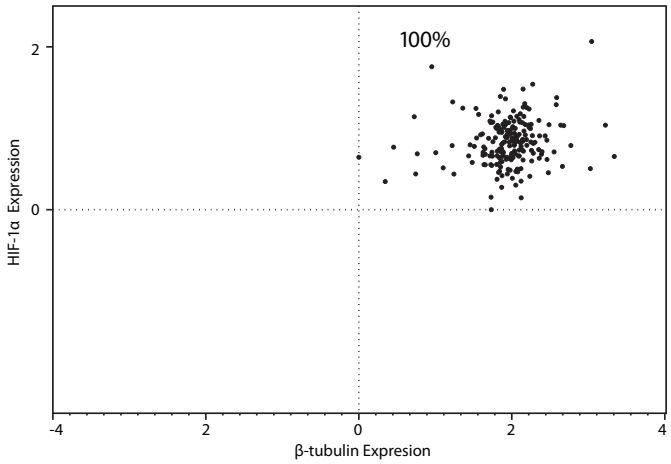
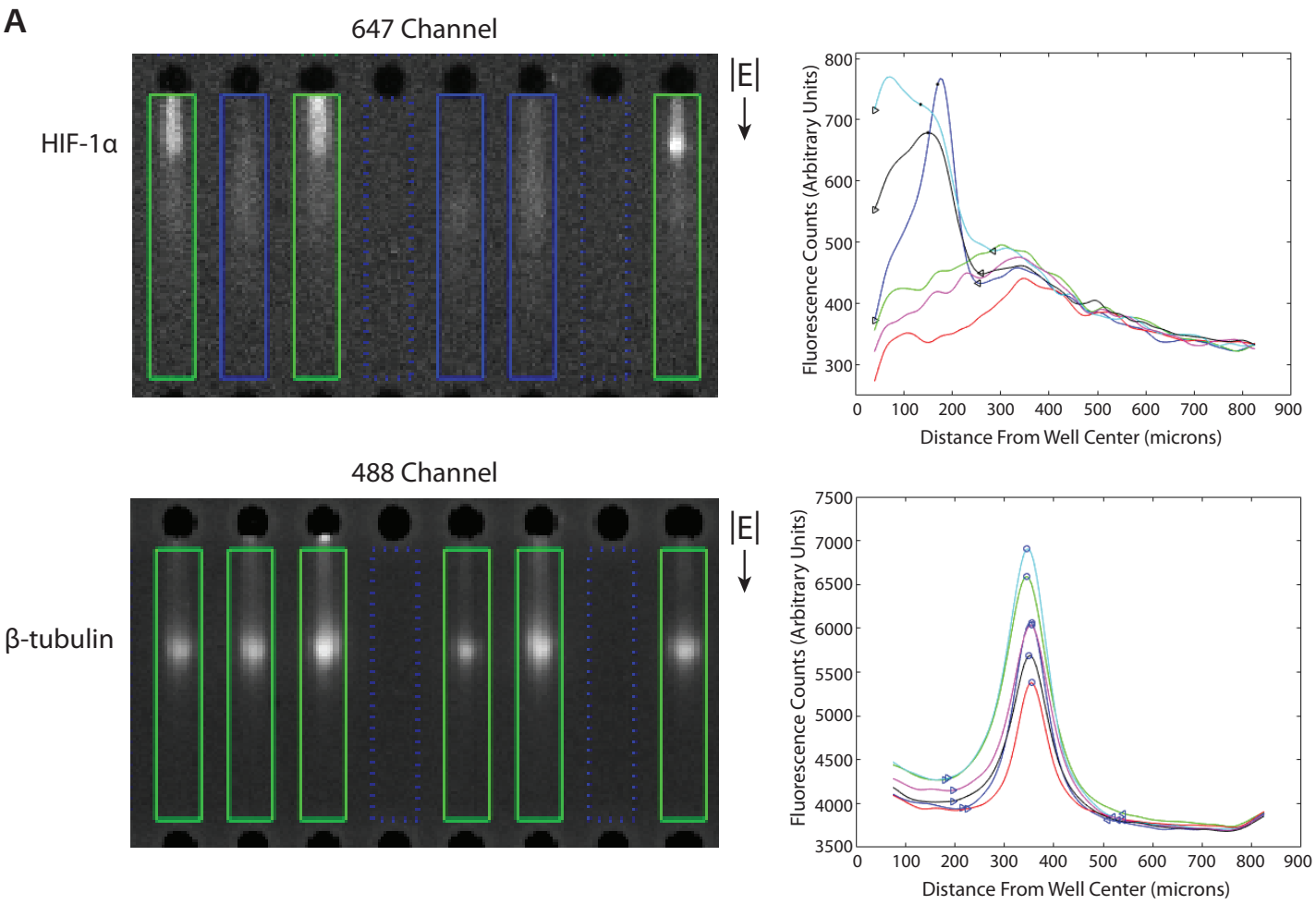



**FIGURE 3. Single-cell RNA-Seq analysis of HIF-1α.** A mixture of DFO-treated and untreated HeLa cells was assayed for expression of HIF-1α. 100% of the cells were found to express HIF-1α RNA. Cell clustering based on β-tubulin expression revealed a single, homogeneous population.

## Cellular Subpopulations Identified by Single-Cell Westerns

RNA-Seq analysis of the HeLa cell mixture showed that 100% of the cells expressed HIF-1α mRNA (**Figure 3**). However, Single-Cell Western analysis with Milo revealed that only approximately 46% of the cells as identified by



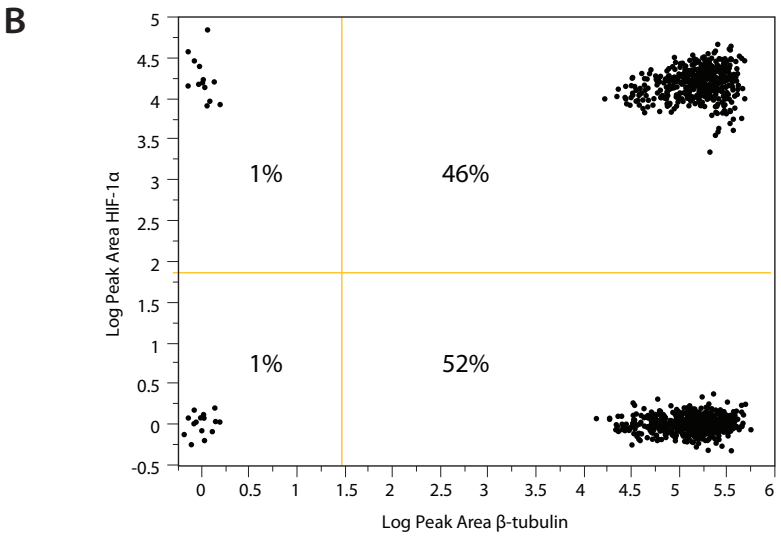




**FIGURE 4. Single-Cell Western analysis of HIF-1α.** A) HIF-1α protein was detected in only a subset of HeLa cells as identified by β-tubulin expression. Example separation images of six cells are shown with HIF-1α protein visualized in the 647 channel and β-tubulin visualized in the 488 channel. Green electrophoresis lanes indicate lanes where peaks were identified by Scout Software, whereas blue lanes indicate lanes with no peaks. Fluorescence intensity plots generated by Scout Software are shown on the right for all β-tubulin+ lanes. B) Bivariate analysis revealed that ~46% of analyzed β-tubulin+ cells were HIF-1α+.

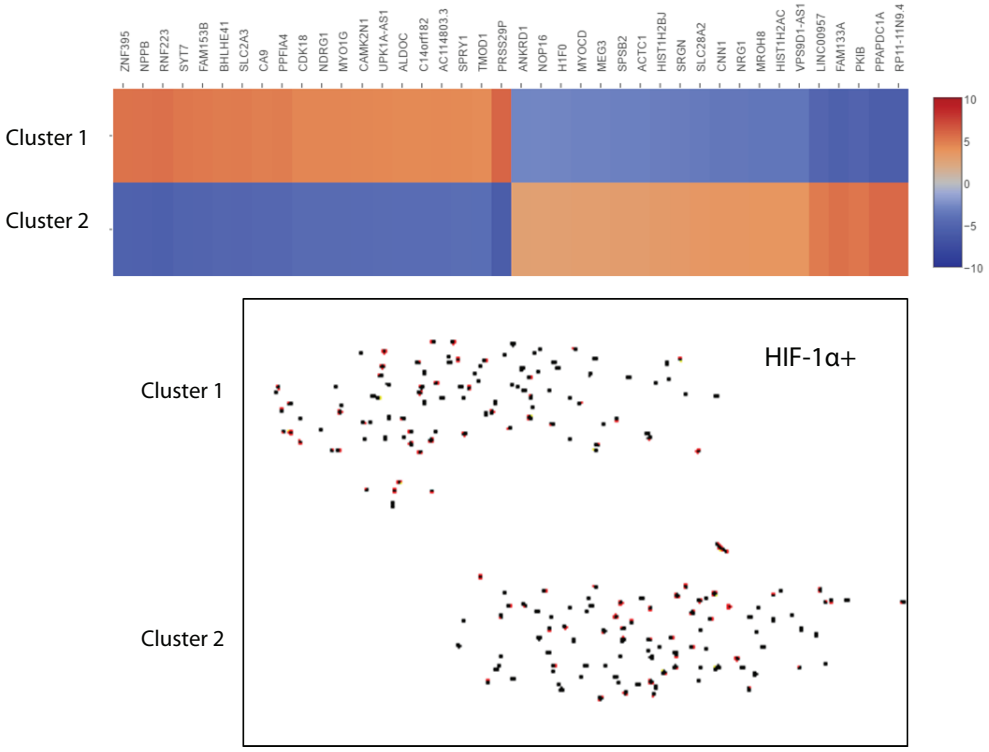



**FIGURE 5. RNA-Seq analysis revealed two distinct cell populations.** A heat map of gene expression shows that the mixture of DFO-treated and untreated HeLa cells contained two populations with distinct gene signatures. Both populations were positive for HIF-1α mRNA. Milo can be used to validate protein expression for these target candidates to understand their functional role in cellular function.

β-tubulin expression stained positive for HIF-1α protein (**Figure 4**). As expected, HIF-1α protein expression was degraded in the population of cells exposed to normoxic conditions. Single-cell protein expression did not correlate with single-cell RNA expression, highlighting the need for both protein and RNA expression information in single-cell gene expression studies.

The single-cell RNA-Seq data also identified two distinct cell populations within HIF-1α expressing cells based on differential expression of genes other than HIF-1α (**Figure 5**), uncovering other genes that may play a key role in hypoxic cellular processes. Milo allows researchers to validate these additional RNA targets revealed by single-cell RNA-Seq experiments with protein expression data which may provide critical insights into the role these genes play in cellular function.

## Conclusion

Validating single-cell RNA sequencing data with single-cell protein expression data is critical when studying gene expression heterogeneity. Single-Cell Westerns offer researchers unrivaled versatility in the diverse targets that can be validated from their sequencing runs. By combining both RNA and protein level expression heterogeneity data, researchers can gain a complete and accurate understanding of the functional changes underpinning disease.

proteinsimple a bio-techne brand

Toll-free: (888) 607-9692
Tel: (408) 510-5500
info@proteinsimple.com
proteinsimple.com



97-3005-00 Rev A