Adapting the Single-Cell Western Protocol to Detect Histone Modifications

Introduction

The Single-Cell Western™ protocol is highly versatile and can easily be adapted for different biological applications and protein targets. Researchers studying signaling proteins or other targets that require treatment of cells prior to analysis can add a drug, cytokine, or other form of pre-treatment directly to scWest chips after cells have been captured and before running the chip on Milo. Cells can also be treated just prior to settling them onto scWest chips. As a result, the flexible Single-Cell Western workflow allows time-dependent experimental manipulations to be easily performed before lysis and electrophoresis.

Here we describe a two-step, on-chip pre-treatment protocol to measure heterogeneity of modified histones — a class of proteins that is challenging to measure but critical in regulating gene expression. This new measurement capability could be key to unlocking new discoveries in the field of epigenetics.

Histone modifications play a critical role in epigentic regulation

Histone post-translational modifications (PTMs) regulate gene transcription by altering the structure of chromatin, a process known as chromatin remodeling. Ever since the human genome was sequenced a little over a decade ago, there has been immense interest in epigenetic regulation of how the DNA code is expressed. Histone-mediated chromatin remodeling plays a major role in this process.

DNA is packaged with histones into structural units called nucleosomes. Each nucleosome is composed of five major histone subtypes: H2A, H2B, H3, H4, and H1. Histones H2A, H2B, H3 and H4 make up the nucleosome core and exist as dimers, while histone H1 acts as a linker that stabilizes the DNA-histone complex (**Figure 1**).

Histone H3 is the most extensively modified of the five major histone subtypes. Mono-, di-, and tri-methylation of histone H3 lysine residues can either activate or repress gene transcription. Tri-methylation of lysine 27 of histone H3 (H3K27me3) represses gene transcription and is thought to play an important role in tumor cell plasticity.

H3K27me3 is transcriptionally repressive and can be difficult to study due to the densely packed heterochromatin. Traditional western blot analysis of histone modifications uses sonication to shear the DNA, disrupt the nucleosome structure, and release the histones from chromatin. Alternatively, histones can be extracted through a multistep process that includes nuclei purification, acid extraction, and TCA precipitation.

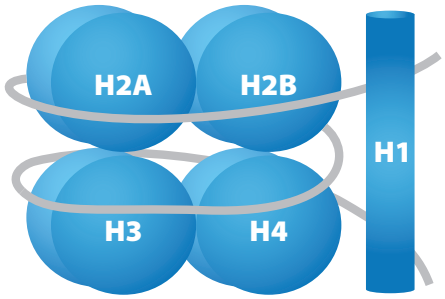

FIGURE 1. Nucleosome structure. DNA wraps around a central core of eight histones and is locked into place by a linker histone (H1).

Modifying the Single-Cell Western protocol to monitor histone PTMs

The open format of scWest chips allows users to modify the Single-Cell Western protocol and develop assays optimized for their targets of interest. In order to make H3K27me3-modified histones physically accessible for Single-Cell Western analysis, we adapted a previously reported in situ chromatin digestion method used for studying DNA-protein interactions via chromatin immunoprecipitation (ChIP).[1] This new method uses a two-step treatment after the cells are settled onto scWest

chips and before they are run on Milo. First, the cellular membrane is permeablized and then the DNA/chromatin is enzymatically digested *in situ*. This method releases chromatin-bound histones and dramatically improves detection of HK27me3 in HeLa cells. On-chip chromatin digestion and variants of this workflow can be used to measure histone modifications in a variety of cell types.

Materials and Methods

CELLS, ANTIBODIES, AND REAGENTS

HeLa cells were purchased from ATCC (Rockville, MD) and maintained in DMEM media. Mouse anti-H3K27me3 antibody was purchased from Abcam (cat# ab6002). Rabbit anti-Histone H3 was purchased from Cell Signaling Technologies (cat# 4499). L-α-lysophosphatidylcholine (LPC) was purchased from Avanti Polar Lipids (cat# 840072). Donkey anti-mouse IgG AF555, donkey anti-rabbit IgG AF647, SuperBlock Buffer (cat# 37515), DNAse I, micrococcal nuclease (MNase), and TCEP-HCl were purchased from Thermo Fisher. Standard scWest chip kits were manufactured at ProteinSimple.

ON-CHIP CHROMATIN DIGEST

HeLa cells were settled onto standard scWest chips at a concentration of 1 x 10^5 cells/mL for 10 minutes. After verifying that 15-20% of the microwells contained a single HeLa cell, the scWest chips were incubated with 1 mL 0.025% LPC in permeablization solution #1 (150 mM sucrose, 80 mM KCl, 35 mM HEPES, 5 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7) at 37 °C for 2 minutes. It is important to reconsitute the LPC from lyophilized powder just prior to use. After permeablizing the cells with LPC, scWest chips were washed with 1 mL permeablization solution #1 (no LPC) and inspected under the brightfield microscope to ensure the cells maintain their morphology. The scWest chips were then incubated with 300 U MNase and 250 U DNase in permeabilization solution #2 (150 mM sucrose, 50 mM NaCl, 50 mM Tris-HCl, 2 mM $CaCl_2$, pH 7) at room temperature for 5 minutes. The chips were then washed with 1 mL Suspension Buffer and run on Milo (**Figure 2**).

RUNNING MILO

Milo Lysis/Run Buffer (R200) was supplemented with 15 mM TCEP-HCl (pH 7). The cell lysis time was set to 10 seconds, electrophoresis was set to 80 seconds at 150 V, and UV capture was set to 4 minutes. The voltage was reduced to 150 V from the 240 V used for the standard Single-Cell Western protocol due to increased conductivity from adding TCEP-HCl to the Lysis/Run buffer.

PROBING AND IMAGING SCWEST CHIPS

After running on Milo, scWest chips were probed with mouse anti-H3K27me3 and rabbit anti-histone H3 primary antibodies diluted in SuperBlock Buffer 1:10 for 2 hours at room temperature. The chips were washed 3 times, 10 minutes per wash with Wash Buffer and then probed with donkey anti-mouse IgG AF555 and donkey anti-rabbit IgG AF647 secondary antibodies diluted in SuperBlock Buffer 1:20 for 1 hour. SuperBlock was used for these experiments, but other blocking buffers may be optimal for other assays. The chips were then washed 3 times for 15 minutes per wash prior to imaging with a GenePix 4400A microarray scanner (100% power, 500 gain). Image files were saved as single-color TIFF (.tif) files.
Note: cytoplasmic proteins commonly used as internal loading controls such as b-tubulin or GAPDH are not compatible with the on-chip chromatin digestion protocol as they can be lost during the membrane permeabilization step.

Data Analysis

Chip images were analyzed using Scout software. Relative H3K27me3 and histone H3 abundance was determined using peak areas calculated by Scout. JMP software was used for graphing signal distributions and statistical analysis. Merged images were created using ImageJ.

FIGURE 2. On-chip chromatin digest protocol.

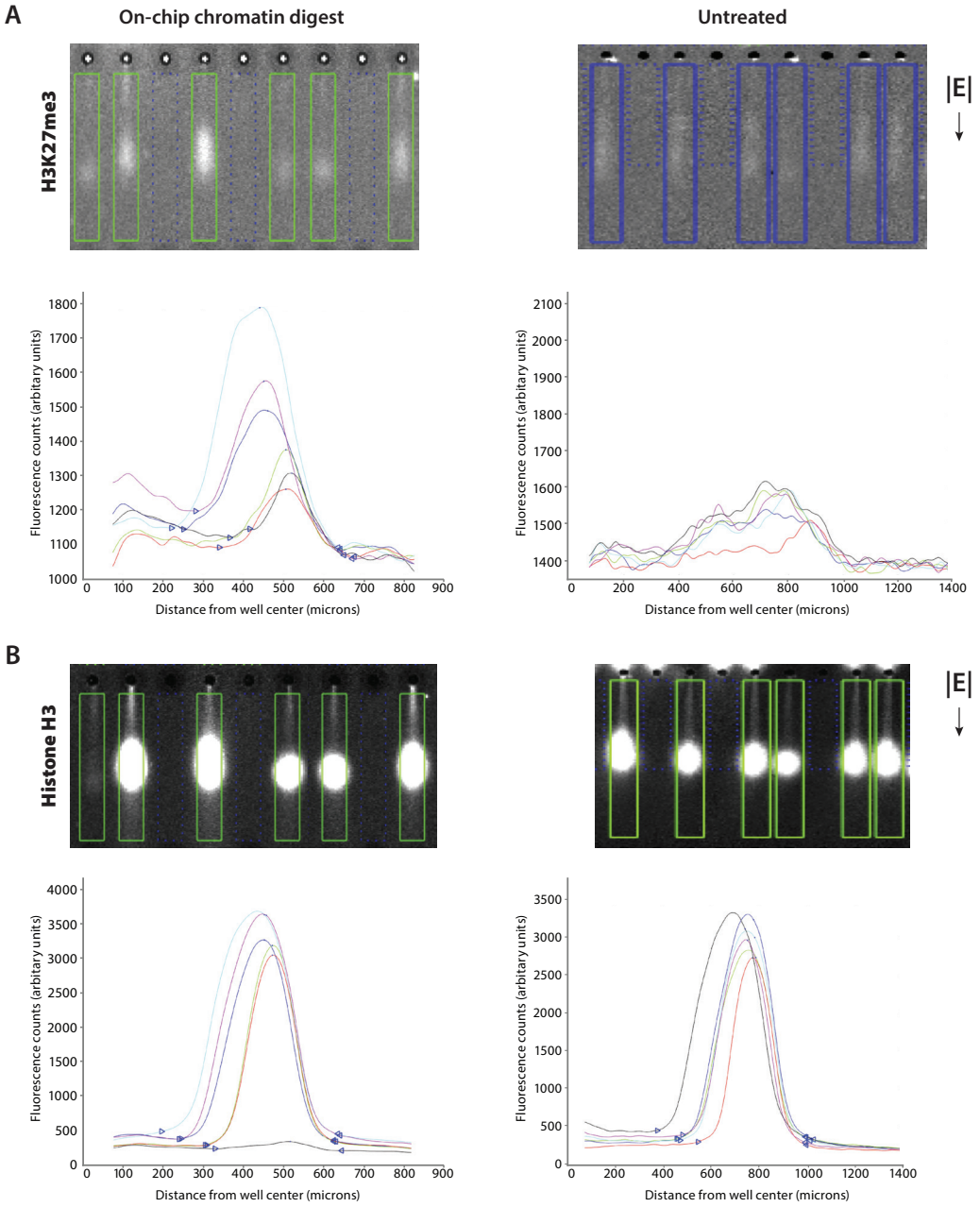

FIGURE 3. **A**) H3K27me3 signals detected in HeLa cells treated with on-chip chromatin digestion are significantly more robust than signals observed in untreated cells. Green electrophoresis lanes indicate peaks are identified in the lane by Scout software, whereas blue lanes indicate that peaks were not detected. **B**) Signals for the histone H3 internal loading control are similar for both treatment groups.

Results and Discussion

H3K27me3 was successfully detected in single HeLa cells treated with the on-chip chromatin digestion protocol (**Figure 3A**). In comparison, H3K27me3 signals in untreated HeLa cells were weak and well below the acceptable peak detection threshold defined by Scout software (**Figure 3A**). Single-Cell Western detection of the histone H3 internal loading control was comparable between both treatment groups (**Figure 3B**). Single-Cell Western detection of H3K27me3 was validated by co-migration with total histone H3 (**Figure 4A**). H3K27me3 peak area, a measure of relative abundance in each cell, spanned approximately one order of magnitude, whereas H3 expression was more uniform in the majority of cells. H3K27me3 was detected in approximately 68% of the cells identified by histone H3 (**Figure 4B**).

This basic on-chip chromatin digestion method can be customized to detect other histone modifications

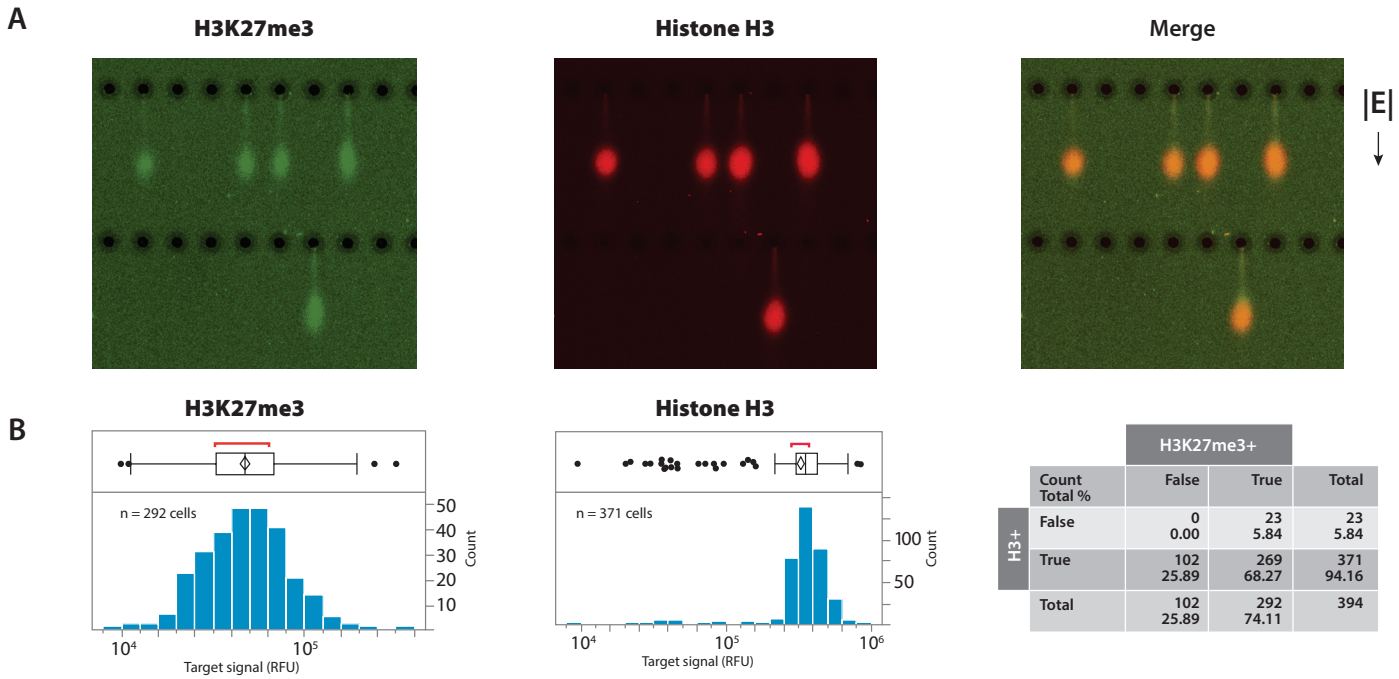

FIGURE 4. **A**) H3K27me3 signal co-localizes with total histone H3. Single-Cell Western detection of H3K27me3 (green) was validated by co-migration with total histone H3 (red). **B**) H3K27me3 and histone H3 signal distributions (RFU) and co-detection analysis. H3K27me3 was detected in ~68% of cells identified by histone H3 expression.

in other cell types. Key parameters to consider are: LPC concentration, MNase concentration and DNase I concentration. If the recommended LPC concentration is too high for a particular cell type it can lyse the cells prematurely, so it is important to visually inspect the chip to determine if cells remain intact after the membrane permeabilization step. If the LPC concentration is too low, the membrane will not be efficiently permeablized and the chromatin-digesting enzymes (MNAse/DNase I) will not enter the cell. Titrating enzyme concentrations may also be necessary depending on the cell type and particular histone modification being investigated. MNase cuts linker DNA between nucleosomes whereas DNAse I cuts along the entire length of the DNA.

Conclusion

The simplicity of the Single-Cell Western assay combined with the open format of scWest chips makes Milo a flexible platform for single-cell protein analysis. Researchers studying protein targets that require pretreatment of cells can easily amend the standard Single-Cell Western workflow to fit their specific needs. We demonstrated that a simple pre-treatment that takes approximately 10 minutes allowed detection of H3K27me3-modified histones in individual cells. This method could potentially be applied to the investigation of other histone modifications and may be useful for any researcher interested in studying epigenetic regulation of gene expression at the single-cell level.

References

1. Assays of nucleosome assembly and the inhibition of histone acetyltransferase activity. (11) Digestion of chromatin; and (12) Purification and characterization of DNA after digestion of chromatin, T Yamasaki, T Murata, C Jin, K Kato, M Noguchi, K Nakade, J Pan, K Nagata, and K Yokoyama, *Protocol Exchange*, 2007; doi:10.1038/nprot.2007.340.

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