Protein Expression Heterogeneity with Milo, the First Single-Cell Western System

Introduction

Every cell is unique, making cell-to-cell heterogeneity important in many areas of biomedicine including cancer pathogenesis, immuno-oncology and regenerative medicine. More and more high-profile publications are using single-cell analysis techniques to reveal variability in cellular response to a drug or stimulus.[1] They're also uncovering variation in drug target expression within a tissue and identifying important subpopulations of cells within complex samples that play key roles in disease progression. Single-cell protein expression information is critical when you need to understand the fundamental composition and behavior of complex biological samples.

Milo, the first and only Single-Cell Western platform out there, lets you do Western blotting at the single-cell level. Now you can run Westerns on thousands of individual cells in parallel and get robust, Western-based information on protein expression heterogeneity in your cells. He also multiplexes so you can measure multiple proteins in each single cell. That means you can get a better understanding of correlations between target expression and characterize cell signaling in specific target-positive subpopulations of cells. Where your target is located in a cell doesn't matter to Milo — his fast, simple workflow lets you measure proteins both on and in each individual cell with the same workflow. It's easy to detect surface proteins and you don't have to worry about fixing and permeabilizing your sample to measure intracellular proteins. As an added bonus, Milo uses conventional Western antibodies which means you can measure diverse protein targets — even ones that don't have good flow cytometry antibodies. The best part? Scout™ Software automates your data analysis and gives you quantitative protein expression measurements in each single cell.

A thousand single cells, just one chip

Milo runs Single-Cell Western assays on scWest chips. This patterned, pre-cast polyacrylamide gel consumable contains 6,400 microwells and captures ~1,000 single cells to run 1,000 Single-Cell Westerns in parallel (**Figure 1**).

All you have to do is load your sample on the scWest chip and put the chip in Milo — he takes it from there. In just five minutes, he'll automatically lyse the ~1,000 cells captured on-chip and do an SDS-PAGE separation on *each single-cell lysate*. Milo then uses light to covalently bind the separated proteins into the gel. This immobilization step gets rid of the time-consuming and sometimes

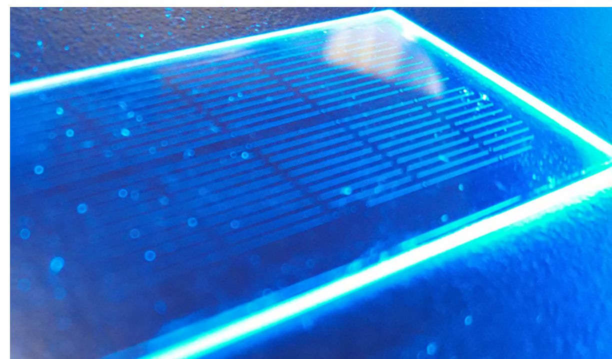

FIGURE 1. Each scWest chip has thousands of microwells that are approximately the size of a single cell patterned into a pre-cast polyacrylamide gel.

FIGURE 2. Single-Cell Western workflow.

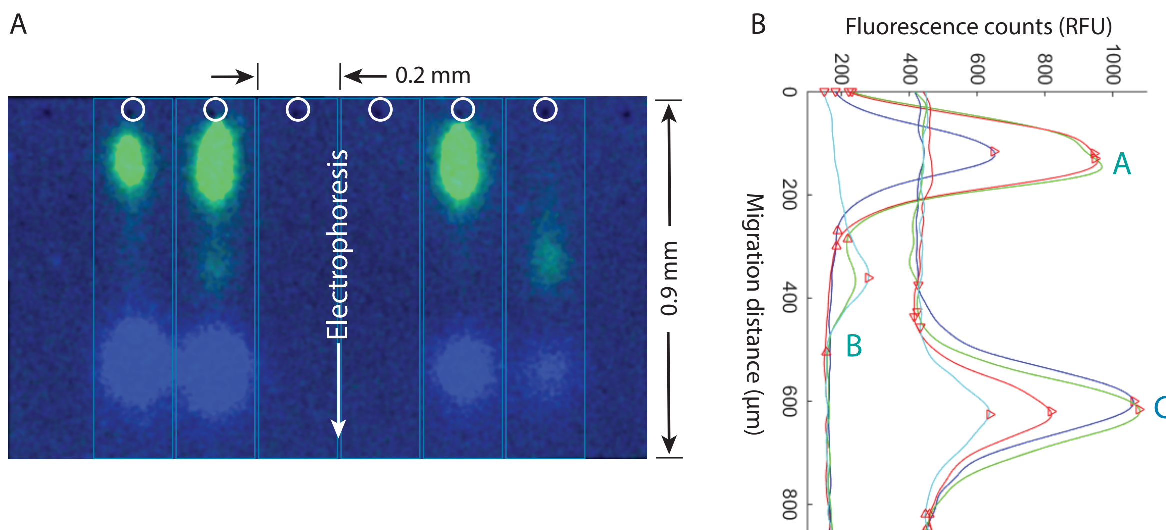

FIGURE 3. Single-Cell Western separations (A) and intensity plots (B) reveal three protein bands (A, B and C) in a HEK293 sample. The migration distance of each band is proportional to the molecular weight of the protein. The area under the curve of each protein peak is proportional to the target abundance.

low-efficiency transfer step that comes with traditional Westerns. After immobilization, just take the chip out and probe it in Milo's benchtop probing chamber using conventional Western antibodies. These are typically unlabeled primaries and fluorescently-tagged secondary antibodies. Then just image the chip fluorescence with any open format microarray scanner. Scout Software automates your data analysis for all 1,000 Westerns so you can get quantitative protein expression results for each single cell.

Count and quantitate at the same time

Each Single-Cell Western uses protein size and antibody binding information for your target of interest to give you a quantitative measure of target abundance in each cell.

Figure 3 shows Single-Cell Western data from a population of HEK293 cells. Every scWest chip has an array of 6,400 single-cell electrophoresis lanes and **Figure 3A** shows six of these lanes. Four cells were captured in this section of the chip, each in a distinct microwell located at the top of each lane. After cell lysis, each single-cell lysate was electrophoretically driven out of the microwell into the gel lanes below, and the proteins were separated according to their molecular weight. This scWest chip was probed for three different proteins (labeled A, B and C). Scout Software created the fluorescence intensity plots in **Figure 3B** which reveal a distinct peak for each of the three protein targets. In this HEK293 sample, some of the single cells express all three proteins, while others express only two and contain different amounts — revealing variable expression across the cell line.

Milo measures two types of heterogeneity in your samples: target expression heterogeneity and cell type heterogeneity. Do you have some cells with very little target and some with a lot? Milo easily measures these cell-to-cell differences and quantifies variations in the amount of protein present in the cells in your sample. Have you ever wondered how many cells in your sample have your target of interest? Milo can do that too! He also enumerates or counts the number of cells in your population that express a particular protein so you can identify subtypes of cells in a mixed sample.

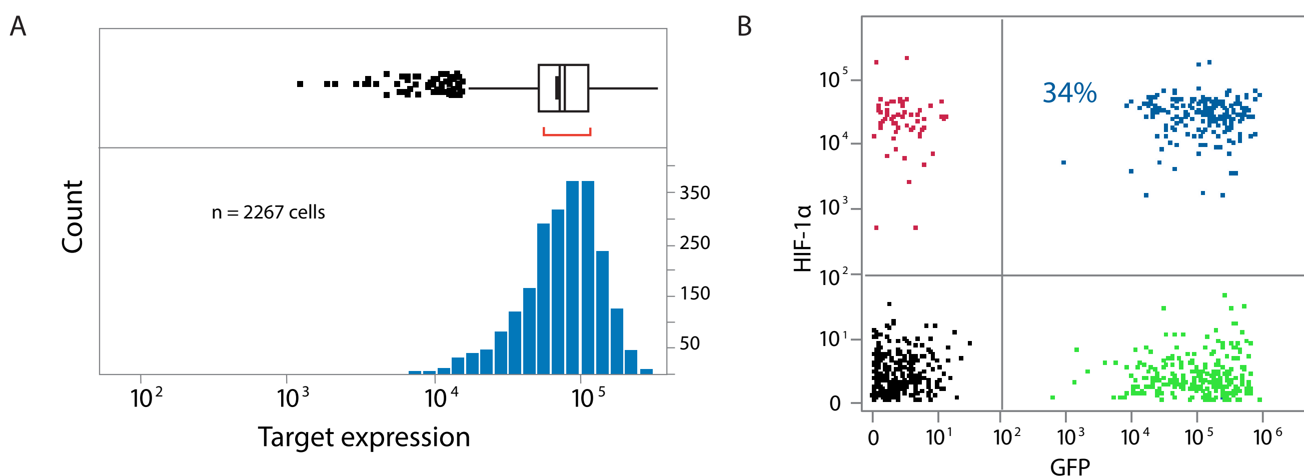

FIGURE 4. Histograms of peak area (A) show expression heterogeneity while scatterplots (B) show how many cells in a mixed population express each target of interest.

Histograms (**Figure 4A**) show variation in the target expression across the population. For example, you could measure a signaling protein that is upregulated in response to a stimulus and see how variable the response to that treatment is across your population.

Scatterplots (**Figure 4B**) can be used to enumerate cell subpopulations. For example, you can probe for a protein that identifies a specific cell type within your mixed sample to see how many cells exhibit that particular phenotype. You can then characterize that subpopulation of cells by measuring expression of a second or third protein target within that subpopulation of cells.

So how does Milo work?

Milo's Single-Cell Western workflow is simple, takes about 4-6 hours, and has only 1-2 hours of hands on time!

PREPPING YOUR SAMPLES

All you need to do is get your sample into a single-cell suspension. Milo uses intact, un-fixed cells so you don't have to lyse your cells or fix and permeabilize them like you would with conventional Westerns or flow cytometry experiments. Just trypsinize adherent cell lines, dissociate tissue samples or collect suspension cells and re-suspend them in Milo's Suspension Buffer at a concentration of 10,000 – 100,000 cells/mL.

LOADING THE SCWEST CHIP

Just pipette 1 mL of your single-cell suspension onto the top of an scWest chip and let it sit for 5-15 minutes. This allows the cells to settle by gravity into the microwells. After that, the microwell occupancy approximately follows a Poisson distribution. That means this finely-tuned cell concentration and cell settling time lets you capture approximately 1,000 single cells (~15% microwell occupancy), with only a few wells that contain two or more cells (**Figure 5**). Visually inspect your chip under a brightfield microscope and count the microwell occupancy for a single block of 400 microwells to estimate the occupancy for the entire chip. This allows you to confirm you've captured single cells in your microwells before you run the chip (**Figure 6**).

AUTOMATE 1,000 SINGLE-CELL SEPARATIONS

After your sample is loaded on the scWest chip, simply put the chip in Milo. Each run only takes 5 minutes and Milo

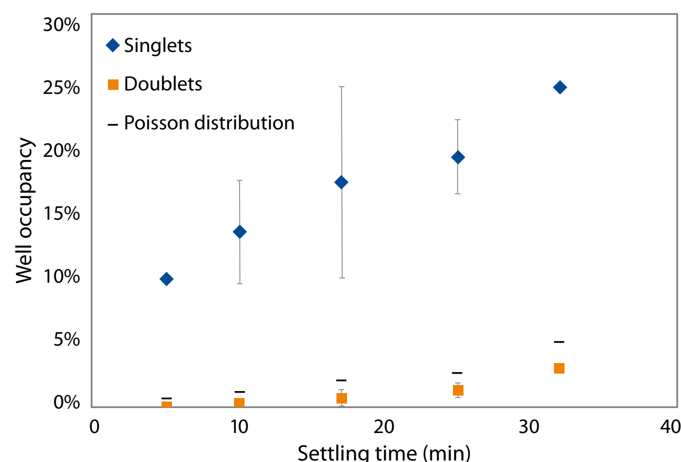

FIGURE 5. Microwell occupancy for increasing cell settling times show strong correlation with expected Poisson capture rates.

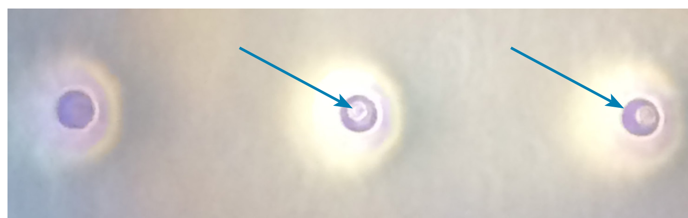

FIGURE 6. Single cells captured in microwells on a scWest chip (occupied wells indicated by an arrow).

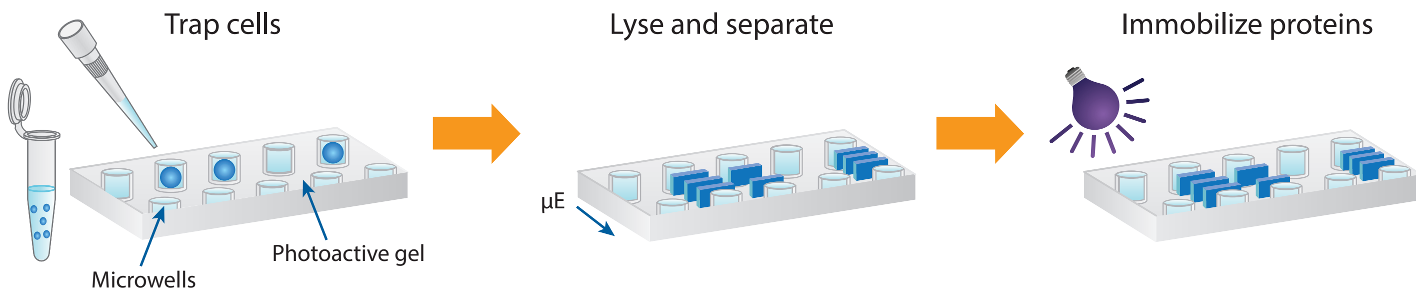

FIGURE 7. Single-Cell Western assay steps.

automates every step (**Figure 7**). He'll chemically lyse the cells, run an SDS-PAGE separation on each single-cell lysate and use light to immobilize separated protein bands into the polyacrylamide gel on-chip.

PROBING THE SCWEST CHIP WITH CONVENTIONAL WESTERN ANTIBODIES

Once Milo finishes the run, just take the scWest chip out and probe it on the benchtop with conventional Western antibodies. Milo doesn't need specialized antibodies or reagents, so you can keep using your favorite antibodies. Probe each chip for 1-2 hours at room temp with unlabeled primary antibodies using the antibody incubation chamber that comes with Milo. You can even probe with a cocktail of primary antibodies to multiplex and detect multiple proteins per cell. After the primary antibody probing and wash steps, probe the chip with fluorescently labeled secondary antibodies for ~1 hour and then wash off any unbound antibody.

IMAGING THE SCWEST CHIP

You can scan scWest chip fluorescence with one of the open format microarray scanners shown in **Table 1**. Make sure to save each chip scan as one or more single-color TIFF images — one for each spectral channel imaged on the chip.

SCANNER NAME	LASER COUNT	COMPANY
SpotLight Turbo	Up to 6	ArrayIT
SureScan	2 (simultaneous)	Agilent
InnoScan 710	2	Innopsys
InnoScan 910	2	Innopsys
InnoScan 1100AL	3	Innopsys
GenePix 4000B	2	Molecular Devices
GenePix 4100A	2	Molecular Devices
GenePix 4300A	Up to 4	Molecular Devices
GenePix 4400A	Up to 4	Molecular Devices
GenePix Professional 4200A	Up to 4	Molecular Devices
ScanArray Express	Up to 5	PerkinElmer
ScanArray Express HT	Up to 5	PerkinElmer
ScanArray 5000	4	PerkinElmer
LS Reloaded	Up to 4	Tecan
PowerScanner	2	Tecan
ArrayIT SpotLight	2	Telechem
GE Typhoon	3	General Electric
G2565CA	2	Agilent
Scanscope FL	4	Aperio

TABLE 1. Compatible microarray scanners.

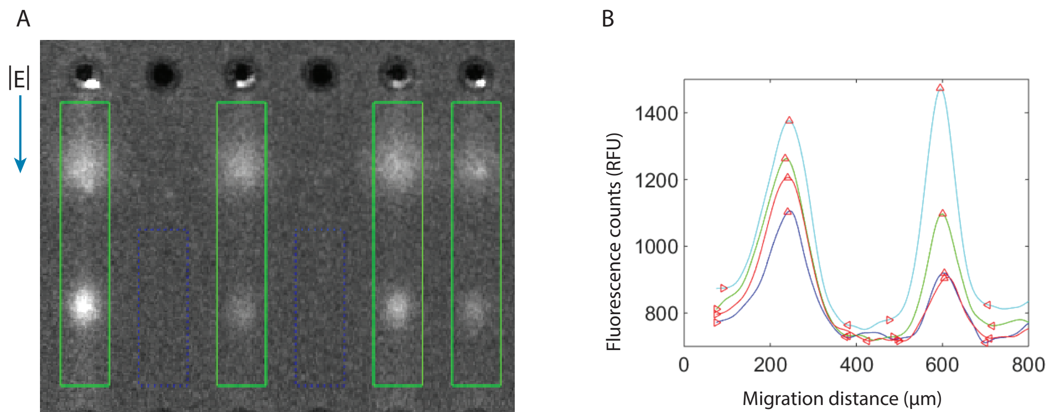

FIGURE 8. Scout Software automates lane finding and peak detection, creates intensity plots and calculates the area under the curve to quantify protein abundance of each target in each cell.

IMAGE ANALYSIS

Scout Software automates scWest image analysis and peak detection for you. It reads in one or more single-color TIFF files per chip and adds them to your chip so you can detect all the proteins in each cell, no matter what color they were scanned in. Next, Scout automatically finds each of the 6,400 single-cell electrophoreses lanes (**Figure 8A**) and automatically detects each peak in each single-cell separation. It then calculates the area under the curve for each peak to quantify target abundance in each cell (**Figure 8B**). You can also use Scout's powerful data curation tools to label peaks, exclude off-target peaks, and export the analyzed peak data as a .csv or .fcs file to plot in your favorite data visualization or graphing tool.

Milo multiplexes too

Milo lets you measure multiple proteins in a single cell using two different methods: spectral multiplexing or size-based multiplexing.

To spectrally multiplex, first probe your targets of interest with primary antibodies raised in different host species — mouse and rabbit for example. Next, probe with host-specific secondary antibodies tagged with different fluorophores, for example donkey anti-mouse Cy3 and donkey anti-rabbit Cy5.

For size-based multiplexing, if there's more than a 30% molecular weight difference between your proteins of interest, you can probe them with antibodies raised in the same host species and image them in the same spectral channel. These targets can be resolved in the SDS-PAGE separation on the scWest chip and differentiated based on molecular weight.

Figure 9 shows Single-Cell Western data for four proteins detected in a single cell. Three different spectral channels were used to detect three species, while β-tubulin and L858R were resolved based on their different molecular weights.

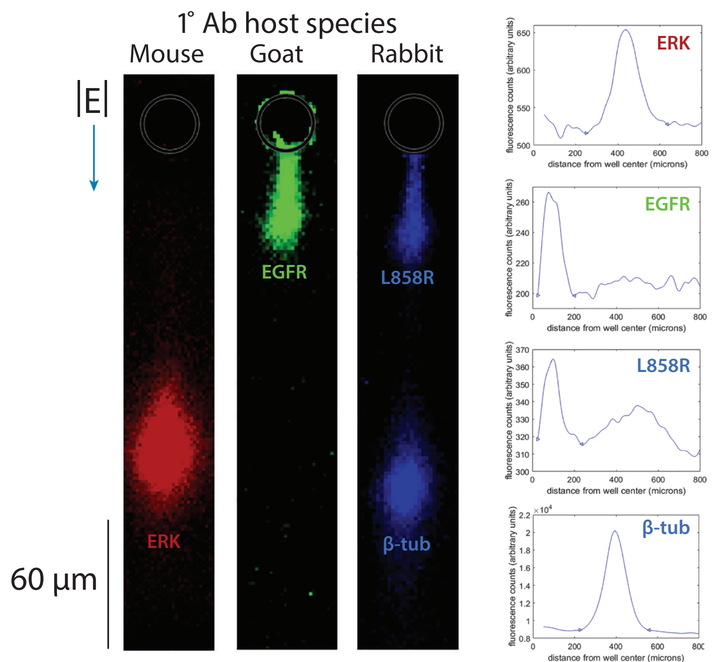

FIGURE 9. Simultaneous measurement of four proteins in a single cell. Different spectral tags were used to label different protein targets and differences in molecular weight were used to resolve different proteins in the same spectral channel.

Detect diverse targets with one workflow

Milo easily measures way more of the proteome than flow cytometry, mass cytometry, and other single-cell protein analysis techniques. You can detect diverse protein targets including cell surface and intracellular or intranuclear proteins.

Milo uses the conventional Western antibodies you're used to so you don't need to find custom or specialized reagents like some other single-cell protein analysis techniques. There are at least 10 times more Western antibodies than antibodies for flow cytometry so now you've got access to a way larger commercial catalog of antibodies to measure your favorite target.

Simplify your phospho-flow experiments to detect phosphorylated proteins, transcription factors and other intracellular signaling proteins with one simple workflow. Milo lyses cells to access intracellular proteins rather than having to get antibodies inside intact cells by fixing and permeabilizing them. That makes your intracellular flow cytometry or phospho-flow assay workflows way more streamlined and lets you do facile single-cell signaling activation measurements.

Milo simultaneously detects cell-surface proteins like a putative drug target and downstream signaling activation like phospho-protein or transcription factor expression at the same time. Because fixation and permeabilization can destroy surface protein epitopes, that can be impossible to do with flow cytometry.

You can also simultaneously detect phospho and total protein expression in the same cell to get information on the extent of phosphorylation over time. When protein isoforms differ in molecular weight, you can use the sizing info Milo gives you to identify protein isoforms bound by the same antibody — which can't be done with flow cytometry!

Archive samples in scWest chips and re-probe months later

Milo's light-activated immobilization step covalently binds your cell lysates into the polyacrylamide gel on the scWest chip. The whole process is unbiased and stable, letting you archive samples after the run for up to 9 months. Now you can go back and reprobe your sample for new targets days, weeks and even months later to hunt down answers to new questions using the same primary sample. You'll also be able to introduce time delays into your experiment. For example, after running a sample on an scWest chip, you can wait to probe it for specific targets until your single-cell RNAseq experiment is done and you know which targets you want to validate with protein information.

Dynamic range and quantitation repeatability

Single-Cell Western assays have a dynamic range of at least 2 orders of magnitude (**Figure 10**), so you can detect both low and high expressing cells in the same sample. You can also probe multiple chips for the same target, to compare multiple samples on different chips with excellent repeatability of the peak signals (**Figure 11**).

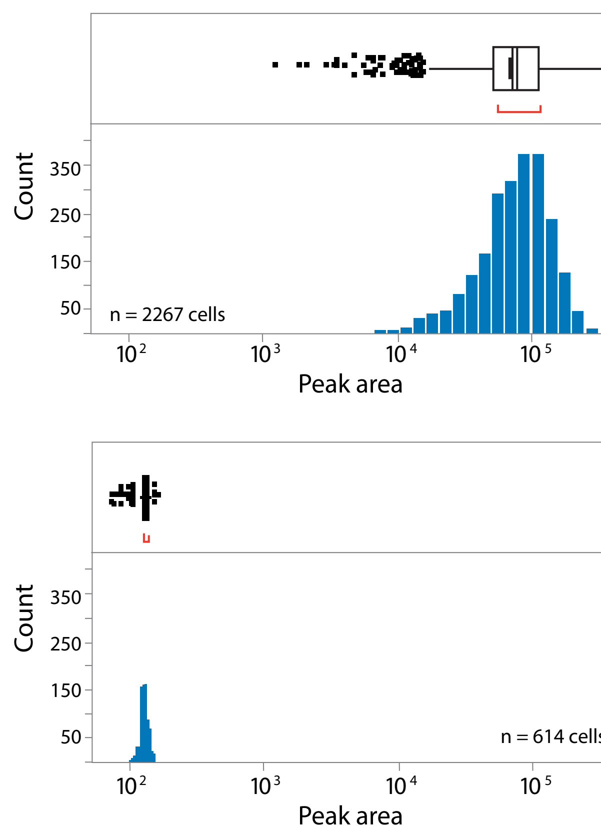

FIGURE 10. Detection of a 3-log dynamic range using samples with low and high expression of the same protein target.

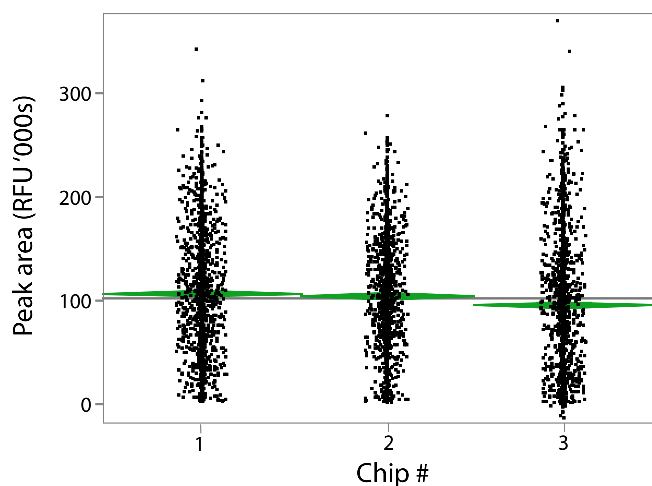

FIGURE 11. Quantitation repeatability. The same sample of GFP-expressing 293 cells was run on three scWest chips and probed for GFP. The CV of the mean peak areas was 6%.

Conclusion

Milo lets you capture ~1,000 single cells and run 1,000 Single-Cell Westerns in parallel. Cell lysis, SDS-PAGE separation and immobilization for every single cell is done on the scWest chip automatically and only takes 5 minutes. After that, probe the chip with commercial Western antibodies. Since you're not stuck with only flow cytometry antibodies, your antibody options go up 10X! You can scan and image scWest chips with a wide range of open-format scanners, and Scout Software does the image analysis, detects multiple proteins per cell, and quantitates protein abundance for you. Plus, you can archive your samples right on the scWest chip and re-probe them again up to nine months later.

Running Single-Cell Westerns with Milo lets you identify subpopulations of cells that can't be resolved with conventional Westerns. You can even resolve protein isoforms that are bound by the same antibody and can't be differentiated in flow cytometry assays because they lack molecular weight sizing information. And if you're doing single-cell RNA experiments, Milo can validate your single-cell RNA data with single-cell protein expression information.

Make measurements you can't make any other way. Milo can detect phosphorylated proteins and transcription factors, not to mention cell-surface proteins with one simple, streamlined workflow. Add a 2 log or better dynamic range to that, and you'll be detecting high and low expressing cells in the same sample in no time. Now you can measure target expression heterogeneity and cell type heterogeneity with the confidence that a robust, Western-based read out provides, and unlock the single-cell proteome at your fingertips.

References

1. Single-cell western blotting, AJ Hughes, DP Spelke, Z Xu, CC Kang, DV Schaffer, and AE Herr, *Nature Methods, 2014*; 11(7):749-55.

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