Multiplexing to the Max: A Guide to Stripping and Reprobing Your Single-Cell Westerns

Introduction

At the core of cell-to-cell heterogeneity are the wide-ranging patterns of protein expression and activity that are responsible for the diversity in individual cell behavior and response. Understanding this variability is critical to understanding the complexity of human biology and the advancement of biomedical research.

More and more, researchers need information about multiple proteins at once. Cell-signaling pathways involve multiple key proteins at any given moment, and cellular responses to novel compounds often include several proteins. Also, the diagnosis of various diseases depends on the status of a handful of proteins. With this demand comes the need for increasingly sensitive, quantitative and reliable multiplexing Western blotting systems. Traditional techniques that examine samples on a population basis can fall short by masking the changes in subpopulations of cells or important rare events. Other single-cell approaches, like flow cytometry, are limited in their ability to easily detect surface, intracellular and/or phosphorylated proteins at the same time; what's more, fluorescent antibody spectral-crosstalk limits the maximum multiplexing level to approximately 12 colors or targets.

Single-Cell Westerns with Milo™ allow you to unlock the single-cell proteome and measure changes in protein expression heterogeneity that aren't possible with other techniques. This technical note will walk you through multiplexing with Milo and provide you with evidence of and guidance for efficient stripping to achieve multiplexing levels that match or exceed those of the most powerful multiplexed flow cytometers.

Meet Milo

Milo is the world's first Single-Cell Western platform. Using scWest chips with cell-sized microwells, he can perform Westerns on 1,000 individual cells simultaneously in a single run. Researchers can now gain selective protein expression information for multiple targets in each cell—all at the same time and irrespective of target location in the cell. Just load your cell suspension onto the chip, lyse the captured cells and Milo will perform an approximately 1-minute, size-based SDS-PAGE separation on each single-cell lysate. You can then probe the scWest chip with your favorite commercially available Western blot antibodies. Then visualize the chip fluorescence using any open format microarray scanner capable of scanning standard glass microscope slides. Finally, Scout™ software analyzes your data to create fluorescence intensity plots that reveal distinct and quantitative peaks for each protein of interest.

Multiplexing with Milo

Milo accomplishes multiplexing via spectral-based or size-based detection capabilities, where one antibody cocktail can be stripped off and the chip reprobed with a new set of antibodies. Spectral-based multiplexing relies on your choice of primary antibodies raised in different hosts and then matching each to an appropriate fluorophore-tagged secondary. For example, mouse and rabbit primary antibodies can be spectrally differentiated by using a donkey anti-mouse Cy3 and donkey anti-rabbit Cy5, respectively. Size-based multiplexing requires that there be more than a 30% difference in molecular weight between your proteins of interest to be adequately resolved by SDS-PAGE separation on an scWest chip. Because of the sufficient gap in molecular weight, probing and imaging can be done using primary antibodies from the same host species or secondary antibodies that fall in the same

spectral channel. **Figure 1** shows Single-Cell Western data for four proteins detected in a single cell using spectral- and size-based multiplexing strategies. Two different spectral channels were used to distinguish the signal for proteins with similar molecular weights (such as ERK and β-tubulin), while β-tubulin and L858R were resolved based on their different molecular weights.

To multiplex to the max with Milo, you'll need to strip the applied antibody cocktail and reprobe for new target proteins, and stripping efficiency is the key to your success. Herein, we demonstrate efficient stripping and reprobing of Milo scWest chips over the course of multiple stripping and reprobing cycles sufficient to permit 10-plex to 12-plex Single-Cell Western assays using a basic two-color scanner — on par with the multiplexing capability of high-end flow cytometers. Using this stripping and reprobing strategy with three or four color scanners could yield even higher multiplexing levels.

Using Jurkat cells, an AML1 rabbit monoclonal antibody (Cell Signaling Technology, PN 4336) was used to detect AML1 protein expression levels imaged with a two-color scanner over the course of five stripping cycles. To calculate stripping efficiency, signal proportions between pre-strip and post-strip conditions were detected using Scout. The median remaining signal, a decimal value, was subtracted from one and multiplied by 100 to be expressed as a percent (see *"Using Scout to calculate stripping efficiency"* below). The median stripping efficiency after each cycle was 98% ± 1%, while the median reprobing efficiency of AML1 across the five cycles was 96% ± 16% (**Table 1**). In a separate set of experiments, the stripping and reprobing efficiency of a β-tubulin mouse monoclonal antibody (GenScript, PN A01717) was evaluated. The median stripping efficiency after each cycle was 99% ± 1.1%. (**Table 2**). The median reprobing efficiency of β-tubulin across the three cycles was 84% ± 19%, while a lower reprobing efficiency was observed in the fourth cycle (**Table 2**). The stripping efficiency remained constant for both targets. There was no trend in reprobing efficiency for AML1, while β-tubulin showed a decline in reprobing efficiency in the final cycle.

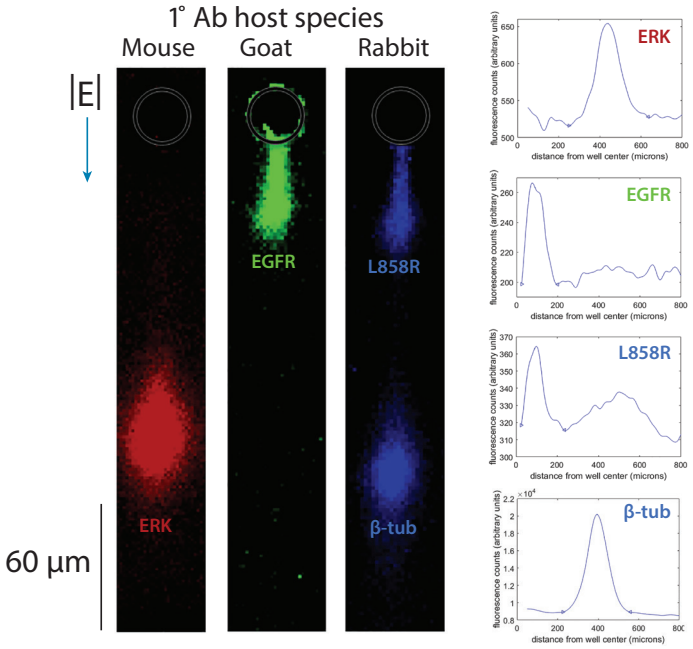

FIGURE 1. Multiplexing with Single-Cell Western. Data for four proteins detected in a single cell. Two different spectral channels were used to detect anti-mouse ERK and anti-goat EGFR, while a third was used to detect β-tubulin and L858R with an anti-rabbit antibody which was resolved based on their different molecular weights.

AML1	MEDIAN STRIPPING EFFECIENCY (%)	MEDIAN REPROBING EFFECIENCY (%)
Cycle 1	98.5	98.8
Cycle 2	98.8	82.0
Cycle 3	98.4	112.1
Cycle 4	97.9	82.1
Cycle 5	97.2	98.0
Average	98.1	95.7

TABLE 1. Stripping and reprobing efficiencies for AML1 over five cycles.

β-TUBULIN	MEDIAN STRIPPING EFFECIENCY (%)	MEDIAN REPROBING EFFECIENCY (%)
Cycle 1	99.4	103.9
Cycle 2	99.4	65.3
Cycle 3	98.9	79.3
Cycle 4	97.9	38.7
Average	99.2	70.5

TABLE 2. Stripping and reprobing efficiencies for β-tubulin over four cycles.

Stripping and Reprobing with Milo

ProteinSimple recommends stripping chips as soon as possible after probing and scanning, but you can store chips in Wash Buffer prior to stripping for up to three days. You should avoid dry storage prior to stripping, which reduces stripping efficiency. Once stripped, chips may be reprobed immediately or dried for long-term storage; be sure to rehydrate dried chips prior to reprobing. Up to nine cycles of stripping and reprobing have been demonstrated in the literature[1,2].

Protocol Materials

PRODUCT NAME	COMPANY	PART NUMBER	DETAILS
scWest Kits Includes: • 10X Suspension Buffer • 5X Wash Buffer • Antibody Diluent 2 • Lysis/Run Buffer	ProteinSimple	• K500 • K600 • K700	• Small scWest kit for small cells • Standard scWest kit • Large scWest kit for large cells
5X Wash Buffer	ProteinSimple	R242	Additional wash buffer
scWest chip canisters (4 pack)	ProteinSimple	035-118	Canister for incubation with stripping buffer
Primary antibodies	Various		
Fluorescently-conjugated secondary antibodies	Various		
Trypsin or another dissociation agent	Various		
10-cm Petri dishes (2 per scWest chip)	Various		
Tris-HCl pH 6.8 buffer	Various		
β-mercaptoethanol (β-ME) 14.3 M	Various		
SDS	Various		
Parafilm M	Various		
Aluminum or heat-tolerant tape	Various		
Tube rack for 50-mL tubes	Various		
Heated water bath	Various		

TABLE 3. Required consumables and reagents.

Stripping and Reprobing Protocol

The Stripping Buffer composition is:

62.5 mM Tris-HCl (pH 6.8)
2% (w/v) SDS (20 g in 1 L)
0.8% (v/v) β-ME (14.3 M)

NOTE: The Tris-HCl and SDS can be prepared in advance and stored as a "Stripping Buffer Base," but the β-ME should be added fresh before each use.

After the initial scan of a probed scWest chip in your microarray scanner, place the chip(s) in a container protected from light (if not stripping immediately, you may store the chips in Wash Buffer for up to three days as previously discussed). Begin by setting a water bath to 60 °C. Place a tube rack inside and adjust the water level to a point where it is roughly 1 cm above the rack. Place weights on top of the rack or otherwise secure the rack to keep it stable within the water bath.

Note that all subsequent procedures should be done within a fume hood (if space allows, the water bath may also be placed within a fume hood). You will need one chip canister (PN 035-118) for each scWest chip that you are stripping. In an appropriate container, aliquot 40 mL of Stripping Buffer Base and 320 µL of β-ME for each scWest chip. For example, for three chips, prepare 120 mL of Stripping Buffer Base and add 960 µL of β-ME. Mix well. Prepare Parafilm and aluminum tape (or another heat-tolerant tape) by cutting them at the appropriate length to wrap around the cap of the chip canister. Aliquot 40 mL of prepared Stripping Buffer into each chip canister. Add one chip to a chip canister, and seal each canister with Parafilm followed by heat-tolerant tape. Place each canister inside the pre-heated water bath and ensure the water level is roughly at cap level for the chip canister. Incubate for 90 minutes.

Once complete, bring all tubes containing chips back to the fume hood and carefully remove each chip, placing them gel-side-up in a Petri dish. Give each chip a quick rinse in 1X Wash Buffer, then wash in four 15-minute cycles using 15–20 mL of 1X Wash Buffer on a shaker. After four washes, the scWest chip is ready to be reprobed with a fresh set of primary antibodies.

Please note that for each chip being stripped and reprobed, approximately 200 mL of 1X Wash Buffer is required to complete the procedure. Although some excess Wash Buffer is provided in the Standard scWest kits, the excess is not enough to strip and reprobe all the chips in a kit. Additional 5X Wash Buffer (PN R252) may be purchased for this purpose. Dispose of all waste in accordance with applicable national, regional and local regulations.

Using Scout to Calculate Stripping Efficiency

Visual inspection of scans post-strip is not a reliable way to troubleshoot stripping and reprobing. Due to the high dynamic range of most microarray scanners, when displaying a scan on a computer screen with automatic contrast adjustment, an image with 90% stripping efficiency will appear very similar to its original image. Therefore, when using visual inspection alone, you may incorrectly conclude that very little stripping has occurred when, in fact, most of the signal has been removed, and the protocol may only need minor tweaking.

Even an scWest chip with approximately 99% stripping efficiency will still have some visible signal (**Figure 2**). Use Scout to quantitatively determine whether the stripping procedure was successful (>98% efficiency).

To calculate stripping efficiency in Scout 2.0, analyze the scanned scWest chip image prior to stripping. Label the detected peaks to indicate the "before stripping" state, for example, "Target-Before." Import a second scWest chip image after the stripping procedure. Create a Peak Table for this post-strip scan, then choose from the menu: Peak Table → Tag Matching Peaks / Stripping Efficiency. Choose to match your "Target-Before" peaks, then create a new "Target-After" tag to apply to the matching peaks. Finally, choose to perform the stripping efficiency calculation. Refer to **Figure 3** for a visual summary of the tag-matching process using Scout.

Any detected peaks in the post-strip image will be tagged "Target-After." For wells where no post-strip matching peak is found, Scout will measure the area in the post-strip image above a line drawn between the start and end points corresponding to the peaks in the pre-strip image.

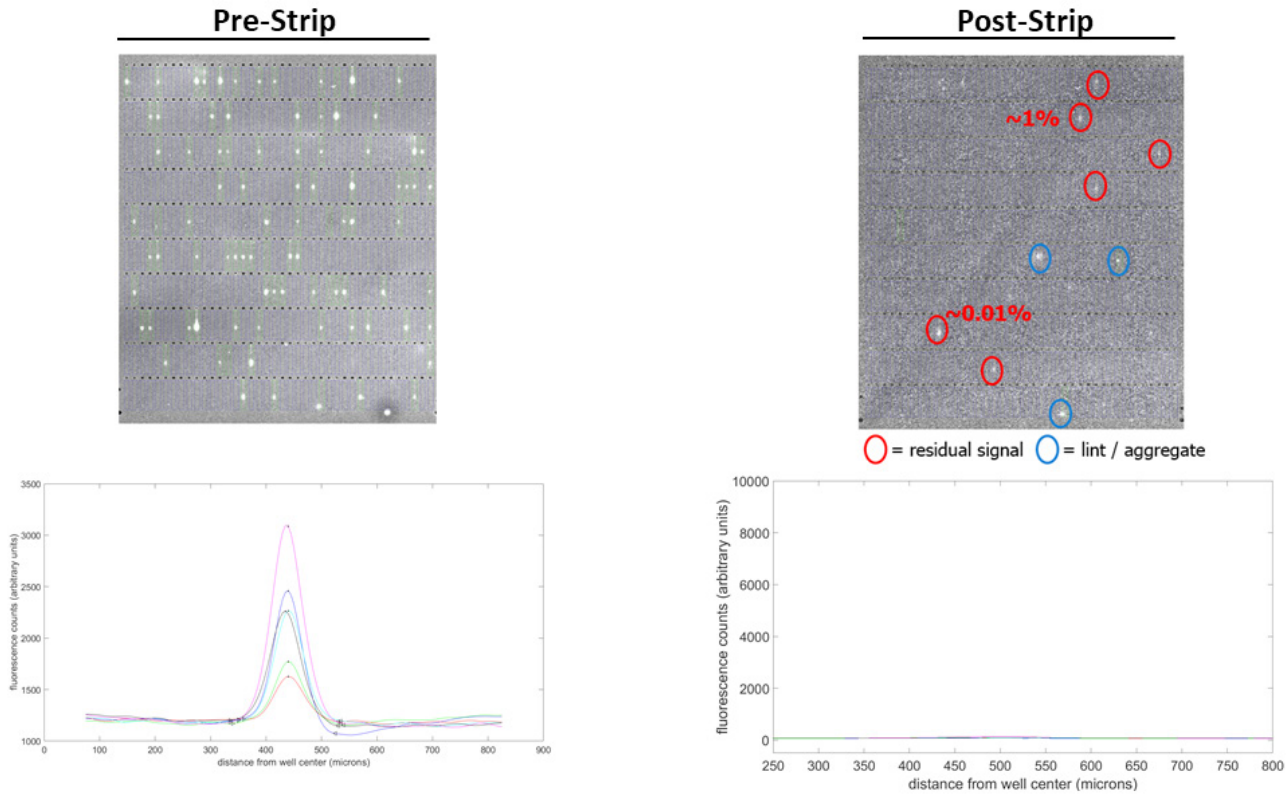

FIGURE 2. scWest chip pre-strip (left) and post-strip (right). Even with an approximate 99% stripping efficiency, some residual signal is visible (red circles).

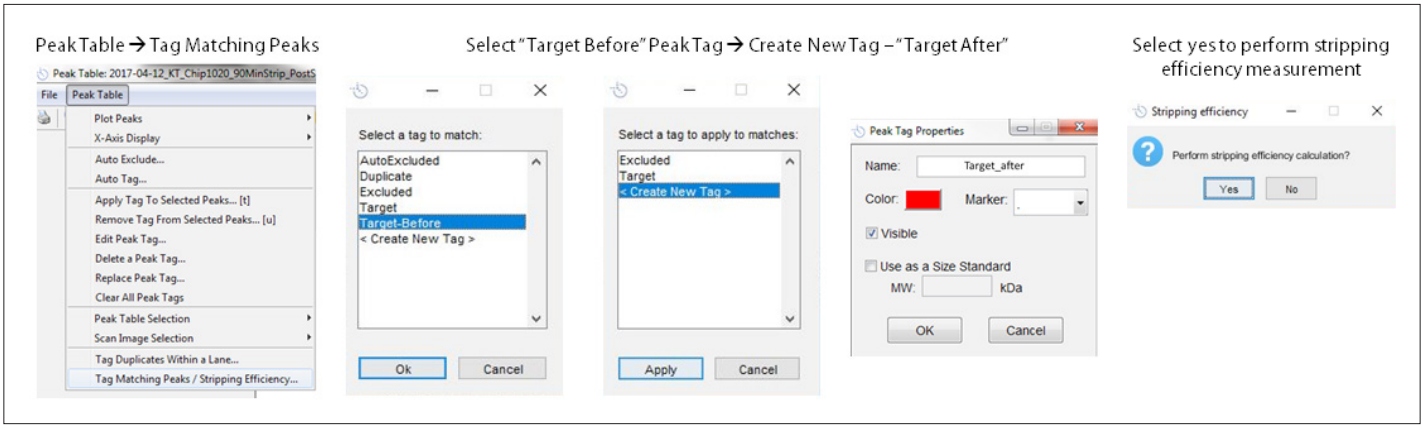

FIGURE 3. Scout 2.0 software clicks to follow when calculating stripping efficiency. Refer to Figure 4 for an example representation of the output generated.

Figure 4 shows example output data from a stripping efficiency calculation generated following the steps in **Figure 3**. Due to variation in scanner output and the forced area calculation described above (which can result in small negative areas when only baseline noise remains), it is normal to see a distribution of stripping efficiency with some measurements slightly higher than 100%.

Assay Development: Strategies and Recommendations

Multiplexing can provide you with the flexibility required to get the most data points out of each sample. Milo can measure approximately four proteins per single cell via spectral and/or size-based multiplexing[2]. When incorporating stripping and reprobing into your multiplexing strategy, we first recommend running scWest chips with a maximum of two protein targets per chip and then imaging via a microarray scanner. After this, follow the outlined stripping procedure above, and check the stripping efficiency of each target using ProteinSimple's Scout software (version 2.0 or greater).

When designing your final multiplexed assay, it's important to be mindful of a couple of things: your target abundance and the stripping efficiency of your antibody-detected target. Stripping efficiency has to do with the affinity of the antibody to your target—low-affinity antibodies will have a high stripping efficiency and vice versa. If both the protein abundance and antibody affinity are equal for your proteins of interest, then the order of protein probing does not matter.

However, as a general rule, it's best to probe for proteins in increasing order of abundance—probe for the low abundance ones first; any target loss due to stripping will affect your ability to detect it most. If your proteins are of similar abundance, you should probe with the lower affinity antibody (higher stripping efficiency) first, so as to minimize the residual signal detected after stripping. These tips help increase the likelihood of detecting multiple proteins before and after stripping. In **Table 4**, we have included several strategies for detecting multiple targets by way of stripping and reprobing based on protein abundance and predicted antibody affinity.

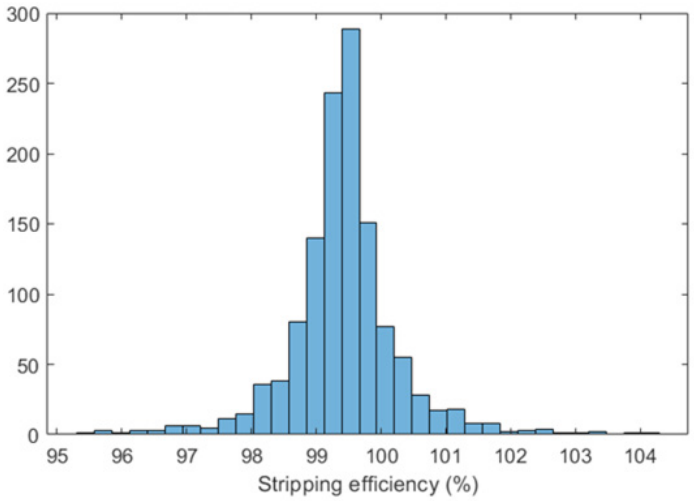

FIGURE 4. Distribution of stripping efficiency calculated in Scout software using a sample chip. Peaks tagged with target = 1283; Matching peaks tagged with target-after = 89; Forced areas calculated when no matching peak found = 1194; Median stripping efficiency = 99.4%; 95% proportion is between 97.1% and 101.7%.

Additional characterization experiments were performed to validate that both AML1 and β-tubulin primary and secondary antibodies are removed during a cycle of stripping and reprobing. Conjugated secondary antibodies used during classic membrane-based stripping and reprobing western blotting protocols often produce spots or speckled patterns with subsequent reprobing. However, with Milo, and as seen in **Figure 5**, a clean signal is observed upon imaging after incubation with only the secondary antibody post-stripping. This confirms both AML1 and β-tubulin primary and secondary antibodies (donkey-anti-rabbit and donkey anti-mouse, respectively) are being removed in the stripping procedure. Stripping efficiency calculations are provided for pre-strip versus post-strip and reprobed (secondary only) versus pre-strip for both AML1 and β-tubulin using Scout software. Please note that dry storage of scWest chips prior to stripping and failure to use fresh β-ME in the Stripping Buffer makeup may reduce stripping efficiency.

Leveraging Milo's multiplexing ability, in **Figure 6**, we demonstrate a four-plexed assay using a basic two-color scanner and one cycle of stripping and reprobing with two sets of antibodies. This scWest chip was probed for four different proteins: AML1, β-tubulin, Histone H3 (Histone H3 [D1H2] XP® rabbit monoclonal antibody, Cell Signaling Technology, PN 4499) and ERK (p44/42 MAPK [Erk1/2] [L34F12] mouse monoclonal antibody, Cell Signaling, PN 4696). **Figure 6** shows the pre-stripping and post-stripping results of probing first for AML1 and β-tubulin and then reprobing for new targets Histone H3 and ERK.

Using Scout, you can take a deeper dive into your multiplexed data to reveal, for example, what percentage of cells co-express the various target proteins. This type of information is especially valuable when measuring, for instance, specific protein expression and signaling events in response to a stimulus, as it will allow for you to observe how the response to treatment varies across your population. For example, in this set of experiments, we elected to analyze AML1 and ERK protein expression in those lanes that are defined as both β-tubulin and H3-positive (**Figure 7**) and revealed four distinct subpopulations of cells.

PROTEIN ABUNDANCE	ANTIBODY STRIPPING EFFICIENCY	DETECTION: CYCLE 1	STRIP	DETECTION: CYCLE 2
Similar	Similar	Either protein		Remaining protein
Similar	Unequal	High stripping efficiency antibody		Low stripping efficiency antibody
Unequal	Similar	Low abundance protein		High abundance protein
Unequal	Unequal	Low abundance protein		High abundance protein

TABLE 4. Multiplexing approaches for stripping and reprobing on Milo.

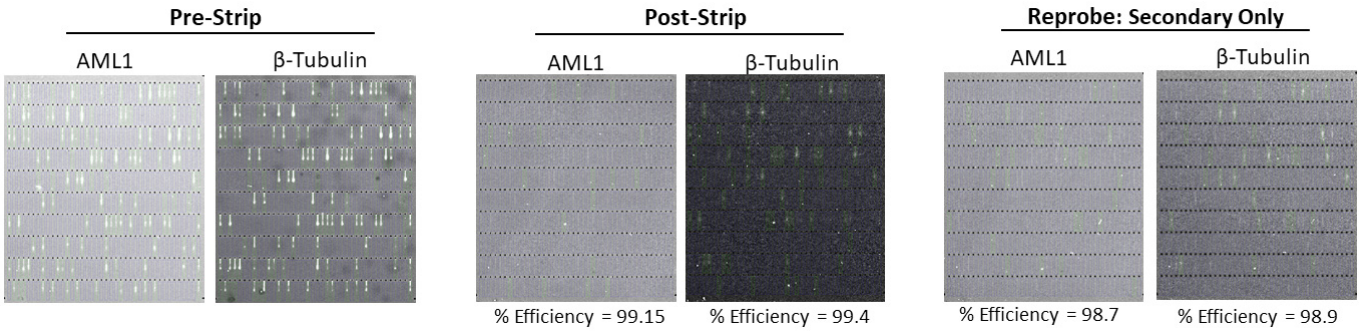

FIGURE 5. AML1 and β-tubulin signal pre-strip, post-strip, followed by reprobing with the corresponding secondary antibody only. The signal observed with the secondary antibody reprobe is negligible, thus confirming both primary and secondary antibodies are being efficiently stripped. Percent stripping efficiency for AML1 pre-strip versus post-strip = 99.15%, and even after reprobing with just the secondary antibody, it is still 98.7% versus pre-strip conditions. Percent stripping efficiency for β-tubulin pre-strip versus post-strip = 99.4% and secondary reprobe versus pre-strip = 98.9%.

Assay Optimization

Although high-efficiency stripping can be achieved for many targets, stripping and reprobing efficiencies may vary for different protein targets. If you have a target that exhibits low-efficiency stripping, you may need to spend some time optimizing your protocol. Points of consideration include increasing the stripping time, increasing the temperature of the water bath or finding a different antibody with a higher stripping efficiency. Finally, consider the order in which targets are probed. To get the most out of multiplexing by stripping and reprobing, save the low-efficiency stripping proteins for last.

Conclusions

Whether you need to conserve limited samples, compare relative abundance of protein isoforms or a phosphorylated form to its total abundance, assess the expression of proteins with a similar molecular weight, or just want to confirm a result with a different antibody, Milo is a powerful solution that lets you do all of this at the single-cell level.

Hypotheses evolve, priorities change, and your samples are precious, making the archive-and-reprobe feature of scWest chips—even months later—a powerful asset for both new protein target discovery and confirmation studies.

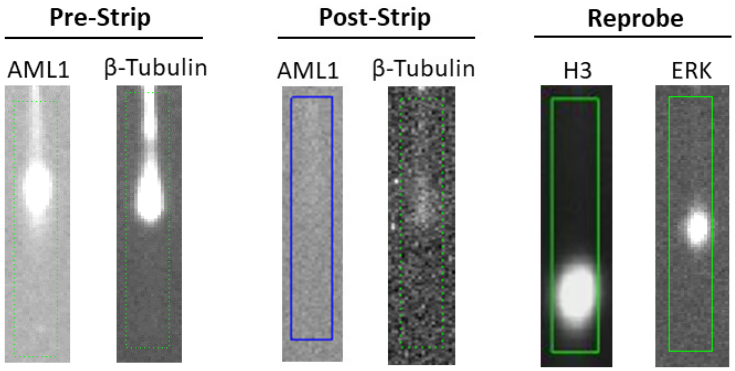

FIGURE 6. Demonstration of a four-plexed assay using a basic two-color scanner. Images shown are of one lane imaged in two different colors (same cell in all images). AML1 and β-tubulin proteins were probed for, stripped and the corresponding lane was reprobed with a new set of antibodies (H3 and ERK, respectively).

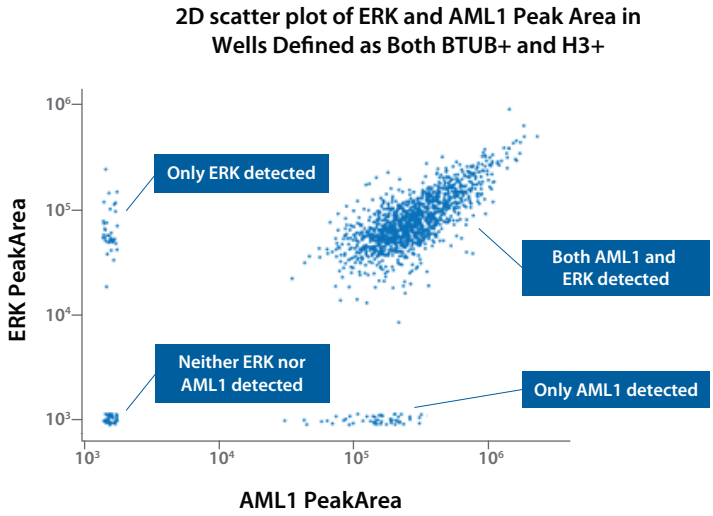

FIGURE 7. 2D scatter plot assessing AML1 and ERK protein expression heterogeneity in lanes having cells positive for both β-tubulin and Histone H3. This type of analysis makes clear the variability of cell populations that exist in a sample.

References

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

2. Single-cell resolution western blotting, C Kang, KA Yamauchi, J Vlassakis, E Sinkala, TA Duncombe, AE Herr, *Nature Protocols*, 2016; 11:1508-1530.

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