Semi-Dry Probing: Improving Probing Uniformity for Single-Cell Western Assays

Introduction

Antibody probing is an important factor that influences Single-Cell Western assay repeatability. In particular, uniformity of antibody probing is important to Milo users in being able to resolve technical variation from the real biological heterogeneity of interest. This tech note describes how a minor modification in the antibody probing procedure used with a standard antibody probing chamber can improve probing uniformity and reduce technical noise.

What is Semi-Dry Probing?

Semi-dry probing is a variation on the method originally recommended for probing full scWest chips using a standard (full chip) antibody probing fixture (P/N A200). The original protocol recommends tapping scWest chips to remove free liquid after they are run in Milo and washed for 2 x 10 minute in Wash Buffer, spotting a droplet of your antibody cocktail on the antibody probing fixture and then laying the chip, gel side down, onto the probing chamber so that the antibody solution wicks across the gel surface (**Figure 1**). This process is similar to the familiar process of applying a coverslip, but failing to remove excess liquid from the surface of the chip can result in nonuniform concentrations of the antibody solution during incubation.

In contrast, semi-dry probing utilizes a centrifugal slide spinner (P/N 110-0006 for 120 V product, 110-0007 for 230 V product) to spin dry scWest chips for 3-5 seconds after they are run in Milo and washed for 2 x 10 minute washes in Wash Buffer. The semi-dry chip is then placed gel side down onto an empty probing chamber and antibody cocktail is injected into the gap between the chamber and the edge of the chip so that the antibody solution fills the chamber completely (**Figure 2**). The semi-dry probing method is recommended for use with the standard (full chip) antibody chamber and it mirrors the recommended protocol for probing with the Three Plex Antibody Probing Chamber (P/N A300). Note that longer spin times or further drying of the chip increases the likelihood of incomplete filling of the probing chamber and/or trapping of air bubbles. Similarly, combining the spin step with the original probing method for antibody filling is not recommended. For more details on the semi-dry probing protocol, please refer to the Milo User Guide.

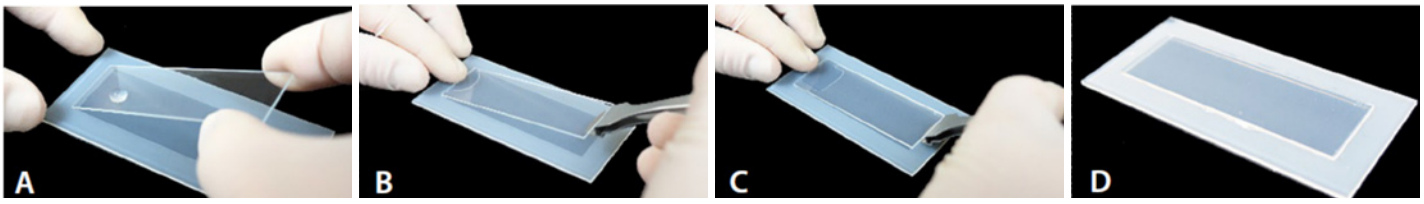


FIGURE 1. Original probing protocol recommended for use with a standard (full-chip) antibody probing chamber. A) Pipette antibody cocktail solution in a bead on the left side of the incubation fixture. B) Gently lower the chip onto the antibody cocktail. C) Stabilize the scWest chip gel side down using the flat edge of tweezers (without gripping with the tweezers). D) scWest chip incubating in antibody cocktail solution with no air bubbles.

FIGURE 2. Semi-dry probing protocol on a standard (full-chip) antibody probing chamber. A) Start with a clean, dry probing chamber on your benchtop. B) Gently lower the semi-dry chip (spun for 3-5 seconds) onto the antibody probing chamber, gel side down. C) Inject antibody cocktail into the gap between the chip edge and the chamber edge in the middle of the long side of the chip. Antibody solution will wick across the chamber until it is completely filled. D) scWest chip incubating in antibody cocktail solution with no air bubbles.

Why Semi-Dry Probing?

REDUCTION IN INTRA-CHIP BACKGROUND CV

A primary benefit of semi-dry probing is that it prevents background signal variation across a scWest chip. Using the original probing method, variation in background signal can be observed on some runs, as seen in **Figure 3A** where background signal on one side of the chip is higher than on the other side. This background variation can occur if there is excess free liquid on the surface of a chip before probing is initiated. In this instance, the buffer droplets on the surface of the wet chip can locally dilute the antibody concentration.

In contrast, the semi-dry probing method can reduce the observed background variation because the spin step consistently removes any excess liquid from the surface of the chip (**Figure 3B**). **Figure 4** shows a quantitative comparison of the mean background signal by block, for all 16 blocks (of 400 microwells) of the scWest chips probed with the original and the semi-dry probing methods. The background signal CV for the original and semi-dry probing methods were 96% and 27%, respectively. The overall mean background level across all blocks is shown as the center line of each orange diamond, while the height of the diamonds indicates the confidence interval for the overall mean.

REDUCTION IN INTRA-CHIP PEAK AREA CV

Similarly, semi-dry probing reduces peak area CV across an scWest chip. To separate out technical noise from biological variation, we can average measured peak area across each block of 400 microwells and then compare

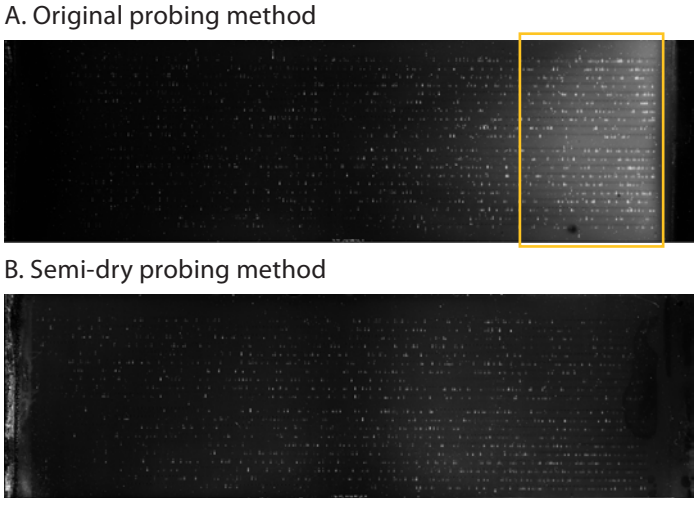


FIGURE 3. Comparison of intra-chip variation in probed signal. A) Excess liquid on the chip surface using the original probing method resulted in the right side of the chip having a higher background than the left side. B) The semi-dry probing method shows uniform background across the full chip surface.

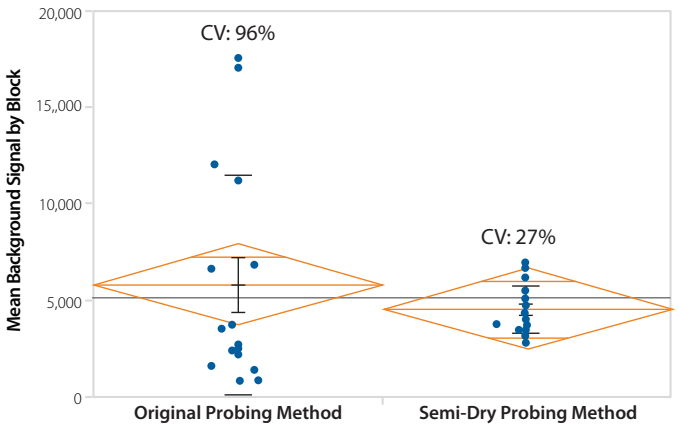


FIGURE 4. Figure 4. Semi-dry probing results in more uniform background signal across the chip. A comparison of mean background signal by block using the original probing method and the semi-dry probing method shows a reduction in CV for mean background signal using the semi-dry probing method. Diamonds show 95% confidence intervals for the overall mean (diamond height) and overall mean values (horizontal centerline in each diamond).

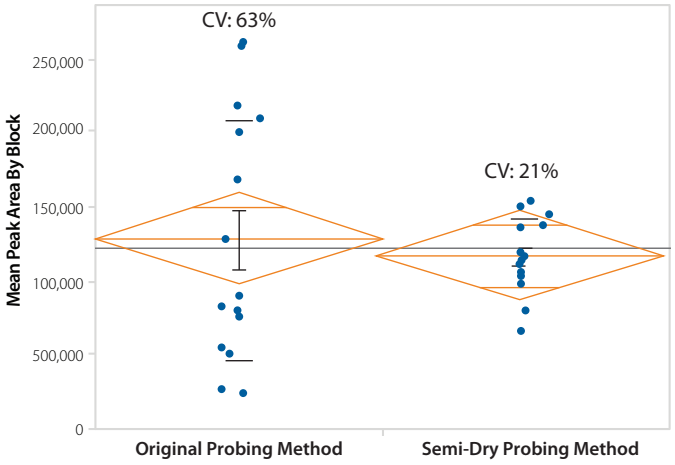


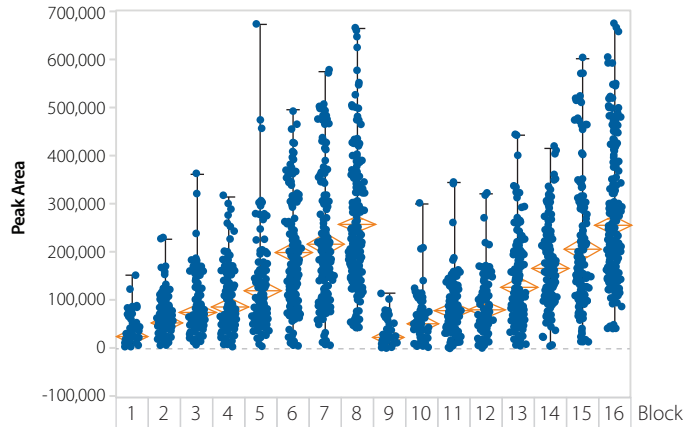
FIGURE 5. Semi-dry probing reduces spatial variation in measured peak area. A comparison of mean peak area by block using the original probing method and the semi-dry probing method shows a reduction in CV for mean peak area using the semi-dry probing method. Diamonds show 95% confidence intervals for the overall mean (diamond height) and overall mean values (horizontal centerline in each diamond).

mean peak areas across all 16 blocks on one chip to look at spatial variation in probing. Figure 5 shows the mean peak area for all 16 blocks on the scWest chips from Figure 3. The CV for the block-averaged peak area was 63% and 21% for the original probing method and the semi-dry probing method, respectively. This demonstrates that antibody probing is more uniform across the chip surface when semi-dry probing is used.

As expected, **Figure 5** also shows that there was no statistically significant difference in block-averaged peak area, indicating that the semi-dry probing method reduces technical noise CV but does not impact repeatability of target quantitation.

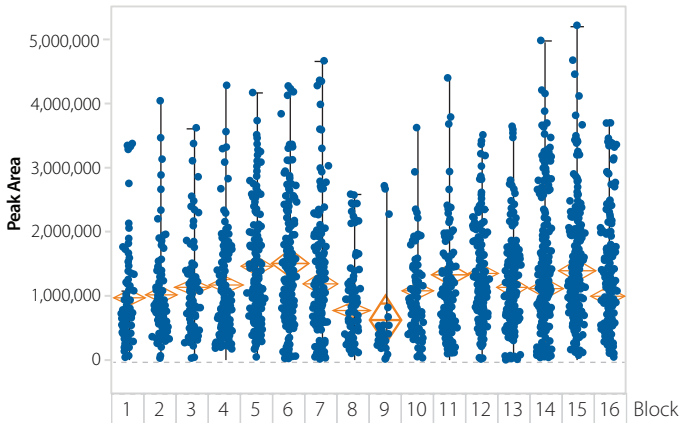
Lowering intra-chip peak CV means the majority of measured variation is due to biological variation in protein expression between cells rather than technical noise contributed by probing variation. **Figure 6** shows a variance components analysis of the two chips from Figure 3. Figure 6B shows that 95% of measured variance is from residual (i.e. well-to-well or biological) variation in the sample while only 4.5% of the overall measured

A. Original probing method



VARIANCE COMPONENTS			
Component	Var Component	% of Total	Sqrt (Var Comp)
Block	6.4093e+11	42.2	800579.47
Within	8.7609e+11	57.8	935998.1
Total	1.517e+11	100.0	1231673.6

B. Semi-dry probing method



VARIANCE COMPONENTS			
Component	Var Component	% of Total	Sqrt (Var Comp)
Block	3.7285e+10	4.5	193093
Within	7.8317e+11	95.5	884696
Total	8.2054e+11	100.0	905789

FIGURE 6. Figure 6. Less than 5% of measured variance is due to spatial location on the chip (probing variation) using the semi-dry probing method. A) Some runs using the original probing method can display spatial variation in peak area. In this chip, 42% of measured variance peak area was due to block location. B) Semi-dry probing improves probing uniformity. In this chip, 4.5% of measured variance was due to block location. More than 95% of measured variance was due to biological variation in the sample.

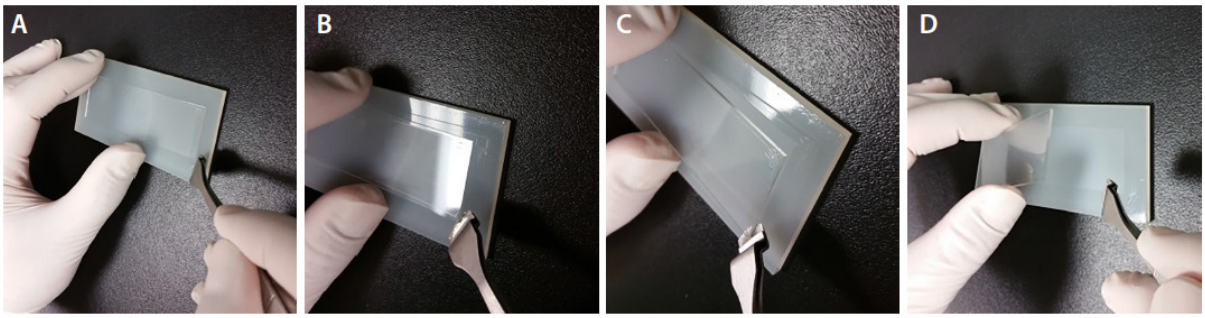


FIGURE 7. Removing the scWest chip from the primary antibody incubation fixture. A) Gently push down on the top corner of the chip with your index or middle finger. B) Lever the tweezers under the opposite corner of the chip without damaging the probing fixture. C, D) Remove the chip by rotating the tweezers under the corner of the scWest chip and pinching the opposite edges of the chip between your index finger and thumb.

variance is from the block (physical location on the chip) due to probing variation. This demonstrates that for chips probed with the semi-dry probing method, less than 5% of measured variance is due to the spatial location on the chip (i.e. probing variation).

MAY REDUCE ANTIBODY CONSUMPTION

Semi-dry probing may also reduce the volume of antibody cocktail required to probe a single scWest chip. The original probing method recommended spotting 80 µL of antibody cocktail onto the probing chamber and then laying down the chip on top of the antibody solution. Semi-dry probing recommends making 80 µL of antibody cocktail but injecting only as much antibody as is required for the solution to completely fill the probing chamber. As antibody probing chambers are used more times, complete filling tends to occur with less volume required. Overfilling the chamber may cause antibody solution to wick outside of the chamber and is not recommended.

Tips & Tricks

Antibody probing chambers should be used as long as they enable the antibody solution to wick completely across the surface of a scWest chip. If incomplete filling is observed, flip the probing fixture over and use the other side or order a new probing fixture (P/N A200).

Take care not to scratch or scuff the fixture as it can interfere with complete and uniform filling of the chamber. Chamber scratching can occur when removing an scWest chip. **Figure 7** shows the recommended protocol to remove scWest chips from the probing fixture to avoid scratching the fixture with the metal tweezers.

Correcting for probing variation in chips probed using the original probing method

While the semi-dry probing method is recommended for future studies, if you already have data from a chip that displays significant probing variation (e.g., Figure 3A), normalizing peak area by the local background (available in data exports from Scout 2.1 and later) can reduce spatial variation in peak area. **Figure 8** shows the mean normalized peak area in each block for the chips shown in Figure 3. You can see that the CV for the original probing method is reduced from 63% (Figure 5) to 25% (Figure 8) when background normalization is used. Meanwhile, the CV for the semi-dry probing method is virtually unchanged at 22% (vs. 21% in Figure 5).

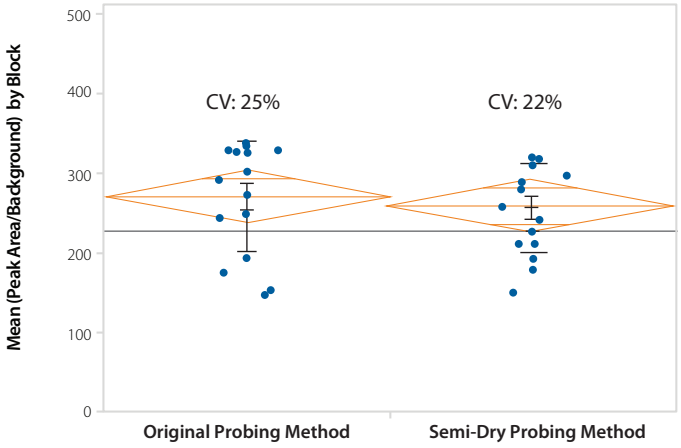


FIGURE 8. Normalizing peak area by background can reduce CV for chips that show significant background variation. A comparison of mean peak area normalized by background using the original probing method and the semi-dry probing method shows a 25% CV for mean normalized peak area using the original probing method. Diamonds show 95% confidence intervals for the overall mean (diamond height) and overall mean values (horizontal centerline in each diamond).

Conclusion

Semi-dry probing is recommended for all Milo users to eliminate technical noise from nonuniform probing that has been observed in some scWest chip runs.

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