Multiplexed Single-Cell Western Analysis of Red Blood Cells for Biomarker Detection in Blood and Neurodegenerative Disorders

Introduction

Diagnostic tests are often developed in blood since it's biological material that is relatively plentiful and easy to obtain compared to, for example, cerebral spinal fluid. Physicians can also request many different tests using one blood draw to help them correctly diagnose their patients. Biomarkers are routinely measured to monitor blood disorders like sickle-cell anemia and thalassemias. However, many diseases lack definitive blood-based biomarkers of diagnostic or prognostic value. In particular, many neurodegenerative disorders such as Parkinson's and Alzheimer's commonly rely on neurological exams that assess motor and sensory skills in order to diagnose disease progression. This can lead to misdiagnoses since these exams are based primarily on expert opinion. Developing simple blood-based tests to objectively detect and determine disease progression would significantly improve patient care and increase chances of detecting neurodegenerative disease early.

In this study, we show that the Milo™ Single-Cell Western platform has the sensitivity required to measure protein expression in single red blood cells (RBCs). We use Milo to detect hemoglobin, a RBC internal control protein, in single RBCs and show how Milo's molecular weight sizing step can resolve different forms of hemoglobin as well as differentiate between specific and non-specific (off-target) binding. Recognizing α-synuclean as a putative biomarker for Parkinson's disease, we also demonstrate a multiplexed α-synuclein and hemoglobin assay in single RBCs that quantifies heterogeneity in the expression of both targets across the cell populations. Together, these results showcase how Milo can be utilized by RBC researchers to study diverse targets relevant to Parkinson's Disease, Sickle Cell Disease, and other RBC disorders.

Single RBC Biomarker Detection

Detecting biomarker levels in an individual red blood cell is challenging due to sensitivity limitations because they are much smaller than most other human cells with a disk diameter of approximately 6-8 µm and thickness of 1-2 µm. It can also be difficult to find antibodies validated for flow cytometry – the most well established single-cell protein analysis technology – for many protein targets of interest. Like all antibody-based measurements, flow-validated antibodies can exhibit non-specific binding that can be impossible to separate from specific binding using flow cytometry. Likewise, because flow cytometry does not separate protein forms by size, detection of specific protein isoforms using flow cytometry would require isoform-specific antibodies which are not readily available. Furthermore, protein dimerization can limit the accessibility of antibody binding sites and lower antibody performance on native flow assays.

Hemoglobin, a protein in red blood cells that carries oxygen throughout the body and can be used as an RBC internal control protein, is particularly challenging to measure in a single RBC using flow cytometry. Detection of hemoglobin in individual RBCs can itself be important in several RBC diseases since disruptions in hemoglobin can lead to serious disorders such as sickle-cell anemia and thalassemias. Hemoglobin detection challenges stem from a lack of highly specific flow-validated antibodies and an inability to differentiate between hemoglobin isoforms which can be associated with disease progression.

Many other putative biomarkers are expressed in RBCs. For example, the deposition of intracellular α-synuclein has been observed in RBCs from patients with the neurodegenerative disorder of Parkinson's disease[1]. The α-synuclein protein has been detected in blood, with more than 99% of the α-synuclein residing in RBCs and

the remaining protein detected in the plasma, platelets and PBMCs[2]. Thus, detection of α-synuclein in RBCs has potential as a diagnostic biomarker for Parkinson's disease, yet detection can be challenging at the single-cell level using conventional tools.

How Milo Achieves High Sensitivity for Single-Cell Protein Measurements

Milo performs single-cell resolution Westerns on ~1,000 single-cells per run and measures protein expression for up to 12 targets per cell[3]. Briefly, a cell suspension is loaded onto an scWest chip at a limiting concentration so that a single cell will settle due to gravity into the microwells patterned into the thin polyacrylamide gel coating the surface of the scWest chip. Cells are then chemically lysed to create a single-cell lysate and a 1 min SDS-PAGE separation resolves proteins in each single-cell lysate according to their molecular weight. Milo then exposes the scWest chip to UV light which covalently immobilizes the separated proteins into the gel. This capture step is highly efficient and eliminates the need for a low efficiency transfer step common in traditional Western blotting. Standard western-validated primary antibodies are then applied to the scWest chip followed by fluorescent secondaries. Then the chip is imaged using any open format microarray scanner and chip images are analyzed using Scout™ Software for quantitative, automated data analysis.

Milo enables high sensitivity single-cell protein measurements by minimizing dilution of single-cell lysates and dramatically reducing protein losses in the western blotting workflow. Lysate dilution is minimized by confining each single-cell lysate to a small reaction volume. Protein losses are minimized by utilizing a high efficiency UV immobilization step to immobilize the sample after separation in order to avoid protein loss associated with the membrane transfer step of traditional westerns. Milo's size-based separation of lysate proteins also gives you molecular weight and antibody specificity information about each protein target for additional confidence in the specificity of target detection. Milo is also flexible so experiments can be tailored to your particular protein and sample of interest.

Materials and Methods

CELLS, ANTIBODIES AND REAGENTS

Human RBCs were purchased from ZenBio (cat# SER-10MLRBC) and used within 3 days of receipt. We recommend analyzing RBC samples as soon as possible after collection to avoid the autofluorescence observed in some older RBC samples. Mouse anti-hemoglobin A (beta chain) antibody was purchased from Rockland (cat #200-301-GS4), rabbit anti-α-Synuclein antibody was purchased from Cell Signaling Technologies (cat# 2642S), and goat anti-hemoglobin (alpha1) antibody was purchased from GeneTex (cat# GTX77484). Alexa Fluor® secondaries purchased from ThermoFisher Scientific include donkey anti-mouse IgG, Alexa Fluor 647 (cat# A-31571) and donkey anti-rabbit IgG, Alexa Fluor 488 (cat# A-21206).

Milo experiments were all run using the Small scWest Kit (PN K500) from ProteinSimple. This kit includes 8 Small scWest chips, 10X Suspension Buffer (SB), 5X Wash Buffer (WB), Antibody Diluent 2, and 8 single-use Run/Lysis Buffer vials.

RUNNING MILO

A suspension of RBCs was made by mixing 1 µL of RBC stock in 14 mL of 1X SB and spinning down at 2,000 RPM for 5 minutes. The SB buffer was aspirated and the cell pellet resuspended with 9 mL of 1X SB to make a 100,000 cell/mL suspension. A cell count should be performed and dilutions adjusted as needed for different starting sample concentrations to achieve a target 100,000 cell/mL suspension. 1 mL of this suspension was settled onto a rehydrated small scWest chip for 5 minutes, after which unsettled cells were washed away using 1 mL of 1X SB. Single-cell occupancy was scored by visually counting 400 wells on the scWest chip using an inverted microscope. Milo captures single cells based on Poisson distribution statistics that control the rate of doublets. Typically, 1,000 captured cells per chip is targeted to produce a maximum number of singlets and minimum number of doublets. The theoretically predicted doublet rate is < 2% of wells when 1000 single-cells are captured.

The scWest chips loaded with RBCs were run on Milo. The cell lysis time was set to 0 seconds followed by 40 seconds of electrophoresis time at 240 V. Protein was then UV captured for 4 minutes. Because RBCs lyse so quickly, a 0 sec lysis time was used to minimize the lysis time and reduce diffusive protein loss out of the top of the well after the cells are lysed and before electrophoresis is initiated. With a 0 sec lysis time programmed into Milo, the effective lysis time was the time between when Lysis/Run buffer was poured into the electrophoresis cell and when the Run button was pushed on Milo (2–3 seconds).

After protein immobilization, scWest chips were probed with rabbit α-synuclein antibody diluted 1:10 in Antibody Diluent 2 and/or mouse Hemoglobin A (beta chain) antibody diluted 1:10 in Antibody Diluent 2 for 2 hours at room temperature. The scWest chips were then washed for 3 x 10 minutes with 1X WB and then probed with donkey anti-rabbit IgG, Alexa Fluor 488 and/or donkey anti-mouse, Alexa Fluor 647 IgG secondary antibodies, diluted to 100 µg/mL in Antibody Diluent 2, for 1 hour at room temperature, in the dark. scWest chips were washed 3 x 15 minutes with 1X WB, dried, and imaged using a GenePix 4400A microarray scanner (Molecular Devices). Images were saved as individual TIFF files for quantitative analysis of target protein peak area using Scout Software.

RESULTS AND DISCUSSION

To assess whether the Single Cell-Western has the sensitivity required to measure protein expression in RBCs, an antibody specific for hemoglobin beta subunit wild type variant A (HbA) was used to probe the RBCs from ZenBio. HbA was successfully detected, with two distinct bands detected due to Milo's ability to separate protein isoforms by molecular weight (**Figure 1A**). The first band (H1) migrated at the expected molecular weight for HbA, increasing confidence that the signal detected was specific. The other band (H2) migrated at a higher molecular weight and could potentially be another hemoglobin isoform or a heme-bound form of HbA. Therefore, the signal for one or more of the specific bands or the ratio between the two can be compared across single cells. In a small number of RBCs, a diffuse, slower band was observed which we attribute to non-specific antibody binding (**Figure 1B**). This band ran at a different molecular weight than the H1 or H2 bands and was eliminated from analysis using Scout software.

Peak area analysis of HbA H1 (**Figure 2A**) and HbA H2 (**Figure 2B**) indicated that in the RBC cell population, there was more HbA H1 expressed compared to HbA H2. There was also slightly more heterogeneity in the levels of HbA H1 compared to HbA H2 between RBCs, with peak

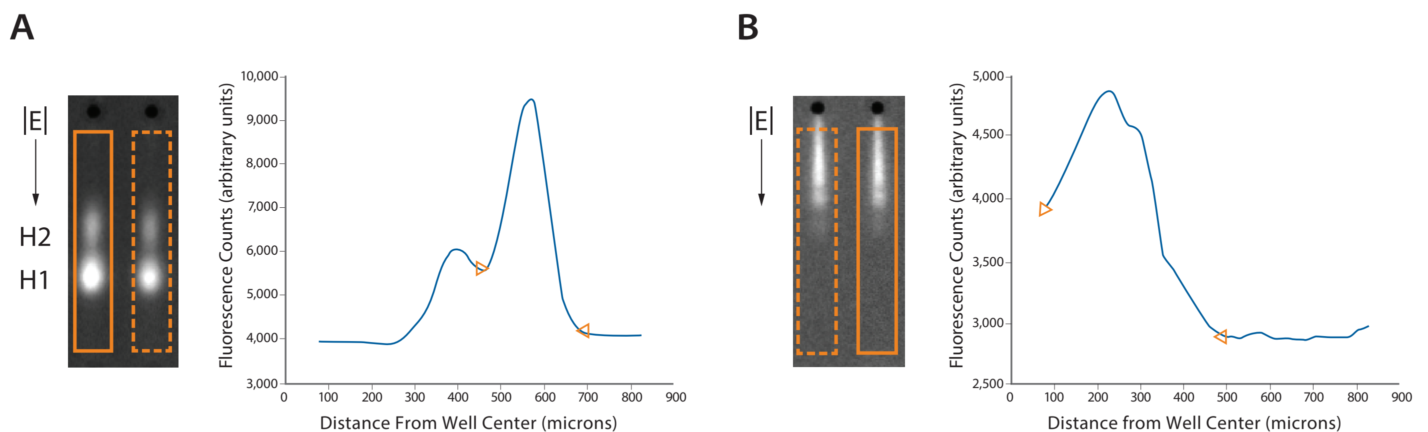
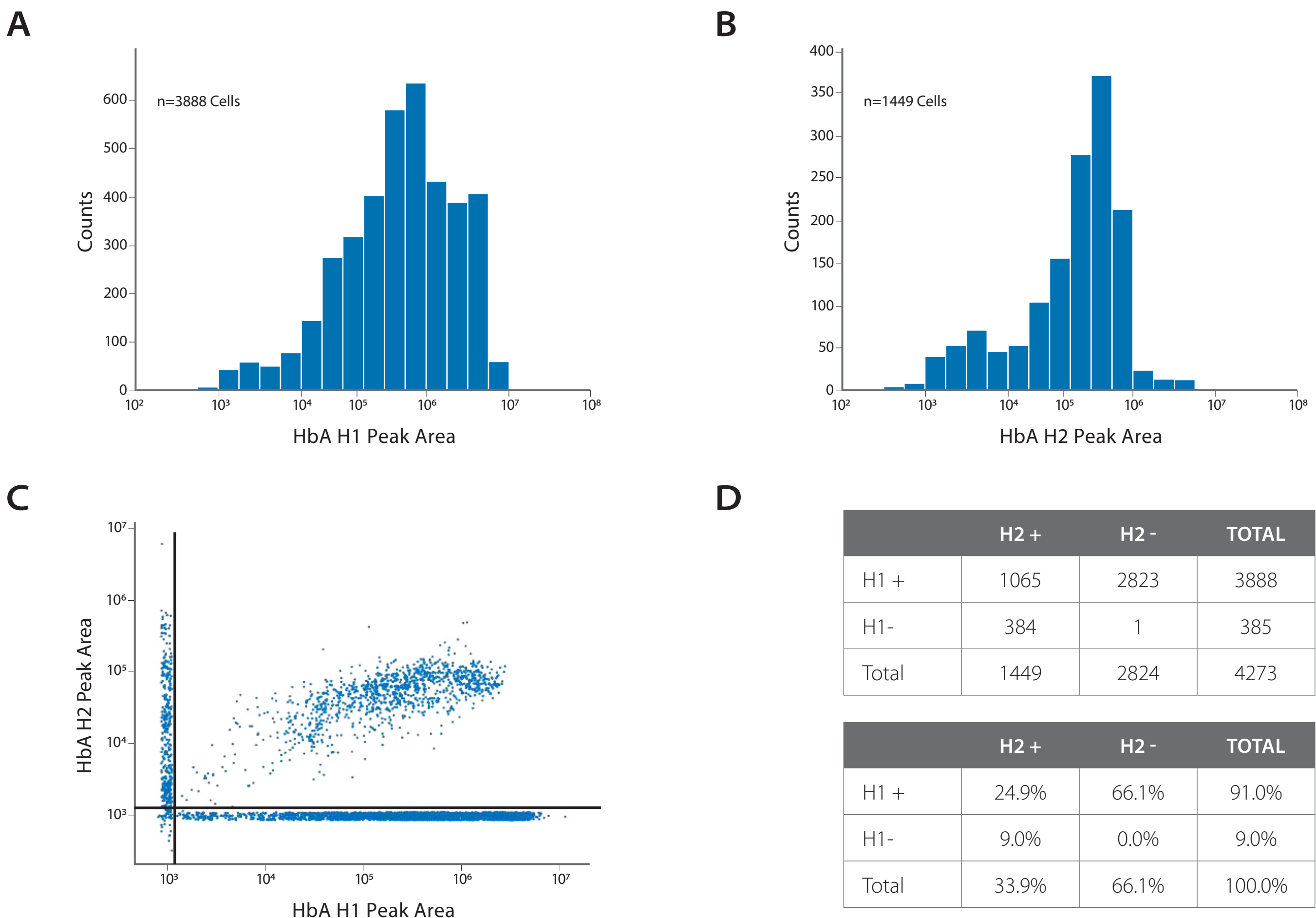


FIGURE 1. A) Milo resolves a dual band pattern for HbA. Image of two lanes where Milo resolves a dual band pattern for HbA (left). The fluorescence intensity plot for the lane highlighted in orange shows two peaks that are distinct in molecular weight (right). Milo's molecular weight sizing step enables resolution of multiple hemoglobin bands so that target abundance can be compared across single-cells for one or more of the specific bands of interest B) Image of two lanes where Milo detects only off-target nonspecific signal detected by the HbA antibody. The fluorescence intensity plot for the lane highlighted in orange shows only a large molecular weight non-specific peak which can be eliminated from analysis. Hemoglobin A primary antibody (Rockland, 200-301-GS4) imaged with Alexa Fluor 647.

areas spanning approximately 4 orders and 3 orders of magnitude, respectively. When comparing the expression of HbA H1 and HbA H2 within a cell (**Figure 2C**), the data showed three clear populations of cells: some that express both H1 and H2 isoforms and populations of cells that express only one hemoglobin form. Interestingly, the hemoglobin isoforms were not both present in every cell. Furthermore, the data suggests more cells contained HbA H1 compared to HbA H2, as 66.1% of the cells were H1+/H2- and only 9% of the cells were H1-/H2+ (**Figure 2D**).

Multiplexing on Milo allows you to detect other proteins of interest in hemoglobin positive cells. For example, Milo can differentiate between normal and a mutant form of hemoglobin, hemoglobin S (HbS), that is found in patients with sickle cell disease (SCD). HbS causes red blood cells to become stiff and crescent-shaped, negatively impacting their ability to carry oxygen. Both forms of hemoglobin can be monitored in the same RBC by multiplexing with Milo using antibodies that specifically recognize the wild type HbA and the mutant HbS.

In this study, HbA was multiplexed with α-synuclein, a potential diagnostic biomarker for Parkinson's disease using antibodies validated for both targets (**Table 1**). In this situation, levels of α-synuclein could be measured as a possible way to determine the Parkinson's disease stage while hemoglobin could be measured as an internal control to identify which well contained an RBC. To ensure signal specificity, HbA and α-synuclein were probed with secondary antibodies conjugated to different Alexa Fluor dyes and imaged in different channels (**Figure 3A**).



	H2 +	H2 -	TOTAL
H1 +	1065	2823	3888
H1-	384	1	385
Total	1449	2824	4273

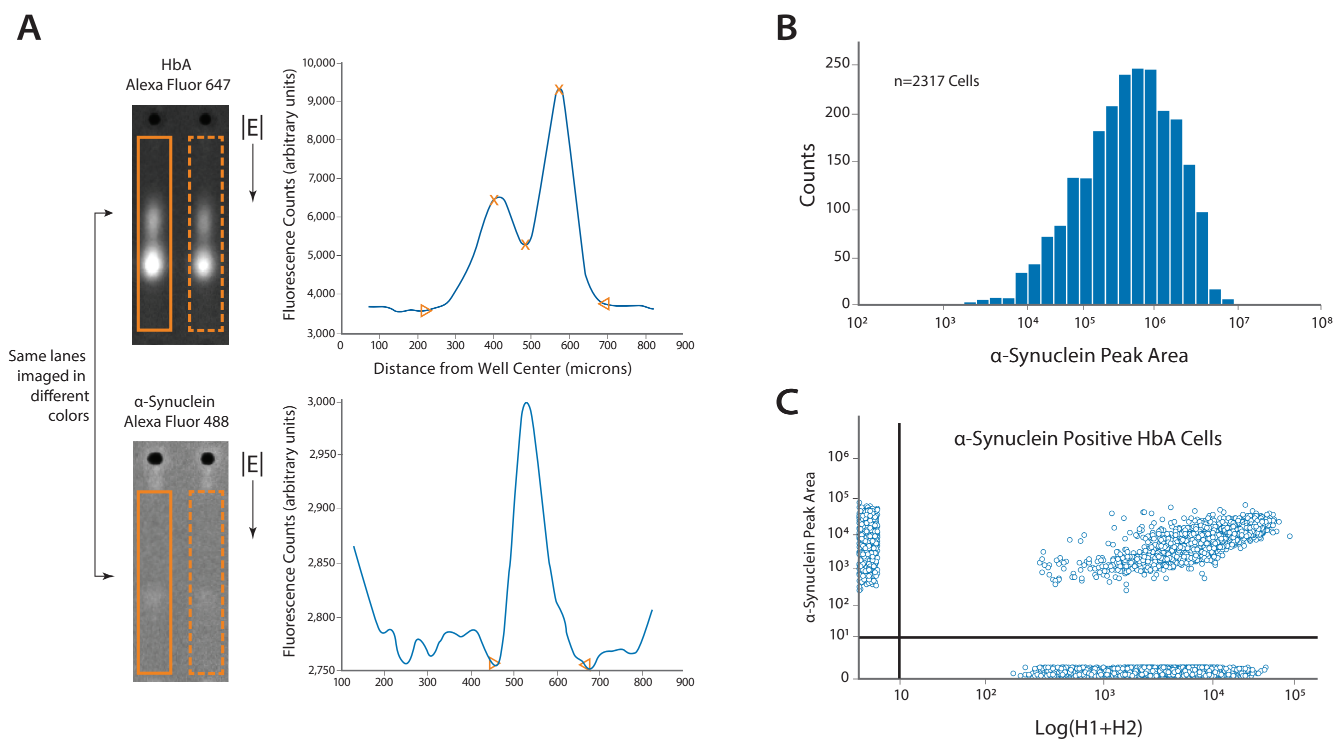
	H2 +	H2 -	TOTAL
H1 +	24.9%	66.1%	91.0%
H1-	9.0%	0.0%	9.0%
Total	33.9%	66.1%	100.0%

FIGURE 2. A) Expression heterogeneity of HbA H1 and B) expression heterogeneity of HbA H2 showed slightly higher expression heterogeneity and protein expression levels for HbA H1 compared to HbA H2. C) Scatter plot of HbA H1 and HbA H2 expression in individual RBCs demonstrates that HbA H1 and HbA H2 are not always co-expressed in cells. In fact, some cells express only one or the other form. D) Quantitation confirms that there are more HbA H1+/H2- cells than HbA H1-/H2+ cells with 66.1% HbA H1+/H2- cells compared to 9.0% HbA H1-/H2+ cells.

Alpha-synuclein heterogeneity in HbA H1 positive cells shows that α-synuclein expression varies almost 1000-fold across the cells analyzed (**Figure 3B**). A scatter plot of α-synuclein peak area vs Log (HbA H1 + HbA H2) indicates that a little less than half of the HbA positive cells also express α-synuclein, with 48.3% of the cells expressing both HbA and α-synuclein (**Figure 3C**). 39.5% of the HbA positive cells did not express α-synuclein and 12.2% of HbA negative cells did express α-synuclein.

PRODUCT NAME	HOST	VENDOR & PRODUCT NUMBER	RECOMMENDED CONCENTRATION
Hemoglobin A (beta chain)	Mouse	Rockland 200-301-GS4	1:10
Hemoglobin, alpha 1	Goat	GeneTex GTX77484	1:10
α-Synuclein	Rabbit	Cell Signaling Technologies 2642S	1:10

TABLE 1. Validated Milo antibodies for Hemoglobin A and α-synuclein and recommended concentrations.



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	H1 & H2+	H1 & H2 -	TOTAL
α-Syn+	2354	594	2948
α-Syn -	1918	0	1918
Total	4272	594	4866

	H1 & H2+	H1 & H2 -	TOTAL
α-Syn+	48.3%	12.2%	2948
α-Syn -	39.5%	0.0%	1918
Total	87.8%	12.2%	4866

FIGURE 3. A) HbA (top) and α-synuclein (bottom) can be multiplexed in Milo by imaging the same lane on a scWest chip in different colors. B) α-synuclein heterogeneity in RBCs in HbA H1 positive cells. C) Scatter plot of α-synuclein peak area vs Log (H1 + H2) peak area demonstrates that 48.3% of HbA positive cells also express α-synuclein. D) Enumeration plots quantify the percentage of cells that co-express each target.

Conclusion

RBC-based biomarkers are important in many different disease areas and hold considerable promise for new, more effective diagnosis and treatment monitoring strategies. In particular, many long-term neurodegenerative disorders are currently diagnosed primarily based on observed symptoms, which eliminates the possibility of detecting disease early when treatments can prevent symptom onset and can result in misdiagnoses. As putative RBC-based biomarkers are identified for these diseases, developing simple blood tests to definitively diagnose diseases is critical in order to effectively diagnose and treat these neurological and other disorders. However, sensitivity and specificity limitations make it challenging to monitor protein biomarker expression in RBCs using traditional protein analysis tools.

The Single-Cell Western addresses these challenges as the assay has the sensitivity and the specificity required to measure expression of HbA and the putative Parkinson's disease biomarker, α-synuclein, in the small RBCs. The capability to separate proteins in a single cell based on molecular weight also gives researchers confidence in the data generated as well as the ability to compare levels of different isoforms or oligomeric forms within a cell or across a population of cells. This is something not possible with flow cytometry, another established single-cell analysis method, since flow cytometry only reports accumulated signals within each cell but cannot resolve different forms. Milo is also compatible with a wider variety of antibodies than are available for flow cytometry, increasing the number of assays possible and decreasing assay development and validation time.

We demonstrated that the Single-Cell Western can multiplex HbA and α-synuclein in a single RBC. This assay brings us closer to monitoring Parkinson's disease using a simple blood test and suggests researchers can use Milo to research and develop new diagnostics and treatments for other RBC-related diseases.

References

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3. Protein expression heterogeneity with Milo, the first Single-Cell Western system. *ProteinSimple Application Note*.

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