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Sickle Cell Imaging Flow Cytometry Assay (SIFCA)

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Abstract

Hemoglobin S polymerization under hypoxic conditions in sickle cell disorders causes characteristic shape changes to human red blood cells. Previous sickling assays used to investigate the efficacy of novel agents to treat these disorders are laborious and observer dependent. Here, we describe a partially automated, high-throughput sickling assay using imaging flow cytometry.

Keywords

Sickle cell disease; Sickling assay; Imaging flow cytometry

1 Introduction

Sickle cell disorders comprise a group of inherited blood diseases that share the genetic mutation HBB Glu6Val, causing the production of the abnormal hemoglobin S (HbS). HbS polymerizes inside red blood cells (RBCs) under hypoxic and acidotic conditions, and the formation of such polymers leads to RBC deformation and to the characteristic elongated shape of a crescent or sickle [1].

RBC sickling is not homogeneous, and the percentage of RBCs undergoing sickling in vitro varies largely across different sickle cell disease genotypes, as well as variations in the concentrations of HbF [2]. Sickling assays have been developed to give an objective measure of the percentage of circulating cells in a given blood sample that sickle upon exposure to conditions known to cause HbS shift to the T-state, which is prone to polymerize and cause RBC sickling. Traditionally, sickling assays rely on the subjective determination of the

percentage of sickling cells by manually counting 200 RBCs through optical microscopy [3, 4, 5, 6]. Therefore, sickling assays based on optical microscopy are cumbersome, time-consuming, and highly observer dependent.

We have, thus, developed an automated, observer-independent sickling assay by applying imaging flow cytometry, which allows software algorithm-driven classification of 20,000 RBCs per sample, yielding a less subjective and more high-throughput assay. This assay has been shown to sensitively detect the known effects of both patient-dependent and -independent variables, such as HbF concentration and sample pH [7].

The SIFCA procedure involves blood sample collection, deoxygenation, imaging flow cytometry, and image analysis. The automated image analysis requires accurate definition of specific masks, features, and image selection.

2 Materials

Prepare and store all solutions and reagents at room temperature unless otherwise specified. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Blood Sample Preparation and Deoxygenation Components

1. Vacutainer EDTA tubes (BD Biosciences Inc.).
2. HS-500 Hemox Solution (TCS Scientific Corporation, New Hope, PA): Store at 4 °C.
3. Phosphate-buffered saline (PBS) 1×.
4. Glutaraldehyde 25 % (Sigma-Aldrich, USA): Vials should be stored frozen at −20 °C or less before use.
5. Glovebox (**see Note 1**) connected to a vacuum pump and a gas cylinder containing 95 % nitrogen/5 % hydrogen mixture, equipped with an oximeter and a 96-well plate shaker. The apparatus is shown in Fig. 1, with the location of valves and gauges for the gas cylinders and vacuum line indicated.
6. 500 µl, 96-well micro-titer plates with adhesive cover.
7. Table-top centrifuge.
8. 1.5 mL polypropylene tubes.
9. 200 µL and 1 mL pipettes and tips.

2.2 Imaging Flow Cytometry Run and Analysis Components

1. PBS 1×.
2. ImageStreamX or MK II imaging flow cytometer (Amnis Corporation, Seattle, WA, USA) equipped with Amnis INSPIRE and Amnis IDEAS softwares.
3. Amnis Speed Beads suspension.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Blood Sample Collection and Preparation

1. Collect 4 mL of peripheral venous blood into Vacutainer EDTA tubes. Sample can be stored at 4 °C for up to 24 h.
2. If sample was stored at 4 °C, place tube on a horizontal shaker at 80–100 rpm until sample is homogenized (approximately 2–5 min).
3. Pipette 10 µL of whole blood into a 1.5 mL polypropylene tube containing 990 µL of Hemox Buffer and mix. This makes a 1 % suspension of blood.
4. Pipette 400 µL duplicates of the 1 % suspension into adjacent wells of a 96-well plate. If more than one sample will be incubated in the same plate, place sample doubles one well apart in both directions, and preferably away from the edges of the plate (Fig. 2).
5. Seal the 96-well plate with an appropriate self-adhesive plastic cover.

3.2 Blood Sample Deoxygenation

1. Before starting, make sure the glovebox is airtight, the gas cylinder contains enough of a 95 % nitrogen/5 % hydrogen mixture, and that the vacuum pump and the oximeter are both operating. Place a 200 µL pipette, 200 µL pipette tips, a new self-adhesive plastic cover, and a proper waste disposal bag for pipette tips and used microplate covers inside the glovebox.
2. Thaw a vial of frozen glutaraldehyde 25 % to room temperature. Total volume of glutaraldehyde should be at least 10 % of the total sample volumes (i.e., 80 µL for two 400 µL samples).
3. Decrease the oxygen concentration inside the glovebox to 2 % by injecting N₂/H₂ mixture and aspirating with the vacuum pump (see **Note 1**).
4. Place the 96-well plate inside the glovebox on top of a shaker and remove adhesive cover.
5. Place the vial containing glutaraldehyde 25 % inside the glovebox and leave cap open (see **Note 2**).
6. Upon opening the glovebox to place the plate and vial, some oxygen may enter the glovebox, so it will be necessary to readjust the oxygen concentration to 2 %.
7. Incubate the 96-well plate at room temperature and 2 % oxygen for 2 h under constant shaking at 300 rpm.
8. Once the incubation is done, turn off the shaker and pipette 40 µL of glutaraldehyde 25 % into each well containing 400 µL of 1 % blood suspension. Mix between seven and ten times with the pipette to sufficiently mix the glutaraldehyde and incubate for 5–10 min.

9. Reseal the plate with a new cover seal and remove from glovebox.
10. Transfer the contents of each well to a properly identified 1.5 mL polypropylene tube.
11. Wash each sample by adding 1 mL of PBS 1×.
12. Centrifuge at $2000 \times g$ for 2 min at 4 °C.
13. Discard supernatant and wash two more times.
14. Resuspend the cell pellet in 100 µL of PBS 1× and store at 4 °C until it is run.

3.3 Imaging Flow Cytometer Sample Run

1. In INSPIRE, for the first run, select the appropriate parameters from the menus to the right: In the Illumination menu, ensure that Brightfield is on and set to 800 for channels 1 and 9, and that the 785 nm laser is set to 0.5; in the Magnification and EDF menu, set the magnification to 60×.
2. Click on “Load”—the equipment will open the sample portal to load the vial containing the sample.
3. Place the open polypropylene tube into the sample portal (see **Note 3**).
4. Click on “OK” when the tube is correctly in place.
5. Once the run starts, wait until the images appear to be in focus and centered (about 30–60 s).
6. Click on “New Histogram”.
7. Click on the “All” population to select it.
8. Choose “Area_Ch01” as the X Axis Feature.
9. This generates a new histogram in the analysis area whose title bar says “All”.
10. Click on “Create Line Region” and draw a line that selects all events with an area between 20 and 350. This eliminates the acquisition of small particles and debris.
11. Name this population “R1.”
12. At this point, if this is the first sample run, save this layout as a template that can be loaded for future runs.
13. Type the name of the file to which images should be saved. Make sure that the number of events acquired is set to 20,000, and that the events to be acquired come from “R1,” not “All,” to minimize acquisition of noise. Designate the destination folder into which files will be saved.
14. Click on “Acquire” to start acquisition.
15. Once acquisition is done, click on “Load” to discard the remainder of the sample and proceed to the next tube. The equipment will return the empty tube before allowing loading of the next sample. Alternatively, click on “Return” to save the remainder of the sample. The equipment will return the tube containing the

remaining cell suspension. Click on “Load” again after removing the tube to allow the next sample to be loaded.

3.4 Imaging Flow Cytometry Analysis: Mask Definition

1. Inside the IDEAS software, click on the “Guided Analysis” menu.
2. Click on “Wizards...,” click on the “Begin Analysis” wizard, and follow the instructions until the wizard ends to identify single, focused cells (see **Notes 4 and 5**).
3. To define new masks, select the “Analysis” menu, then click on “Masks...” to open a dialogue box (see **Note 6**).
4. On the left side, a box will show a list of masks already available. To create a new mask, click on “New.”
5. Click on “Function” to open a dialogue box called “Define Mask Function”.
6. Under “Function,” select “Object.”
7. Under “Mask,” select “M01.”
8. Under “Channel,” select “Ch01.”
9. Under “Image,” select “Ch01.”
10. The name of the mask will, by default, become the definition of the mask just created.
11. Click on “OK.”
12. This returns you to the previous box, where it will show the definition of the mask. It should show “Object(M01, Ch01, Tight)”.
13. Click on “OK” to include the new mask. It will show up as the last mask on the list to your left.
14. Click on “Function” to open the dialogue box called “Define Mask Function.”
15. De-select “Link Inputs.”
16. Under “Function,” select “System.”
17. Under “Mask,” select “Object(M01, Ch01, Tight).”
18. Under “Channel,” select “Ch01.”
19. Under “Image,” select “Ch01.”
20. Under “Weight,” select “80.”
21. Click on “OK.”
22. This returns you to the previous box, where it should show the definition of the mask as “System(Object(M01, Ch01, Tight), Ch01, 80).”
23. Click on “OK” to include the new mask.

24. Click on “Function,” and a dialogue box called “Define Mask Function” will open up.
25. De-select “Link Inputs.”
26. Under “Function,” select “Range.”
27. Under “Mask,” select “System(Object(M01, Ch01, Tight), Ch01, 80).”
28. Under “Image,” select “Ch01.”
29. Under “Area,” set a Minimum value of 350, and a Maximum value of 5000 (the latter should be default).
30. Under “Aspect Ratio,” check that Minimum value is 0, and Maximum value is 1.0 (default).
31. Click on “OK.”
32. This returns you to the previous box, where it will show the definition of the mask as “Range(System(Object(M01, Ch01, Tight), Ch01, 80), 350–5000, 0–1).”
33. Click on “OK” to include the new mask.
34. Click on “Function” to open the dialogue box called “Define Mask Function.”
35. De-select “Link Inputs.”
36. Under “Function,” select “Range.”
37. Under “Mask,” select “Object(M01, Ch01, Tight).”
38. Under “Image,” select “Ch01.”
39. Under “Area,” set a Minimum value of 350, and a Maximum value of 5000 (the latter should be default).
40. Under “Aspect Ratio,” check that Minimum value is 0, and Maximum value is 1.0 (default).
41. Click on “OK.”
42. This returns you to the previous box, where it should show the definition of the mask as “Range(Object(M01, Ch01, Tight), 350–5000, 0–1).”
43. Click on “OK” to include the new mask, and it should show up as the last mask on the list to your left. Figure 3 depicts the sequential creation of user defined masks and demonstrates how these tighter masks yield more accurate analysis of single cells and their feature values.

3.5 Features Definition

1. Select the “Analysis” menu, then click on “Features...” to open a dialogue box.
2. On the left side, a box will show a list of features already available. To create a new feature, click on “New.”

3. Select the Feature type “Spot Count” from the pull-down menu next to “single.”
4. Select the Object M01 mask from the pull-down menu.
5. Click on “Set Default Name” (typically, it will be Spot Count_Object M01) and click “OK” (see **Note 7**).
6. Repeat steps 3–5 for all three new masks: System(Object(M01, Ch01, Tight), Ch01, 80), Range(System(Object(M01, Ch01, Tight), Ch01, 80), 350–5000, 0–1), and Range(Object(M01, Ch01, Tight), 350–5000, 0–1).
7. Click on “Close,” and the software will calculate the values for each of the features and each cell (see **Note 8**).

3.6 Image Selection

1. To define the “Single cells” gate, click on “New Scatterplot.”
2. Choose “Area_M01” as the X Axis Feature, and “Aspect Ratio_M01” as the Y Axis Feature from the pull-down menus.
3. Click on “Create Rectangle Region” or “Create Polygon Region” to draw a region that gates the main cell population, tight enough to exclude particles and debris to the left, and the larger cell aggregates to the right. Name this region “Single Cells.” One example using a rectangular region is shown in Fig. 4a (see Note 9). It is important that cells with low aspect ratio be included in this gate, as cells which are sickled will have lower aspect ratios.
4. To define the “Cells in focus” gate, click on “New Histogram.”
5. Click on the “Single Cells” population to select it.
6. Choose “Gradient RMS_M01_Ch01” as the X Axis Feature.
7. This will generate a new graph in the analysis area whose title bar says “Single cells.”
8. Click on “Selected Bin” in the “Population” pull-down menu.
9. Click on “New Line Region” and draw a line that selects the right portion of the curve containing cells in focus, including the whole right tail. Name this population “Cells in focus.” This should look like Fig. 4b.
10. Click on the bins to check the focus of the selected cells to define the cutoff value that selects focused cells. You can manually adjust the value of the X Coordinate through the “Regions...” dialogue box under the “Analysis” menu, or by adjusting the left edge of the region (see **Note 10**).
11. To define the “Cells of interest” gate, click on “New Scatterplot.”
12. Click on the “Cells in focus” population to select it.
13. Choose “Spot Count_ Range (System(Object(M01, Ch01, Tight), Ch01, 80), 350–5000, 0–1)” as the X Axis Feature, and “Spot Count_ Range (Object(M01, Ch01, Tight), 350–5000, 0–1)” as the Y Axis Feature from the pull-down menus.

14. Click on “New Rectangle Region,” and draw a rectangle that gates cells that have an X and Y value equal or less than 1.
15. Name this population “Cells of interest.” This should look like Fig. 4c.
16. To define the “One spot” gate, click on “New Scatterplot.”
17. Click on the “Cells of interest” population to select it.
18. Choose “Spot Count_ Object M01” as the X Axis Feature, and “Spot Count_ System (Object (M01, Ch01, Tight), Ch01, 80)” as the Y Axis Feature from the pull-down menus.
19. Click on “New Oval Region,” and draw a circle that gates the single dot containing cells that have an X and Y value equal to 1.
20. Name this population “One spot.” This should look like Fig. 4d. This is the population of cells that will be classified according to shape.

3.7 Final Cell Shape Analysis

1. Inside the Features dialogue box, click on “New.”
2. Select the Feature Type “Shape Ratio.”
3. Select the “Range(System(Object(M01, Ch01, Tight), Ch01, 80), 350–5000, 0–1)” mask.
4. Click on “Set Default Name” and click on “OK.”
5. Repeat the same procedure for the “M01” mask, creating the “Shape Ratio_M01” feature.
6. Click on “New Scatterplot.”
7. Click on the “One spot” population to select it.
8. Choose “Shape Ratio_M01” as the X Axis Feature, and “Shape Ratio_ Range(System(Object(M01, Ch01, Tight), Ch01, 80), 350–5000, 0–1)” as the Y Axis Feature from the pull-down menus.
9. Use the “New Rectangle Region” tool to create two square regions: one top-right region, named “Normal cells”, and one bottom-left region, named “Abnormal cells.”
10. Select “Regions...” from the “Analysis” menu.
11. Click on “Normal cells.”
12. Type in new values for the X Coordinates (top 0.5, bottom 1) and Y Coordinates (top 1, bottom 0.5).
13. Click on “Abnormal cells.”
14. Type in new values for the X Coordinates (top 0, bottom 0.5) and Y Coordinates (top 0.5, bottom 0) and click on “Close.” The resulting plot should look like Fig. 4e.

15. Click on the “Σ” icon on the top right corner of this graph. This will show the statistics for this graph, with the “Count” and “%Gated” values for each population (see Notes 11 and 12).
16. The “% Gated” value will yield what percentage of the cells with an adequate image (i.e., a focused, single cell image) had their shape classified as “normal” or “abnormal”. Cells outside these gates are classified as “indeterminate.”
17. The SIFCA Abnormal:Normal ratio can be calculated by dividing “Abnormal cells” Count by the “Normal cells” Count (see **Notes 13 – 15**).

4 Notes

The glove box (Plas Labs, CAT# Z563013; see Fig. 1) contained a gas analyzer (ServoFlex miniMP 5200 by Servomex) to detect concentrations of oxygen, and was equipped with a standard vacuum pump (model number 5KC37NN76X, General Electric). The oxygen concentration inside the glovebox was decreased to 2 % by injecting a mixture of N₂/H₂ and aspirating oxygen with the vacuum pump. We find that determining the optimal rate of gas injection that balances the aspiration by the vacuum pump makes the deoxygenation process less cumbersome and prevents glove damage from excessive pressure. Lowering the oxygen concentration to 2 % with the vacuum pump on and gas cylinder open will result in a final oxygen concentration lower than desired, so, in our experience, reaching 2.5 % is enough to turn off gas and vacuum and wait for the system to equilibrate. Usually, before the system stabilizes, reaction between oxygen inside the glovebox and hydrogen in the gas mixture will yield water vapor and cloud the glovebox while the oxygen concentration continues to drift down.

2. This allows the glutaraldehyde to equilibrate with the hypoxic conditions.
3. Polypropylene tubes with hinged lids should be loaded well open to avoid obstruction. In our experience, many red blood cell samples will be too concentrated to be run as prepared, generating an “insufficient volume” error upon loading the sample. Adding additional 50–100 µL of PBS while mixing the sample may prevent rejection by the equipment when trying to load. Removing any bubbles with the pipette at this point is also crucial to avoid bubbles into the cytometer.
4. Since this assay does not use fluorescent antibodies, there is no need to create a new Compensation Matrix. If prompted about the channels used in the experiment, click on “Skip” until the user exits the wizard, since only bright-field images will be used (Channels 01 and 09).
5. If “View” is set to “All Channels,” the user should be able to see all the 12 channel images for each cell. Select “Ch01” from the pull down menu next to “View” to see only one bright-field image of each cell. For a better view of the images with uniformly shaped fields of view: click on “Image Properties.” On the “Display Properties” tab, define Display Width and Display Height (lower right corner of the window) as 100, and click on “OK.”

6. IDEAS automatically generates some masks, such as M01, M02, and M03. For the SIFCA, you will need to create new masks. You can name them with simpler names, but make sure not to get them mixed up when creating the features.

7. Although IDEAS also creates several features automatically, you will need to create new features to complete the SIFCA analysis. You may choose to name the features differently, but in this protocol, we have chosen to stick to the default names generated by the software.

8. Every time a new feature is created and the user clicks on “Close,” the software will calculate the values for each cell. This will take some seconds, and can take a couple minutes depending on how many features have been created at once, and how many images have been acquired.

9. The X and Y values that define this gate may change from day to day, and from instrument to instrument depending on fluidics. Every time a region is created, the software will automatically suggest a name (e.g., R1, R2, R3). If the user clicks on “OK” by accident, the name can be changed later by accessing the same dialogue box.

10. The remaining coordinates do not need to be changed, providing the whole right tail of the curve has been selected when defining the line region. In the example shown in Fig. 2, the X value used was 41.

11. The user can visualize the separated populations of cells by selecting the “Abnormal” and “Normal” cell populations from the “Population” pull-down menu.

12. If either of these values are not shown by default, right-click on the plot or table to open a dialogue box for “Statistics” and make sure “Count” and “%Gated” are checked.

13. For “% Gated” values to be accurate, make sure the “One spot” population corresponds to a “%Gated” value of 100 %.

14. Alternatively, the SIFCA Abnormal:Normal ratio can be obtained by dividing “%Gated” of “Abnormal cells” by “Normal cells” values.

15. Higher SIFCA Abnormal:Normal ratios are obtained in samples containing a larger number of sickling cells, i.e., samples subjected to lower pH, and samples from patients with lower HbF content.

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References

1. Barabino GA, Platt MO, Kaul DK. Sick cell biomechanics. *Annu Rev Biomed Eng.* 2010; 12:345–367. DOI: 10.1146/annurev-bioeng-070909-105339 [PubMed: 20455701]

2. Brittenham GM, Schechter AN, Noguchi CT. Hemoglobin S polymerization: primary determinant of the hemolytic and clinical severity of the sickling syndromes. *Blood*. 1985; 65:183–189. [PubMed: 3965046]
3. Charache S, Conley CL. Rate of sickling of red cells during deoxygenation of blood from persons with various sickling disorders. *Blood*. 1964; 24:25–48. [PubMed: 14194270]
4. Bergeron MF, Cannon JG, Hall EL, Kutlar A. Erythrocyte sickling during exercise and thermal stress. *Clin J Sport Med*. 2004; 14:354–356. [PubMed: 15523207]
5. Horiuchi K, Ohata J, Hirano Y, Asakura T. Morphologic studies of sickle erythrocytes by image analysis. *J Lab Clin Med*. 1990; 115:613–620. [PubMed: 2341764]
6. Obata K, Mattiello J, Asakura K, Ohene-Frempong K, Asakura T. Exposure of blood from patients with sickle cell disease to air changes the morphological, oxygen-binding, and sickling properties of sickled erythrocytes. *Am J Hematol*. 2006; 81:26–35. DOI: 10.1002/ajh.20514 [PubMed: 16369974]
7. van Beers EJ, Samsel L, Mendelsohn L, Saiyed R, Fertrin KY, Brantner CA, et al. Imaging flow cytometry for automated detection of hypoxia-induced erythrocyte shape change in sickle cell disease. *Am J Hematol*. 2014; 89:598–603. DOI: 10.1002/ajh.23699 [PubMed: 24585634]

Figure 1. Glovebox used for sample deoxygenation

The apparatus is shown with the location of valves and gauges for the gas cylinders and vacuum pump indicated.

Figure 2.
Schematic representation of sample arrangement in a 96-well plate.

Figure 3. Bright-field imagery depicting creation and purpose of user-defined masks

The left column shows the bright-field image with no mask. The second column shows the default mask M01. The third column, “System80,” shows the user defined System (Object (M01, Ch01, Tight), Ch01, 80) mask. The right column, “RangeSystem,” shows the user defined Range (System (Object(M01, Ch01, Tight), Ch01, 80), 350–5000, 0–1) mask. Each image contains a small particle next to a single cell. The default mask includes the small particle and thus elongates the masked area, resulting in skewed feature values, such as aspect ratio and shape ratio. Use of the System (Object (M01, Ch01, Tight), Ch01, 80) mask achieved a tighter fit to the cell and disconnected the small particle mask from the cell mask.

Use of the Range(System(Object(M01, Ch01, Tight), Ch01, 80), 350–5000, 0–1) mask allowed for a tight mask of the cell image, but no longer masked the small particle, thus providing a more accurate measurement of the feature values of the cell. Using these masks for generating the spot count and shape ratio features enabled better precision in analysing frames containing only one object as well as more accurate shape ratios.

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Figure 4. Example of the graphs obtained in the SIFCA analysis

(a) Shows the gate defining single cells. (b) Displays the definition of focused images. (c) Shows gating out images with more than one cell per image, and (d) depicts the refinement of this selection, yielding the “one-spot” population. (e) Shows the final analysis gates, defining “normal cells” and “abnormal cells.” In (a), (b), and (e), each dot represents one individual event. In (c) and (d), the dots represent a population of events containing various numbers of masked events. The 1 spot population was gated and used for further analysis. The use of the spot count feature with multiple masks allowed for the elimination of artifacts from the analysis, such as events having a cell with a particle next to it, thereby giving the masked event a longer shape ratio.