



# Studying T Cells N-Glycosylation by Imaging Flow Cytometry

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## ABSTRACT

Imaging flow cytometry is an emerging imaging technology that combines features of both conventional flow cytometry and fluorescence microscopy allowing quantification of the imaging parameters. The analysis of protein posttranslational modifications by glycosylation using imaging flow cytometry constitutes an important bioimaging tool in the glycobiology field. This technique allows quantification of the glycan fluorescence intensity, co-localization with proteins, and evaluation of the membrane/cytoplasmic expression. In this chapter we provide the guidelines to analyze glycan expression, particularly the  $\beta_{1,6}$  GlcNAc branched N-glycans, on the membrane of intestinal T cells from inflammatory bowel disease patients.

**Key words** N-Glycosylation, T-Cell Receptor, T-cells, Ulcerative colitis, Imaging flow cytometry

## INTRODUCTION

Glycosylation is an important protein posttranslational modification being fundamental for the regulation of proteins biological functions both in homeostasis and in pathological conditions. The development of novel accurate assays for analysis of this key biological process is of utmost importance in the glycobiology research field. Flow cytometry is a widely used technique that allows measuring fluorescence intensity in many events, but that lacks the possibility of analyzing imaging parameters. On the other hand, fluorescence microscopy is not ideal to analyze and quantify parameters from a large number of cells. The invention of the ImageStream system, which combines features of fluorescence microscopy and flow cytometry, overcomes these limitations opening new opportunities to explore a range of applications with utility in the glyco-science research field. This technique presents an extraordinary throughput, thus allowing fast acquisition of imaging data [1]. Furthermore, the IDEAS data analysis software has the capacity to calculate several quantitative features that allow the graphical identification of populations based in several variables (fluorescence intensity, cell size, shape, texture, co-localization of multiple probes, among others) [1]. Therefore, imaging flow cytometry technique becomes a remarkable tool for studies related with cellular morphology, internalization, cell signaling, co-localization, and trafficking (see specific applications in: <https://www.amnis.com/applications.html>). Additionally, we can even create/define masks to easily identify our targets, for instance, to identify co-localization of probes at the cell membrane.

Taking advantages of the combined parameters of imaging flow cytometry, we have analyzed the levels of membrane TCR branched N-glycans comparing different ulcerative colitis (UC) patients and controls. Furthermore, we provide here an example for analysis of co-localization between a lectin-recognizing glycan and a protein receptor (TCR). Using imaging flow cytometry we evaluated the co-localization of the L-PHA lectin (Phaseolus Vulgaris Leucoagglutinin lectin that specifically recognizes the  $\beta_{1,6}$  GlcNAc branched N-glycan structures) with surface TCR  $\alpha/\beta$  on purified intestinal lamina propria T lymphocytes. Interestingly, this bioimaging technique has validated results obtained from the canonical molecular techniques used to assess proteins glycosylation (such as Western blot and immunoprecipitation approaches) [2]. In this chapter we provide a comprehensive approach on how to use imaging flow cytometry in glycoscience, particularly in studying N-glycosylation on the membrane of T cells.

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## MATERIALS

### Reagents for Cell Staining

1. Phosphate buffered saline (PBS) 1×.
2. FACS Buffer: PBS 1× with 0.1 % sodium azide and BSA 2 %.
3. Antibodies: anti-TCR  $\alpha/\beta$  mAb (clone BW242/412, mouse IgG2b) conjugated with R-phycoerythrin (PE) (MiltenyiBiotec); Fluorescein Phaseolus vulgaris Leucoagglutinin (L-PHA/FITC) (Vector Laboratories); mouse IgG2b anti-human conjugated with PE (Immunotools); Streptavidin-conjugated fluorescein isothiocyanate (FITC) (Caltag Laboratories).

### Equipment

Imaging flow cytometer equipped with one laser and one camera (ImageStreamX, from Amnis, now part of Millipore). This equipment can have more lasers and cameras for more complex analysis.

## METHODS

This Imaging flow cytometry protocol involves three main parts: (1) Staining of cells with the probes of interest; (2) acquisition of data on an imaging flow cytometer; (3) data analysis and statistics.

### Cell Staining

Cells can be stained with a protocol typically used for traditional flow cytometry, but a careful titration of the labeling agents (dyes or antibodies) is even more crucial. As with any imaging experiment, one should aim for good signal-to-noise ratio while avoiding saturation. This is particularly important when dealing with more than one fluorophore excited by the same laser: the settings that can be changed during acquisition (laser power and selected filters) will affect visualization of all stainings, and thus it is crucial to tune labeling for optimal imaging. The following protocol is described for already optimized dilutions of probes (see Note 1).

Each experiment must include different samples: the staining of interest, a negative control (isotype control if using antibodies) and single stained cells for compensation, when analyzing more than one color.

1. After isolating lamina propria T lymphocytes (LPLs), following an adapted protocol [3], wash cells with RPMI by centrifuging at 800×g, 5 min, 4 °C.
2. Remove supernatant.
3. Resuspend pellet with 1 ml FACS buffer and keep on ice.

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4. Divide resuspended pellet in 4 Eppendorf tubes (see Note 2) for: (a) negative control, (b) double staining (anti-TCR and L-PHA), (c) single staining TCR, and (d) single staining L-PHA, for compensation.

5. Centrifuge at 300xg, 10 min, 4 °C.

#### Blocking

6. Discard supernatant.

7. Resuspend samples with 500 µl of FACS buffer. 8. Incubate for 30 min, on ice.

9. Centrifuge at 300xg, for 10 min, 4 °C.

10. Discard supernatant.

#### Staining

11. Resuspend samples in 100 µl FACS buffer.

12. Add 10 µl PE-conjugated anti-TCR antibody to tubes b and c, or the equivalent amount of isotype control to the negative control sample (a).

13. Incubate for 30 min, on ice and in the dark.

14. Wash with 500 µl FACS buffer by centrifuging at 300xg, 10 min, 4 °C.

15. Repeat washing step (see Note 3).

16. Prepare the lectin 4 µg/ml: 1 µl L-PHA-FITC in 500 µl FACS buffer.

17. Incubate samples b and d with 50 µl of prepared lectin and control a with 50 µl of FACS buffer.

18. Incubate on ice for 30 min, in the dark.

19. Prepare a streptavidin-FITC 1 µg/ml solution (from stock 1000 µg/ml): 1 µl Streptavidin-FITC in 1000 µl FACS buffer.

20. Incubate samples a, b, and d with 50 µl of streptavidin solution.

21. Incubate on ice for 30 min, in the dark.

22. Wash with 500 µl FACS solution by centrifuging at 300xg, 10 min, 4 °C.



23. Repeat washing step.

Fixation (after staining, see Note 4).

24. After the previous step, discard supernatant.

25. Fix cells with 100  $\mu$ l formaldehyde 4 %, 20 min, room temperature, in the dark.

26. Centrifuge at 300 $\times$ g, 10 min, 4 °C.

27. Wash with 500  $\mu$ l PBS 1 $\times$ .

28. Centrifuge at 300 $\times$ g, 10 min, 4 °C.

29. Repeat the washing step.

30. Discard supernatant.

31. Resuspend in 60  $\mu$ l of PBS 1 $\times$  (see Note 5) and maintain at 4 °C, in the dark until acquisition in the ImageStream cytometer (see Note 6).

## Acquisition of Data by Imaging Flow Cytometry

Images of isolated cells can be acquired on a 6-Channel ImageStreamX imaging flow cytometer equipped with one laser (Amnis, EMD Millipore), using the INSPIRE software.

1. Initialization of equipment.

Initialize the ImageStream by following your facility's rules: typically, you need to ensure all containers are filled with appropriate solutions (beads, sterilizer—you can use 10 % bleach, cleanser, debubblers—you can use 70 % isopropanol, rinse—ultrapure water, sheath—filtered PBS 1 $\times$ , also add some bleach to the waste container) and you may need or not to calibrate.

Preparation of samples immediately before acquisition:

2. To filter samples, start by adding a 2  $\mu$ l drop of PBS 1 $\times$  to the inside edge of a 1.5 ml tube.

3. Place a 70  $\mu$ m cell strainer on top of the tube.

4. While exerting some pressure, pipette the cell suspension on the cell strainer, aiming for the place where you added the PBS 1 $\times$  drop (see Note 7).

## Sample Acquisition

1. Start by acquiring the sample with all stainings, to allow you to adjust the equipment settings.

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2. Create a classifier based on the bright-field image, so that only events with an area above a certain size will be included in the analysis. In this case, we choose an area higher than 20, measured in the bright-field image.
3. Adjust the laser power and choose the best filters for your image. These settings should be defined in such a way that images are not saturated (see Note 8).
4. Once all settings are defined, you can start acquiring your data. Ensure that you set up the number of events to acquire to at least 10,000 events.
5. Analyze firstly your stained samples and negative controls, and then proceed for the single stained controls for compensation.
6. Before acquiring samples for compensation enable the option "Comp settings," and change the classifier for the channel you will be visualizing (as you will not be able to see any bright-field image).
7. After finishing acquisition, proceed with sterilization and cleaning protocols for your equipment.

## Data Analysis and Statistics

Analysis was performed with IDEAS 5.0 (Amnis, EMD Millipore) as follows (Fig. 1):

1. A compensation matrix can be created by choosing "create a new matrix" under "Compensation," and following the wizard. Select the files corresponding to single stained controls. At the end, verify whether images are properly compensated by using the "preview images" tool in the "create compensation matrix" dialog box.
2. For analysis, several wizards are available. Here, we advise starting with the co-localization wizard. Briefly, this wizard guides the user through the most common steps: uploading of raw data files (.rif) and compensation matrix to create compensated data files (.cif) and data analysis files (.daf); gating focused events; gating single cells (see Note 9); gating in populations of interest (in this case, TCR-FITC+ cells); calculation of co-localization, or the Bright Detail Similarity between the two probes (L-PHA and anti-TCR) (see Note 10).
3. Besides calculating co-localization, one can proceed to other analysis in the same file. In this case, the intensity of L-PHA staining specifically located at the membrane of TCR  $\alpha/\beta$  positive events can also be determined. For that, it is necessary to create a new mask by going to Analysis and then Masks (see Note 11). Here, a new mask to depict the cell membrane was created. This mask uses the functions dilate and erode ("dilate and not erode") and can be based in the bright-field or in a fluorescence image of the membrane.
4. Then create a new feature by going to Features under the Analysis menu. Here, we were interested in evaluating the intensity of L-PHA staining specifically in the cell membrane. A histogram plot for this new feature can then be generated.



5. Finally, create a statistics report to include all relevant information: percentage of TCR+ cells, mean bright detail similarity, mean fluorescence intensity of L-PHA at the membrane, etc.

6. An interesting feature of the IDEAS software is the possibility to easily analyze many files with one template. If you have many files to analyze, save the analysis file as a template and perform a batch analysis, by going to Tools, choosing batch data file and defining the input files and template to use.

## Notes

1. If you do not have any information about using your probe with the available imaging flow cytometer, you should start by titrating it. Perform the protocol described herein, but with different concentrations of probe. The ideal concentration of probe(s) allow you to visualize cells with a good, but not saturated signal (see Note 8), using the same settings for all probes.

2. Ideally, cells should be counted and divided equally for each condition. But when having a limited number of cells, cell suspension can be divided in the 4 Eppendorfs, as following: 500  $\mu$ l—double staining TCR-PE/L-PHA; 166  $\mu$ l—single staining TCR-PE; 166  $\mu$ l—single staining L-PHA- FITC; 166  $\mu$ l—negative control (isotype + Streptavidin-FITC).

3. Samples single staining TCR-PE (tube c) should be resuspended in 60  $\mu$ l of PBS 1 $\times$  and kept on ice until FIXATION.

4. Fixation is a critical step that should be optimized to determine if it is better to fix before or after staining with the specific probes. Furthermore, it must be tested what is the best for fixation (paraformaldehyde, formaldehyde, methanol), since it may influence cells morphology and protein distribution, affecting the quality of images and consequently evaluation of the parameters of interest.

5. Samples are resuspended in 60  $\mu$ l of PBS 1 $\times$  because the ImageStreamX will run samples with a minimum of 50  $\mu$ l. The number of cells per sample advised by the manufacturer is approximately  $1 \times 10^6$  cells (up to  $5 \times 10^6$  cells) in a final volume of 50  $\mu$ l, in a 1.5 ml microcentrifuge tube, but we found that samples with a lower number of cells can still be analyzed, although acquisition will take a long time.

6. Ideally acquisition in the ImageStream cytometer should be performed immediately after staining. As this is not always practical, fixation allows keeping the cells in cold PBS 1 $\times$  for a couple of days. Imaging should however be performed as soon as possible.

7. Samples with such a small volume are troublesome to filter. Add a 2  $\mu$ l drop of PBS 1 $\times$ , that may stay at the edge of the tube or flow down. Either way, it will create a channel for fluid to flow through by capillary action. It is important to avoid having fluid all around the tube as the sample will then not flow through. The first time you do this start by training with water.

8. During acquisition, you can plot in real time the highest intensity in one pixel of your event vs the area of each event on the bottom graphs of the acquisition windows. This allows you to visualize whether your images are saturated.

9. When performing analysis with the IDEAS software an interesting feature is the fact that you can always observe the images to which a dot in a dot plot corresponds. This becomes extremely useful when deciding on where to draw a gate, as it is possible to visualize whether that dot is a cell, cell debris, a doublet, etc.

The bright detail similarity is one of the many built in features that can be used with IDEAS. This index gives a measure of the co-localization of two probes in a defined region. It computes the log transformed Pearson's correlation coefficient of the bright spots with a radius of 3 pixels or less within a masked area in the two input images. More details on how this feature functions can be found in [4].

10. It must be pointed out that this bright detail similarity gives a measure of whether two molecules are within the same area of the cells, but due to resolution limitations, it does not indicate whether these molecules interact with each other. For that, other techniques are necessary, such as immunoprecipitation [2] or FRET.

11. If you use a wizard that automatically creates a mask, it is advisable to confirm whether this mask is being correctly applied. For that, go to image properties (icon is the symbol for brightness and contrast manipulation), create a new view that includes your channel of interest with the mask created, go back to the global window and choose that view. Click then on the mask icon and scroll through your cells to see how the mask is being applied (Fig. 2).

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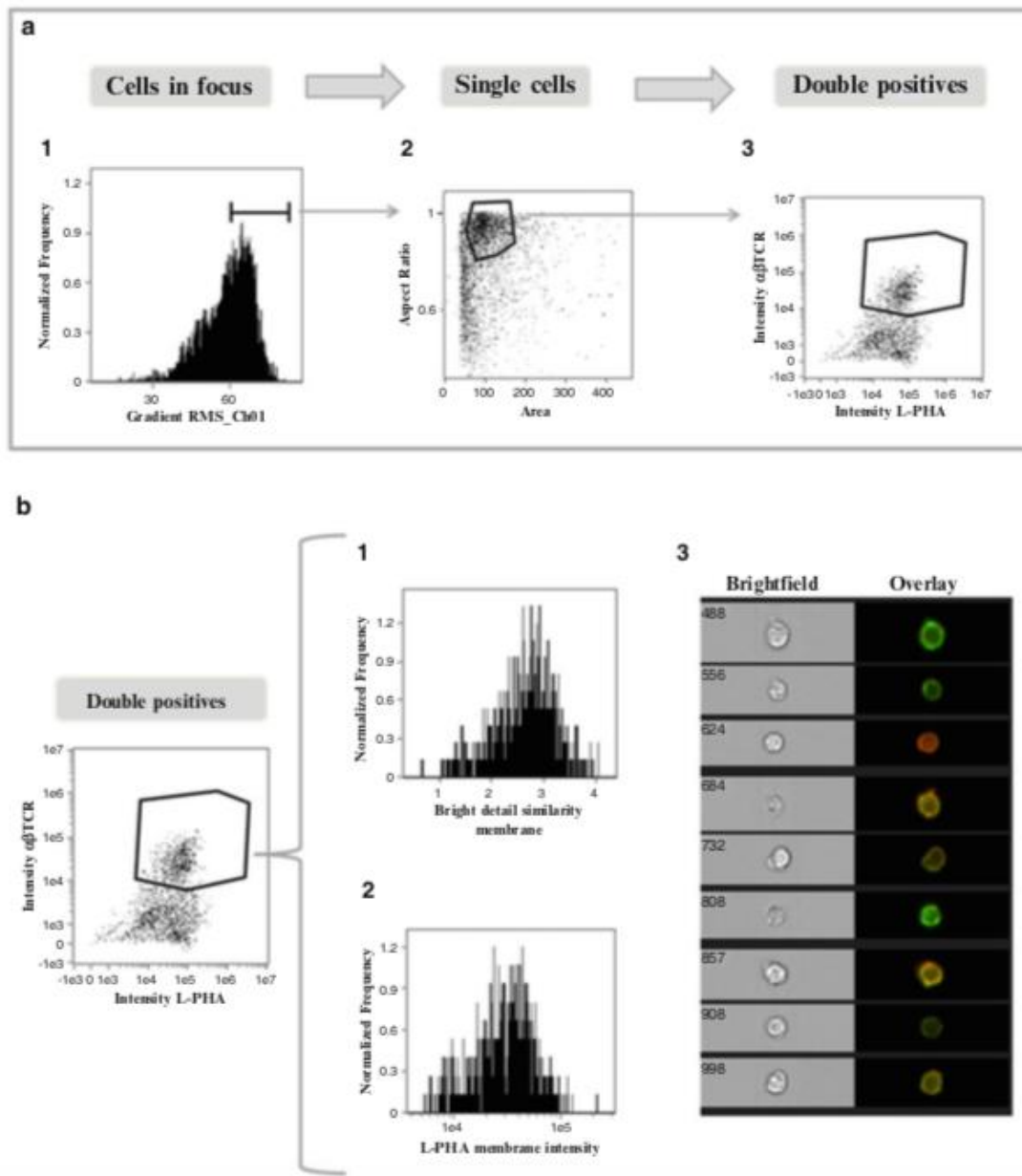


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**Fig. 1** Schematic representation of the analysis using an example of data acquired after staining for L-PHA and TCR. (a) Sequence of analysis steps. (1) A histogram for gradient RMS in bright-field images (Channel 01) can be used to gate for cells in focus. (2) A scatter plot of aspect ratio/area in Channel 1 (bright-field) is used to gate for single cells, within focused events. (3) The intensity of  $\alpha/\beta$  TCR and L-PHA staining is used to gate on double positive cells within focused single cells. (b) Graphic representation of parameters analyzed from the double positive cells selected. (1) The level of co-localization at the membrane was quantified as the bright detail similarity. (2) Histogram represents the intensity of L-PHA staining on the membrane of  $\alpha/\beta$  TCR+ L-PHA+ cells. (3) Panel showing examples of single cell bright-field (Ch01) and merged (TCR in red and L-PHA in green) images, showing co-localization between TCR  $\alpha/\beta$  and L-PHA

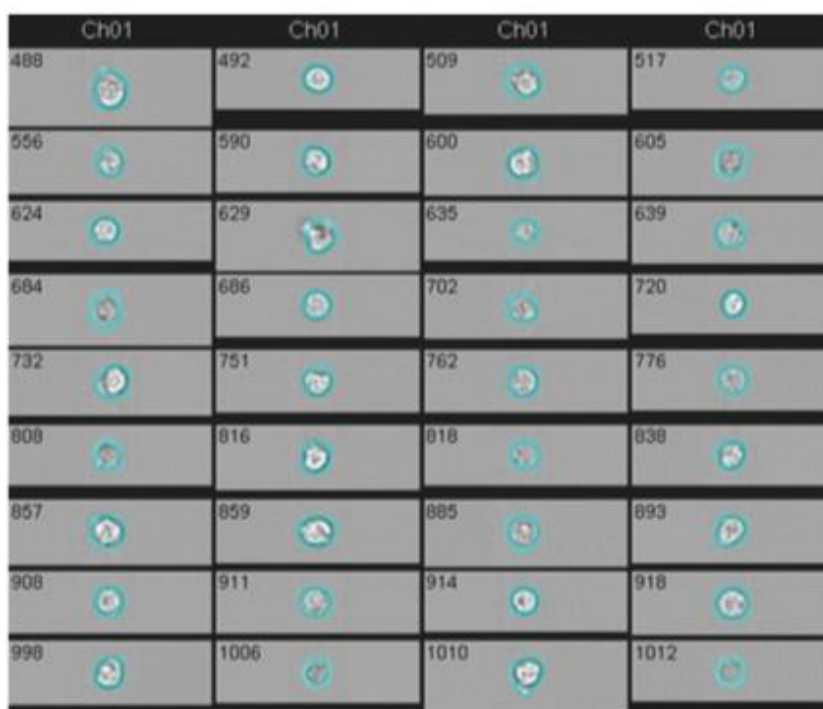


Fig. 2 Panel with examples of bright-field images (channel 1, Ch01) with indication of the membrane mask create (in light blue). By creating masks, with IDEAS 5.0 (Amnis, EMD Millipore), we can restrict the analysis to a specific location. The mask membrane created allows quantification of L-PHA staining on the cell membrane of TCR  $\alpha/\beta$  + cells from ulcerative colitis patients and controls