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Flow Cytometry as a Tool for Measurement of Steroid Hormone Receptor Protein Expression in Leukocytes

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Abstract

Measurement of protein expression in live, intact cells using flow cytometry (FC) has been employed for several decades in the areas of immunology, cell biology, and molecular biology. More recently, this technique has found appreciation in applied scientific fields, including cancer biology and endocrinology, to serve as a tool for identifying cells more likely to respond to specific treatments. FC, also referred to as fluorescence-activated cell sorting (FACS), is an antibody-based method that provides the user with an ability to identify proteins expressed on surfaces of cells as well as in the cytoplasm, including steroid hormone receptors. This technique is most useful for examining specific cell types in a heterogeneous population and therefore can be used to identify cells more likely to respond to treatments based on expression of the appropriate receptor. Isolation of purified subpopulations for further manipulation and investigation of functional capacity is also possible using a cell sorter, which uses similar technology to isolate cells for use by the researcher. This is especially important for studying responses of less abundant cell populations in tissues that express high levels of a target protein or receptor of interest. Furthermore, FACS analysis is clinically useful to identify and isolate responsive cell populations, which may be less appreciable in whole tissues because of the diluting effects of surrounding, nonresponding cell types. Immune cells are commonly utilized as a source of cell populations in the FC technique and have previously been shown to express steroid hormone receptors and respond to steroid hormone treatment. Here, we demonstrate that FC is a useful tool for identifying immune cells expressing steroid hormone receptor protein. This method can also be easily expanded to include other, nonimmune cell populations to address specific research questions related to steroid hormone receptor biology.

Keywords

Flow cytometry; Steroid hormone receptors; Leukocytes

1. Introduction

Flow cytometry was first developed by researchers at Stanford University who set out to create a system for identifying and isolating live cells that could be used for further culturing and manipulation (1). This method creatively uses fluorescently-tagged antibodies to identify cells expressing a protein or receptor of interest (information on fluorochromes currently available for use is described and reviewed in **ref.** (2)). This method may also be similarly used with green fluorescent protein (GFP)-tagged antibodies. Samples are analyzed in a flow cytometer using lasers that can detect an array of information about cell. Detectors aimed directly in line with a single laser beam (forward scatter, FSC) determine its cell size while detectors aimed perpendicular to the laser beam (side scatter, SSC) assess granularity

within the cytoplasm of cells (3–5). Fluorescent detectors within the flow cytometer are used to determine amount of fluorescence emitted by cells, which indicates level of protein expression. Currently, approximately 12 fluorochromes can be analyzed at one time.

Flow cytometry is particularly valuable as it can determine not only whether a cell is expressing the protein of interest but also indicates the amount of protein expressed by a single cell on the basis of intensity of fluorescence. In addition, flow cytometry can be used to study expression of proteins on the surface of cells as well as those localized within the cytoplasm, which can be achieved using a simple permeabilization step. Given the powerful tool of flow cytometry, it is possible to determine specific cell populations within a tissue expressing such intracellular proteins as steroid hormone receptors to determine cells most relevant and more likely to respond to steroid hormone treatment. Previous studies by our group and others indicated usefulness of this technique in analyzing steroid hormone receptor expression in patient samples, cell lines, and murine models (6–10). Another advantage to this technique is that expression of proteins can be correlated with degree of activation, maturation, or differentiation of given cell types (11). Finally, if complex mixtures of cells are present, flow cytometry can be used to sort subpopulations of cells and identify steroid hormone receptors expressed by specific cell types for further investigation. We describe here a detailed method for assessing expression of steroid hormone receptor protein in immune cells collected from rats using flow cytometry and demonstrate the feasibility of looking at other cell populations using this method.

2. Materials

2.1. Isolation of Cells

1. *Conditioned medium*: RPMI 1640 (Mediatech; Herndon, VA) containing 10% charcoal-stripped serum (CSS) (Biomeda; Foster City, CA) (*see* Note 1); 2% L-glutamine and 2% penicillin–streptomycin (both from Sigma; St. Louis, MO).
2. *Cultured Cell Collection*: Polypropylene tubes (15 mL, Corning brand; Fisher Scientific; Morris Plains, NJ), conditioned medium, phosphate-buffered saline (1× PBS).
3. *Tissue Collection*: Polypropylene tubes (15 mL, Corning brand; Fisher Scientific; Morris Plains, NJ), conditioned medium, 1× PBS.
4. *Tissue Dissection*: scalpel, forceps, stainless steel surgical blade (10, Feather brand; Fisher Scientific; Morris Plains, NJ), polystyrene tissue culture dish (60 × 15 mm style; BD Biosciences; San Diego, CA), 1× PBS.
5. *Tissue Digestion*: deoxyribonuclease I from bovine pancreas, type IV (Sigma), collagenase from *Clostridium histolyticum*, type IV (Sigma), hyaluronidase from sheep testes, type IV (Sigma).
6. *Cell Filtration*: Polypropylene tubes (50 mL, Corning brand; Fisher Scientific), cell strainer (70 μm; BD Biosciences; San Diego, CA), 1× PBS.
7. *Immune Cell Isolation (removal of unwanted or nonimmune cell populations)*: BioWhittaker ACK Lysing Buffer (Cambrex; Walkersville, MD) to remove erythrocytes or Ficoll-Hypaque solution (Sigma-Aldrich, St. Louis, MO) to remove erythrocytes and granulocytes.

¹Charcoal-stripped serum (CSS) is used as some components of sera have demonstrated hormone-mimicking properties. CSS prevents unwanted changes in cellular activity that could potentially skew results of the experiment.

2.2. Sample Preparation for Flow Cytometer

1. Polystyrene tubes (5 mL) (Falcon brand; BD Biosciences; San Diego, CA)
2. *FACS Buffer*: 1× PBS, 2% CSS and 0.2% sodium azide.
3. *Primary Antibodies*: purified anti-rat glucocorticoid receptor (GR, recognizes and binds to amino acid residues 346–367) (reconstituted in PBS to 100 µg/mL, diluted 1:5,000); purified anti-rat progesterone receptor (PR, recognizes and binds to amino acid residues 533–547) (reconstituted in PBS to 100 µg/mL; diluted 1:5,000); and purified anti-rat androgen receptor (AR, recognizes and binds to amino acid residues 321–572) (reconstituted in PBS to 100 µg/mL; diluted 1:5,000) – all purchased from Affinity Bioreagents (Golden, CO); phycoerythrin (PE)-conjugated anti-rat CD45 expressed by leukocyte (immune cell) populations (0.1 mg/mL; BD Biosciences; San Diego, CA). Isotype control antibodies included the following: purified mouse IgG (PR control) (0.1 mg/mL; diluted 1:5,000) (BD Biosciences); purified rabbit IgG (GR and AR control) (1 mg/mL; diluted 1:10000) (R&D Systems; Minneapolis, MN); PE-conjugated mouse IgG1 (CD45 control) (0.1 mg/mL) (BD Biosciences).
4. *Secondary Antibodies*: fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (for GR and AR); fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (for PR).
5. Cytofix/Cytoperm solution and wash buffer (BD Biosciences, San Diego, CA).
6. Rat serum (Sigma) to prevent nonspecific binding of antibodies within the cytoplasm of cells.
7. Paraformaldehyde solution (4%) to generate fixed cells if cells cannot be examined within 24–48 h of collection and staining with antibodies.

2.3. Data Collection and Analysis

1. BD FACSCalibur cytometer (BD Biosciences), with computer interface (Macintosh or PC).
2. Isotonic saline for FACSCalibur sheath reservoir and 10% bleach in deionized water for FACSCalibur waste reservoir.
3. *Data retrieval software (any of the following)*: BD CellQuest (BD Biosciences), BD CellQuest Pro (BD Biosciences), or FlowJo (Tree Star, Inc.; Ashland, OR).
4. *Highlighting of specific cell populations using a color coding system*: Paint-A-Gate software (BD Bioscience), which provides figures readily available for use in presentations.
5. *Statistical analysis software*: A number of different statistical packages, including basic functions included in Microsoft Excel, can be used to analyze data following acquisition in the flow cytometer.

3. Methods

3.1. Generating Single-Cell Suspensions

Live cells obtained from culture techniques or isolated from tissues can be easily prepared for analysis of protein expression using flow cytometry. Frozen tissues can also be considered for this method but should be allowed to grow overnight (at least 24 h) in tissue culture conditions to ensure nonviable cells are removed from the sample. Lymphoid organs, such as bone marrow and spleen, provide a plentiful source of immune cells. Other tissues,

such as liver and kidney, can also be used for analysis of immune cells, although they have markedly reduced populations of immune cells. In addition, immune cells can be readily isolated from peripheral blood but require measures to remove erythrocytes in order to eliminate this unwanted cell population. The following includes detailed information on generating a single cell suspension to prepare for use in a flow cytometer. For cells that have been cultured, these can be collected and counted as dictated in **Steps 3–6**:

1. Collect tissue from animals and place in separate 15-mL polypropylene tubes with 5 mL conditioned medium in each tube.
2. Transfer tissue to 15 mm culture dish (BD Biosciences) for dissection using scalpel and forceps. For spleen, continual application of pressure to tissue using forceps alone is sufficient to generate a single cell suspension. For other lymphoid tissues and non-lymphoid tissues, use of a scalpel with a surgical steel blade (#10) can generate smaller pieces of tissue. Supplementing the sample with 1× PBS (up to an additional 2 mL) to culture dish will further promote a single-cell suspension. If necessary, digestion with DNase I, collagenase, and hyaluronidase (all from Sigma) for at least 1 h at 37°C may be necessary to further break up tissue. Prior to enzymatic digestion, centrifuge sample for 5 min (900 × g) at 25°C to pellet cells. Remove supernatant by decanting, and resuspend pellet in enzymes to ensure maximal digestion. After digestion step, add 5 mL of conditioned medium to each tube to neutralize enzyme activity.
3. Following dissection and digestion of tissue or collection of cells from culturing conditions, it is important to filter sample to ensure a uniformly single-cell suspension and to remove any clumps or tissue debris that could potentially clog the flow cytometer. Transfer sample into a 50-mL polypropylene tube with a 70 µm cell strainer (BD Biosciences) placed in the tube opening (*see* Note 2). Mix sample (pipetting up and down to loosen clumps of cells), and slowly pour sample over cell strainer. To remove remaining debris, fill tube to 40 mL total volume using 1× PBS. Discard cell strainer.
4. For removal of only erythrocytes (retains polymorphonuclear and mononuclear immune cell populations), centrifuge samples for 5 min (900 × g) to pellet cells. Resuspend cell pellet in ACK Lysing Buffer for 10 min at 37°C. Centrifuge cells for 5 min (900 × g) at 25°C to pellet cells, and remove supernatant by decanting. Resuspend pellet in 10 mL conditioned media, depending on the concentration of cells, to prepare for cell counts (*see* Note 3).
5. For removal of erythrocytes and granulocytes (retains mononuclear immune cell populations), place 10 mL of Ficoll-Hypaque solution in empty 50-mL polypropylene tube for each sample. *Very* slowly add sample to the tube, ensuring the Ficoll layer is not disturbed (*see* Note 4). Samples should be carefully placed into centrifuge (not disturbing Ficoll layer) and centrifuged 30 min (900 × g) at 4°C. Samples should then be carefully removed from centrifuge, and the layer containing immune cells (cloudy region just above Ficoll layer) can be collected and transferred to empty 50-mL tube (*see* Note 5). 1× PBS should be added to tube

²Cell strainer may need to be moistened using 1× PBS prior to addition of sample to ensure even distribution and maximal filtration across unit.

³If cell collection results in high cell number yield, it will be important to dilute samples prior to obtaining cell counts. For this, a small portion (~50 µL) can be collected from a well-mixed sample and added in PBS to make the appropriate dilution (i.e., 1:20 dilution factor) prior to obtaining cell counts and determining cell viability.

⁴To ensure Ficoll layer is not disturbed, it might be useful to tilt 50-mL tube at an angle (approximately 30–45°) prior to adding sample. If Ficoll layer is disturbed during addition of sample, it will likely reduce viability as Ficoll is toxic to cells.

⁵To facilitate collection from tubes after Ficoll separation, it may be necessary to remove approximately 10–15 mL of supernatant (contains dead cells and tissue debris) before collecting leukocytes in cloudy region just above Ficoll layer.

to fill to a total volume of 50 mL to neutralize effects of Ficoll contaminant in the collected cell suspension. Centrifuge samples for 10 min ($900 \times g$) at 4°C to pellet cells. Remove supernatant by decanting, and resuspend cells in 10 mL conditioned media, depending on concentration of cells, to prepare for cell counts.

6. Cells should be counted (using trypan blue for verifying cell viability) to determine numbers of viable cells available for analysis of protein expression using flow cytometry (*see* Note 3). A range of cell numbers is expected, depending on tissue type. Lymphoid tissues (i.e., spleen) will yield higher numbers ($\sim 2.0 \times 10^8$), whereas non-lymphoid tissues (i.e., liver) will likely have fewer total leukocyte numbers ($\sim 4.0 \times 10^7$).

3.2. Labeling of Samples for Use in Flow Cytometer

1. Flow cytometry entails the use of fluorochrome-conjugated antibodies (*see* Note 6). Therefore, it is recommended that researcher conduct this portion of the experiment in a room with very little light or with limited exposure to light. Using lighted biosafety cabinet during addition of antibodies is acceptable as exposure is minimal.
2. Determine the number of polystyrene tubes required for experiment. This should include a tube containing cells only to measure autofluorescence (no antibodies); a tube containing directly-conjugated isotype control antibodies, if primary antibody is conjugated with fluorochrome; if secondary anti-body is conjugated, separate tubes containing sample with primary antibody as well as sample with primary and secondary fluorochrome-conjugated antibody should be prepared to serve as proper controls. For multiple fluorochrome-conjugated antibodies used in a single sample (i.e., FITC and PE antibodies together), tubes containing isotype control antibodies with directly-conjugated fluorochromes should be prepared with samples requiring both directly-conjugated fluorochromes and those requiring secondary antibodies. Remaining tubes should account for specific proteins or receptor of interest for analysis. An example is provided in Table 1 (*see* Note 11).
3. For each polystyrene tube, transfer 1.0×10^6 cells of sample. If cell suspension contains less than 1.0×10^6 cells/mL, centrifuge sample for 5 min ($900 \times g$) at 4°C to pellet cells. Remove supernatant by decanting, and resuspend cell pellet in FACS buffer at 1.0×10^6 cells/mL (*see* Note 7).
4. After samples have been transferred to polystyrene tubes, add 2 mL FACS buffer to each tube to wash cells. Centrifuge tubes for 5 min ($900 \times g$) at 4°C to pellet cells.
5. Remove supernatant by decanting, resuspend pellet in 2 mL FACS buffer (second wash step), and centrifuge tubes 5 min ($900 \times g$) at 4°C to pellet cells.
6. Remove supernatant by decanting, and vortex tubes (gently) to loosen cell pellet (*see* Note 8).
7. Using separate pipette tips, add 10 μl PE-conjugated isotype control (mouse IgG) or anti-rat CD45 antibodies to appropriate tubes (*see* Table 1). Thoroughly mix

⁶One of the major limitations of flow cytometry is antibodies recognizing a protein or receptor of interest must be available for use. Without an appropriate antibody that is specific to the protein, it is not possible to utilize this method to measure protein expression.

¹¹It would be useful to include a sample that does not express the steroid hormone receptor of interest (serves as a negative control) and a sample that is known to express the steroid hormone protein of interest (serves as a positive control) for each experiment.

⁷If 1×10^6 cells/tube are not available due to the number of tubes required for appropriate analysis or a low cell yield, the researcher may use as few as 0.5×10^6 cells but no less than 0.25×10^6 cells per tube to ensure sufficient numbers of cells for analysis.

⁸Another common practice to loosen cell pellet prior to addition of antibodies is to grinch (scrape tube across grate of biosafety cabinet) instead of using a vortex.

antibody with sample by pipetting up and down. Although this protocol includes information for isolating leukocyte populations, other markers for specific immune cell populations or other cell types can also be used to identify expression of steroid hormone receptors in cells.

8. Place all tubes (including tube containing sample for measuring autofluorescence) on ice (4°C) for 20 min in a dimly lit room to provide ample time for antibody to bind to CD45⁺ cells (expressed by leukocyte populations).
9. Remove tubes from ice, add 2 mL FACS buffer to each tube to wash away excess antibody. Centrifuge tubes for 5 min (900 × g) at 4°C to pellet cells.
10. Remove supernatant by decanting. Loosen cell pellet by gently vortexing to prepare for intracellular labeling of steroid hormone receptors. Cells labeled only for determining expression of cell surface molecules or not receiving anti-bodies (autofluorescence) can remain on ice during intracellular labeling portion of the experiment.
11. Add 0.25 mL CytoFix/CytoPerm solution (BD Biosciences) to each tube for intracellular staining of steroid hormone receptors (*see* Table 1), and mix with resuspended cells. This solution will permeabilize and fix cells (*see* Note 9). Place tubes on ice for 15 min.
12. Remove cells from ice. Wash cells by adding 2 mL CytoFix/CytoPerm wash buffer (BD Biosciences) to each tube.
13. Centrifuge tubes for 5 min (900 × g) at 4°C to pellet cells. Remove supernatant by decanting. Loosen cell pellet by gently vortexing to prepare for intracellular labeling of steroid hormone receptors.
14. Using separate pipette tips, add 10 µl of rat serum to each tube. Serum provides a blocking mechanism that will inhibit nonspecific binding of antibodies to other intracellular proteins. Mix serum with cells by thoroughly pipetting up and down. Place tubes on ice for 10 min.
15. Using separate pipette tips, add 10 µl of purified isotype control (mouse IgG, rabbit IgG) or anti-rat steroid hormone receptor (PR, GR, AR) antibody to appropriate tube (*see* Note 10). Mix antibody with cells by thoroughly pipetting up and down. Place tubes on ice for 10 min (*see* Note 11).
16. Using separate pipette tips, add 10 µl of FITC-conjugated goat anti-mouse or anti-rabbit antibody to appropriate tubes (secondary antibody). Mix antibody with cells by thoroughly pipetting up and down. Place tubes on ice for 10 min.
17. Remove cells from ice. Wash cells by adding 2 mL FACS buffer. Centrifuge tubes for 5 min (900 × g) at 4°C to pellet cells. Remove supernatant by decanting. Loosen cell pellet by gently vortexing.
18. If cells will be analyzed in flow cytometer within 48 h, add 1.0 mL FACS buffer to each tube. Cover with aluminum foil to prevent exposure to light.
19. If cells will be analyzed more than 48 h from time of staining, it is recommended to resuspend all samples in 1.0 mL of a 4% paraformaldehyde solution to fix cells.

⁹To permeabilize cells for intracellular staining, use of 0.3% saponin in 1× PBS can be substituted for the CytoFix/CytoPerm solution.

¹⁰For this protocol, only one steroid hormone receptor is analyzed for each tube. It is possible to analyze multiple steroid hormone receptors expressed by an individual cell but will likely result in problems with nonspecific binding due to multiple antibodies being introduced into the cytoplasmic region of cell. It is, therefore, highly recommended that the researcher analyze only one intracellular protein at a time in a single sample.

Although samples undergoing intracellular staining for steroid hormone receptor expression have been fixed with CytoFix/CytoPerm solution, resuspending all samples in 4% paraformaldehyde solution will provide consistency with analysis.

3.3. Acquisition of Steroid Hormone Receptor Expression Data Using Flow Cytometer

1. Turn on FACSCalibur (BDBiosciences) and computer that will receive data from flow cytometer (*see* Note 12). The flow cytometer valve should be set to “Standby.” For most units, the flow cytometer must be turned on prior to the computer to ensure the computer recognizes it is connected to the instrument (*see* Note 13). The researcher should verify which equipment should be turned on first.
2. Check the sheath-fluid reservoir to ensure it contains a sufficient amount of isotonic saline and the waste reservoir to ensure waste has been removed and contains no more than 75 mL of a 10% bleach solution.
3. Launch CellQuest software on computer interface to begin acquisition of samples.
4. Choose the Dot Plot option under Plots menu (displays two parameters of information on a single frame); Select Acquisition. The X parameter should be FSC (Forward Scatter), and the Y parameter should be SSC (Side Scatter). Repeat this process to generate another dot plot on the screen. Choose FL2 (displays PE fluorescence) for X parameter and FL1 (displays FITC fluorescence) for Y parameter. If only one parameter of information is needed, the user may choose to select the Histogram option under Plots menu (displays a single parameter of information on frame). Choose parameter of information to display on the Y-axis.
5. Choose the Connect to Cytometer option under the Acquire menu to connect computer to the flow cytometer. An Acquisition Control box will appear on the Desktop.
6. Choose the Acquisition and Storage option under Acquire menu to select total number of cells to be collected for each sample by the flow cytometer.
7. Choose the Counters option under Acquire menu to see number of cells being collected as the machine acquires data.
8. Choose the Parameter Description option under Acquire menu to create a folder containing data files for each sample and provide specific information associated with the particular sample.
9. Choose the Detectors/Amps option under Cytometer menu to change settings on machine. This will require use of samples of a known size, intracellular granularity, and positive expression of protein of interest to compare with samples for each experiment.
10. Move sample arm of the flow cytometer to remove tube containing deionized water (dH₂O). Gently vortex Autofluorescence Sample (contains no antibodies) to resuspend cells and generate a single-cell suspension. Place Autofluorescence Sample on flow cytometer stage, and reposition sample arm to ensure the machine acquires the data on sample. The Autofluorescence Sample provides the user with

¹²There are a variety of flow cytometry instruments available from BD Biosciences, including FACScan, FACSaria, FACSCanto, and several other instruments that can be selected based on the needs of the researcher. In addition, there are other companies, including Beckman-Coulter, that offer a variety of flow cytometer instruments.

¹³In the event the computer does not recognize the flow cytometer, restart both the computer and flow cytometer in the appropriate order.

an opportunity to determine the most appropriate settings to account for sample-specific differences in flow cytometer readings.

11. Turn valve on flow cytometer to the Run position, and click on the Acquire button within Acquire Control box on the Desktop to begin collecting data. For Autofluorescence sample, the Setup box should be selected to provide an opportunity to change settings in the Detectors/Amps fields.
12. After settings are determined and saved, remove check mark from Setup box to begin collecting data to be saved on the file created for each sample. It may be useful to create a gate around a group of cells using a Polygon tool (found in the Tool Palette of flow cytometer software) to select a specific set of cells to be analyzed. For example, cells of a larger size or cells positive for expression of a specified parameter may be sufficient for purposes of the experiment). The user may also choose to select total number of cells for collection, based on gated population by indicating this within the Acquisition & Storage option under Acquire menu.
13. Rate of cell collection (Low, Med, Hi) by the flow cytometer may vary based on number of cells in each tube. No more than 1,500 events/second should be collected to prevent clogging machine (*see* Note 15).
14. Once the indicated number of events (cells) has been collected, the Autofluorescence Sample may be removed and replaced with next sample. Proceed with subsequent samples until a file has been generated for each sample collection.
15. Be sure to clean the machine with a tube containing the appropriate cleaning solution (10% bleach or other solution) for at least 5 min. Return tube containing dH₂O water to the stage of flow cytometer.

3.4. Data Analysis

1. Data analysis can be performed with a number of different software platforms currently available for use with flow cytometry data, including CellQuest and CellQuest Pro (BD Biosciences) and FlowJo (Tree Star, Inc.). In addition, Paint-A-Gate (BD Biosciences) allows the user to select specific cell populations using different colors to more prominently feature groups of cells (Fig. 1).
2. The selected software package allows the user to open files generated from a flow cytometer for subsequent analysis. Some software packages may require conversion of files prior to analysis. This, supplied application is usually with the flow cytometry software package.
3. Data can be generated into dot plot or histogram formats for viewing (Fig. 2 and Fig. 3). There are also options to choose a contour plot or density plot, depending on needs of the researcher (*see* Note 14).
4. Each software package will provide flow cytometer-generated statistics on the collected data, which is easily accessible by selecting information under Stats

¹⁵FACSCalibur may require lines to be flushed if no events are being acquired by the flow cytometer. This may be due to the machine being clogged by clumps of cells or air bubbles that have formed. Other problems preventing the flow cytometer from acquiring events include a cracked sample tube (transfer cells to new tube); sheath reservoir too low or not properly capped (check fluid amount and placement of sheath cap); or waste reservoir being full (remove waste fluid).

¹⁴If the fluorochrome is not stably coupled to the antibody, it will not be possible to identify fluorescence and measure amount of receptor expressed by cells. This could result in the generation of falsely negative data. Background fluorescence is sometimes higher in fixed cells, which occurs with use of the CytoFix/CytoPerm solution for intracellular staining of steroid hormone receptors. Therefore, titration of antibody to find optimal concentration for each sample may be necessary. It will be important to evaluate data generated from the flow cytometer and analyzed by software to gauge appropriate concentration for each experiment.

menu. Information includes data on quadrants within a dot plot. Statistics include percentiles, raw numbers, and mean fluorescent intensity values for a sample (Fig. 4).

5. After data from software has been collected, values generated can undergo statistical analysis using any standard statistical package. For example, FlowJo has a feature to allow the user to transfer data into Microsoft Excel.

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References

1. Herzenberg LA, Sweet RG, Herzenberg LA. Fluorescence-activated cell sorting. *Sci Am* 1976;234(3):108–117. [PubMed: 1251180]
2. Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M, Herzenberg LA. The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. *Clin Chem* 2002;48(10):1819–1827. [PubMed: 12324512]
3. McCoy JP Jr. Basic principles of flow cytometry. *Hematol Oncol Clin North Am* 2002;16(2):229–243. [PubMed: 12094472]
4. McCoy JP Jr, Carey JL, Krause JR. Quality control in flow cytometry for diagnostic pathology. I. Cell surface phenotyping and general laboratory procedures. *Am J Clin Pathol* 1990;93(4 Suppl 1):S27–S37. [PubMed: 1690504]
5. McCoy JP Jr, Overton WR. A survey of current practices in clinical flow cytometry. *Am J Clin Pathol* 1996;106(1):82–86. [PubMed: 8701938]
6. Berki T, Kumanovics G, Kumanovics A, Falus A, Ujhelyi E, Nemeth P. Production and flow cytometric application of a monoclonal anti-glucocorticoid receptor antibody. *J Immunol Methods* 1998;214(1–2):19–27. [PubMed: 9692855]
7. Marchetti D, Van NT, Gametchu B, et al. Flow cytometric analysis of glucocorticoid receptor using monoclonal antibody and fluoresceinated ligand probes. *Cancer Res* 1989;49(4):863–869. [PubMed: 2492208]
8. Butts CL, Shukair SA, Duncan KM, et al. Progesterone inhibits mature rat dendritic cells in a receptor-mediated fashion. *Int Immunol* 2007;19(3):287–296. [PubMed: 17289656]
9. Butts CL, Shukair SA, Duncan KM, Harris CW, Belyavskaya E, Sternberg EM. Effects of dexamethasone on rat dendritic cell function. *Horm Metab Res* 2007;39(6):404–412. [PubMed: 17578756]
10. Butts CL, Shukair SA, Duncan KM, Harris CW, Belyavskaya E, Sternberg EM. Evaluation of steroid hormone receptor protein expression in intact cells using flow cytometry. *Nucl Recept Signal* 2007;5:e007. [PubMed: 17710123]
11. Boldizsar F, Palinkas L, Czompoly T, Bartis D, Nemeth P, Berki T. Low glucocorticoid receptor (GR), high Dig2 and low Bcl-2 expression in double positive thymocytes of BALB/c mice indicates their endogenous glucocorticoid hormone exposure. *Immunobiology* 2006;211(10):785–796. [PubMed: 17113916]

Fig. 1. Flow cytometer events displayed in a color-coded fashion. Paint-A-Gate software (BD Biosciences) allows the user to display groups of cells using a coloring system. In this dot-plot example, a population of immune cells (CD45⁺) collected from rat spleen is subdivided into cells expressing glucocorticoid receptor (CD45⁺ GR⁺, *light blue*), not expressing GR (CD45⁺ GR⁻, *yellow*), and nonimmune cells (CD45⁻, *dark blue*) (see *Color Plates*).

Fig. 2. Steroid hormone receptor expression in leukocytes displayed in dot-plot format expression by CD45⁺ leukocytes obtained from rat spleen was analyzed using Cellquest Pro (BD Biosciences). Dot-plots exhibit two parameters: (A) Autofluorescence sample (no antibodies added); (B) expression of GR and AR by CD45⁺ cells and the appropriate isotype control (Rabbit IgG); and (C) expression of PR by CD45⁺ cells and isotype control (Mouse IgG). *Cross-hatch lines* (to determine positive and negative cells) were established on the basis of isotype controls. Upper right-hand quadrant represents cells expressing both CD45 and steroid hormone receptor of interest.

Fig. 3.

Steroid hormone receptor expression in leukocytes displayed in histogram format. In this example, expression of the receptor for the steroid hormone progesterone (PR) by CD45⁺ leukocytes obtained from rat spleen is shown using CellQuest Pro (BD Biosciences). Histograms exhibit a single parameter. PR expression is compared with the isotype control antibody (Mouse IgG), which provides information on background measure of fluorescence.

Total Events: 20000

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	1	0.01	0.01	24.36	85.05
UR	4094	29.74	20.47	400.97	776.75
LL	100	0.73	0.50	13.52	29.00
LR	9573	69.53	47.87	200.54	30.87

Fig. 4.

Statistical information automatically generated by flow cytometry software provides different information about cells. In this example, quadrant statistics is provided for Fig. 3.2b. Quadrant statistics gives information generated from dot-plots, including percentiles for each quadrant (representing positive and negative expression of parameters); mean fluorescence intensity (representing average amount of receptor expressed by individual cells); and raw numbers of events (cells) collected for each quadrant. Shown is information generated from the sample analyzing CD45 and GR expression. Fluorescence intensity can only approximate receptor number and indicate cells expressing more or less of a receptor or protein of interest, unless sample with cells expressing known receptor number is used for comparison.

Table 1

Sample list for steroid hormone receptor labeling of immune cells using flow cytometry

Tube no.	Fluorochrome I (FITC)	Fluorochrome II (PE)	Comments
1	None	None	Autofluorescence sample
2		Mouse IgG	Isotype control for CD45
3		CD45	Expressed on surface of leukocytes
4	Purified mouse IgG	CD45	Control for primary antibody
5	Purified mouse IgG/ FITC Goat anti-mouse IgG	CD45	Isotype control for PR
6	Purified PR/FITC goat anti-mouse IgG	CD45	Identifies cells expressing PR
7	Purified rabbit IgG	CD45	Control for primary antibody
8	Purified rabbit IgG/ FITC goat anti-mouse IgG	CD45	Isotype control for GR and AR
9	Purified GR/FITC goat anti-mouse IgG	CD45	Identifies cells expressing GR
10	Purified AR/FITC goat anti-mouse IgG	CD45	Identifies cells expressing AR

The following serves as an example of tubes and the appropriate antibodies to be added for cell labeling. This list includes a tube to measure autofluorescence (no antibodies added) as well as isotype controls and tubes to receive antibodies specific to the steroid hormone receptor of interest