Evaluation of steroid hormone receptor protein expression in intact cells using flow cytometry

Cherie L. Butts, Shetha A. Shukair, Kristina M. Duncan, Christopher W. Harris, Elena Belyavskaya and Esther M. Sternberg✉

✉ Corresponding Author: sternbee@mail.nih.gov

Section on Neuroendocrine Immunology and Behavior, National Institute of Mental Health/NIH, Bethesda, Maryland, USA

Several methods are currently employed to evaluate expression of steroid hormone receptors in tissues and cells, including real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR) and western blot assays. These methods require homogenization of cells, thereby preventing evaluation of individual cells or specific cell types in a given tissue sample. In addition, methods such as real-time RT-PCR assess mRNA levels, which may be subject to posttranslational modifications that prevent subsequent production of functional proteins. Flow cytometry is a fluorescence-based technique commonly used to evaluate expression of cell surface and intracellular proteins. This method is especially useful as it allows for single-cell analysis and can be utilized to determine the amount of receptor expressed by individual cells. Flow cytometry is commonly used to analyze immune cell activity and determine functionality based on changes in expression of cell surface molecules, as well as intracellular proteins (such as cytokines). Here, we describe a method to identify protein expression of steroid hormone receptors by rat leukocytes from different organs (spleen, liver and thymus) using flow cytometry. We examined expression of glucocorticoid receptor (GR), androgen receptor (AR) and progesterone receptor (PR) by cells at these sites and were able to demonstrate expression of receptors, as well as the intensity of expression of each receptor. This method is useful for rapid, high throughput measurement of steroid hormone receptors at the protein level in single, intact cells and would be valuable to determine which cells are more likely to respond to steroid hormone treatment.

Introduction

Flow cytometry is a well established technique for examining and sorting fluorochrome-labeled cells. It provides simultaneous assessment of a multitude of characteristics of single, intact cells (live or fixed) using a laser beam and various detectors [Dive et al., 1992; Kuru et al., 2001; Lewis and Van Epps, 1983; Weyde et al., 1997; Zohar et al., 1997]. Individual cells are distinguished based on size, cytoplasmic granularity and positive or negative expression of different receptors using fluorochromes conjugated to an antibody that recognizes the protein of interest [Kim et al., 2003; Pharaboz et al., 1986]. Typical fluorochromes include fluorescein isothyocyanate (FITC), phycoerthrin (PE) and peridinin chlorophyll protein (PerCP), which emit green, orange and red fluorescence, respectively.

Different detectors are used to analyze light scattering properties of each cell. Detectors aimed directly in line with the laser beam (forward scatter, FSC) determine the size of the cells; while detectors that are aimed perpendicular to the laser beam (side scatter, SSC) assess the granularity within the cytoplasm of cells [McCoy, 2002; McCoy et al., 1990; McCoy and Overton, 1996]. Additional detectors are used to determine the amount of fluorescent intensity emitted by individual cells, which corresponds to the number of receptors expressed by the cells. The fluorescent data is collected and then translated into two forms of information: one set of

information indicates the proportion of the total number of cells expressing a particular receptor (percent expression); while the second set of information furnishes the number of receptors expressed by an individual cell based on the brightness of fluorescence emitted by fluorescently-tagged antibodies (mean fluorescent intensity). Data can be plotted into a single-dimension histogram that demonstrates size, granularity or expression intensity of an antibody. Multiple parameters can also be displayed using a dot plot, which shows expression of up to two fluorochrome-labeled receptors, size (FSC) versus granularity (SSC), or a combination of these factors. In addition, it is possible to select information (termed gating) based on one of these parameters and show a histogram or dot plot of other parameters.

Flow cytometry is commonly used to examine immune cell activity, measuring changes in expression of activation markers (i.e., proteins expressed when immune cells are stimulated) on the surface of cells and changes in expression of intracellular proteins associated with specific types of immune responses, especially cytokine production [McCoy and Overton, 1994; McCoy, 2001; Weyde et al., 1997]. Previous studies have shown the feasibility of using flow cytometry to measure steroid hormone receptors. Van, et al. [Van et al., 1984] used fluoresceinated estradiol to identify estrogen receptors using flow cytometry, and Marchetti, et al. [Marchetti et
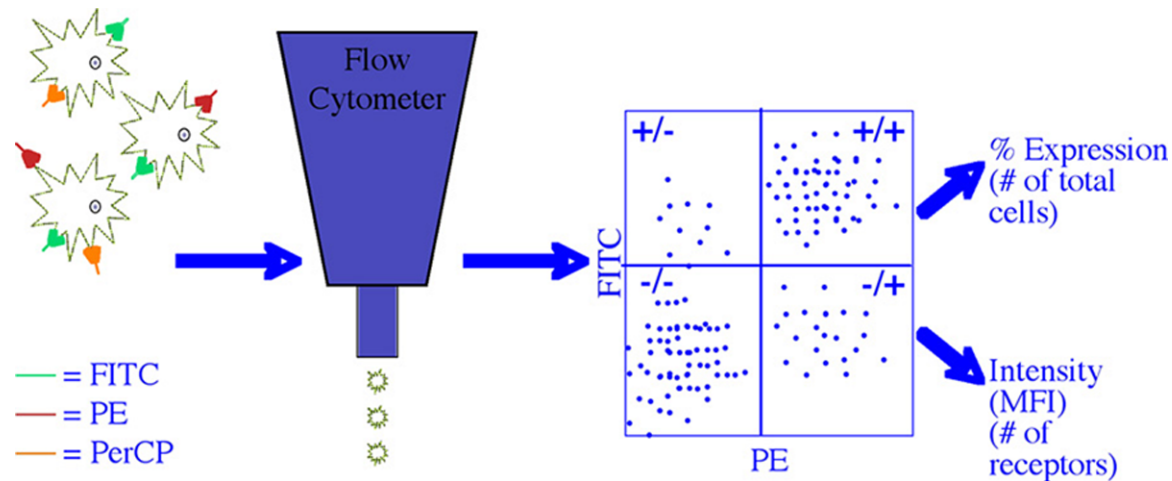
Figure 1.   Diagram of flow cytometry analysis of proteins expressed by cells. Flow cytometry is a fluorescence-based method that allows single-cell analysis of both extracellular and intracellular protein expression. Cells are stained with fluorochrome-labeled antibodies, collected in a flow cytometer, and analyzed using flow cytometry data analysis software such as CellQuest Pro. The flow cytometer is able to provide information on the proportion of cells expressing a specific receptor, as well as the relative number of receptors (mean fluorescent intensity - MFI) expressed by a single cell or population of cells.

al., 1989] generated a monoclonal antibody to the human glucocorticoid receptor (GR) to demonstrate the feasibility of using flow cytometry to measure GR on a T-cell acute lymphoblastic leukemia cell line. Berki, et al. [Berki et al., 1998] used flow cytometry to demonstrate GR expression in Hep G2 cells (human hepatocellular carcinoma cell line) and cells from mouse liver and thymus. Gotovac, et al. [Gotovac et al., 2003] used flow cytometry to evaluate differences in GR expression in patients with posttraumatic stress disorder compared to healthy volunteers, and, more recently, analyzed GR expression in different leukocyte populations of patients [Vidovic et al., 2007].

Here, we describe a method for evaluating protein expression of different steroid hormone receptors in rat leukocytes using flow cytometry and provide information on both proportions of cells expressing steroid hormone receptors, as well as the intensity of a particular steroid hormone receptor expressed by immune cells. Using real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and fluorescent microscopy methods, we have previously detected mRNA and protein, respectively, for steroid hormone receptors in these cells [Butts and Sternberg, 2004]. In order to demonstrate that this technique can be used in a more generalized manner in the study of nuclear hormone receptors, we also include analysis of cells collected from different organs and show that this method is not exclusively useful for a particular tissue type.

Reagents and instruments

Flow cytometer

To analyze the samples, a BD FACSCalibur multicolor flow cytometer (BD Biosciences; San Diego, CA) equipped with a 488-nm laser capable of detecting and distinguishing fluorescence emissions was chosen. This system combines dual-laser technology and powerful software to provide high throughput capacity for studies using whole cell suspensions that can be analyzed using

an automated sample loader or manual sample loading (Figure 1). For these studies, the manual sample loading option was utilized. BD Biosciences has a reputation for providing quality instrumentation that is used in both clinical and research laboratories.

Analysis software

Following collection of samples using a BD FACSCalibur cytometer, data was analyzed using BD CellQuest Pro (BD Biosciences; San Diego, CA). BD CellQuest Pro software is user-friendly companion software to the BD FACSCalibur that allows acquisition of information on cells collected by the cytometer followed by subsequent formatting and analysis of data.

Antibodies

Antibodies that recognize and bind to amino acid residues 346-367 of the rat glucocorticoid receptor (GR) (100 μg/ml, diluted 1:5000); amino acid residues 533 to 547 of the rat progesterone receptor (PR) (100 μg/ml; diluted 1:5000); and amino acid residues 321 to 572 of the rat androgen receptor (AR) (100 μg/ml; diluted 1:5000) were purchased from Affinity Bioreagents (Golden, CO). Antibodies to rat CD45 (0.1 mg/ml) were purchased from BD Biosciences (San Diego, CA). Isotype control antibodies included the following: purified mouse IgG (PR control) (0.1 mg/ml; diluted 1:5000) (BD Biosciences); purified rabbit IgG (GR and AR control) (1 mg/ml; diluted 1:50000) (R&D Systems; Minneapolis, MN); mouse IgG1 (CD45 control) (0.1 mg/ml) (BD Biosciences).

Methods

Isolation of spleen, liver and thymus tissue

8-11 week-old female Fischer (F344/NHsd) rats were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals were maintained in pathogen-free facilities, and all procedures were performed on approved protocols in accordance with the NIMH Animal Care and Use Committee (ACUC). Animals were sacrificed by

decapitation to obtain spleen, liver and thymus tissue, which were collected in RPMI 1640 (Mediatech; Herndon, VA) containing 10% charcoal-stripped serum (CSS) (Biomeda; Foster City, CA), 2% L-glutamine, and 2% penicillin-streptomycin (both from Sigma; St. Louis, MO) (conditioned medium). Charcoal-stripped serum was used, as some components of medium have been shown to demonstrate hormone-mimicking properties. Red blood cells were lysed with ACK lysis buffer containing ammonium hydroxide. Single cell suspensions of spleen, liver and thymus cells were obtained using mechanical shearing methods followed by passing cells through a cell strainer (70 μm). Granulocytes and red blood cells were removed using a Ficoll-Hypaque solution (Sigma-Aldrich, St. Louis, MO).

Figure 2.   CD45 expression in spleen, liver and thymus cells.
Histograms generated by flow cytometry analysis software show expression of CD45 by freshly-isolated rat cells from spleen, liver and thymus. Light-colored lines represent cells stained with isotype control antibodies. Dark, bold lines represent cells stained with anti-rat CD45 antibodies. Each graph is representative of 5 experiments.

Analysis of hormone receptor expression

Cells from spleen, liver and thymus (1×10^6/tube) were collected in polystyrene Falcon tubes (BD Biosciences, San Diego, CA) and washed with FACS buffer containing PBS (Molecular Biologicals, Inc.; Columbia, MD), 2% CSS (Biomeda), and 0.2% sodium azide (Sigma). Cells were centrifuged for 5 minutes at 2000 rpm. Supernatant was removed from tubes, followed by resuspension of cells and incubation with 10μl PE-labeled anti-rat CD45 antibody. Tubes were placed on ice and in the dark for 30 minutes to ensure sufficient time for antibody to bind to cells. Although studies have shown that fluorescent antibody labeling of cells can be performed at room temperature [Sheikholeslami et al., 2006], it is recommended that these experiments be done at colder temperatures to ensure that the fluorochrome does not lose intensity during cell preparation.
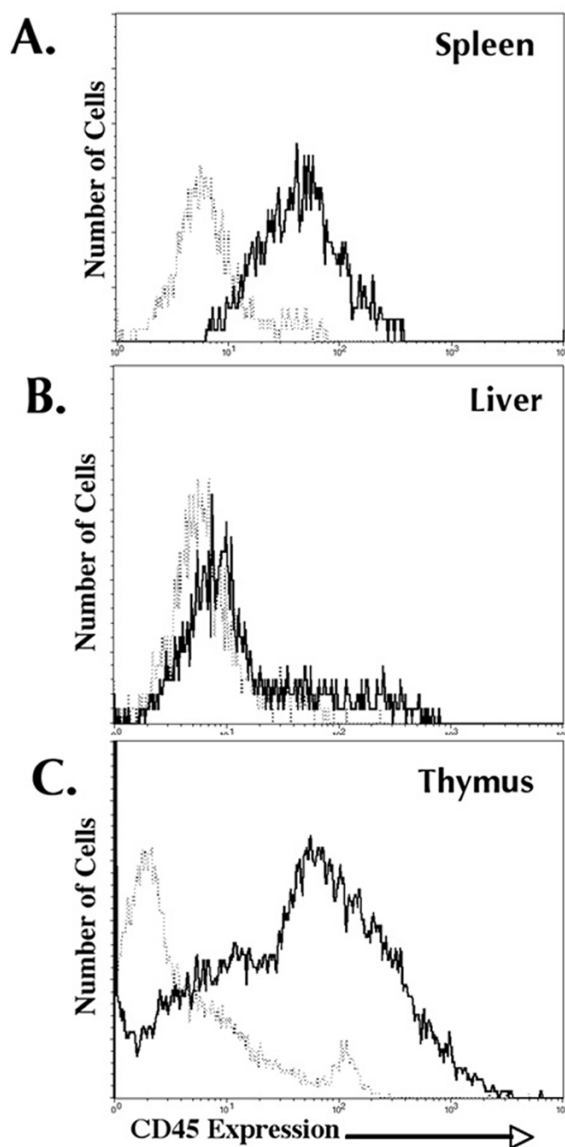
Cells were washed with FACS buffer to remove excess antibody and centrifuged for 5 minutes at 2000 rpm. Cells were then treated with Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA) for 20 minutes to permeabilize cells and washed with Cytofix/Cytoperm wash buffer to remove excess solution. Cells were incubated with 10μl horse serum for 10 minutes. Incubation of permeabilized cells with horse serum is a commonly used method for preventing nonspecific binding of antibodies to intracellular proteins [Zhong et al., 1999]. 10μl purified antibodies to rat progesterone receptor (PR), androgen receptor (AR) or glucocorticoid receptor (GR) (all from Affinity Bioreagents) were added for 10 minutes; separate tubes were incubated with 10μl purified irrelevant antibodies to mouse IgG as an isotype control for PR or rabbit IgG as an isotype control to AR and GR. A secondary anti-mouse or anti-rabbit antibody conjugated to FITC was then added for an additional 10 minutes to each tube. All incubations were done on ice (4°C). Experiments included analysis of only one steroid hormone receptor expressed by cultured cells per sample. Cells were collected using a BD FACSCalibur (BD Biosciences, San Diego, CA) and analyzed with BD CellQuest Pro analysis software (BD Biosciences).

Results

Identification of immune cells using flow cytometry

In order to evaluate expression of steroid hormone receptors by rat leukocytes, it was important to first identify immune cells from each sample. CD45 is a protein marker that is expressed on the surface of all leukocytes. Therefore, we used an antibody that is specific for this receptor to identify immune cell populations from each of the organ sites. As shown in Figure 2, histograms show proportions of CD45-expressing cells (CD45^{+}) from spleen (Figure 2A), liver (Figure 2B) and thymus cells (Figure 2C). Each organ site had varying proportions of CD45^{+} cells. Each histogram also included the appropriate isotype control for the CD45 antibody to account for any background effect of the flow cytometer and analysis software.

Organ Site	Spleen	Liver	Thymus
Isotype Control	26.77	36.26	27.91
GR	318.83	358.52	443.72
AR	225.99	184.72	195.57
Isotype Control	17.42	42.31	37.00
PR	128.24	81.18	95.03

Table 1.   Mean fluorescent intensity of steroid hormone receptor expression.   Mean fluorescent intensity (MFI) values of leukocytes expressing steroid hormone receptors are provided from dot plots shown in Figure 3. The table represents data automatically generated by the analysis software and shows the MFI values for CD45^{+} cells that express the glucocorticoid receptor (GR), androgen receptor (AR) and progesterone receptor (PR) – termed double-positive cells. The MFI values for the isotype control antibodies are also shown for comparison. The values reported for this table are representative of 7 separate experiments.

Steroid hormone receptor expression in leukocytes

Following identification of the CD45^{+} population of cells in each organ site, we next determined the expression of steroid hormone receptors by this cell population. For these experiments, we chose to examine glucocorticoid receptor (GR), androgen receptor (AR) and progesterone receptor (PR) expression by these cells. We used dot plot diagrams, as these allow the visualization of expression of two receptors simultaneously. Figure 3 shows expression of GR, AR and PR in CD45^{+} cells from spleen, liver and thymus. GR was expressed in approximately 50-60% of cells from each site. AR was expressed by approximately 25-40% of cells; and PR was expressed in approximately 20-30% of CD45^{+} cells.

Intensity of steroid hormone receptor expression in leukocytes

We were able to show that CD45^{+} cells expressed each of the steroid hormone receptors examined. We next wanted to determine the amount of steroid hormone receptor expressed by each cell population. In order to do so, we utilized the statistics automatically generated by the CellQuest Pro program. This analysis software is able to provide values for the mean fluorescent intensity (MFI) of the receptors based on the number of fluorochrome-labeled antibodies that bind to each cell and are captured by the flow cytometer. The MFI represents the average amount of intensity of each labeled cell and thereby indicates which cell populations have higher or lower expression of the specific receptor analyzed. The software is able to generate the MFI data for a dot plot based on the receptor shown on the X-axis and Y-axis, and further segregates this information for cells falling within a specific quadrant, which is determined by the cross hatches. Cross hatches are identified using fluorescently-labeled isotype control antibodies to the receptor of interest. Table 1 shows the mean fluorescent intensity (MFI) for the population of CD45^{+} cells shown in Figure 3 that expressed the particular steroid hormone receptor. The data in Table 1 includes the MFI for the receptors of interest and are compared with the isotype control.

Discussion

Flow cytometry is an important tool for determining protein expression of cell surface receptors and has also been shown to be useful for identifying expression of intracellular proteins in permeabilized cells [Yole et al., 2007]. Therefore, flow cytometry can be a potentially useful tool in the field of steroid hormone receptor biology by providing a technique for analyzing steroid hormone receptors at the protein level within intact cells [Thomas et al., 2007]. Previous studies have indicated the usefulness of this technique in analyzing specific hormone receptors in patient samples, cell lines and murine models [Berki et al., 1998; Marchetti et al., 1989]. We have expanded on previous studies and shown that multiple steroid hormone receptors can be analyzed in rat leukocyte populations using this technique. Here, we show identification of one steroid hormone receptor expressed by a single cell, although it is feasible to analyze multiple intracellular proteins using flow cytometry. This type of analysis has not been previously reported and may pose increased difficulties due to nonspecific binding of antibodies to intracellular proteins.

In addition to providing information on cells expressing specific receptors, this method gives an indication of the number of receptors expressed by an individual cell, thus providing a means to quantify receptors on each cell. Another advantage of this technique is that expression of proteins of interest can be correlated with the degree of activation, maturation or differentiation of given cell types, as previously reported by Boldizsar, et al. [Boldizsar et al., 2006] in their analysis of glucocorticoid receptors expressed by murine thymocytes. Finally, if complex mixtures of cells are present, flow cytometry can be used to sort subpopulations of cells and therefore identify steroid hormone receptors expressed by specific types of cells. This method is, however, limited in its inability to specify the location within the cell (nucleus, cytoplasm, mitochondria) of intracellular proteins that are identified.

Here, we show that flow cytometry can be used to identify intracellular expression of steroid hormone receptors by rat leukocytes. We are able to demonstrate expression of multiple steroid hormone receptors, including the glucocorticoid receptor, androgen receptor and progesterone receptor, from a variety of cell sources and organs. Using real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and fluorescent microscopy methods, we have previously detected mRNA and protein, respectively, for these receptors in cells [Butts et al., 2007]. This method could, therefore, be useful to answer a variety of scientific questions related to steroid hormone receptor biology, including determining the responsiveness of specific cell types to steroid hormone treatment. This method would, therefore, be a very useful
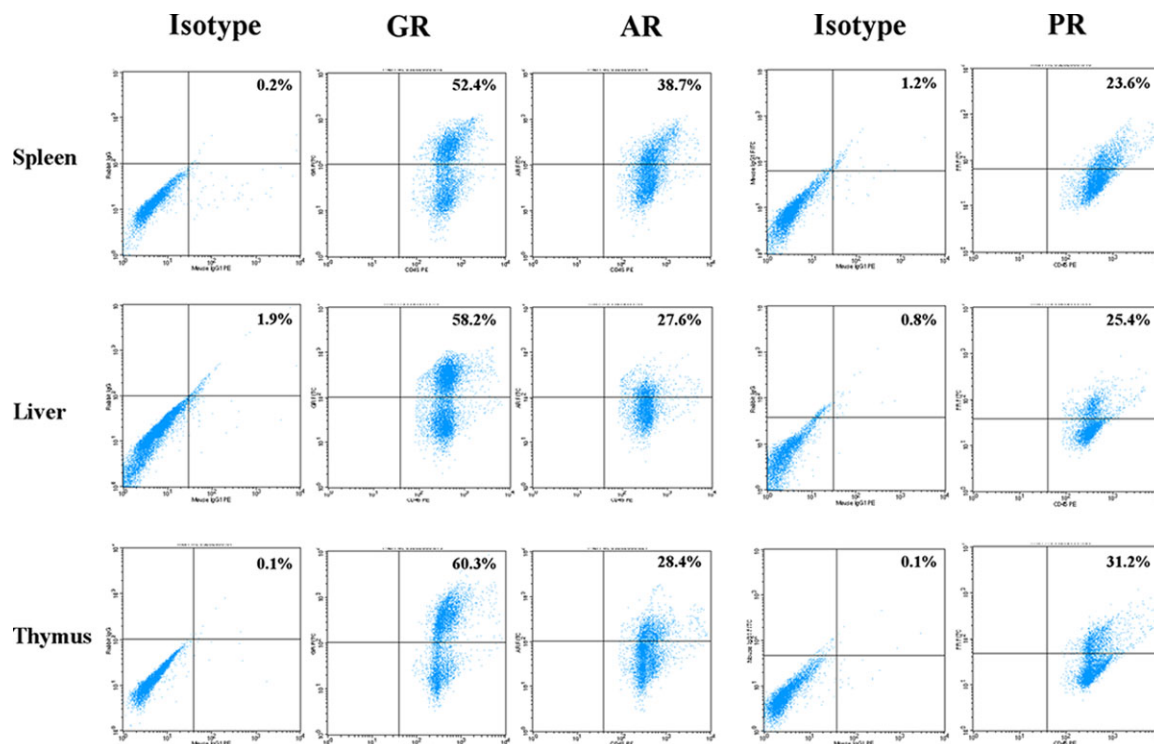
Figure 3.   Steroid hormone receptor expression by rat leukocytes. Expression of steroid hormone receptors by CD45^{+} rat leukocytes from spleen, liver and thymus organs is analyzed using flow cytometry. Dot plots show glucocorticoid receptor (GR), androgen receptor (AR) and progesterone receptor (PR) expression by these cells – termed double-positive cells. Isotype controls for GR and AR (Rabbit IgG) and PR (Mouse IgG) are shown for comparison. Cross hatch lines were determined by comparing with isotype control antibodies. Percentages represent the percent of CD45^{+} cells expressing the particular steroid hormone receptor examined. Each graph is representative of 7 separate experiments.

tool for rapid, high throughput measurement of steroid hormone receptors at the protein level in single, intact cells.

Acknowledgements

References

Berki, T., Kumanovics, G., Kumanovics, A., Falus, A., Ujhelyi, E. and Nemeth, P. (1998) Production and flow cytometric application of a monoclonal anti-glucocorticoid receptor antibody *J Immunol Methods* **214**, 19-27.

Boldizsar, F., Palinkas, L., Czompoly, T., Bartis, D., Nemeth, P. and Berki, T. (2006) 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* **211**, 785-96.

Butts, C. and Sternberg, E. (2004) Different approaches to understanding autoimmune rheumatic diseases: the neuroimmunoendocrine system *Best Pract Res Clin Rheumatol* **18**, 125-39.

Butts, C. L., Shukair, S. A., Duncan, K. M., Bowers, E., Horn, C., Belyavskaya, E., Tonelli, L. and Sternberg, E. M. (2007) Progesterone inhibits mature rat dendritic cells in a receptor-mediated fashion *Int Immunol* **19**, 287-96.

Dive, C., Gregory, C. D., Phipps, D. J., Evans, D. L., Milner, A. E. and Wyllie, A. H. (1992) Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry *Biochim Biophys Acta* **1133**, 275-85.

Gotovac, K., Sabioncello, A., Rabatic, S., Berki, T. and Dekaris, D. (2003) Flow cytometric determination of glucocorticoid receptor (GCR) expression in lymphocyte subpopulations: lower quantity of GCR in patients with post-traumatic stress disorder (PTSD) *Clin Exp Immunol* **131**, 335-9.

Kim, E. Y., Zeng, Q. T., Kuo, F. C., Rawn, J. and Mentzer, S. J. (2003) Visual representation of cell subpopulation from flow cytometry data *AMIA Annu Symp Proc*, 893.

Kuru, L., Parkar, M. H., Griffiths, G. S. and Olsen, I. (2001) Flow cytometry analysis of guided tissue regeneration-associated human periodontal cells *J Periodontol* **72**, 1016-24.

Lewis, S. L. and Van Epps, D. E. (1983) Demonstration of specific receptors for fluoresceinated casein on human neutrophils and monocytes using flow cytometry *Inflammation* **7**, 363-75.

Marchetti, D., Van, N. T., Gametchu, B., Thompson, E. B., Kobayashi, Y., Watanabe, F. and Barlogie, B. (1989) Flow cytometric analysis of glucocorticoid receptor using monoclonal antibody and fluoresceinated ligand probes *Cancer Res* **49**, 863-9.

McCoy, J. P., Jr. and Overton, W. R. (1996) A survey of current practices in clinical flow cytometry *Am J Clin Pathol* **106**, 82-6.

McCoy, J. P., Jr. (2002) Basic principles of flow cytometry *Hematol Oncol Clin North Am* **16**, 229-43.

McCoy, J. P., Jr., Carey, J. L. and Krause, J. R. (1990) Quality control in flow cytometry for diagnostic pathology. I. Cell surface phenotyping and general laboratory procedures *Am J Clin Pathol* **93**, S27-37.

McCoy, J. P., Jr. and Overton, W. R. (1994) Quality control in flow cytometry for diagnostic pathology: II. A conspectus of reference ranges for lymphocyte immunophenotyping *Cytometry* **18**, 129-39.

McCoy, P. (2001) T-cell receptor analysis by flow cytometry *Cytometry* **44**, 369.

Pharaboz, M. O., Cordier, G. and Andre, J. (1986) Flow cytometry analysis of cells dispersed from the MtTF4 tumor whose growth is inhibited by estradiol treatment *Mol Cell Endocrinol* **48**, 229-37.

Sheikholeslami, M. R., Jilani, I., Keating, M., Uyeji, J., Chen, K., Kantarjian, H., O'Brien, S., Giles, F. and Albitar, M. (2006) Variations in the detection of ZAP-70 in chronic lymphocytic leukemia: Comparison with IgV(H) mutation analysis *Cytometry B Clin Cytom* **70**, 270-5.

Thomas, P., Pang, Y., Dong, J., Groenen, P., Kelder, J., de Vlieg, J., Zhu, Y. and Tubbs, C. (2007) Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor α subtypes and their evolutionary origins *Endocrinology* **148**, 705-18.

Van, N. T., Raber, M., Barrows, G. H. and Barlogie, B. (1984) Estrogen receptor analysis by flow cytometry *Science* **224**, 876-9.

Vidovic, A., Vilibic, M., Sabioncello, A., Gotovac, K., Rabatic, S., Folnegovic-Smalc, V. and Dekaris, D. (2007) Circulating lymphocyte subsets, natural killer cell cytotoxicity, and components of hypothalamic-pituitary-adrenal axis in Croatian war veterans with posttraumatic stress disorder: cross-sectional study *Croat Med J* **48**, 198-206.

Weyde, J., Wassermann, K. and Schell-Frederick, E. (1997) Analysis of single and double-stained alveolar macrophages by flow cytometry *J Immunol Methods* **207**, 115-23.

Yole, M., Wickstrom, M. and Blakley, B. (2007) Cell death and cytotoxic effects in YAC-1 lymphoma cells following exposure to various forms of mercury *Toxicology* **231**, 40-57.

Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., Buechler, P., Isaacs, W. B., Semenza, G. L. and Simons, J. W. (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases *Cancer Res* **59**, 5830-5.

Zohar, R., Sodek, J. and McCulloch, C. A. (1997) Characterization of stromal progenitor cells enriched by flow cytometry *Blood* **90**, 3471-81.