Flow Cytometry Analysis of Intracellular Protein

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1. Introduction

The technique of measuring intracellular protein levels using flow cytometry is a very rapid method to detect protein on a single-cell level.

The demand for determining protein levels in a continuously decreasing amount of input cells is currently being developed by scientists and medical doctors. Another demanding area is the splitting of active and inactive (phosphorylated and unphosphorylated, respectively) forms of the protein being monitored, e.g., during different phases of the cell cycle, differentiation or carcinogenesis. The Western blot technique has been typically used for this purpose. The utilization of multi color flow cytometry allows for measurements of multiple proteins in parallel, regardless of the protein length. As the technique evolves, it is possible to obtain information on over 13 parameters per cell (Krutzik et al., 2004).

Measuring the phosphorylation status of specific proteins using this technique is very efficient. It eliminates a number of problems related either to Western blotting or ELISA.

Compared to the Western blot, this progressive method has much lower requirements on the number of input cells (only 1×10^4 cells are needed for determining the concentration of a given protein), it requires less time for processing and, last but not least, is also much more cost effective. Furthermore, it allows researchers to distinguish the various cell populations in the sample without complicated cell separation, if the appropriate antibodies are used. This is beneficial for searching for cell populations and their reactions on external stimuli, such as changed cultivation conditions during in vitro experiments or influencing the organism during in vivo experiments.

In this chapter, the application and pitfalls of flow cytometric intracellular protein measurements are illustrated by the data of our original research of porcine monocytes and CD34+ hematopoietic stem/progenitor cells. In short, a successful utilization of this progressive technique depends on correctly performing the 4 following steps:

1. Precise experimental planning to obtain a defined cell population using this technique.
2. Effective fixation and permeabilization of the cell population.
3. Choosing the optimal isotype control.
4. Selecting the optimal system of primary and secondary antibodies.

2. Fixation and permeabilization

Intracellular flow cytometry, in comparison with conventional cell surface labeling methods, requires fixation and permeabilization of the cells before staining of intracellular antigens (Robinson et al., 1993). Moreover, a variety of commercial kits for fixation and permeabilization are available.

The first step in the population analysis is high quality fixation. A crosslinking reagent (typically formaldehyde) could be used for this purpose. For every given cell population, it is necessary to test the adequate concentration of the solution. The most common formaldehyde concentration is about 4%. Other fixation limiting factors are time and temperature. The incubation time, according to the cell population type, varies between 8 and 15 minutes. The incubation temperature is 37 °C. A variety of commercial kits for fixation and permeabilization are available.

The following step is for permeabilization with detergents (Triton X 100 or saponin) or alcohol (ethanol or methanol). Although the protocols that have been used to stain phosphoepitopes for flow cytometry differ from one to another, they rely on two primary permeabilization reagents – saponin or methanol.

Saponin permeabilization

Saponin is a mixture of terpenoid molecules and glycosides that permeabilize cells by interacting with cholesterol present in the cell membrane (Melan et al. 1999). This creates pores in the plasma membrane that are large enough for entry of fluorophore-conjugated antibodies. Because the intracellular proteins can leak out of saponin-treated cells, they must be first exposed to a crosslinking reagent, such as formaldehyde, to cross-link proteins and nucleic acids into a cohesive unit within the cell. Saponin has become the detergent of choice for cytokine staining, and several groups have utilized it for permeabilization in phospho-epitope staining protocols (Pala et al., 2000). It is typically used at concentrations ranging from 0.1% to 0.5%, similar to cytokine-staining procedures.

Three commercially available kits (Leukoperm, Serotec, UK; Fix & Perm, An Der Grub, Austria; IntraStain, DAKO Cytomation, Denmark) along with combinations of 2 or 4% paraformaldehyde with 0.1 or 0.05% saponin were tested for fixation and permeabilization of isolated pig's peripheral blood mononuclear cells or whole blood leukocytes (Zelnickova et al., 2007).

The fixation and permeabilization process could lead to non-specific binding of primary or secondary antibodies. In comparison to all three tested commercial kits, a combination of paraformaldehyde and saponin caused an increase in non-specific binding of antibodies. The intensity of fluorescence of the negative peak of paraformaldehyde/saponin fixed cells was evidently higher in comparison with the negative peak of cells fixed with the use of commercial kits.

The fixation and permeabilization process could lead to elevation of autofluorescence of cells. The autofluorescence of cells was at the lowest level in all tested kits. In contrast, the combination of paraformaldehyde and saponin in all concentrations caused an increase of autofluorescence. The autofluorescence in samples fixed with 4% paraformaldehyde was higher than with 2% paraformaldehyde.

The fixation and permeabilization process could damage the light scatter properties of the cells. The light scatter characteristics of all tested cells were comparable after any type of fixation and permeabilization. The light scatter characteristics were always changed in comparison with fresh preparations. However, well distinguishable and bounded subpopulations of mononuclear cells and neutrophils were obtained by adjusting the settings in the side and forward scatters.

Some protocols use labeling of intracellular molecules in the whole blood instead of isolated blood leukocytes. In our laboratory, the lysis of red blood cells (RBC) is always performed before starting the staining procedure. However, in order to make the processing of large amounts of samples as quick as possible, the hemolysis is commonly performed in the 96-well plate in which the cultivation was previously performed. The volume of the hemolytic reagent is therefore relatively small and the hemolysis is not complete. Therefore, it is advantageous if the fixation and permeabilization procedure leads to the lysis of these contaminating erythrocytes. It was found that only the IntraStain kit and paraformaldehyde/saponin fixation and permeabilization completely lysed RBC in the samples. The other two kits did not induce a lysis of porcine RBC (although complete lysis of human RBC was obtained by these kits in preliminary experiments, which is in accordance with the manufacturer's instructions).

The problem can occur when the lysis of RBC is performed before fixation and permeabilization. The lysis of RBC induced by ammonium chloride solution (the lysing reagent commonly used in our laboratory to lyse porcine RBC) before fixation and permeabilization with commercial kits strongly changed the light scatter characteristics of white blood cells (WBC). This was apparent, especially in neutrophils, which completely fused with the lymphocyte population. Moreover, the population of lymphocytes was much more dispersed as shown by the side scatter measurements. If the lysis of RBC was performed before their fixation and permeabilization with the IntraStain kit, and the cells were washed twice in CWS solution immediately after lysis, the scatters were not altered. If these washing steps were tested with the other two kits, the light scatters were changed. Thus, the lysis of RBC cannot be achieved with the Fix&Perm or Leukoperm kits without alteration of light scatter characteristics of WBC.

Finally, the effect of different fixation/permeabilization reagents on IFN-γ and TNF-α staining was tested. Generally, we can say that commercial kits mostly gave better results compared with paraformaldehyde/saponin. IntraStain and Leukoperm gave better results than Fix&Perm in some cases. If the use of a combination of paraformaldehyde/saponin is considered, 0.05% saponin should be avoided, especially in combination with 2% paraformaldehyde. Saponin in a concentration of 0.1% in combination with 4% paraformaldehyde slightly increased the percentages of positive cells in comparison with 2% paraformaldehyde. However, these differences were nonsignificant.

Consistent with the above mentioned parameters, the IntraStain kit gave the best results compared to the other two tested kits, as well as when compared to paraformaldehyde/saponin. Therefore, this kit was chosen for fixation and permeabilization of porcine leukocytes for experimentation.

Since a wide range of different fixation and permeabilization reagents that were not tested in our study are currently available, the above mentioned parameters can serve as a particular

protocol of intracellular cytokine detection and also as a suggestion for optimization of the fixation, permeabilization and cell surface labeling procedures for any laboratory.

2.1 Methanol permeabilization

Alcohol permeabilization has typically been used for the analysis of DNA by flow cytometry (Ormerod et al., 2002), but can be successfully applied to phospho-epitope staining as well (Krutzik et al., 2003). It is thought that alcohols fix and permeabilize cells by dehydrating them and solubilizing molecules out of the plasma membrane. Proteins may be made more accessible to antibodies during the process and cells are permeabilized to a greater extent than with saponin, allowing efficient access to the nuclear antigens.

Another option is to use commercially available kits. Currently, there are a large number of them available on the market. A fact that needs to be taken into account when using the commercial kits is that even if the kit works well, the method cannot be excessively modified. This could be reflected in the scale of results.

By using one of the best available kits for permeabilization, BD Fix&Perm, with methanol permeabilization during "The decrease in p-CrkL levels upon imatinib treatment" experiment, we came to the following conclusion:

The method (permeabilization using BD Fix&Perm) is less sensitive. It does not recognize the difference between 0 μM and 5 μM imatinib (IM). The speed (2 hours) is an advantage and there is a smaller amount of necessary input material (cells) as well. Although the peaks are sharp, it seems to be more difficult for the antigens to get into the cell, because of less aggressive permeabilization. The methanol permeabilization is much more sensitive. It is able to recognize differences between 0 μM and 5 μM IM. It is more time consuming (5 hours) and requires a higher amount of input material (cells). The significant advantage is the customizability of the method according to user needs (Figure 1).

3. Isotype control

The selection of the appropriate isotype control is an important element in flow cytometry experiments. Isotype controls are antibodies of the same isotype as the target primary antibody. They are of unknown specificity or are raised against antigens known to be absent in target cells. Isotype controls are used to estimate non-specific staining of primary antibodies. Several factors can contribute to the levels of this "background" staining, including Fc receptor binding, non-specific protein interactions, and cell autofluorescence. These factors may vary depending on the target cell type and the isotype of the primary antibody. Therefore, isotype controls need to be properly chosen. Isotype control antibodies ideally match the primary antibody's host species, isotype, and conjugation format. For example, if the primary antibody is an APC-conjugated mouse IgG2a, then it will be necessary to choose an APC-conjugated mouse IgG2a isotype control. Thus, isotype control is supposed to have all the non-specific characteristics of the target primary antibody and it is able to accurately determine the level of specific staining. Various monoclonal antibody idiotypes are used in flow cytometry applications: most frequently, IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA; less frequently IgD, IgE, IgG2c, Ig kappa, and Ig lambda are applied. Designing the experiment, this isotype and origin species of the primary antibody must be known to find a suitable isotype control. The appropriate isotype control is subsequently

looked up in a company product list according to the desired isotype (IgG1, IgG2a, IgM, etc.), reactivity (mouse, human, rat, etc.), and conjugate (FITC, PE, APC, etc.). In addition, recommended isotype controls can often be found on the Data Sheets for primary

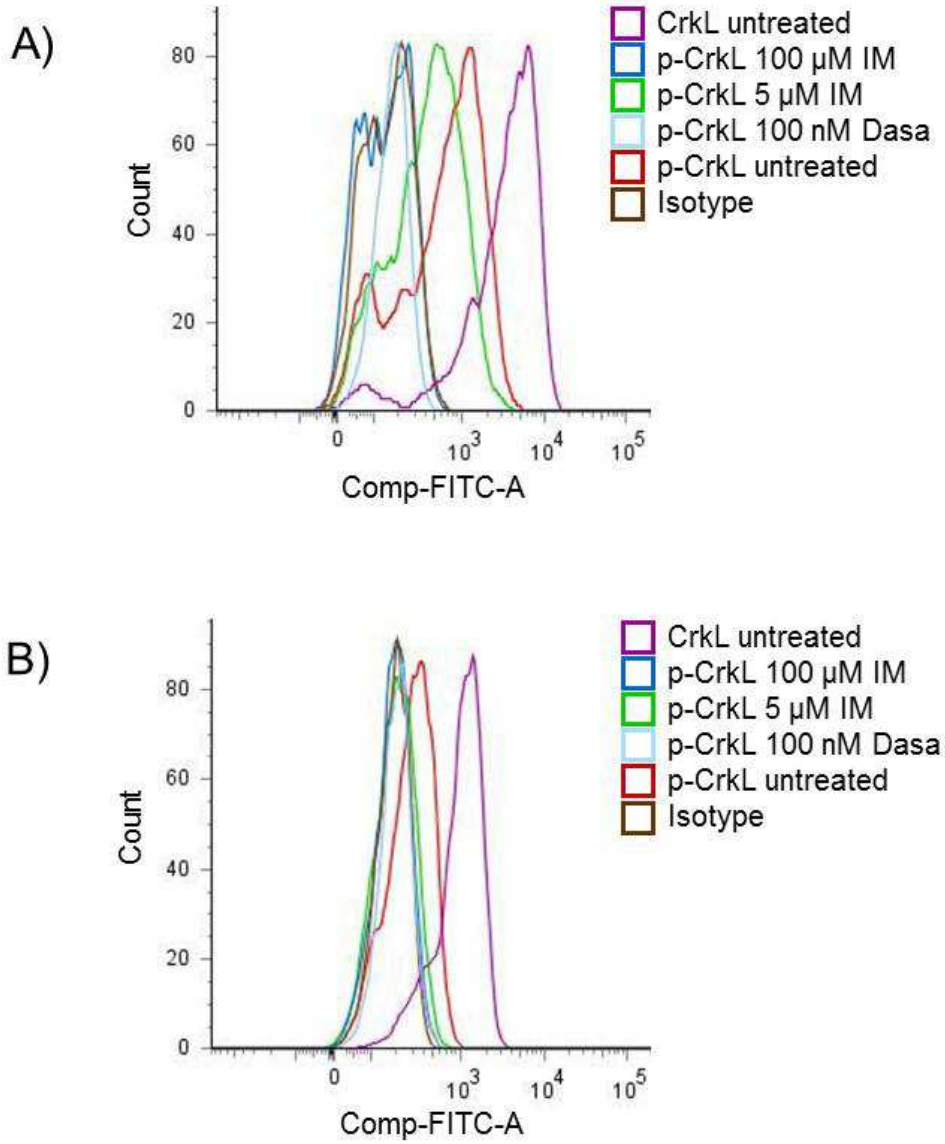


Fig. 1. The difference between permeabilization protocols using (A) methanol and (B) the BD Fix&Perm kit. Permebilization with methanol is more sensitive and allows researchers to distinguish between individual MFI peaks. Fix&Perm kit is less time consuming with relatively low sensitivity

antibodies. Isotype control antibodies are commercially available for both direct and indirect immunofluorescence in the form of fluorochrome-conjugated antibodies and unconjugated antibodies, respectively. During the flow cytometry analysis, the idiotype control antibody is diluted to the same concentration as the specific primary antibody, and is used to stain the sample of negative control cells. This negative control serves to determine the amount of non-specific "background" fluorescence. It allows for setting a threshold of negativity of stained cells. Any event generating a signal above this baseline is considered to be specifically labeled with the target primary antibody.

The isotype control plays an important role during the processing of final measurements. The level of monitored protein is determined as the geometric mean of fluorescent intensity (MFI) of labeled sample, minus the isotype control (O'Gorman et al., 1999, Holden et al., 2006,Hulspas et al., 2009).

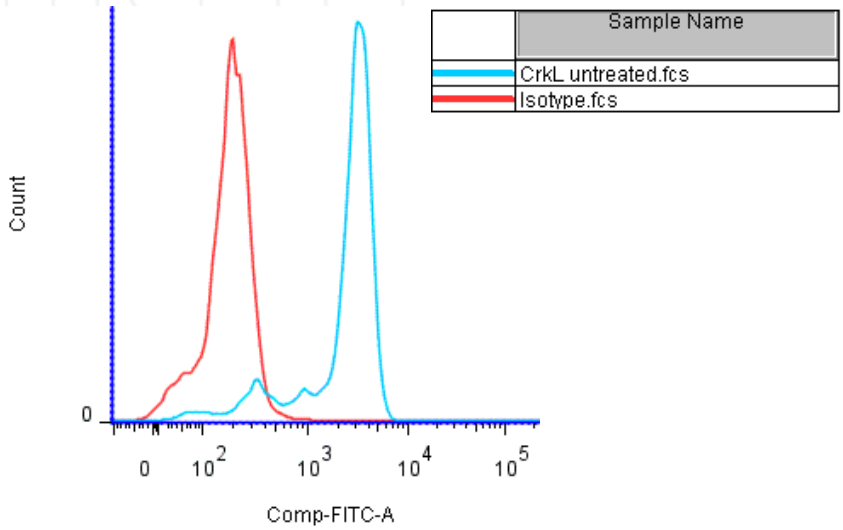


Fig. 2. Representative FACS plot showing isotype and CrkL MFI peaks visualized by a FITC-conjugated secondary antibody in CD34+ cells isolated from peripheral blood of a newly diagnosed CML patient. The MFI peak of isotype indicates the FITC unspecific background fluorescence. These were visualized using FlowJo software

4. Intracellular antigens for flow cytometry

The most common way to visualize of the complex between a monoclonal antibody and an antigen in flow cytometry is to covalently bind the antibody to different fluorescent molecules (fluorophores). After exposure to radiation from an excitation source, these fluorophores emit photons with longer wavelengths. Currently, there is a wide range of commercially available fluorophores starting from the small polycyclic molecules, such as fluorescein isothiocyanate (FITC), cyanines, and dyes of the Alexa series, through fluorescent phycobilipoproteins whose best-known representatives are phycoerythrin (PE), allophycocyanin (APC). New way in flow cytometry is also Qdot® nanocrystals –

nanometer-scale semiconductor particles with unique fluorescence properties (eBioscience , Molecular Probes®)..

The world leader in bringing innovative diagnostic and research tools to different specialists are Beckman Coulter and eBioscience as the major companies producing unconjugated and conjugated antibodies for flow cytometry. BD Biosciences offers a number of Alexa Flouro (AF®) 488/647/700-, APC-, FITC-, PE-, and Pacific BlueTM- conjugated antibodies of BD PharmingenTM and BD PhosflowTM brands used for multicolor flow cytometry (BD Biosciences, 2011).

A particularly strong area of leukemia immunophenotyping that contains a broad panel of research products has been built up by (DAKO, 2010). DAKO offers various types of reagents for use in flow cytometry, including primary single-color antibodies conjugated with a single fluorochrome; MultiMix™ Dual-Color Reagents based on the combination of two or more antibodies labeled with FITC and RPE; and MultiMix™ Triple-Color Reagents based on the combination of three antibodies labeled with fluorescein isothiocyanate (FITC), R-phycoerythrin (RPE) and allophycocyanin (APC) or FITC, RPE and RPE-Cy5. Other products for use in flow cytometry are isotype reagents, secondary antibody conjugates, streptavidine conjugates and other accessories. There are also several kits available, e.g. an apoptosis kit used for flow cytometric distinction between viable cells in single cell suspensions or an Enumeration of Stem Cells Kit used for optimal enumeration of CD34+ hematopoietic stem /progenitor cells (DAKO, 2010).

Another strong company in the production of high-quality activation-state antibodies is Cell Signaling Technology. They offer a number of primary and conjugated antibodies as well as antibody-related kits. Conjugated antibodies are conjugated with AF®, PE or biotin (Cell Signaling Technology, 2011)

There are many other companies not mentioned in the table, including R&D Systems, Miltenyi Biotec and BioLegend, that offer a wide range of unconjugated and conjugated monoclonal antibodies and kits. For example, R&D Systems offers a wide range of biotin-, fluorescein-, PE-, PerCP-, AF® 488-, or APC- conjugated monoclonal antibodies specifically designed to monitor protein expression by flow cytometry (R&D Systems, 2011).

Generally, the problem with labeling various molecules for flow cytometry in animal species other than mice is the poor availability of directly conjugated primary antibodies. Therefore, indirect labeling is commonly used. This includes labeling cells with a non-conjugated antibody and subsequent visualization with a secondary fluorochrome-conjugated antibody. This indirect labeling is limited by the subclasses of primary antibodies, which should not share the same subclass. This limits the use of the antibodies, especially in the case of multicolor labeling.

When intracellular labeling is combined with cell surface marker labeling in pigs, the problem with sharing the same subclasses is more noticeable since all the anti-porcine cytokine antibodies used share the most common mouse IgG1 subclass. The cell surface molecules are always labeled prior to the labeling of the intracellular cytokine in our protocol. Accordingly, the anti-cytokine antibody must be directly conjugated with fluorochrome (such as anti-TNF-α or anti-IFN-γ) or it must be biotinylated (such as anti-IL-2 or anti-IL-10) or pre-labeled in some other way (Zelnickova et al., 2008).

Intracellular marker	Type	Clone name	Isotype	Reactivity	Labelling	Source	Ref.
Cytokines							
IL-2, IL-4, TNF- α, etc.	mAb	5344.111, 3010.211, 6401.1111, …	Ms IgG_1,κ	Hu	FITC/ PE/APC/…	BD Biosciences	Karanikas et al., 2000
IFN- γ	mAb	P2G10	Ms IgG_1,κ	Pig	PE-conjugate	BD Biosciences	Gerner et al., 2008
Cell cycle or Apoptosis							
p53	mAb	DO7	Ms IgG_2b	Hu	nonconjugated	Dako	Millard et al., 1998
bcl-2	mAb	124	Ms IgG_1	Hu	FITC-conjugated	Dako	Millard et al., 1998
rb	mAb	G3-245	Ms IgG_1	Ms, Qua, Mn, Mk, Rat, Hu	nonconjugated	BD Biosciences	Millard et al., 1998
MDR1	mAb	JSB-1	Ms IgG_1	Hu, Rat, Ms	nonconjugated	Millipore	Millard et al., 1998
PCNA	mAb	PC10	Ms IgG_{2a}	Hu	nonconjugated	Dako	Millard et al., 1998
caspases	pAb	-	Rab IgG	Hu, Rat, Ms	PE-conjugated	BD Biosciences	Belloc et al., 2000
perforin	mAb	dG9	Ms IgG_{2b}	Hu	PE-conjugated	BD Biosciences	Gerner et al., 2008
Ki67	mAb	B56	Ms IgG_1,κ	Ms	FITC-conjugated	BD Biosciences	Gerner et al., 2008
Bax, Bcl-x_L, Mcl-1	pAb	-	Rab IgG	Ms/ Rab/ Hu	unconjugated	Santa Cruz Biotechnology	van Stijn et al., 2003
Viral particles							
HIV	mAb	unknown	$MsIgG_{1b12}$	Hu	unknown	Denis Burton	Mascola et al., 2002
Receptors or their proteins, enzymes							
Steroid hormone receptor proteins	mAb pAb	*(various)*	*(various)*	*(various)*	*(various)*	Afinity bioreagents	Butts et al., 2007
Cox1/2	mAb	AS70, AS67	Ms IgG_1,κ	Hu	FITC, PE	BD Biosciences	Ruitenberg et al., 2003
Estrogen Receptor α	mAb	1D5	Ms IgG_1	Hu	unconjugated	Dako	Cao et al., 2000
Phospho-proteins							
Akt	mAb	5G3	Ms IgG_1	Hu, Ms, Rat, Hm	unconjugated	Cell Signaling Technology	Tazzari et al., 2002
	pAb	-	Rab IgG	Hu, Ms, Rat, Hm, Mk, Chick, Dm, Bov, Pig, Dog			
MEK1/2	pAb	-	Rab IgG	Hu, Ms, Rat, Mk, Sc	unconjugated	Cell Signaling Technology	Chow et al., 2001
ERK1/2	mAb	20A	Ms IgG_1	Hu, Ms, Rat	AF-conjugated	BD Biosciences	Krutzik et al., 2003
JNK	mAb	41	Ms IgG_1	Hu	unconjugated	BD Biosciences	Krutzik et al., 2003
p38 MAPK	mAb	30	Ms IgG_1	Hu, Rat, Ms	unconjugated	BD Biosciences	Krutzik et al., 2003
Stat1	mAb	14	Ms IgG_1,κ	Hu, Ms	unconjugated	BD Biosciences	Krutzik et al., 2003
Stat3	mAb	D3A7	Rab IgG	Hu, Ms, Rat, Mk	AF-conjugated	Cell Signaling Technology	Kalaitzidis et al., 2008
Stat4	pAb	-	Rab IgG	Hu, Ms	unconjugated	Invitrogen	Uzel et al., 2001

Table 1. continues on next page

Intracellular marker	Type	Clone name	Isotype	Reactivity	Labelling	Source	Ref.
Stat5	mAb	47	Ms IgG_1	Hu	AF-conjugated	BD Biosciences	Krutzik et al., 2003
Stat6	mAb	18	Ms IgG_{2a}	Hu	PE-conjugated	BD Biosciences	Krutzik et al., 2003
CD79a	mAb	HM57	Ms IgG_1	Hu	PE-conugated	Dako	Gerner et al., 2008
mTOR	mAb	D9C2	Rab IgG	Hu, Ms, Mk, (Rat)	unconjugated	Cell Signaling Technology	Kalaitzidis et al., 2008

Hu – human; Ms – mouse; Mk – monkey; Hm – hamster; Dm – drosophila melanogaster; Bov – bovine; Sc – saccharomyces cerevisiae; Mk – mink; Qua - quail

Table 1. Intracellular antigens for flow cytometry

A method for the direct labeling of antibodies that share the same subclass was tested. Because it was necessary to label relatively small amounts of antibodies, the Zenon-labeling technology was chosen.

The Zenon reagents are provided by Invitrogen. This labeling involves the binding of Fab fragments of the fluorochrome-labeled, subclass-specific secondary antibody to the primary antibody, prior to the labeling of the cells. The excess of the fluorochrome-labeled secondary antibody is neutralized by addition of irrelevant mouse antibody in excess, which is supplied within the Zenon kit. The disadvantage of these reagents is the relatively high price. The price is the same for all fluorochromes; however, the number of reactions that can be performed by the kit differ among fluorochromes. Packages with Alexa Fluor, FITC, Texas Red and Pacific dyes contain reagents for 50 rounds of labeling, packages with phycobiliprotein dyes such as R-PE or APC contain reagents for 25 rounds of labeling, and packages with tandem dyes contain reagents for only 10 rounds of labeling. Therefore, it is advantageous to choose Zenon reagents containing Alexa Fluor dyes.

The other disadvantage of the Zenon labeling is that the fluorescence yield of Zenon-labeled antibodies is slightly lower compared to classical indirect labeling. Therefore, antibodies against strongly expressed markers should be preferably stained with the Zenon reagents. (Ondrackova et al., 2010, 2011)

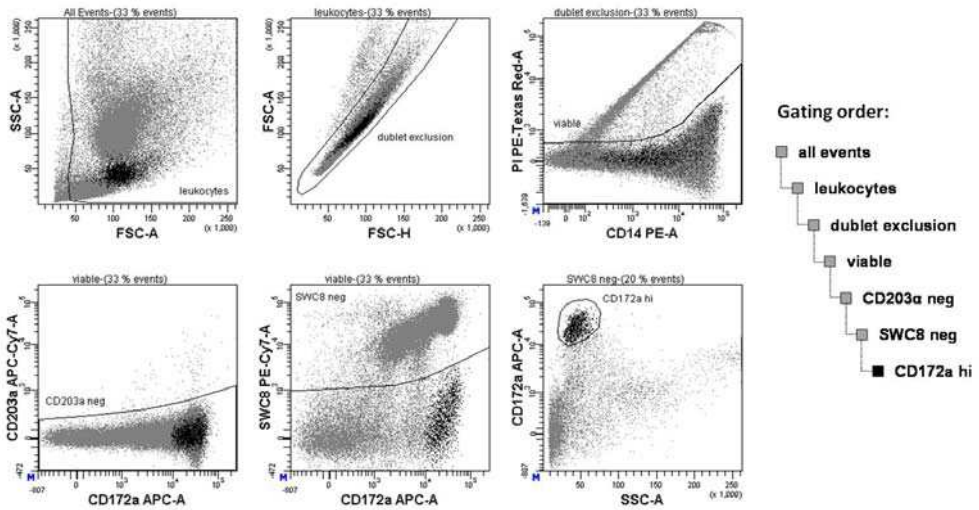
General protocol for cell surface staining followed by intracellular labeling

1. Place the samples of cell suspensions into a U-bottom 96-well plate, spin the plate, remove as much supernatant as possible, and vortex the plate.
   If viability staining with permanent dye combined with intracellular staining is performed, then:
2. Add the viability staining dye, incubate according the manufacturer's instructions, vortex the plate, and wash once.
   The cell surface staining is performed as follows:
3. Add the cocktail of primary antibodies against cell surface molecules in a total volume of 10 µl (dilute antibodies in CWS) + 10 µl of heat-inactivated, filtered goat serum, vortex the plate, incubate for 15 min at 4°C, and wash twice.
4. Add a secondary antibody cocktail in total volume of 25 µl (dilute antibodies in CWS), vortex the plate, and incubate for 20 min at 4°C.
   If the cell surface molecules are to be labeled with Zenon reagents or if intracellular staining follows, then:

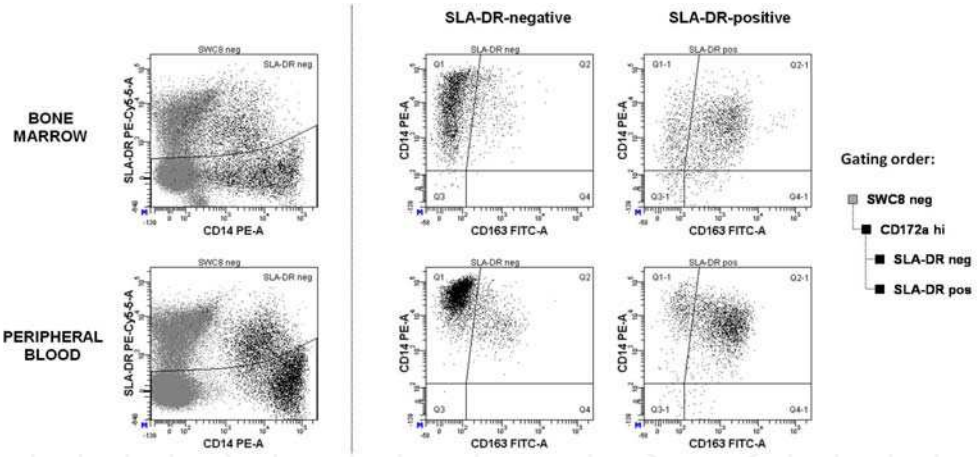
5. Wash once and vortex the plate thoroughly because the plate in the next step cannot be vortexed due to the risk of the samples overflowing into the neighboring wells.
6. Add 100 µl of heat-inactivated, filtered mouse serum diluted 1:10 in CWS, incubate for 20 min at 4°C, and wash once.
   Labeling cell surface molecules with Zenon-labeled antibodies now follows, but this step can be omitted if it is not required:
7. Add the cocktail of Zenon-labeled antibodies against cell surface molecules in a total volume of 10 µl, incubate for 15 min at 4°C, vortex the plate, and wash –twice.
   Staining of intracellular molecules with directly-labeled, Zenon-labeled, or unlabeled antibodies follows, but these steps can be omitted if they not required:
8. Add 30 µl of Solution A from the IntraStain kit, vortex thoroughly to allow complete hemolysis of contaminating red blood cells, incubate for 15 min at room temperature, and wash –twice.
9. Add primary antibodies against intracellular molecules (directly-labeled, Zenon-labeled, or unlabeled) diluted in Solution B from the IntraStain kit in a total volume of 20 µl, vortex the plate, incubate for 20 min at room temperature, and wash twice.
10. Add secondary antibodies diluted in Solution B from the InraStain kit and CWS (ratio of Solution B and CWS 1:1) in total volume of 25 µl, incubate for 20 min at room temperature, wash twice.
11. Resuspend samples in CWS in the volume that is required for the subsequent measurement and measure by the appropriate method.

CWS solution: PBS containing 1.84 g/l EDTA, 1 g/l NaN3, 4 ml/l gelatin from cold water fish skin

Wash definition: Add as much CWS into each well as possible, spin 3 min at 500 g, remove much supernatant as possible, and vortex.



(a)



(b)

Fig. 3. Seven color flow cytometry for identification of porcine monocyte subpopulations using two Zenon-labeled antibodies

The red blood cells from the whole peripheral blood or from the bone marrow sample were lysed with ammonium chloride solution. The following fluorescent staining was performed:

Excitation wavelength	Primary antibody					Secondary antibody / Zenon reagent / viability stain				
	Target molecule	Clone	Manufacturer	Amount per well	Stained at point	Class/ subclass	Fluorochrome	Manufacturer	Dilution	Stained at point
488	CD163	2A10/ 11	VMRD, USA	0.1 µl	3)	IgG1	AlexaFluor488	Invitrogen, USA	1:750	4)
488	CD14	MIL-2	Serotec, UK	1 µl	3)	IgG2b	AlexaFluor647	Invitrogen, USA	1:750	4)
488						Propidium iodide				11)
488	SLA-DR	MSA3	VMRD, USA	0.05 µl	3)	IgG2a	PE-Cy5.5	Invitrogen, USA	1:100	4)
488	SWC8	MIL-3	Dr. J.K. Lunney *	1 µl	3)	IgM	DyLight405	GeneTex, USA	1:500	4)
640	CD172α	DH59 B	VMRD, USA	0.1 µl	7)	Zenon IgG1 **	AlexaFluor647	Invitrogen, USA		
640	CD203α	PM 18-7	Serotec, UK	0.1 µl	7)	Zenon IgG1 ***	APC-AlexaFluor750	Invitrogen, USA		

* a generous gift from Dr. J.K. Lunney, Animal Parasitology Institute, Beltsville, MO, USA
** labeling with the Zenon® AlexaFluor647 Mouse IgG1 Labeling Kit perform as follows: 0.1 µl of anti-CD172α and 0.5 µl of Solution A of the Zenon Kit, mix well, incubate 10 min at 4°C, then add 0.5 µl of Solution B of the Zenon kit, mix well, incubate 10 min at 4°C
*** labeling with the Zenon® APC-AlexaFluor750 Mouse IgG1 Labeling Kit perform as follows: 0.1 µl of anti- CD203α and 0.5 µl of Solution A of the Zenon kit, mix well, incubate 10 min at 4°C, then add 0.5 µl of Solution B of the Zenon kit, mix well, incubate 10 min at 4°C
The measurement was performed by using BD FACSAria I flow cytometer (Becton Dickinson, USA).

The gating strategy for identification of monocytes in the bone marrow is depicted (A). Briefly, the leukocytes were gated according their light scatter properties (upper left dot-plot). The dublets of cells were excluded from the further analysis (upper middle dot-plot). The viable (propidium iodide-negative) cell were gated (upper right dot-plot). The CD203α-positive macrophages were excluded (lower left dot-plot). The monocytes were identified as SWC8-negative (lower middle dot-plot) CD172α-positive cells (lower right dot-plot).

Then monocyte subpopulations from the bone marrow and peripheral blood were identified based on expression of SLA-DR, CD14 and CD163 (B). The SLA-DR-positive and negative monocytes were gated (left dot-plots). Then SLA-DR-negative (middle dot-plots) and SLA-DR-positive (right dot-plots) monocyte subpopulations were depicted in CD163 vs. CD14 dot-plots.

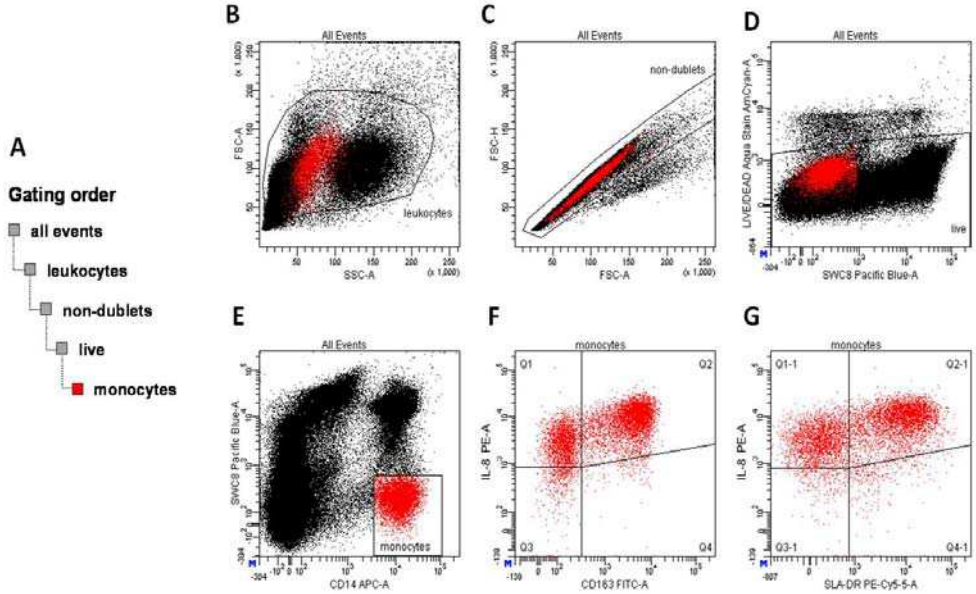


Fig. 4. Six color flow cytometry for measurement of IL-8 production by monocyte subpopulations using the Zenon-labeled anti-IL-8 antibody

The gating order for evaluation of IL-8 production by monocyte subpopulations is depicted (A). Briefly, the leukocytes were gated according their light scatter properties (B). The dublets of cells were excluded excluded from the further analysis (C). The viable (LIVE/DEAD® Fixable Aqua Dead Cell Stain-negative) cell were gated (D). The monocytes were identified as SWC8-negative CD14-positive cells (E). The IL-8 production by CD163-positive and negative monocytes (F) and by SLA-DR-positive and negative monocytes (G) was then evaluated.

The whole peripheral blood diluted 1:1 with RPMI 1640 was stimulated for 2 hours with LPS (1 µg/ml) in the presence of brefeldin A (10 µg/ml). The red blood cells were lysed with ammonium chloride solution. The following fluorescent staining was performed:

Excitation wavelength	Primary antibody					Secondary antibody / Zenon reagent / viability stain				
	Target molecule	Clone	Manufacturer	Amount per well	Stained at point	Class/subclass	Fluorochrome	Manufacturer	Dilution	Stained at point
405	SWC8	MIL-3	Dr. J.K. Lunney *	1 µl	3)	IgM	DyLight405	GeneTex, USA	1:500	4)
405						LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit **				2)
488	CD163	2A10/11	VMRD, USA	0.1 µl	3)	IgG1	AlexaFluor488	Invitrogen, USA	1:750	4)
561	IL-8	8M6	Serotec, UK	0.2 µl	9)	Zenon IgG1 ***	R-PE	Invitrogen, USA		
561	SLA-DR	MSA3	VMRD, USA	0.05 µl	3)	IgG2a	PE-Cy5.5	Invitrogen, USA	1:100	4)
640	CD14	MIL-2	Serotec, UK	1 µl	3)	IgG2b	AlexaFluor647	Invitrogen, USA	1:750	4)

* a generous gift from Dr. J.K. Lunney, Animal Parasitology Institute, Beltsville, MO, USA
** diluted 1:1000 in PBS, 10 µl / well, incubation15 min at 4°C
*** labeling with the Zenon® R-Phycoerythrin Mouse IgG1 Labeling Kit perform as follows: 0.2 µl of anti-IL-8 and 4 µl of Solution A of the Zenon kit, mix well, incubate 10 min at 4°C, then add 4 µl of solution B of the Zenon kit, mix well, incubate 10 min at 4°C, then add 11.8 µl of Solution B of IntraStain kit
The measurement was performed by using BD LSRFortessa flow cytometer (Becton Dickinson, USA)

5. Intracellular measurement of the p-CrkL and CrkL levels using flow cytometry

5.1 Theoretical background

Chronic myeloid leukemia (CML) is a myeloproliferative disorder of hematopoietic stem cells that is characterized by the presence of the BCR-ABL fusion gene, which encodes the constitutively active BCR-ABL tyrosine kinase (Daley et al., 1990). Currently, the tyrosine kinase inhibitor imatinib (IM) (a potent inhibitor of BCR-ABL) is used as a first line therapy for CML patients (Baccarani et al., 2009).

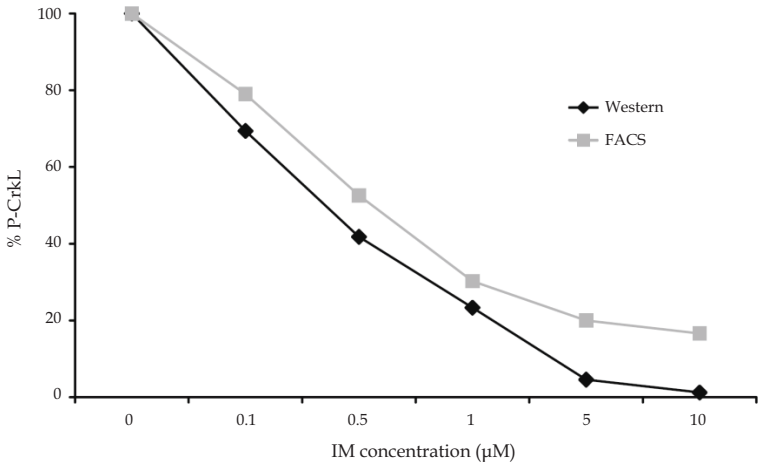


Fig. 5. The equivalence between flow cytometry and Western blot methods, in p-CrkL reduction in a BCR-ABL positive K562 cell line after 48 h treatment with imatinib (Hamilton et al., 2006)

The CrkL protein is a downstream signaling substrate of BCR-ABL, and its tyrosine phosphorylation (p-CrkL) serves as a specific indicator of BCR-ABL kinase activity in CML cells (Nichols et al., 1994; Patel et al., 2006). Recent studies have revealed that p-CrkL can act as a prognostic marker for imatinib treatment response of CML patients using either western blotting (White et al., 2005) or flow cytometry (Lucas et al., 2010). However, certain discrepancies have been found in the literature concerning the predictive value of p-CrkL in different cell types (mononuclear cells or CD34+ cells) used for analysis (Khorashad et al., 2009).

The technique for measuring p-CrkL levels using flow cytometry was originally described by Hamilton et al., (Hamilton et al., 2006) and the equivalence between flow cytometry and western blot methods was demonstrated (Figure 5).

5.2 The technique of intracellular p-CrkL and CrkL measurement by flow cytometry

Mononuclear cells (MNCs) were isolated from PB of newly diagnosed CML patients using Histopaque-1077 density gradient centrifugation (Sigma–Aldrich, St. Louis, MO, USA) and subsequently enriched for CD34+ cells using magnetic-activated cell sorting (MACS) with a CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. CD34+ cells ($5x10^4$) were incubated for 16 h with 0, 0.5, 1.5, and 5 µM imatinib in 1 ml of serum-free medium (SFEM), supplemented with StemSpan CC100 cytokine mixture (StemCell Technologies, Köln, Germany) as previously described (Koutna et al., 2011). Then the cells were washed in phosphate buffered saline (PBS) and fixed with 4% formaldehyde for 10 min at 37°C, then washed in PBS and permeabilized by 90% methanol at 4°C for 30 min.

After permeabilization, the cells were washed in PBS and incubated with primary unlabeled antibody for 30 min at 4°C in 100 µl of FACS incubation buffer (0.5% bovine serum albumin in PBS). The concentration of primary antibodies was 12 µg/ml (p-CrkL, Cell Signaling Technology, Danvers, MA, USA; CrkL, Santa Cruz biotechnology, Santa Cruz, CA, USA; isotype control anti-normal-rabbit IgG G, R&D Systems, Minneapolis, MN, USA). The cells were washed in FACS incubation buffer and incubated with FITC-conjugated anti-rabbit IgG secondary antibody (Sigma-Aldrich) in a concentration of 10 µl/ml.

All samples were measured on a FACSCanto II Flow Cytometer (Becton Dickinson). For data analysis, BD FACSDiva (Becton-Dickinson) and FlowJo (Tree Star, Ashland, USA) software were used. The viable cell population was gated according to forward scatter and side scatter parameters. The level of p-CrkL and CrkL in the viable cells was determined as the geometric mean fluorescence intensity (MFI) of the p-CrkL- or CrkL-labeled sample minus the MFI of the isotype control (Figure 6).

The $IC50^{imatinib}$ was defined as the concentration of imatinib that caused a 50% decrease in the amount of p-CrkL compared to the untreated control (Figure 7) (White et al., 2005). The p-CrkL/CrkL ratio was calculated by dividing the concentrations of p-CrkL by those of CrkL and multiplying by 100 in untreated cells (Lucas et al., 2010). The p-CrkL ratio was assessed as a percentage of p-CrkL in the samples treated with a maximal imatinib concentration (5 µM) relative to the untreated control (Figure 7) (Khorashad et al., 2009).

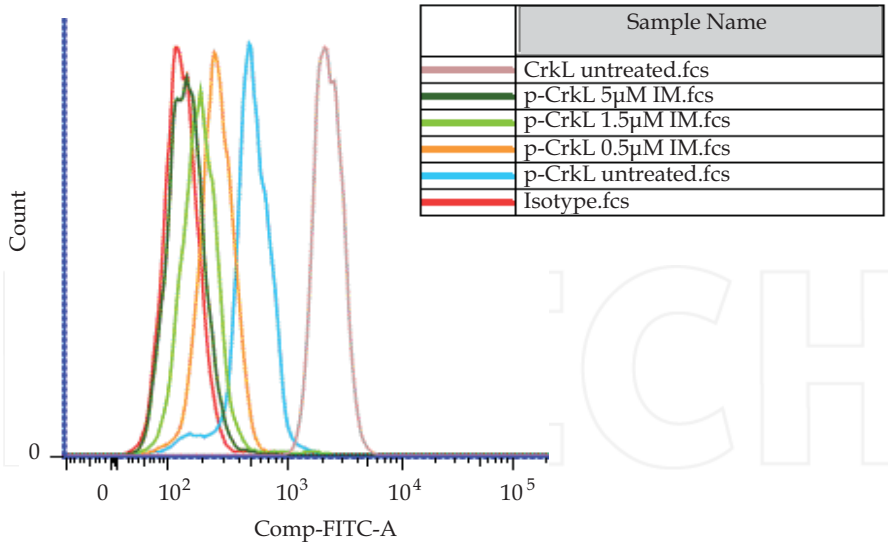


Fig. 6. Representative FACS plot showing isotype, p-CrkL and CrkL MFI peaks in CD34+ cells isolated from peripheral blood of a newly diagnosed CML patient. Changes in p-CrkL MFI peaks following *in vitro* imatinib (IM) treatment are detectable and were visualized using FlowJo software

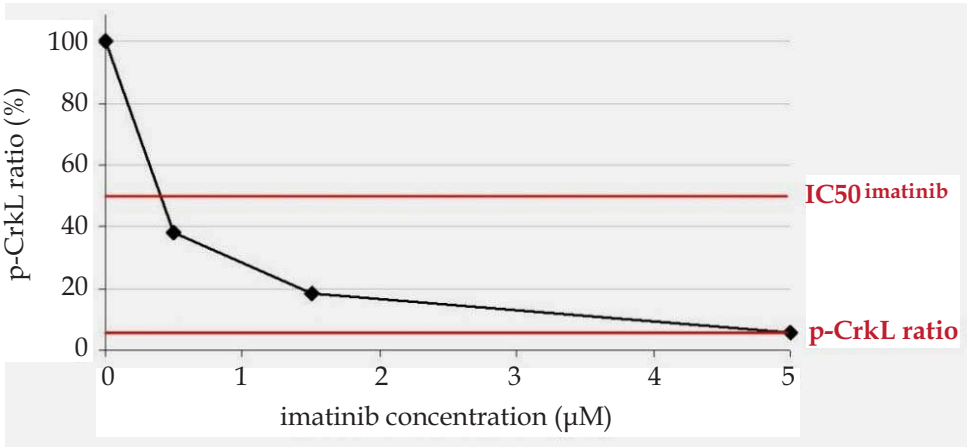


Fig. 7. The graph of p-CrkL decrease upon *in vitro* imatinib treatment. MFI peaks quantification was calculated in FlowJo software

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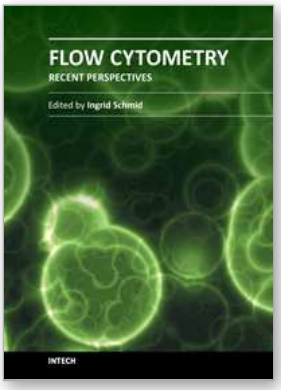

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"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

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