There are many protocols for staining cells for flow cytometry. Protocols may to need be optimized for different cell types, targets, or applications. This is our basic protocol for extracellular staining of cell surface epitopes in suspension cells for flow cytometry.

1. Reagents

	BD Bioscience	Home made	Biolegend	Miltenyi Biotec
Stain Buffer	Stain Buffer with BSA (554657) Stain Buffer with FBS (554656)	PBS pH 7.4 0.2% BSA	Cell Staining Buffer (420201)	-
Red Blood cell Lysis Buffer	Lysis Buffer (555899)		10X Red Blood Cell (RBC) Lysis Buffer (420301)	Red Blood Cell Lysis Solution 10× (130-094-183)
fixable dead cell dye	Fixable Viability Stain	-	Zombie Dye	Viobility™ Fixable dyes
Non fixable dead cell dye	PI (556463) 7ADD (55925)	-	PI (421301) 7-AAD (420404)	IP (130-093-233) 7AAD (130-111-568)
FCr block	Human BD Fc Block 564219 Mouse BD Fc Block 553141	-	Human TruStain FcX™ (422302) Mouse TruStain FcX™ (anti-mouse CD16/32) Antibody 101320	FcR Blocking Reagent, human (130-059-901) FcR Blocking Reagent, human (130-092-575)
Fixation Buffer	BD Cytofix (554645)	0.5-2% paraformaldehyde	Fixation Buffer (420801)	-
Brilliant Stain Buffer	563794	-	-	-

2. Procedure

Notes : In general, cells should be centrifuged sufficiently so the supernatant fluid can be removed with little loss of cells, but not so hard that the cells are difficult to resuspend.

We recommend staining with ice-cold reagents/solutions and at 4°C, as low temperature and presence of sodium azide prevent the modulation and internalization of surface antigens. Internalization can cause a loss of fluorescence intensity.

Cell suspension preparation

1. If necessary, resuspend cells with **Stain Buffer**.
2. Wash the cells twice in cold Stain Buffer and pellet the cells by centrifugation at 300-350*g* at 4°C for 5 min. Discard supernatant.

Red Blood Lysis Buffer (Optional)

3. If necessary (e.g. spleen), resuspend pellet in 1X **Red Blood cell Lysis Buffer**. Incubate on ice according to manufacture informations (volume and time). Stop cell lysis by adding Staining Buffer to the tube. Centrifuge for 5 minutes at 350xg and discard supernatant. Repeat wash as in step 2.

Fixable Live/Dead Dye (Optional)

4. To exclude dead cells from analysis, resuspend cells in at 1-10x10^6 cells/ml in **PBS or other protein-free** buffer and stain cells with a **fixable dead cell dye**, according to the product protocol (10-15 minutes at room temperature or on ice protected from light). Wash cells twice with 2 ml of Stain Buffer.

   Don't forget to keep some unstained cells for controls (unstained cells and single stained cells) before this step !

   Note: If cell fixation will not be performed,* a non-fixable dead cell stain*, such as PI or 7-AAD, can be added prior to analysis (see section 12).

Cells Aliquot

5. Count viable cells and resuspend in Cell Staining Buffer at 5-10 x 10^6 cells/ml.
6. **Distribute 100µl/tube of cell suspension (5-10 x 10^5 cells/tube) into 12 x 75mm plastic tubes.**

Fc Block (Optional)

7. To block non-specific Fc-mediated interaction, add a certain volume of Fc Block per 10^6 cells per tube (or per well) and incubate for 10 min at room temperature. (follow manufacture instructions for time, volume and temperature). It is not necessary to wash cells between these blocking and immunostaining steps.

Antibodies staining

8. Add fluorescent antibodies to their predetermined optimal quantity in Stain Buffer (**BD Horizon Brilliant Stain Buffer should be used anytime two or more BD Horizon Brilliant dyes**

are used in the same experiment) and add small aliquots (e.g., 10 µl) of the diluted antibodies to the tubes or microwells that contain the target cell suspensions and incubate for 30 minutes on ice, protected from light.

Example of creating a 5-Color Fluorescent Antibody Cocktail containing 2 different Brilliant Violet™ Conjugates
Final Volume per Test = 90 µL

		Total Number of Tests			
	Volume/Test (µL)	1	3	5	10
Brilliant Stain Buffer	50	50	150	250	500
Reagent 1 (BV)	5	5	15	25	50
Reagent 2 (BV)	5	5	15	25	50
Reagent 3	5	5	15	25	50
Reagent 4	5	5	15	25	50
Reagent 5	20	20	60	100	200
Total Volume	**90**	**90**	**270**	**450**	**900**

Add desired volume of Reagent Cocktail (90 µL in this 5-color example) to all tubes or wells using the protocols for staining human cells described above.

9. Wash the cells two times with either 200-µL (for plates) or 1-mL (for tubes) volumes of Stain Buffer. Centrifuge cells at 300*g* for 5 minutes.
10. Carefully aspirate (for microwell plates or tubes) or invert and blot away (for tubes) supernatants from cell pellets.
11. Tap tubes/plates to loosen the cell pellet and Resuspend the cell pellet in either 200-µL (for microwell plates) or 0,5 mL (for tubes) volumes of Stain Buffer.

DNA Dye for dead cell exclusion (Optional)

12. Prior to analysis, add **non-fixable dead cell stain**, such as PI or 7-AAD. Incubate on ice following manufacture instructions. Do not wash off PI after staining,

FACS analysis

13. Analyze stained cell samples by flow cytometry.

Notes : If analysis must be delayed, then the stained cells can be fixed with **Fixation Buffer** for 30 minutes at 4°C, washed, resuspended in Stain Buffer, and then stored at 4°C (protected from light). The fixed cells should be analyzed as soon as possible. We have not tested all fluorescently conjugated antibodies for this fixation. Therefore, researchers may need to verify if this fixation will affect antibody binding and fluorescence intensity.