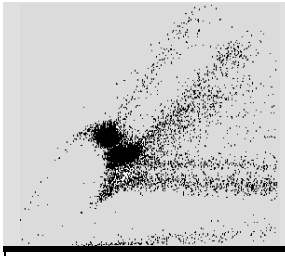

THE GOODELL LABORATORY

Author	K. Karen Lin	March 30, 2009
Title	**Intracellular Staining of HSCs (sparse cells) with Carrier Cells**	
Introduction	This protocol describes an intracellular staining technique, adapted from the protocol developed by Dr. Garrett Heffner (Weissman Lab), and is particularly useful for the following scenarios, 1) Small number of purified HSCs (~1000 cells) with carriers 2) Surface marker staining followed by intracellular staining 3) Intracellular staining with phospho-proteins The fluorophores are interchangeable. The standard analysers compatible with this particular protocol are a LSRII with a blue laser, red laser, a violet laser, and an UV-laser (BD Biosciences) as well as an Imagestream with comparable lasers (Amnis).	
Materials	**1. 16% Paraformaldehyde (PFA) solution (Electron Microscopy Sciences, 15710)** **2. 100% Ethanol** **3. 1% BSA in PBS** **4. 30% Hydrogen peroxide solution (Sigma, H1009)** **5. Barcode Dyes with desired concentration** 1) Alexa 488 (Invitrogen A-20000) 2) Pacific Blue (Invitrogen P-10163) **6. Your antibodies** 1) Note that to date, ONLY FITC and Pacific Blue conjugated-antbodies (among the fluorophore collection in our lab, including FITC, PE, PE-Cy7, PECy5, APC, and APC-Cy7) are found to survive the "quenching process" that is necessary to eliminate the HRP activity of endogenous hydroperoxidase. To perform any intracellular staining in HSC, pre-sorting cells is highly recommended. 2) Fluorophores for the intracellular staining: Alexa647 7. **Tyramide Signal Amplification (TSA) Reagents**: TSA Kit is available from Invitrogen with Horseradish Peroxidase (HRP) conjugated goat anti-rabbit IgG antibody (TSA Kit with Alexa Fluor 647 Tyramide, Cat No. T20926).	
Protocol		*Notes*
1.	Fix the cells-of-interest: sort cells (or cell cultures) into a fixed volume. Add appropriate 16% PFA into cell suspension to 4% PFA. Tap the tube to mix the cells and incubate at RT for 10 minutes (or on ice for 30 minutes)	

2.	Permeabilization: Add 100% ICED EtOH directly to the tube to make 90% EtOH solution. Incubate on ice for 15 minutes. Wash with PBS, spin cells down.	If you have been sorting cells for the whole day, you could consider stopping the incubation here, and storing the samples in a -20 freezer. Note that you need to mix the EtOH with the cell suspension THOUROUGHLY; otherwise the cells will be frozen and disrupted.
3.	Barcoding: A multiple color barcoding strategy could be employed by using combinations of barcoding dyes (Krutzik and Nolan, 2006). In general, cells are resuspended in 30% EtOH. Barcoding dye should be added in a 1/100 ratio (10ul to an 100ul cell suspension) to the desired concentration. Incubate under room temperature for 10 minutes. Wash with 10X volume of BSA-PBS, spin down the cells.	-BSA-PBS= 1% of BSA in PBS -Barcoding dyes usually come in powder forms. These fluorescent dyes should be resuspended in Methanol, and dried back into powders with a Speed-vac. In our lab, the barcoding dyes are aliquoted at 25ug/tube, and stored in the -20^{o}C freezer. For experiments, the barcoding dye aliquots are resuspended in DMSO for the staining process.
4.	In the mean time, wash and spin down the carrier cells as well. Mix these un-labeled carrier cells (~5x 10^4 cells) with the target cells.	Carrier cells in our lab are spleenocytes that have been fixed and permeablized with 90% EtOH. These carriers can be stored in the EtOH solution at -20^{o}C for at least half an year.
5.	Quenching the residual hydrogen peroxidase with 1% H2O2 in 1% BSA-PBS buffer. Incubate at room temperature for 30 minutes. Wash with 10X volume of BSA-PBS buffer, spin down (2200rpm, 6 minutes).	

6.	Staining with the 1st antibody. Incubate under room temperature for 30 minutes. Wash with 10X volume of BSA-PBS buffer, spin down (2200rpm, 6 minutes).	Primary antibodies should be titered for appropriate dilution.
7.	Staining with HRP-conjugated secondary antibody. Incubate at room temperature for 30 minutes. Wash with 10X volume of BSA-PBS, spin down (2200rpm, 6 minutes)	HRP-conjugated secondary antibodies that have been tested: 1) goat anti-rabbit antibody 1:400 (Santa Cruz, sc-2065, Lot G3103) 2) goat anti-rabbit antibody 1:200 (Invitrogen, included in the TSA kit, T20926)
8.	Developing color with Tyramide Signal Amplification (TSA) kit: 1) Prepare 100X solution A: add 1ul of 30% H2O2 into 200 ul of H2O2 2) Prepare FRESH solution B: -1X solutionA -1X Tyramide (100X dilution) - in amplification buffer (100ul/reaction) 3) Staining with 100ul/reaction of solution B, incubate for 5 minutes, stop reaction by adding 10X volume of BSA-PBS 4) Spin down (2200rpm, 6 minutes), resuspend in 350ul BSA-PBS for flow analysis.	

References.

Krutzik, P.O., and Nolan, G.P. (2006). Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. Nature methods *3*, 361-368.