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Flow Cytometry Research Group 2015 Study

Evaluating the Effects of Cell Sorting on Gene Expression

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Flow Cytometry Research Group

Abstract

The Flow Cytometry Research Group has continued with the goal of establishing best practice guidelines for cell sorting conditions that minimize cell stress, perturbation, or injury to the sorted cell populations. In prior FCRG studies, gene expression changes in Jurkat T lymphoblast cells were measured following cell sorting with different system pressures and nozzle sizes where minimal effects observed resolved over time in culture. Last year's study examined the effect sorting has on primary cells (C57Bl/6 mouse splenic B lymphocytes). B lymphocytes were isolated using multiple flow sorters with 100 micron nozzle size/20 psi pressure or 70 micron nozzle size/70 psi pressure sorter configurations. Genome-wide gene expression analysis was performed on selected samples using affymetrix microarrays and a small number of candidate genes were identified as responding differentially in high or low pressure conditions. In the latest study, additional samples from the same batch of sorting runs were assayed by eBioscience QuantiGene Plex (QGP) to validate the significance of the candidate genes identified in microarray data. Since the QGP assay is a highly multiplexed bead based assay, additional genes known to respond to cell stress and damage were also evaluated for changes as a result of cell sorting. Details of the study and results will be presented along with future plans.

Background

- Mouse B cell samples from a previous data set, ES cells and dendritic cells were evaluated for gene expression changes after cell sorting on different instruments using the settings listed.
- Analysis was done using a custom QuantiGene 60-Plex assay designed to include genes previously found to be differentially expressed in B cells after sorting at different pressures (microarray candidate) and genes involved in cell stress pathways.

QuantiGene Custom 60-Plex

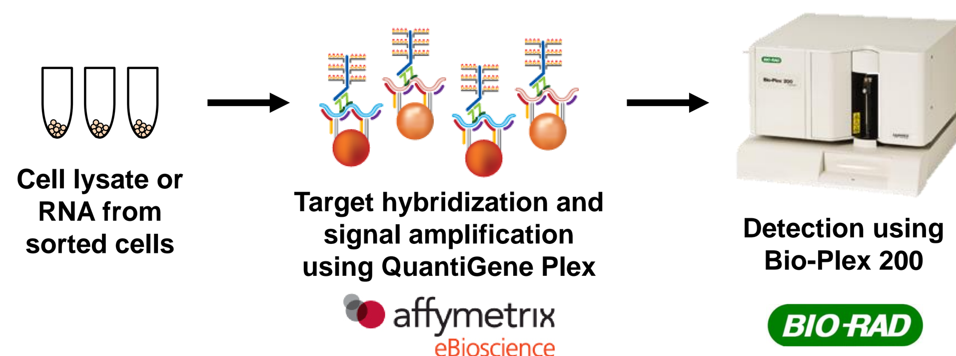
Symbol	Functional Category
Aen	apoptosis
Apaf1	apoptosis
Bax	apoptosis
Bbc3	apoptosis
Cflar	apoptosis
Cyfip2	apoptosis
Fas	apoptosis
Phlda3	apoptosis
Tnfrsf10b	apoptosis
Traf4	apoptosis
Unc5b	apoptosis
Xiap	apoptosis
Dram1	autophagy
Prkab1	autophagy
Prdm1	cAMP & MAPK pathway
Btg2	cell cycle arrest
Cdkn1a	cell cycle arrest
Fbxw7	cell cycle arrest
Actb	control
Gapdh	control

Symbol	Functional Category
Hprt	control
Tbp	control
Ubc	control
Ywhaz	control
Ddb2	DNA repair
Polh	DNA repair
Rrm2b	DNA repair
Xpc	DNA repair
Atf4	heat shock & upr
Atf6	heat shock & upr
Atf6b	heat shock & upr
Bid	heat shock & upr
Calr	heat shock & upr
Ddit3	heat shock & upr
Dnajc3	heat shock & upr
Hsp90aa1	heat shock & upr
Hsp90b1	heat shock & upr
Hspa4	heat shock & upr
Hspa5	heat shock & upr
Xbp1	heat shock & upr

Symbol	Functional Category
Pank1	metabolism
Abcg1	microarray candidate
Fos	microarray candidate
Gm129	microarray candidate
Klf4	microarray candidate
Plk2	microarray candidate
Rgs1	microarray candidate
S1pr3	microarray candidate
Il1a	NFkB pathway
Il6	NFkB pathway
Tnf	NFkB pathway
Alox5	ROS control
Fdxr	ROS control
Ppib	ROS control
Sesn1	ROS control
Sesn2	ROS control
Egr1	shear stress responsive
Gpr87	survival
Tnfsf13b	survival
Triap1	survival

Note – The chosen genes within the apoptosis, autophagy, metabolism, cell cycle arrest, ROS control, DNA repair & survival categories are human p53 transcriptional targets. NFkB and cAMP/MAPK pathway genes were chosen as possible upstream and downstream transcriptional targets of microarray candidate genes.

QuantiGene Assay Workflow

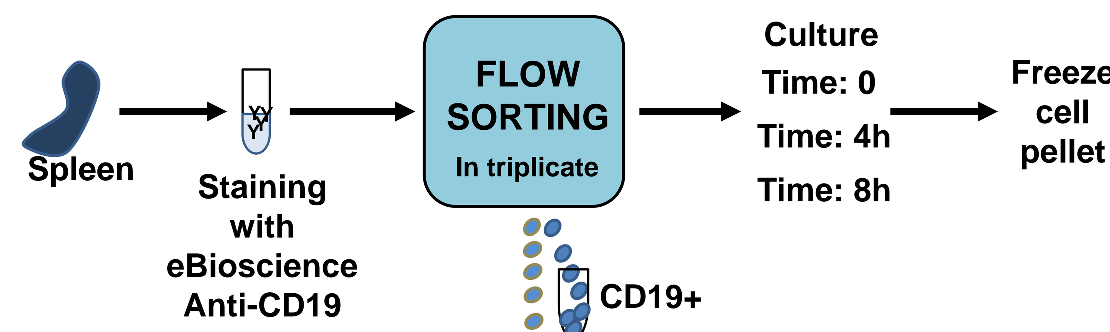


Note – For lysates, cells were used at a concentration of 1500/μl. For RNA, 100 ng was used for each replicate. Some replicates were pooled to achieve this amount.

Mouse Splenic B Cells

- 3 sites (5 total instruments)
- C57Bl/6 male mice CD19+ B cells isolated via cell sorting
- Cells collected for microarray (2014/2015 work) and Quantigene-Plex (QGP) validation of microarray results

BD FACSAria II (2 sites)	BD Influx	BC MoFlo Astrios or XDP
100 micron/20-25 psi	100 micron/20 psi	100 micron/20-25 psi
70 micron/60 psi	70 micron/60 psi	70 micron/70 psi



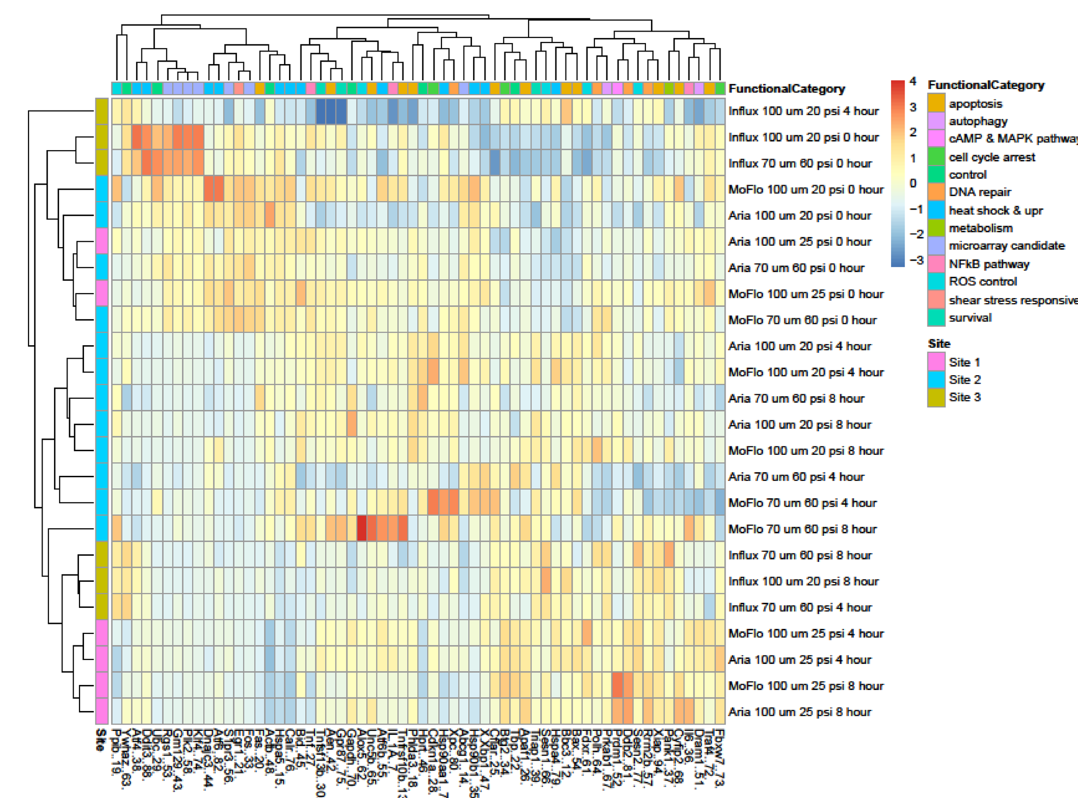


Figure 1 – Gene expression heatmap from B cell QGP assays. Euclidian distance clustering of samples and genes allows visualization of differences in gene expression profiles across samples. Data is from averaging triplicate samples and duplicate technical QGP replicates. Expression data was normalized to the geometric mean of 6 housekeeping genes and then standardized by column prior to clustering using the R statistical computing language. Thirteen of the 60 genes tested were below the limit of detection.

Observations:

- Samples at time 0 (no culture period) tend to cluster together and represent the initial state of B cells prior to sorting/culture.
- Samples from each site tend to cluster together as expected since each site used its own Bl6 mouse for splenocyte harvest.
- No clear correlation of sorter configuration with gene expression is observed indicating most changes are associated with inter-site effects (different animals) or placing cells into an in-vitro culture environment.
- Most gene expression changes observed are associated with decreased expression that occurs compared to time 0 samples. We speculate this is a result of culturing primary cells without activation such that cells are overall less metabolically active compared to the time 0 samples harvested from their native environment in the spleen. These results are consistent with microarray data from previous work.
- Microarray candidate genes are observed to be modulated in a manner consistent with original microarray data, but from this analysis we observe that differential expression of the candidate genes is primarily associated with site 3's animal having a unique time 0 expression profile and not a result of differential expression associated with a particular sorter/nozzle configuration.

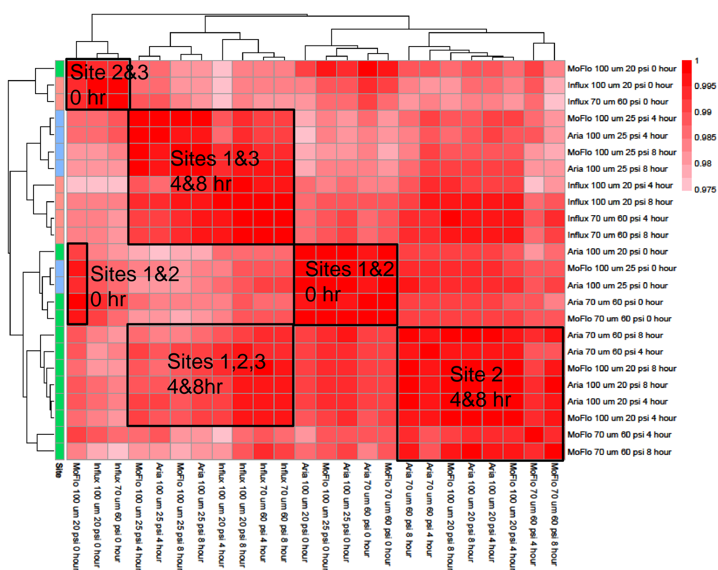


Figure 2 – Peason correlation of gene expression profiles for B cell QGP samples reveals a high degree of similarity across samples (all correlation values >0.975).

High degress of correlation among high pressure or low pressure sorted samples is not observed. However, tight correlation of sites and time points was seen (annotations and labels) and is consistent with the observations made for figure 1 above.

Mouse V6.5 ES cells

- 1 site
- V6.5 ES cells sorted vs unsorted triplicate samples
- Cells collected for RNAseq (pending) and QuantiGene Plex (QGP)

Cell line	V6.5 mouse ES
Culture media	NB27 +2i feeder free
Sorter	BC MoFlo Legacy
Nozzle/ Sheath pressure	100 micron/20 psi
Sheath fluid	Leinco S632 ClearSort

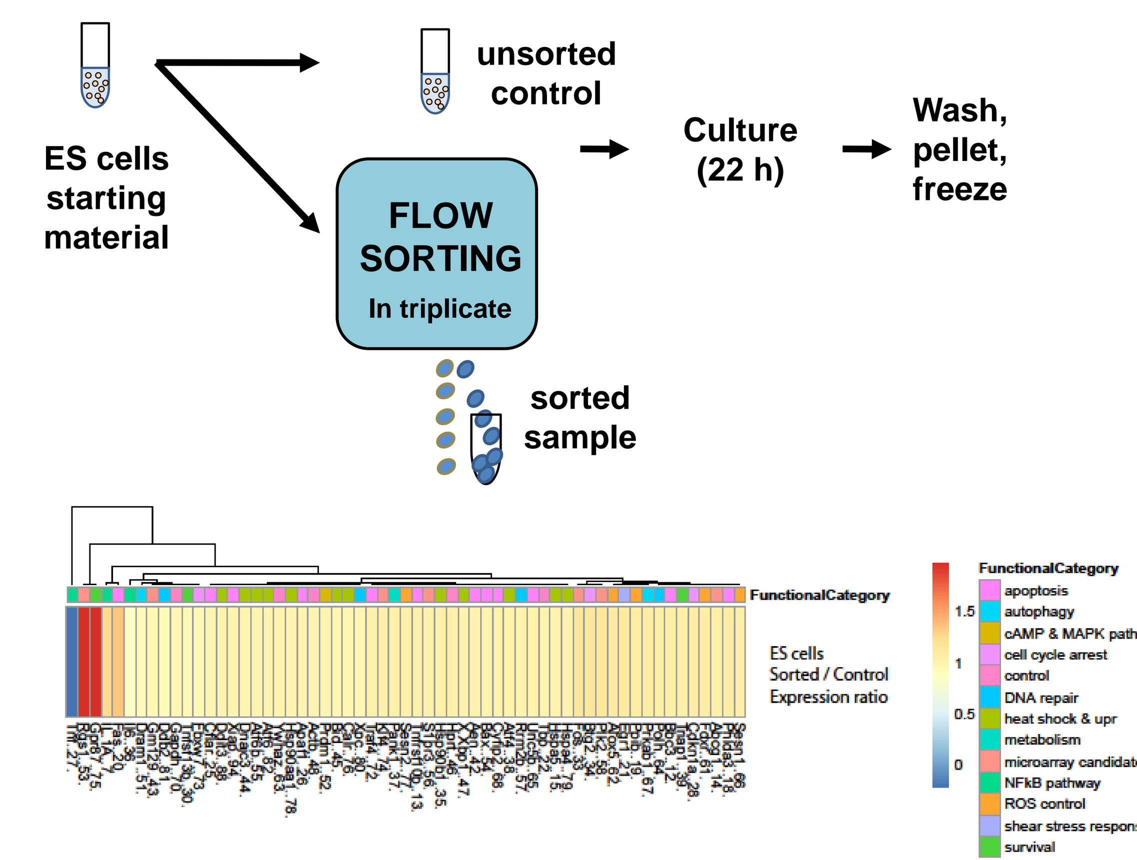


Figure 3 – Heatmap of fold changes of gene expression in sorted / control samples for ES cells. Only 3 out of the 60 genes tested were below the limit of detection (LOD) in the ES cell samples. Of the differentially expressed genes in this data set, both Fas and IL-1A are above the LOD for the assay.

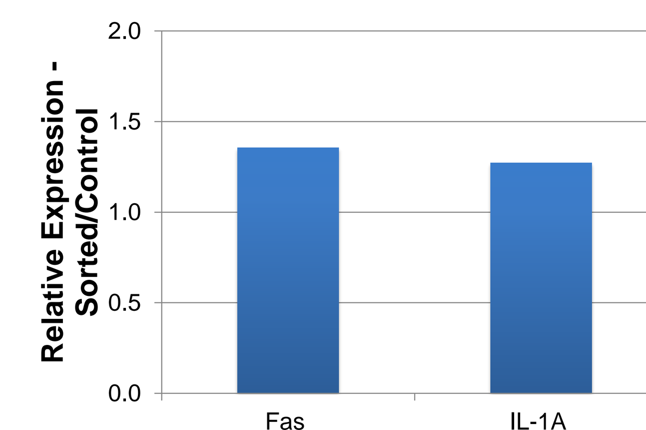


Figure 4 - Relative expression of Fas and IL-1a genes in ES cells shows a slight increase in expression after cell sorting.

Assay Panel Pathway Illustrations

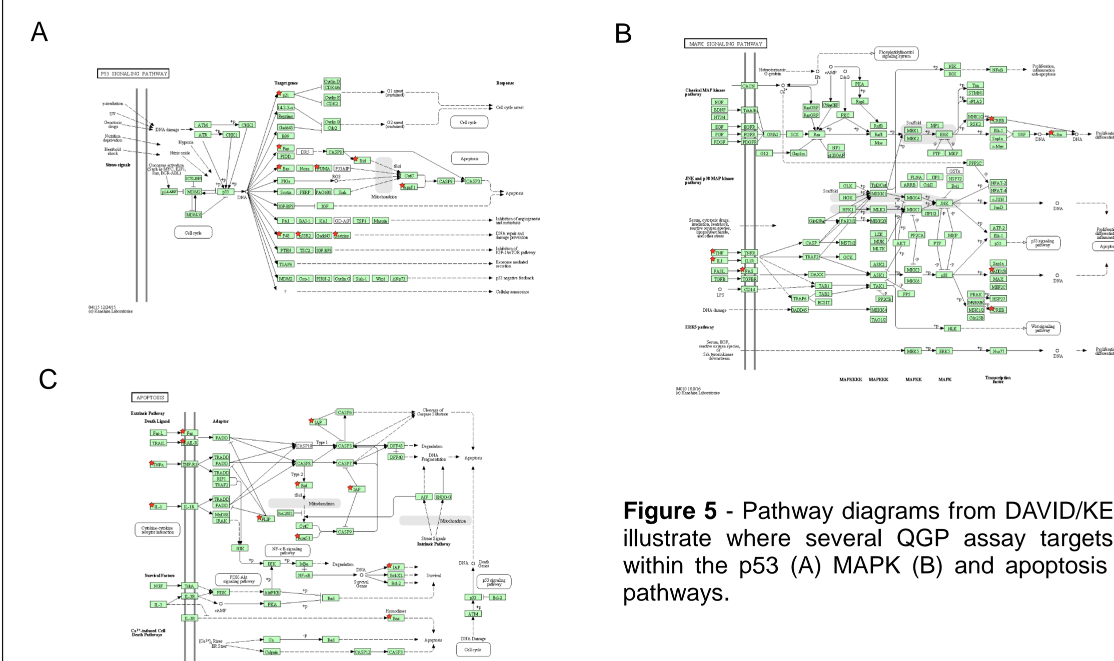
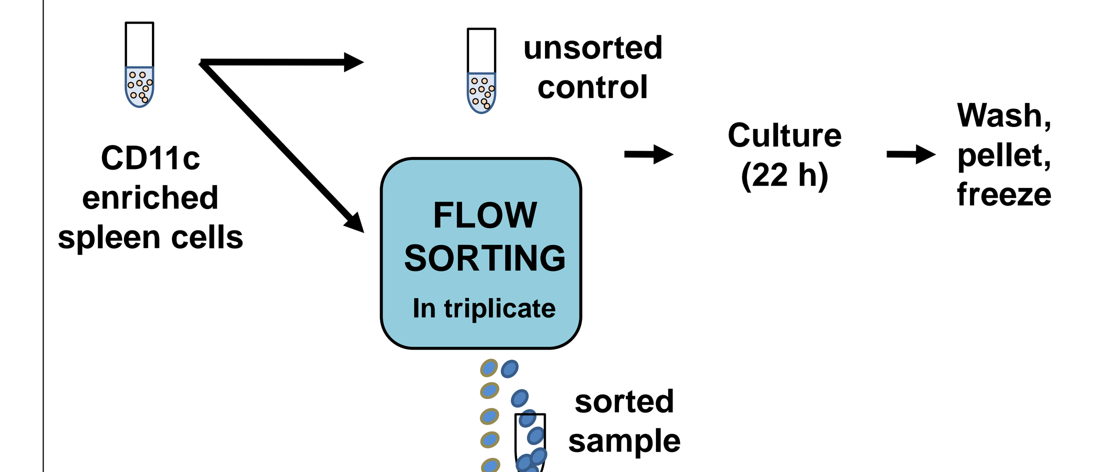


Figure 5 - Pathway diagrams from DAVID/KEGG illustrate where several QGP assay targets lie within the p53 (A) MAPK (B) and apoptosis (C) pathways.

Mouse Splenic Dendritic Cells

- 1 site
- C57BL/6 CD11c+ DCs sorted vs unsorted triplicate samples
- Cells collected for RNAseq (pending) and QuantiGene Plex (QGP)

Cell type	C57BL/6 splenic DCs
Culture media	RPMI +L-glut, BME, & 10% FBS
Sorter	BC MoFlo Legacy
Nozzle/Sheath Pressure	70 micron/60 psi
Sheath fluid	Leinco S632 ClearSort



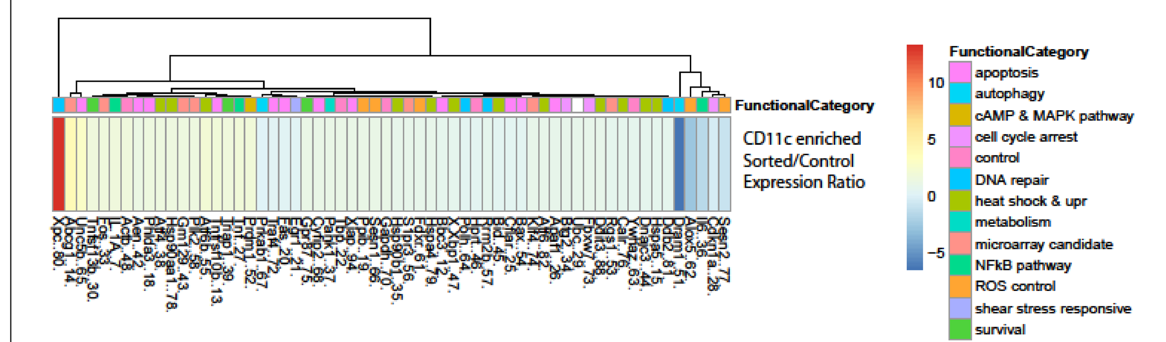


Figure 6 – Heatmap of fold changes of gene expression in sorted / control samples for CD11c enriched cells. Due to low abundance of input material from these samples, 45 out of 60 targets were below the LOD including all of the differentially expressed genes within this analysis set.

We believe the high levels of endogenous Rnases present in splenic tissue caused exceptionally high levels of degradation not experienced in other samples. Bioanalyzer data from a replicate set, not shown here, indicates a RIN of 5-6 for CD11c enriched and cultured cells when RNA was extracted using a column based total RNA kit. However, ES cells extracted in the same manner from similar cell numbers had RIN values of >9.

Conclusions and Future Directions

Conclusions

- For all of the cell types tested, there were a minimal number of genes changed as a result of cell sorting across all instruments and settings.
- Genes previously identified by microarray to be differentially expressed in sorted B cells, were validated using the QuantiGene Plex assay.
- The QuantiGene plex assay is a simple and flexible alternative to qPCR since sample input can be simplified to using cell lysate and avoid RNA and cDNA processing steps.

Acknowledgements

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