The New ABRF Flow Cytometry Research Group

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Introduction

The Flow Cytometry Research Group (FCRG) is the latest addition to the ABRF RG family. This RG is currently in its first year and has 10 members, several of whom are new to the ABRF but have been very active in and come from the flow cytometry core community. The FCRG has submitted a 3 year research plan that will characterize alterations in both gene expression and ultimately cellular function as a result of the stresses imparted by cell sorting. We will use a variety of cell types, lasers, and sorters to identify optimal conditions and eventually Best Practices for minimal cellular system disruptions. Integration of flow cytometry with other core technologies and ABRF RGs will become even more critical as many new technologies will fully take advantage of the sample processing capability of cell sorting allowing higher resolution targeted downstream molecular applications such as single cell gene expression. The new FCRG will seek to foster collaboration, integration and synergy between experts of diverse technologies the very factors that will become increasingly vital to successful research.

Methods

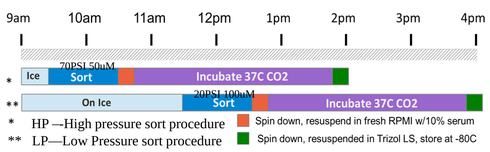
Cell Culture and Sorting

Beckman Coulter Quanta SC

- Jurkat cell line cultured to log phase 95% viable by PI and Trypan blue. Size concentration, and uniformity confirmed on Quanta SC (Beckman Coulter) cytometer. Sorter sterility was assessed by standard microbiological methods.
- Post-sort viability assessed with Celigo-- Bright field, Hoechst, and PI fluorescence image analysis.
- Immediately before each sort condition, an aliquot from Master Stock was filtered through 30µm mesh. Unsorted control samples, were adjusted to simulate the media condition of sorted samples.
- Sort and control samples were kept at room temp (RT) for the duration of the specific sort.
- Prior to sort, Master Cell Stock was kept on ice:
  - » High Pressure sort performed early in the day-- 30 min on ice
  - » Low pressure sort performed 3 hrs later- --------210 min on ice
- Following sort cells–both sorted and unsorted control-were pelleted by centrifugation, re-suspended in fresh growth media, cultured at 37° C. 3 hrs before harvested for RNA
- Cell pellets were homogenized in Trizol LS and stored at -80° C.
- RNA was isolated from Trizol, processed with RNeasy Mini Column system (Qiagen) and quantified using Nanodrop ND1000, followed by assessment on the Agilent 2100 Bioanalyzer.

RNA Processing and Data Analysis

RNA was converted to labeled cDNA, fragmented and, hybridized to the GeneChip Human Gene 2.0 ST arrays using the standard WT protocol from Affymetrix. Resulting CEL files were exported to GeneSpring GXv12.5, quantile normalized using Plier16 and baseline transformed to the median of all samples. The entity list was then filtered to remove those with signal in the bottom 20th percentile across all samples and further refined to exclude entities >25% CV across all replicates in a condition. This target set was interrogated for entities with >1.5 fold differential expression and statistical significance ($p<0.05$, Benjamini Hochberg FDR corrected) between the conditions being compared.

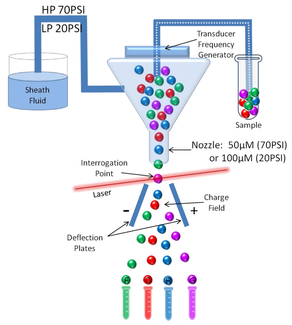


A single Master Stock of cultured cells was harvested, pooled and stored on ice prior to sort. One aliquot was taken from the Master Stock for HP sort. The remaining Master Stock remained on ice until an aliquot was taken for the HP sort –LP ice time 30 min., --HP 120 min. Sort performed at ~25°C (RT). Each sort condition embedded 3 replicate processes. Control (unsorted) cells were removed from ice with the sort aliquot, held at RT during the sort, then processed parallel to sorted sample (spin/Trizol).

How Does Cell Sorting Work?

FACS—Fluorescent Activated Cell Sorting enables purification of very specific cell subsets

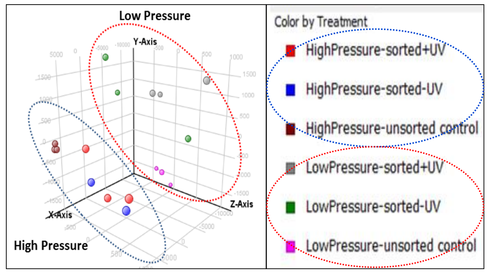
- The stream is separated into droplets.
- Droplets containing the target cells are electrically charged below the interrogation point.
- Charged plates deflect the differentially charged droplets into a tube.



- Functional or Static analysis– cells can be live, functional and pure or can feed directly into molecular analysis.
- Highly pure subsets are routinely used in static gene and protein analysis. This purity reduces interfering signals (noise) from irrelevant cell populations that confound the exquisitely sensitive bio-analytic tools available to researchers today.
- Small nozzle sizes and high system pressure alone may be traumatic to cells.
- Rapid depressurization at the nozzle tip could destabilize a cell.
- The small nozzle/high pressure used in the extreme condition for this study would not typically be employed to harvest live cells.
- Live sorts of Non-hematopoietic cells usually employs 100, 120 or 150µM nozzles at 20, 15 or 12 PSI respectively.

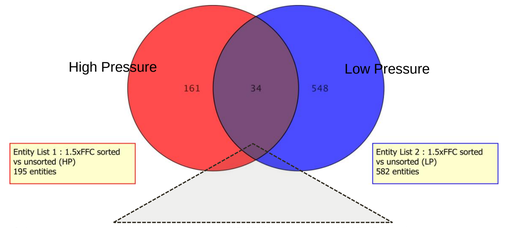
Results

Alterations in gene expression was seen not only between sort conditions their respective controls but also between the controls at the two time-points.



3D Gene Expression principle component plot of flow sorted Jurkat cells following 3 hr post-sort incubation in complete RPMI growth media. Jurkat cells were sorted at either high (70PSI/50µM nozzle) or low (20PSI/100µM nozzle) pressure settings. Both pressure/nozzle conditions included either shielding or exposure to UV laser—a hypothesized trigger of differential expression. Data clearly indicate differential gene expression for sorted cell populations regardless of pressure compared to their respective unsorted control. Additionally, even more striking differences are seen between the 2 unsorted controls, indicating an underappreciated effect of the duration of cell storage on ice while waiting to be sorted.

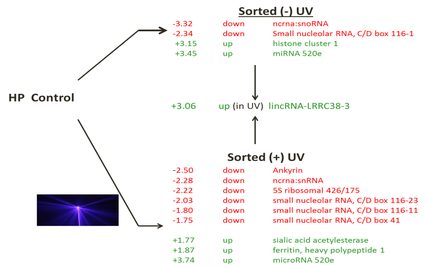
Both High and Low Pressure Sorting Triggers Differential Gene Expression Compared to Unsorted Cells



Transcript	HP-UV/Control	LP-UV/Control
microRNA 520e (metastasis-suppressive)	3.45	1.66
microRNA 128-2 (Inhibits Angiogenesis)	1.51	1.96
AFF2 intronic transcript 1 (non-protein coding)	1.51	1.53
ELKS/RAB6-interacting/CAST family member 1	1.66	1.56
immunoglobulin lambda variable 1-50 (non-functional)	1.50	1.66
immunoglobulin heavy variable 2-26	-1.50	-1.39
zinc finger protein 36, C3H type-like 2 (early response)	-1.54	-1.70
small nucleolar RNA, C/D box 32B	-2.39	-1.54
DNA-damage-inducible transcript 4 (Regulates cell growth and survival)	-2.27	-1.55

34 of the probed cDNA exibited similar response to both sorting processes.

Few Genes Showed Changes With High Pressure Configuration Regardless of UV Exposure. Most were ncRNAs

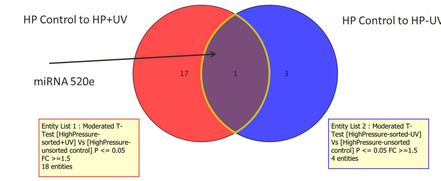


Low Pressure Control vs Low Pressure (-) UV 1.75 fold cut off

-4.34	down	linc-POMZP3
-2.37	down	linc-LRRC8D
-2.23	down	KIAA1731
-2.14	down	5S ribosomal
-2.02	down	linc-PRH2-1
-1.92	down	coiled-coil domain containing 17
-1.83	down	Possible miRNA
-1.80	down	linc-ANO5-3
-1.80	down	snoRNA
1.76	up	Mdm2, p53 E3 ubiquitin protein ligase
1.76	up	poly(A) polymerase beta
1.76	up	linc-GTF2H2-2
1.77	up	small nucleolar RNA, C/D box 32B
1.78	up	linc-STAT4-2
1.8	up	linc-SCAMP1-2
1.81	up	transforming growth factor, beta 1
1.81	up	taste receptor, type 2, member 19
1.83	up	CTGLF6
1.83	up	fibroblast growth factor 1
1.88	up	neurexophilin
1.9	up	immunoglobulin heavy variable 1-18
1.91	up	linc-ANKRD50-3
1.92	up	proline-rich coiled-coil 2A
1.96	up	nuclear receptor co-repressor 2
1.97	up	coagulation factor VII
1.98	up	midnolin
2.01	up	microRNA 4644
2.1	up	solute carrier family 25
2.12	up	linc-ACTL7A-7
2.19	up	snRNA

The Effects of UV Seems Minimal on Jurkat Cells

- Number of RNAs with 2 fold change-up or down
- Common RNAs in overlap



Maximum effect is between HP and LP Controls! Longer duration on Ice appears to skew gene expression

A----High pressure vs low Pressure controls-Down regulated

-10.5	jun proto-oncogene	-2.3	receptor-interacting serine-threonine kinase 4
-7.7	heat shock 70kDa protein 1B/A]	-2.3	THAP domain containing 9 | SEC31 homolog -2.3
-5.4	inhibitor of DNA binding 2, dominant negative	-2.3	myosin, heavy chain 9, non-muscle
-5.2	FBJ murine osteosarcoma viral oncogene homolog	-2.3	immediate early response 3
-5.2	serpin peptidase inhibitor, clade H (heat shock protein 47),	-2.3	hemoglobin, alpha 2 | hemoglobin, alpha 1
-5.0	hairy and enhancer of split 1, (Drosophila)	-2.3	immediate early response 3
-4.7	microRNA 3143	-2.3	secretoglobin, family 1D, member 4
-3.9	growth arrest and DNA-damage-inducible, beta	-2.2	H1 histone family, member X
-3.6	distal-less homeobox 2	-2.2	immediate early response 3
-3.5	SMAD family member 7	-2.2	v-ski sarcoma viral oncogene homolog (avian)
-3.5	early growth response 1	-2.1	microRNA 3142
-3.3	serpin peptidase inhibitor, .3.3 ubiquitin C	-2.1	ring finger protein 151
-3.2	v-maf musculoaponeurotic fibrosarcoma oncogene -3.2	-2.1	activating transcription factor 3
-3.1	heat shock 70KDa protein 6 (HSP70B')	-2.1	Kruppel-like factor 16
-3.0	metallothionein 1E	-2.1	ATPase, Na+/K+ transporting, alpha 3 polypeptide
-2.9	solute carrier family 30 (zinc transporter), member 1	-2.1	protein kinase D2
-2.8	BCL2-associated athanogene 3	-2.1	KH-type splicing regulatory protein | microRNA 3940
-2.8	hairy/enhancer-of-split related with YRPW motif 1	-2.0	interferon regulatory factor 2 binding protein-like
-2.6	adenoceptor beta 2, surface	-2.0	zinc finger protein 777
-2.6	serum/glucocorticoid regulated kinase 1	-2.0	sterile alpha motif domain containing 1
-2.6	docking protein 2, 56kDa	-2.0	PR domain containing 9
-2.5	regulator of G-protein signaling 2, 24kDa	-2.0	BCL6 corepressor
-2.5	tubulin, beta 4A class IVa		
-2.5	jun D proto-oncogene		
-2.4	CAP-GLY domain containing linker protein 3		
-2.4	microRNA 4739		
-2.4	polymerase (RNA) II (DNA directed) polypeptide A,		
-2.4	host cell factor C1 (VP16-accessory protein)		
-2.4	T cell receptor alpha joining 21		
-2.4	ataxin 2-like		
-2.3	microRNA 449c		

18 histone
82 ncRNA c/d box, sno, Linc

B----High pressure vs low Pressure controls up-regulated

2.0	zinc finger protein B3	2.2	ring finger protein 146
2.0	zinc finger protein 555	2.2	chondroitin sulfate proteoglycan 4 pseudogene
2.0	AFF2 intronic transcript 1 (non-protein coding)	2.2	zinc finger protein 117
2.0	phosphodiesterase 3B, cGMP-inhibited	2.2	baculoviral IAP repeat-containing protein 1-like
2.0	taxilin gamma 2, pseudogene	2.2	zinc finger protein 681
2.0	zinc finger protein 33B	2.2	G protein-coupled receptor 52
2.0	FBXO36 intronic transcript 1 (non-protein coding)	2.2	importin 5 pseudogene
2.0	RNA, Ro-associated Y4 pseudogene 23	2.2	oculomedin
2.0	aminopeptidase puromycin sensitive pseudogene	2.3	zinc finger and BTB domain containing 26
2.0	Rho GTPase activating protein 11B	2.3	ASH1L intronic transcript 1 (non-protein coding)
2.0	speedy homolog E7	2.3	protein tyrosine phosphatase, non-receptor type 20C
2.1	chromosome 22 open reading frame 42	2.3	taste receptor, type 2, member 20
2.1	coiled-coil domain containing 71	2.3	taste receptor, type 2, member 30
2.1	zinc finger protein 627	2.4	taste receptor, type 2, member 14
2.1	CD99 molecule pseudogene 1	2.4	EYA3 intronic transcript 1 (non-protein coding)
2.1	family with sequence similarity 133, member B	2.5	ZNF346 intronic transcript 1 (non-protein coding)
2.1	regulator of G-protein signaling 11	2.5	RSF1 intronic transcript 2 (non-protein coding)
2.1	family with sequence similarity 45, member A	2.5	chromosome 15 open reading frame 29
2.1	EF-hand domain family, member A2	2.7	COX assembly mitochondrial protein 1 homolog (S. cerevisiae)
2.1	phosphatidylinositol 3-kinase-related kinase	2.7	chromosome 9 open reading frame 3
2.1	chromosome 1 open reading frame 132	2.7	glucuronidase, beta pseudogene 3
2.1	glucuronidase, beta pseudogene 4	2.9	taste receptor, type 2, member 19
2.1	MORC family CW-type zinc finger 3	4.1	ATG10 intronic transcript 1 (non-protein coding)
2.1	coiled-coil domain containing 144A		
2.1	tripartite motif containing 61		

23 miRNA, 26 linc, 28 ncRNA-miRNA locus, 31 unknown ncRNA, 45 snoRNA, 6 rRNA, 1 C/D box

Conclusions and Future Directions

- The process of sorting seems to have an effect on gene expression. Differences in sort pressures as well as exposure to UV seemed to have a moderate effect on expression.
- The incubation on ice for the duration of the sort also seems to have an effect on expression.
- Mostly differential expression was seen in ncRNA including lincRNAs and snRNA.
- Other cell types as well as other sorting platforms must be evaluated.

Acknowledgements

Affymetrix, Inc.
Stowers Institute Cytometry and Tissue Core Facility
Marcy Kuentzel Center for Functional Genomics-SUNY Albany

Selected References

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