Single Cell Sorting and the Bioinformatics Pathway

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The Flow Cytometry Research Group A (Roughly Half) Decade in Review

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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

- 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.

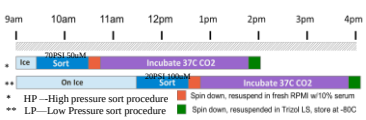
Beckman Coulter Quanta SC

- 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.

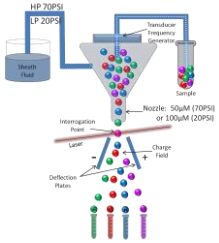


A single Master Stock of cultured cells was harvested, pooled and stored on ice prior to sort. One aliquot was taken from the Master Stock for HP sort. The remaining Master Stock remained on ice until an aliquot was taken for the HP sort –LP ice time 30 min., --HP 120 min. Sort performed at ~25°C (RT). 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

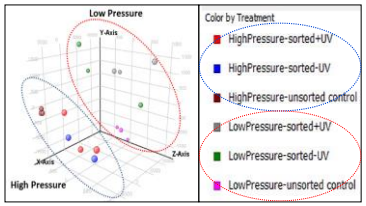
- The stream is separated into droplets.
- Droplets containing the target cells are electrically charged below the interrogation point.
- Charged plates deflect the differentially charged droplets into a tube.



- 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.

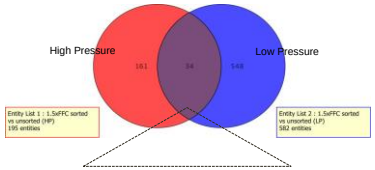
Results

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.

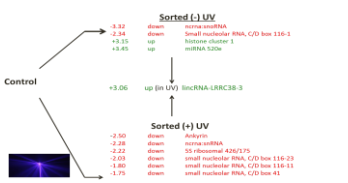
Both High and Low Pressure Sorting Triggers Differential Gene Expression Compared to Unsorted Cells



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

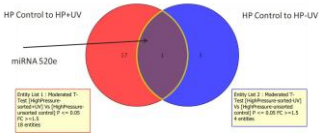


Low Pressure Control vs Low Pressure (-) UV 1.75 fold cut off

-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	CTSLF6
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

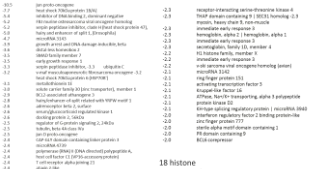
The Effects of UV Seems Minimal on Jurkat Cells

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



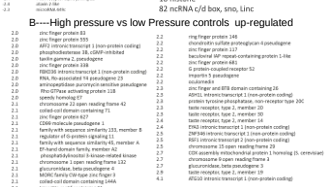
Maximum effect is between HP and LP Controls! Longer duration on Ice appears to skew gene expression

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



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

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



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

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UNIVERSITY OF MICHIGAN MEDICAL SCHOOL

Flow Cytometry Research Group 2014 Study

Evaluating Effects of Cell Sorting on Cellular Integrity and Gene Expression

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Introduction

During the past year the Flow Cytometry research Group has continued on its goal to establish best practice guidelines for cell sorting conditions that minimize cell stress, perturbation, or injury to the sorted cells.

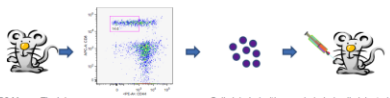
Towards this goal the group has followed up on an observation from our initial study that showed poor cell recovery when a clonal population of cells (Jurkat) was sorted aggressively under intentionally adverse sorting conditions (excessive pressure as well as undersized sorting orifice). In this follow-up study we sought to identify unique qualities of the cells that survived the adverse sorting conditions, in the hope that this may prove to be a useful test method for assessing deleterious effects of cell sorting across a wide variety of cell types.

To address this question, six FCRG member-sites received a distribution of the same Jurkat cell population and using different instrumentation and sorting conditions, sorted these cells for subsequent cell cycle analysis, post-sort viability, and recovered cell counts. In addition, one site submitted parallel samples for microarray analysis.

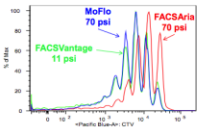
Background

Previous studies by individuals in the FCRG have revealed detrimental effects on cell function after sorting using cuvette versus jet-in-air sorters.

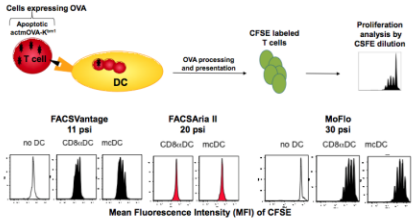
Study 1-Decreased proliferation of transplanted T cells



Mice were sacrificed after 1 week and lymph node and spleen were evaluated for total number of CellTracker Violet labeled cells and the degree of proliferation. The cells sorted on the jet-in-air MoFlo and FACSVantage (low pressure control) proliferated to a greater degree than those sorted on the FACSAria cuvette sorter indicating an increase in cell injury when sorting using a cuvette.



Study 2-Decreased function of dendritic cells



Dendritic cells sorted on the jet-in-air FACSVantage and MoFlo were able to process and present antigen to CFSE labeled T cells resulting in proliferation while those sorted on the FACSAria cuvette sorter displayed a decrease in antigen presenting function.

Jurkat Cell Study

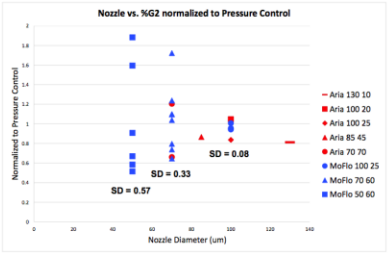
Jurkat cells were evaluated after cell sorting by analyzing cell cycle profile and gene expression changes.

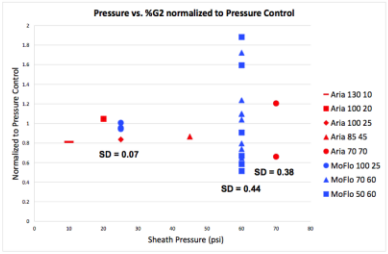
Sample treatments included:

- **Unsorted Control** - Cells that were kept on ice for the duration of the sort
- **Pressure Control** - Cells that were mounted on the sorter and exposed to pressure, but not sorted
- **Sorted Sample** - Cells that were collected after sorting

Cell Cycle Analysis

Preliminary evidence revealed a loss of cells in G2 phase of the cell cycle after sorting under harsh conditions. To determine if cell cycle profile changes are an indicator of adverse sorting conditions, Jurkat cells were distributed to several sites and sorted using a variety of instruments and settings. Control cells were exposed to pressure but not sorted. Viability data was obtained before and after sorting. Cells were ethanol fixed, shipped to a participating site, stained with propidium iodide and analyzed for cell cycle profile. Data points are grouped based on the instrument, nozzle size and sheath pressure respectively (see legend).





The frequency of cells in G2 from the sorted sample was normalized to that of the pressure control. When comparing this normalized value to the diameter of the nozzle tip or to the sheath pressure there is a larger variation using a smaller nozzle diameter and/or a higher pressure.

Gene Expression Data

Flow sorting is often upstream of functional or gene expression studies. We wanted to understand the degree, if any, to which flow sorting may induce changes in gene expression and minimize these effects when possible through use of optimal conditions. Jurkat cells, a robust transformed cell line, were sorted on a MoFlo cell sorter using a 50 um nozzle tip at 60 psi, pelleted and resuspended in culture media and incubated for the times indicated. Gene expression changes were determined using Affymetrix Primeview microarrays and data was analyzed using the TAC software.

Sorted sample vs. unsorted control at 4 and 8 hours

4 Hours

Fold Change (linear)	Gene Symbol	Description
(+)2.04	KMT2C	lysine (K)-specific methyltransferase 2C
-2.04	ACTG2	actin, gamma 2, smooth muscle, enteric
-2.04	TMPO	thymopoietin
-2.1	NNT	nicotinamide nucleotide transhydrogenase
-2.14	HTR2B	5-hydroxytryptamine (serotonin) receptor 2B, G protein-coupled
-2.15	SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)
-2.21	FKBP4	FK506 binding protein 4, 59kDa
-2.22	RRN3 , RRN3P1 , RRN3P2	RRN3 RNA polymerase I transcription factor homolog
-2.27	STEAP1	six transmembrane epithelial antigen of the prostate 1
-2.27	RGS18	regulator of G-protein signaling 18
-2.41	ACTA2	actin, alpha 2, smooth muscle, aorta
-2.45	HSPA4L	heat shock 70kDa protein 4-like
-2.52	CCNB1IP1	cyclin B1 interacting protein 1, E3 ubiquitin protein ligase
-2.83	SCG2	secretogranin II
-3.01	HYI	H1 histone family, member 0
-5.26	HSPA6 , HSPA7	heat shock protein 6 ,protein 7
-19.9	HSPA1A , HSPA1B	heat shock protein 1A ; 1B

8 Hours

Fold Change (linear)	Gene Symbol	Description
-2.01	ACTA2	actin, alpha 2, smooth muscle, aorta
-2.17	ANKRD37	ankyrin repeat domain 37
-2.19	ACTG2	actin, gamma 2, smooth muscle, enteric
-2.25	VEGFA	vascular endothelial growth factor A
-2.38	PTPN3	protein tyrosine phosphatase, non-receptor type 3
-2.54	VEGFA	vascular endothelial growth factor A
-2.86	DDIT4	DNA-damage-inducible transcript 4
-3.31	VEGFA	vascular endothelial growth factor A

Sorted sample vs. pressure control at 4 and 8 hours

4 Hours

Fold Change (linear)	Gene Symbol	Description
(+)2.23	QSER1	glutamine and serine rich 1
(+)2.06	SBK1	SH3-binding domain kinase 1
(+)2.05	SCRIB	scribbled planar cell polarity protein
(+)2.04	TXLNG2P	taxilin gamma 2, pseudogene
(+)2.03	SFT2D3 , WDR33	SFT2 domain containing 3 ; WD repeat domain 33
(+)2.01	SPEN	spen homolog, transcriptional regulator (Drosophila)
-2.01	SGK494 , SPAG5	uncharacterized serine/threonine-protein kinase SgK494 ; sperm associated antigen 5
-2.05	HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
-2.06	NNT	nicotinamide nucleotide transhydrogenase
-2.16	SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)
-2.19	PRC1	protein regulator of cytokinesis 1
-2.31	BTBD1	BTB (POZ) domain containing 1

8 Hours

Fold Change (linear)	Gene Symbol	Description
No changes	NA	NA

Pressure control vs. unsorted control at 4 and 8 hours

4 Hours

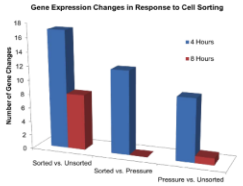
Fold Change (linear)	Gene Symbol	Description
-2.03	CHORDC1	cysteine and histidine-rich domain (CHORD) containing 1
-2.05	HSPA4L	heat shock 70kDa protein 4-like
-2.07	JUN	jun proto-oncogene
-2.14	H1F0	H1 histone family, member 0
-2.16	RRN3 , RRN3P1 , RRN3P2	RRN3 RNA polymerase I transcription factor homolog
-2.41	CCNB1IP1	cyclin B1 interacting protein 1, E3 ubiquitin protein ligase
-2.59	SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1) ;
-6.54	HSPA6 , HSPA7	heat shock 70kDa protein 6 (HSP70B') ; heat shock 70kDa protein 7 (HSP70B)
-16.5	HSPA1A , HSPA1B	heat shock 70kDa protein 1A ; heat shock 70kDa protein 1B

8 Hours

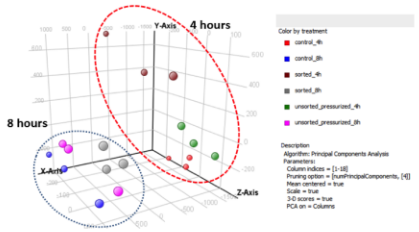
Fold Change (linear)	Gene Symbol	Description
-2.05	VEGFA	vascular endothelial growth factor A

Gene Expression Data

Gene expression changes in Jurkat cells that were sorted and re-cultured for 4 hours are minimal and decrease substantially after 8 hours of culture. Indicating a minimal effect caused by the sorting process and that Jurkat cells can recover upon exposure to normal culture conditions.



Principle Component Plot of Microarray Data



A principle components analysis of the microarray data suggests that the sorting introduces some cellular changes at the transcriptional level but these changes substantially decrease after a recovery period.

Conclusions

- Functional data from certain cell types reveals detrimental effects from cell sorting using a cuvette style instrument.
- Cell cycle profile changes are highly variable in Jurkat cells sorted using smaller nozzles and/or higher pressures.
- The highest number of up-regulated genes was detected 4 hours after sorting when comparing sorted cells to those exposed to pressure only (sorted sample vs pressure control) indicating an immediate gene expression response from the sort.
- There is a minor effect of pressurizing the cells which causes only down regulation of genes (pressure control vs. unsorted)
- From these data it appears that most changes come from the actual sorting process. However, it is important to note that the overall effect of sorting is surprisingly minimal and that Jurkat cells can recover after a resting period.

Acknowledgements

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Flow Cytometry Research Group 2015 Study

Evaluating the Effects of Cell Sorting on Gene Expression

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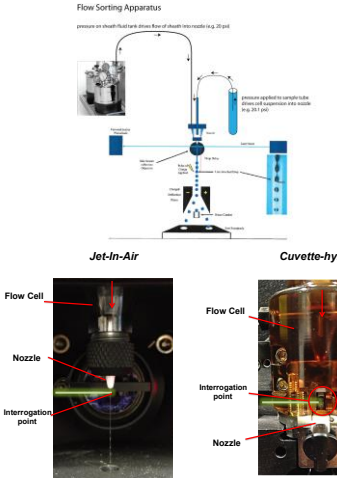
Introduction

The Flow Cytometry Research Group has continued with the goal to establish best practice guidelines for cell sorting conditions that minimize cell stress, perturbation, or injury to the sorted cell populations. In past FCRG studies, gene expression changes in sorted Jurkat cells, a human lymphoblastic T cell line, were correlated to nozzle size and sort pressure. The current study examined the effect sorting has on primary cells (C57Bl/6 mouse splenic B lymphocytes). B lymphocytes were isolated using multiple flow sorters under gentle (100 micron nozzle size/20 psi pressure) and stressful (70 micron nozzle size and 70 psi pressure) sort conditions. The sorts were performed using several instrument types to compare the differences in instrument designs (cuvette hybrid and jet-in-air) in addition to differences in sort conditions. Gene expression was assessed using Affymetrix Mouse Gene ST 2.0 microarrays using targets prepared from the NuGEN Pico reagents and Qiagen Micro minelute columns

Background

When considering how to set up a cell sorter one of the significant variables that can have an effect on functional ability as well as cell health is the nozzle size and related pressure. A smaller nozzle requires a higher pressure be applied in order to generate a stable stream, with the opposite being true for a larger nozzle. A larger nozzle is thought to lead to a more gentle, but slower sort. This effect can be tested and is one of the goals of the current study.

There are also two common types of cell sorters, the Jet-in-air and Cuvette systems. The primary difference between the two systems is where the sample is excited. In the jet-in air system the sample stream is excited after it has passed out of a nozzle, whereas in the the cuvette system the excitation occurs while inside a quartz cuvette. Evidence has shown that this seemingly minor difference can lead to dramatic differences in cell health. Testing this effect is one of the ongoing goals of this research group.

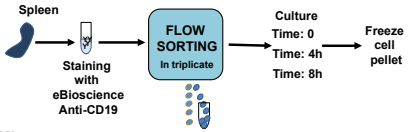


2014-2015 Mouse B Cell Study

At 5 different sites (7 total instruments), primary cells from the spleen of a C57Bl/6 mouse were dissociated and CD19+ B cells were isolated via cell sorting. The B cells were evaluated after cell sorting by analyzing gene expression changes. RNA was generated from a selection of the sorted cells, amplified and analyzed via microarray.

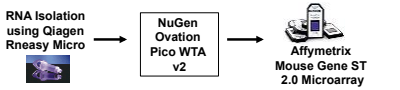
- **Sorters:** BD FACSAria II (4 sites) – Cuvette-hybrid system
  BD Influx (1 site) – Jet-in-air system
  BC MoFlo Astrios (2 sites) – Jet-in-air system
- **Sort Conditions**: High Pressure – 70uM nozzle, 60-70psi
  Low Pressure – 100uM nozzle, 20-25psi
- **Culture Conditions:** 0, 4, and 8 hrs in culture post sort

Cell Sorting:



Microarray: 2 different sites and 3 different instruments

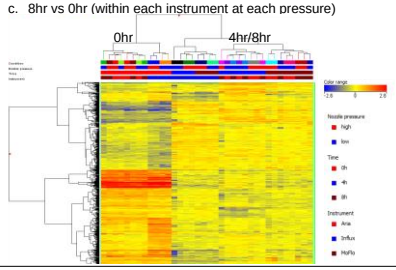
- 36 chips: 3 instruments, 3 time points, 2 conditions, 2 replicates



	FACSAria	Influx	MoFlo Astrios
Low Pressure	100micron/25 psi	100micron/20 psi	100micron/20 psi
High Pressure	70 micron/60 psi	70 micron/60 psi	70 micron/70 psi

Gene Expression Analysis Criteria:

1. Bottom 20th percentile probes across all samples were filtered out.
2. Remove any entities that had >25% CV
3. Differential expression using 2-way ANOVA
   a. Either between 4hr or 8hr as compared to the 0hr time point within each instrument and at both pressures
   b. Or between the different pressures at 0hr time points within each instrument
4. A 2-fold cutoff was applied to each comparison
5. Lists of differentially expressed entities were generated for the following comparisons.
   a. 0hr low vs 0hr high (within each instrument)
   b. 4hr vs 0hr (within each instrument at each pressure)
   c. 8hr vs 0hr (within each instrument at each pressure)



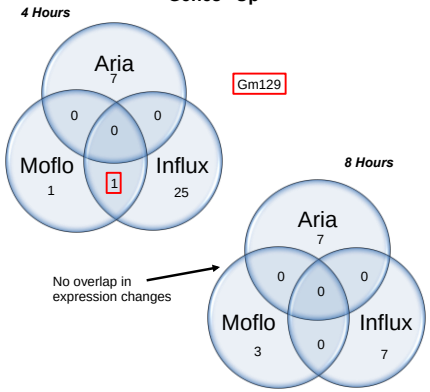
Pressure Induced Changes - Part 1

The analysis of these data was further focused on the gene expression variations between the "high" and "low" pressure conditions. To do this we took the fold change from 0hr to 4 or 8hr at low pressure conditions and compared that to the fold change from 0hr to 4 or 8hr at high pressure conditions.

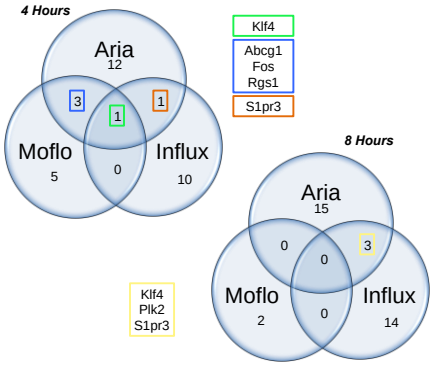
Number of Genes/Group

Gene response	4 hour			8 hour		
	FACSAria	Influx	MoFlo Astrios	FACSAria	Influx	MoFlo Astrios
Up	7	26	2	7	7	3
Down	17	12	9	18	17	2
	Cuvette	Jet-in-Air		Cuvette	Jet-in-Air	

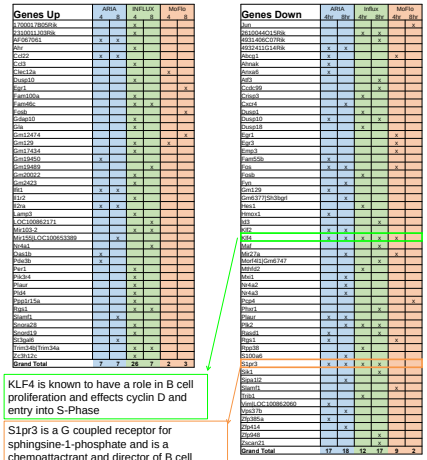
Genes "Up"



Genes "Down"



Pressure Induced Changes - Part 2



KLF4 is known to have a role in B cell proliferation and effects cyclin D and entry into S-Phase

S1pr3 is a G coupled receptor for sphingsine-1-phosphate and is a chemoattactrant and director of B cell trafficking

Conclusions and Future Directions

Conclusions

- Cell sorting causes relatively few gene expression changes with a limited amount of overlap between instrument and time point.
- In agreement with past FCRG studies, although there were some alterations in gene expression, most of those changes had subsided with extended culture times.
- While gene expression changes were minor, cell viability was decreased after culture showing that cell sorting can have deleterious effects on cells (data not shown).
- Initial data (n=1) supports anecdotal evidence that sorting with the MoFlo Astrios has less effect on cells.

Future Directions

- These data represent a small portion of the total samples collected this year.
- Gene expression changes will be further explored using PCR with attention paid to differences between instrument types as well as continued exploration of the effects of pressure conditions.
- The FCRG plans to publish the results of this, and past years, studies.
- Please consider taking part in the FCRG survey (3 questions), regarding this project and future directions:

Acknowledgements

We sincerely appreciate the support of Affymetrix/eBioscience for Mouse Gene ST 2.0 microarray and the CD19 antibody. Qiagen for the Rneasy Micro Columns. NuGen for the Ovation Pico WTA reagents. Marcy Kuentzel for microarrays analysis at the Center for Functional Genomics at SUNY Albany.

Evaluating Cell Sorter Cleaning Procedures Across ABRF-FCRG Institutions by Testing for Common Contaminants (Poster # 22)

Roxana del Rio, University of Vermont; Kathleen Brundage, West Virginia University; Alan Bergeron, Dartmouth College; Andrew Box, Stowers Institute for Medical Research; Matt Cochran, University of Rochester Medical Center; Monica DeLay, Cincinnati Children's Hospital; Maris Handley, Massachusetts General Hospital; E. Michael Meyer, University of Pittsburgh Cancer Institute; Alan Saluk, The Scripps Research Institute; Peter Lopez, New York University Langone Medical Center.

The Association of Biomolecular Resource Facilities

Flow Cytometry Research Group

BACKGROUND

- Cell sorting plays an important role in many *in vitro* and *in vivo* studies, including genomic studies in which single cell isolation is required.
- Then, it is critical that during the passage of the cell through the sorter that there is minimal contact with eukaryotic and prokaryotic cells and debris.
- Any cell product that come together with sorted cells has the potential to affect their functional properties (i.e. activation, proliferation), or unwanted nucleic acids may be amplified during downstream assays.

JUSTIFICATION

- As ABRF-Flow Cytometry Research Group, we are interested in developing best practices for maintaining a "clean" sorter.
- The short term goal for this study is to determine how "clean" sorters are using regular cleaning procedures. The long term goal is to provide recommendations on how to improve (if necessary) aseptic sorting procedures.

METHODS

1) Participants: 8 FC Shared-Resource Labs (SRL); 19 instruments tested (5 BD Aria I, 7 BD AriaII, 2 BC MoFlo, 2 BC Astrios, 1 BD Influx, 1 BioRad S3, 1 PL Avalon).
2) Pre-sorted sample (from sheath tank and/or stock bottle) and post-sorted stream were collected on aseptic conditions and distributed to 2 labs to perform tests.
3) The first test-lab performed endotoxin (ThermoFisher Sci, Cat. 88282; colorimetric), and RNase (ThermoFisher Sci, Cat. AM1964; fluorometric) assays; the second test-lab evaluated bacteria and fungus contamination assays (ThermoFisher Sci/Molecular Probes, Cat. 7028; fluorometric). Additionally, we surveyed the standard cleaning regimen that each supplier FC-SRL does in a regular basis.

RESULTS

Figure 1.- Detection of Bacteria and Fungus by Flow Cytometry: Syto9 (nuclei staining); Calcofluor (fungal cell walls); WGA-TR (bact)

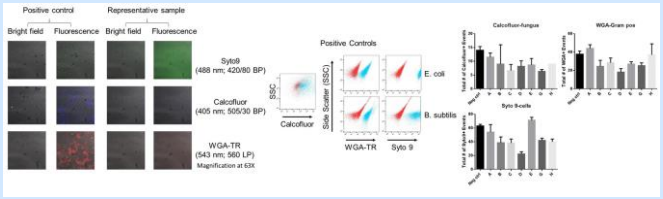


Figure 2.- Detection of RNase: at 5 and 20 minutes after addition of substrate

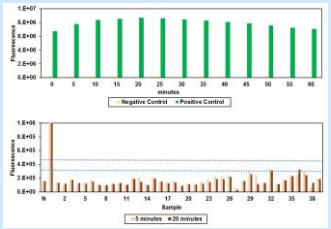


Table I.- Detection of Endotoxin

Site	Number of Instruments	Bacteria/Yeast	Pre-instruments	Post-instruments
A	1	B/Y	+	+
B	1	B	-	-
C	4	Y	+	+ (A&S) – (M&I)
D	3	No	+	+ (all)
E	2	B	-	-
F	2	No	-	-
G	5	B/Y	+ 1x PBS - Water	(+) all
H	1	No	-	+

Table II.- Shared-steps on cleaning procedures between participating labs

Common cleaning procedures:
1) Autoclave sheath tank (and ethanol tank) at least every other month. Rinse tanks with 10% bleach may be recommended.
2) Depending upon the system, every week run through sorter bleach and sterile water (in some cases ethanol as well).
3) Every other month replace filters and sample lines.
4) Before and after sorting, flush sample line with Contrad [illegible]

CONCLUSIONS

• In general, there is not a common procedure to keep sorters clean of contaminants. Instead, we have shown that different aseptic practices used among participating labs keep sorters clean.

•The sheath fluids used were either hand-made or by different manufacturers (ThermoFisher, Leinco, Sigma, Hospira, and BioSource). No difference on sterility/cleanliness was detected.

•Regardless of the cleaning procedure utilized, instruments are consistently free of RNases, fungus and bacteria (cells).

•Our results showed that endotoxin (a component of the membrane of Gram-negative bacteria), it is a common contaminant found on sheath tank and/or PBS (general) reservoir. However, it is most likely to be detected in instruments that sort microorganisms (bacteria) than in instruments that do not sort bacteria.

•The presence of endotoxin on stream/sorted fluid is regardless of the cleaning procedure utilized.

FUTURE DIRECTIONS

- Instruments that were positive for endotoxin will be re-tested in-house (second test).
- Instruments tested positive for second time will follow a protocol of decontamination suggested by McIntyre, C et al (Application Note, BD Biosciences, Nov 2009), followed by a third test.
- We expect to test for mycoplasma as wells, since mycoplasma is a common contaminant on cultures and can be easily pass into a sorter instruments.

Supported by the ABRF. Special thanks to ThermoFisher Sci and Lonza for donating reagents and to all participating Flow Cytometry-Shared Resource Labs

MEDICAL SCHOOL
UNIVERSITY OF MICHIGAN

Flow Cytometry Research Group 2016-2017 Study

Endotoxin Contamination of Cell Sorters: Evaluating Cleaning and Testing Procedures

Laura Blunk, Stowers Institute for Medical Research; Dave Adams, University of Michigan, Alan Bergeron, Dartmouth College; Kathleen Brundage, West Virginia University; Karen Clise-Dwyer, MD Anderson Cancer Center; Matt Cochran, University of Rochester Medical Center; Monica DeLay, Cincinnati Children's Hospital; Roxana Del Rio Guerra, University of Vermont; Maris Handley, Massachusetts General Hospital; Peter Lopez, New York University Langone Medical Center; E. Michael Meyer, University of Pittsburgh Cancer Institute; Alan Saluk, The Scripps Research Institute

Abstract

Cell sorting plays an important role in many *in vitro* and *in vivo* studies. Sorted cells are often placed back into culture for expansion, used for *in vitro* experiments or adoptively transferred into animals. Thus, anything that the cells come into contact with while passing through the cell sorter has the potential to affect their function, including cytokine production or proliferation. This is particularly true for endotoxin, a lipopolysaccharide derived from gram-negative bacteria, which can elicit a variety of direct and indirect cellular responses, depending on cell type. Every flow cytometry shared resource lab has its own routine cleaning procedures for sorters. However, endotoxin is not commonly considered and is not usually included in testing as a contaminant in the cell sorter fluidics. To investigate the prevalence of endotoxin contamination samples were collected from sorters in various cell sorting facilities across the USA. In addition, a hydrogen peroxide cleaning procedure was tested by a subset of the facilities to determine its effectiveness in eliminating endotoxin contamination. The results will be presented here.

Background

Endotoxin aka lipopolysaccharide (LPS) is a large molecule consisting of a lipid and O-linked polysaccharide. It is found in the outer membrane of gram negative bacteria and is typically released upon the death of the bacteria. It is known to elicit an immune response even at very low levels (0.02ng/ml)[1]. Macrophages, dendritic cells and B cells are particularly sensitive to endotoxin. LPS activates these cells by binding to a receptor complex made up of TLR4, CD14 and MD-2. If these cell types are passed through a sorter that is contaminated with endotoxin they could become inadvertently activated, thus affecting the results of downstream assays.

Last year the FCRG began a study to look at "how clean is your sorter really?". The idea behind the study was that anything (i.e. RNAses, endotoxins, bacteria and fungus) that a cell comes in contact with during the sorting process can affect the results of downstream assays. In last year's screen of FCRG members' sorters a number of them had endotoxin contamination. For this year, we chose to expand the testing to sorters in other facilities across the USA. In addition, to remove endotoxin from contaminated sorters a H_2O_2 protocol based on two publications[2,3] was tried. H_2O_2 was chosen because it oxidizes the endotoxin thereby inactivating it.

Participants Profile

47 sorters from 17 Flow Cytometry Core Facilities in USA
Bacteria/Yeast run in 27 instruments
Instrument Types:

Astrios – 6	Jazz - 1
FACS Aria – 29	MoFlo - 2
Fusion – 1	S3 – 5
Influx – 3	

Protocol for Collecting and Testing Samples for Endotoxin Contamination

1. Sorters were started up as normally done following standard startup protocol for the lab
2. A sterile 10 ml pipet was used to collect samples from the sheath tank connected to the instrument
3. After turning on the sorter and fluidics following normal procedures, 10 ml of sheath fluid was collected in a single 15 ml tube of sheath fluid by placing a sterile 15 ml conical tube under the fluid stream exiting the nozzle
4. Samples were shipped on wet ice or with freezer packs to the testing lab
5. Endotoxin levels in the samples were determined using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher catalog #88282)
   Detection range: 0.01 – 0.1 ng/ml LPS

Figure 1. Sixty-two Percent of the 0-5 Year Old Sorters had Detectable Endotoxin Levels

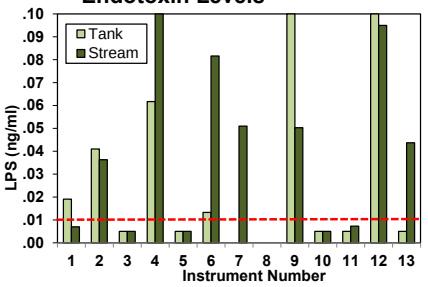


Figure 2. Seventy-eight Percent of the 6-10 Year Old Sorters had Detectable Endotoxin Levels

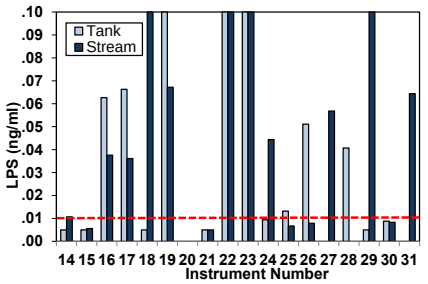
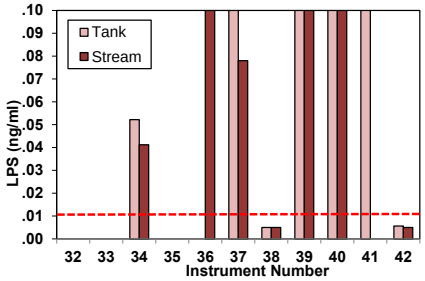


Figure 3. Fifty-Five Percent of the > 10 Years Old Sorters had Detectable Endotoxin Levels



NOTE: The age of 5 sorters were not reported. Of those five, 3 had endotoxin levels of > 0.1 ng/ml and 2 had 0.068 and 0.076 ng/ml in the samples collected from the stream of the instrument.

Parameters That Did Not Correlate with Endotoxin Results

1. Date of last PM (preventative maintenance)
2. Source and type of sheath (homemade vs company)
3. Recent contamination
4. Cleaning methods including bleach, water, ethanol, detergent and sporacidin
5. Modifications to the fluidics system of the instrument
6. Whether or not bacteria or yeast have been run through the instrument

Protocol for Removing Endotoxin from a Sorter

1. Remove sort nozzle (on ARIAs only) and bypass sheath filter
2. Fill sheath tank halfway with 1% H_2O_2 (30% H_2O_2 stock diluted with sterile H_2O)
3. Perform a fluidics startup
4. Load tube containing 1% H_2O_2 on sample loader
5. Perform a "Clean Flow Cell" procedure 2-3 times
6. Turn on stream
7. Run tube of 1% H_2O_2 at highest flow rate for 2h
8. Empty sheath tank, fill with 1% H_2O_2 and let sit for 2h then rinse thoroughly with sterile water and fill with sheath
9. Perform "Fluidics Startup" – repeat 3 times
10. Load tube containing sterile sheath on sample loader
11. Perform "Clean Flow Cell" procedure 5 times
12. Turn on stream
13. Run tube of sterile sheath at highest flow rate for 3 h
14. Perform an ethanol fluidics clean
15. Replace H_2O_2/H_2O saturated sheath filter with a brand new filter

Results of H_2O_2 Cleaning

Table 1. Cleaning #1 – Endotoxin Levels in the Stream

	Pre-clean	Day 3 Post clean	Day 65 Post Clean
Sorter A	0.054 ng/ml	BDL*	0.089 ng/ml
Sorter B	0.089 ng/ml	BDL*	0.087 ng/ml
Sorter C	0.082 ng/ml	0.091ng/ml	0.052 ng/ml

*Below Detection Limit (0.01ng/ml)

Table 2. Cleaning #2 – Endotoxin Levels in the Stream

Stream	Pre-clean	Day 10 Post clean
Sorter A	Not Done	0.070 ng/ml
Sorter B	Not Done	0.082 ng/ml
Sorter C	Not Done	0.072 ng/ml

Table 3. Cleaning #3 – Endotoxin Levels in the Sheath Tank & Stream of Sorter D

	Pre-clean	Day 1 Post clean	Day 6 Post Clean	Day 10 Post Clean
Tank	>0.100 ng/ml	BDL*	BDL*	>0.100 ng/ml
Stream	>0.100 ng/ml	0.100 ng/ml	Not done	Not done

*Below Detection Limit (0.01ng/ml)

Conclusions

1. Many sorters in core facilities have some level of endotoxin contamination.
2. There does not appear to be any specific pattern or explanation on why some instruments are contaminated.
3. Cleaning with H_2O_2 does not always remove endotoxin but when it does clean the instrument does not stay clean long.

Acknowledgements

We would like to thank all the individuals who provided samples for our study.

References

1. Schwarz H., Schmittner M., Duschl A. and Horejs-Hoeck J. 2014. Residual endotoxin contaminations in recombinant proteins are sufficient to activate human CD1c$^+$ dendritic cells. PLOS One. 9(12):e113840.
2. McIntyre C.A. and Reinin G. 2009. Reduction in endotoxin levels after performing the prepare for aseptic sort procedure on the BD FACSAria II flow cytometer. BD Application Note.
3. Lin S.M., Svoboda K.K.H., Giletto A., Seibert J. and Puttaiah R. 2011. Effects of hydrogen peroxide on dental unit biofilms and treatment water contamination. European Journal of Dentistry. 5:47-59.

Information Dissemination

Sorting for RNA

What are Best Practices?

- Fixed
- Unfixed

But What About

What are Best Practices?

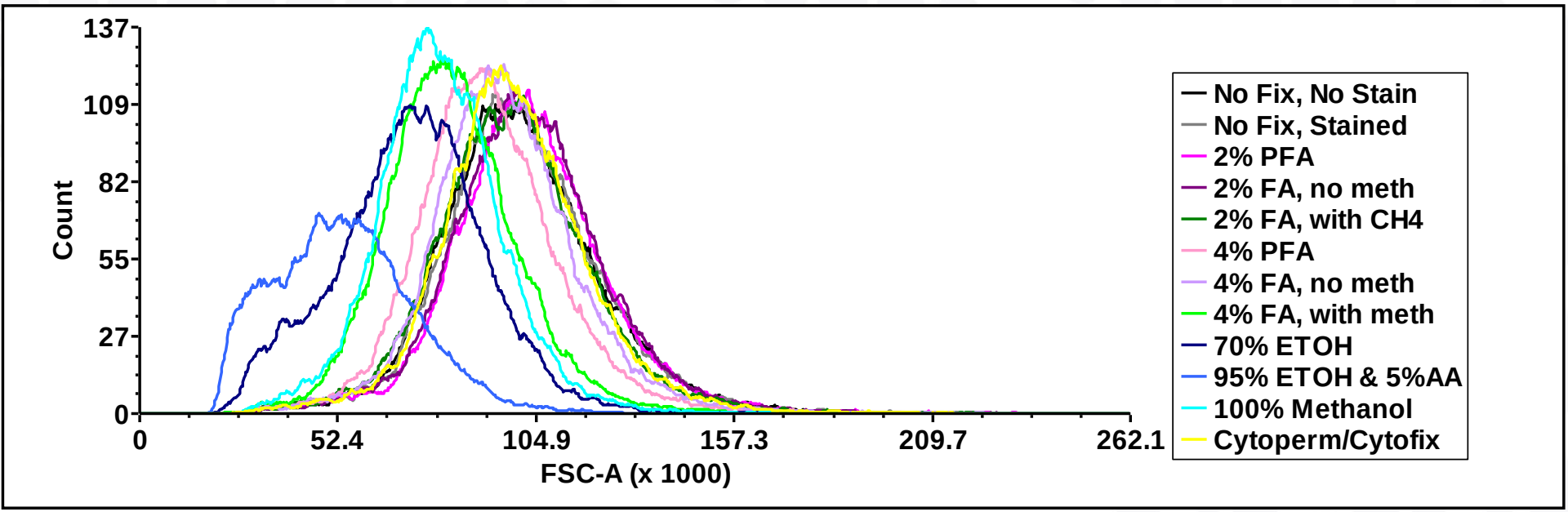
- Fixed
- Unfixed
- Unsorted Control

But What About

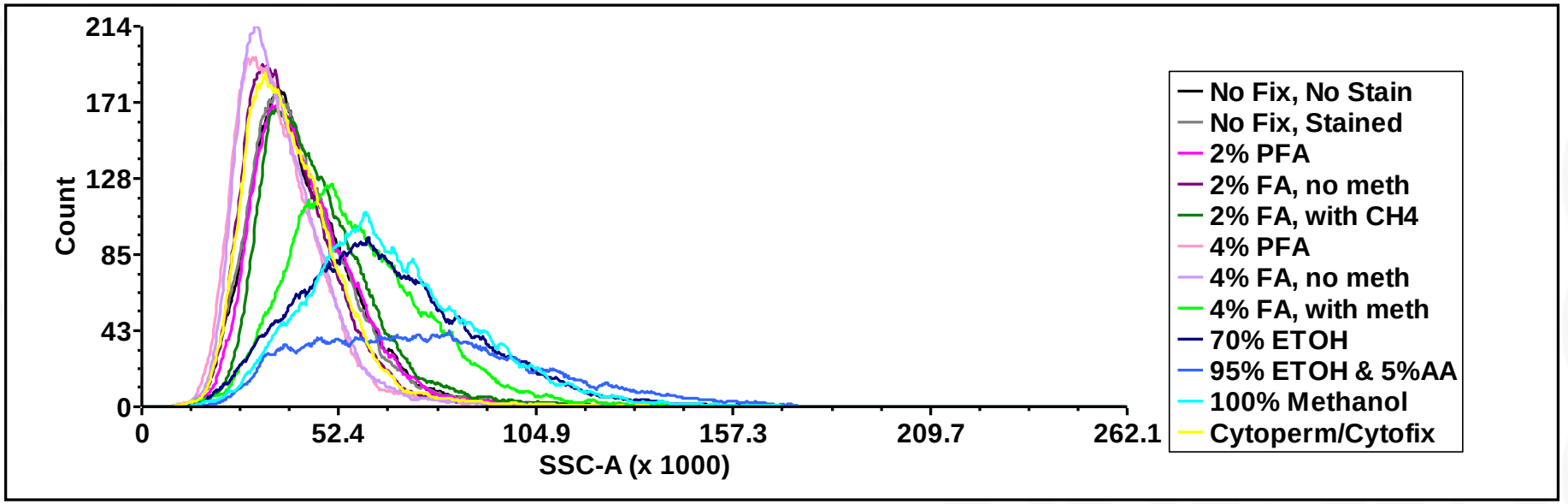
What are Best Practices?

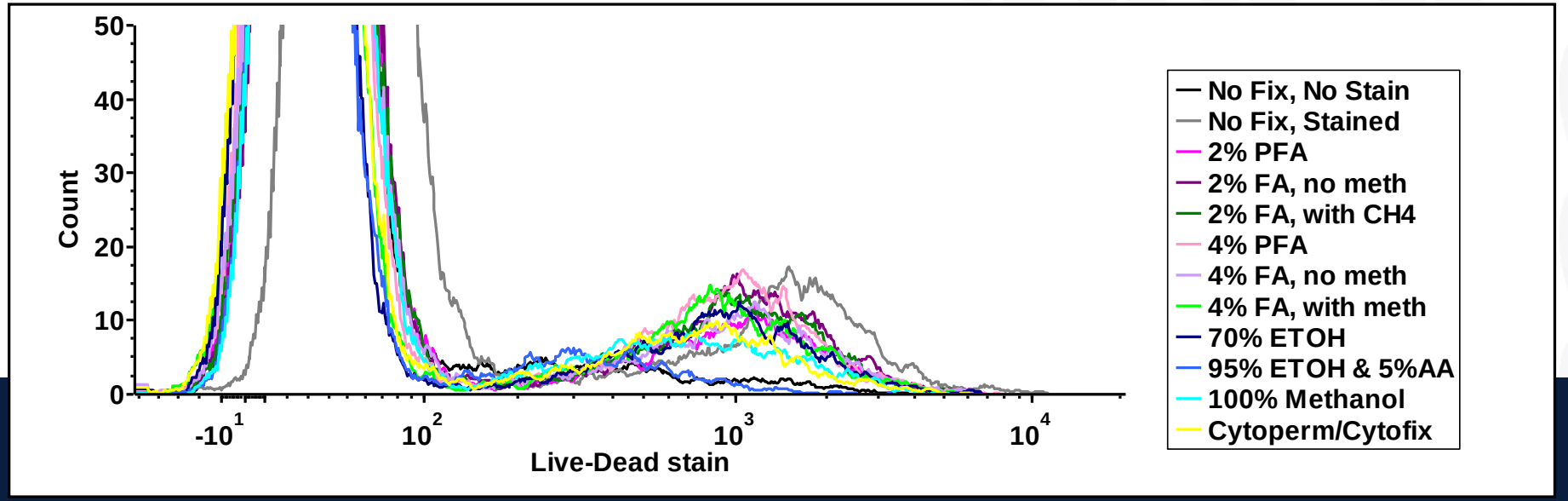
- Fixed
  - Paraformaldehyde
  - Formaldehyde
  - Ethanol
  - Methanol
  - Zinc-based
  - Cytoperm/Cytofix

Effects of Fixation on Cells



Effects of Fixation on Cells





Other Issues

- HL60 Cells Genetically Verified (Reproducibility)
- Holding Times (Match Conditions)
- Storage Temperatures
- Buffers and Protein Matching
- Sort Conditions
  - Nozzle
  - Pressure
  - Temperature

And So We Begin

- HL60 Cells Made Available to Each Team
- Internal Controls for Each Team Generated
  - Fixed
  - Unfixed
  - Unsorted
- RNA Harvested and Enumerated
- Stay Tuned

Acknowledgements

We would like to thank all the individuals and vendors who have assisted with our studies over the years.

Please consider joining the FCRG. Contact sheets available in the exhibit hall, speak to a member, or shoot me an email (davadams@med.umich.edu)