Flow Cytometry Research Group 2018-2019 Study

A Multi-Core Study on How Different Fixation Methods Prior to Sorting Impact the Purity, Quality, and Yield of RNA From Sorted Cells

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Abstract

Increasingly, Flow Cytometry Shared Resource Facilities are asked to sort fixed cells for RNA isolation either in bulk or at the single cell level. With so many fixation methods in the literature the Flow Cytometry Research Group (FCRG) decided to perform a systematic evaluation of the reported fixation methods to assess how the different fixatives affected the quality of RNA isolated from sorted cells. Based on the literature, four different common chemical fixatives were analyzed using the human cell line HL-60. The assessment included paraformaldehyde fixation, alcohol fixation (methanol and ethanol), zinc fixation, and three commercial reagents. Each method was tested at two separate shared facilities and for some methods different variations of the fixation procedure (i.e., time, temperature, and dilution) were also tested. The protocol involved fixing the cells first. The next day, fixed cells were sorted into lysis buffer. RNA isolated from the cells was assayed to determine purity, quality, and concentration. Each condition had sorted and not-sorted samples. A NanoDrop was used for purity and a Bioanalyzer for quality and concentration. Few fixed samples (sorted or not) returned any intact RNA, pointing to the unreliability of many common fixation methods. Sorting did correlate with decreased RNA yield, although the cause has yet to be determined.

Concentration and Purity of Extracted RNA

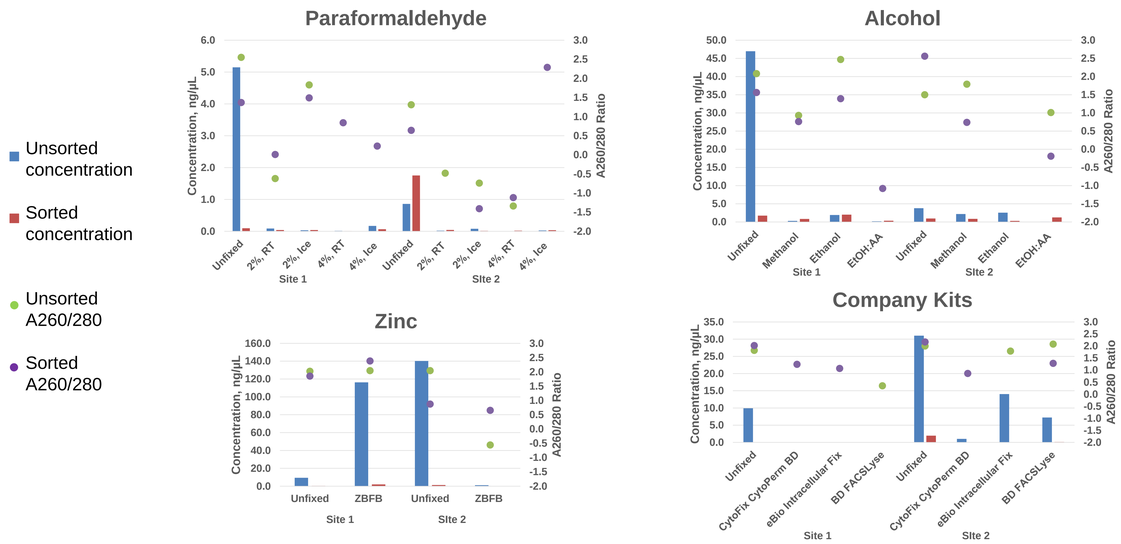

Figure 1. Concentration of RNA (ng/µL) is shown with bars. Purity as measured by A260/280 ratio is shown with dots (optimal for RNA is ~2.0).

Cell Staining & Sorting Protocol

Before fixation:

1. HL-60 cells were grown in RPMI+10%FBS at 37ºC and 5% CO_2.
2. Cells were stained in 1X PBS+0.5%BSA with Viobility 488/520 Fixable Dye (Miltenyi Biotec #130-109-812) according to the manufacturer's protocol.
3. Cells were washed, resuspended at 1x10^7/mL, and put on ice.
4. Each site set aside a pair of samples for unfixed controls (sort/no-sort).

After fixation:

1. Sorters were started up following standard startup protocol for the lab using a 100 µm tip and 1X PBS.
2. All samples were filtered at 40-70 µm before sorting.
3. The same day as fixation, 2x10^5 cells from one unfixed sample were added directly to RLT Plus buffer (Qiagen #1053393) 1:3.5 vol/vol as an unsorted control, and 2x10^5 dye- unfixed cells were sorted into RLT Plus buffer at 1:3.5 vol/vol.
4. Fixed samples were stored overnight. The next day 2x10^5 cells from each fixation condition were added to RLT Plus buffer as unsorted controls, and 2x10^5 dye- cells were sorted from the other samples into RLT Plus buffer (the Site 1 ZFNB-fixed sample was sorted into 1:1 ZFNB-glycerol instead).
5. All samples were stored at –80ºC before shipment to the RNA testing site on dry ice.

Fixation Protocols

1. Paraformaldehyde (PFA) (defined as methanol-free formaldehyde) – Cells were pelleted and resuspended to 1x10^6 cells/ml with PFA under the following conditions: 2% PFA at RT, 2% PFA on ice, 4% PFA at RT, and 4% PFA on ice. After a 20 min incubation, cells were washed with PBS-BSA.
2. Three alcohol-based fixatives were tested: 100% methanol, 70% ethanol, and 95:5 ethanol:acetic acid (EtOH:AA) mix. For methanol-fixed samples, cells were pelleted, resuspended in 4 mL 100% methanol, and incubated on ice for 15 min. For ethanol-fixed samples, cells were pelleted, resuspended in 5 mL 70% ethanol, and incubated on ice for 30 min. For EtOH:AA-fixed samples, cells were pelleted, resuspended in 20 mL 95:5 EtOH:AA mix, incubated at -20ºC for 15 min, centrifuged at 120 g for 10 min at 4ºC, resuspended a second time in 20 mL 95:5 EtOH:AA, and incubated at -20ºC for 15 min. All fixed samples were washed with PBS-BSA, resuspended at 2x10^6 cells/mL PBS-BSA, and stored for 18-24 h at -80ºC (methanol) or -20ºC (ethanol or EtOH:AA).
3. Zinc-based – Samples were suspended in 1 mL Zinc Buffer [ZFNB: 0.1 M Tris-Cl, ph 7.8; 0.05% (v/v) calcium acetate; 17.16 mM zinc trifluoroacetate; 0.5% (v/v) zinc chloride] and stored overnight at -20ºC 1:1 in glycerol. Prior to sorting, cells were washed with PBS-BSA.
4. Commercial reagents – Cells were fixed with one of the following kits per manufacturer's recommendations: BD Cytofix/Cytoperm (#554714) for 20 min at 4ºC, eBioscience Intracellular Fix (#88-8824- 00) for 20 min at RT, and BD FACS/Lyse (#349202) for 10 min at RT. All samples were washed twice with PBS-BSA.

				RNA Integrity Number	
Fixation Method	Sorter	Fix	Sort	Site 1	Site 2
Paraformaldehyde	1. Astrios	No, on ice	No	9.6	9.9
	2. FACSAria II	No, on ice	Yes	0.0	10.0
		2% fix RT	No	2.1	1.0
		2% fix RT	Yes	1.0	1.0
		2% fix ice	No	1.1	1.0
		2% fix ice	Yes	1.0	1.0
		4% fix RT	No	1.0	0.0
		4% fix RT	Yes	0.0	1.0
		4% fix ice	No	2.5	1.1
		4% fix ice	Yes	2.4	1.0
Alcohol	1. FACSAria II	No	No	9.4	10.0
	2. FACSAria III	No	Yes	10.0	10.0
		Methanol	No	3.9	8.5
		Methanol	Yes	2.2	4.5
		Ethanol	No	3.4	8.3
		Ethanol	Yes	2.3	3.3
		Ethanol/Acetic Acid	No	8.8	1.0
		Ethanol/Acetic Acid	Yes	2.3	10.0
Zinc	1. FACSAria III	No	No	10.0	10.0
	2. FACSAria II	No	Yes	9.5	2.5
		ZBFB	No	6.1	1.0
		ZBFB	Yes	7.8	7.8
Commercial Kits	1. FACSAria II	No	No	10.0	9.5
	2. FACSAria III	No	Yes	9.6	10.0
		CytoFix CytoPerm BD	No	1.0	2.3
		CytoFix CytoPerm BD	Yes	0.0	1.0
		Ebio Formaldehyde?	No	1.0	2.4
		Ebio Formaldehyde?	Yes	1.0	1.0
		BD FACSLyse	No	1.0	2.4
		BD FACSLyse	Yes	0.0	1.0

RNA Quality

RIN 8.0-10.0

Degraded RNA

No RNA

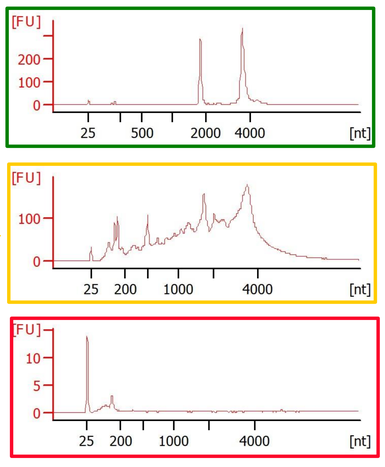

Figure 2. RNA Integrity Numbers (RIN) for each sample are indicated in the table. RIN values 8-10 indicate intact RNA, values between 3 and 8 represent degraded RNA, and <3 represent the absence of RNA. To the right are examples of the Bioanalyzer traces for the 3 groups.

RNA Testing

1. RNA extraction was performed using the RNeasy Plus Micro Kit (Qiagen, Valencia, CA) per manufacturer's recommendations.
2. RNA purity testing was performed with a NanoDrop.
3. RNA quality and yield testing was performed with the Agilent Bioanalyzer 2100 system.

Conclusions

- Highest RNA quality (as indicated by RIN) was attained from unfixed samples. Thus, our results indicate that all fixation methods had an affect on the quality and yield of the samples whether sorted or non-sorted.
- Our results indicate the need for identifying the method of fixation and the method of isolation of RNA for a specific cell type. Certain methods of fixation may work best for a particular cell type or tissue. Further processing of the sample for RNA isolation needs to take into account the fixation method. Determining these factors prior to sorting would be highly recommended by the FCRG.
- Our results indicate that a significantly lower RNA yield from sorted cells should be expected.

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