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Establishing methods to assess epigenetic signatures in archived study tissues

Maureen Malloy[1], Gleta Carswell[2], Brian Bennett[3], Pierre Bushel[3], Leah Wehmas[2], Susan Hester[2], and Brian Chorley[2]

[1]Oak Ridge Institute for Science and Education, Oak Ridge, TN [2]Office of Research and Development, US EPA, RTP, NC

[3]National Institute of Environmental Health Sciences, RTP, NC

Brian N. Chorley | chorley.brian@epa.gov | 919-541-2329

www.epa.gov

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Abstract

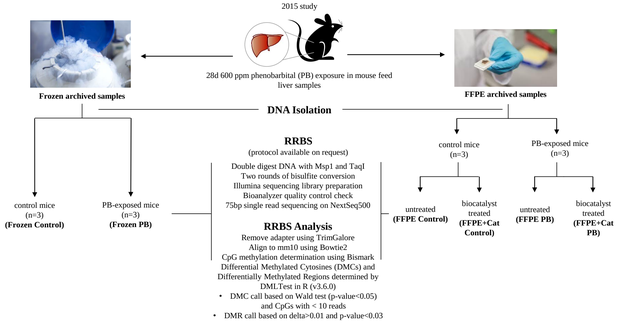
Revisiting archived studies using new methods on preserved samples will aid in a molecular understanding of adverse chemical effects, thereby reducing animal use, enhancing chemical hazard prioritization, and providing weight of evidence for regulatory decision making. Alterations in DNA methylation can occur with environmental chemical exposures, persist over time, and result in adverse health outcomes. In this pilot work, we tested the ability of Reduced Representation Bisulfite Sequencing (RRBS) to measure DNA methylation in formalin fixed paraffin embedded (FFPE) tissue samples relative to paired frozen controls. The 5 yr old liver tissue samples were obtained from male mice treated with 600 ppm phenobarbital (PB), a known mediator of DNA methylation changes and potential tumorigen, or vehicle control in drinking water for 28 d. Sequencing results indicate that FFPE samples contained significantly higher CpG sites relative to frozen samples (average 8.6 and 4.4 million reads, respectively with p=0.010; Kruskal-Wallis rank sum test); however, with higher read depth criteria (≥ 5X CpG site coverage), the remaining read counts for each sample type were statistically similar. In addition, read count variability in FFPE samples was significantly higher (p-value = 1.767e-11; Kruskal-Wallis rank sum test) than in frozen samples, overall indicating poorer quality sequencing data associated with FFPE. Only a few differentially methylated regions (DMRs) due to PB exposure were quantified in either sample type (4 and 1 DMRs, FDR p<0.05 for frozen and FFPE, respectively). When assessed with less stringency (uncorrected p<0.03 and methylation changes of >1%), 644 and 963 DMRs were identified for frozen and FFPE samples, respectively; however, only 30 DMRs were shared between sample types. In addition, genes linked to DMR regions overlapped less than 2% of previously published DMR genes due to short-term PB exposure in mouse liver, regardless of sample source. This indicates the study sample size (n=3 per condition) was of insufficient power to determine true DMRs. Follow-up studies in progress will address this issue. With sufficient power, we estimate that these FFPE-sourced data will accurately represent data from frozen/fresh tissue, thereby unlocking epigenetic-based data from archived tissue to refine mechanistic understanding of susceptibility to chemical-mediated toxicity. ***This poster does not necessarily reflect EPA policy.***

Introduction & Methods

Biorepositories contain millions of preserved tissue samples from human, rodent, and alternative models. These samples often come from informative studies covering hundreds of compounds with data on study methods, animal condition, and results. Revisiting these experiments using new methods on preserved samples will aid in a molecular understanding of adverse chemical effects. These advances will also help in translating chemical effects on the animal to those that occur in cell culture without the need for new and costly experiments. This reduces animal use, helps prioritize hazard, and assist risk assessment. ***We propose to perform Reduced Representation Bisulfite Sequencing (RRBS) to measure DNA methylation in preserved formalin-fixed paraffin-embedded (FFPE) mouse liver tissues. DNA methylation are known to respond to environmental exposures and these changes can persist over a lifetime, or even across generations, and may be key biological events for adverse health outcomes.***

DNA analyses (e.g., assessing DNA methylation, hydroxymethylation) is difficult because of damage induced by the formalin-fixation process. Thus, method optimization is required to access these valuable FFPE resources. Here we attempted to improve the sequencing quality by using a nucleic acid demodification (biocatalyst) method that we have successfully used to improve gene expression measurements (RNA sequencing) in FFPE tissue (see Wehmas et al. 2018). Five-year old matched frozen and FFPE mouse liver samples were tested from phenobarbital (PB) treated mice, a known exposure that acutely alters DNA methylation patterns (Lempiainen, et al. 2013; Thomson et al. 2013).

Study Design and Sample Source



Results

Sample Source	DNA input (ng)	No. of reads in millions	Mapping efficiency (%)	Unique CpGs at 1X depth in millions	Unique CpGs at 5X depth in millions	Unique CpGs at 10X depth in millions	Unique CpGs at 25X depth in millions
Frozen (n=6)	500 (300 MspI/ 200 TaqI)	283.8 (47.3 avg)	65.08%	4.4 +/- 0.2	1.6 +/- 0.1	1.3 +/- 0.1	0.7 +/- 0.1
FFPE (n=6)	500 (300 MspI/ 200 TaqI)	630.4 (105.0 avg)	71.22%	8.6 +/- 2.5 * +	2.9 +/- 2.0 +	1.5 +/- 1.0 +	0.6 +/- 0.3 +
FFPE + catalyst (n=6)	500 (300 MspI/ 200 TaqI)	326.7 (54.5 avg)	69.83%	6.8 +/- 2.1 * +	1.7 +/- 0.9 +	0.9 +/- 0.6 +	0.4 +/- 0.4 +

Table 1. Sequencing and CpG call metrics associated with frozen, FFPE, and FFPE + Cat sourced samples. * = significantly different from frozen (p<0.05, Kruskal-Wallis rank sum test) + = variability different from frozen (p<0.05, Kruskal-Wallis rank sum test)

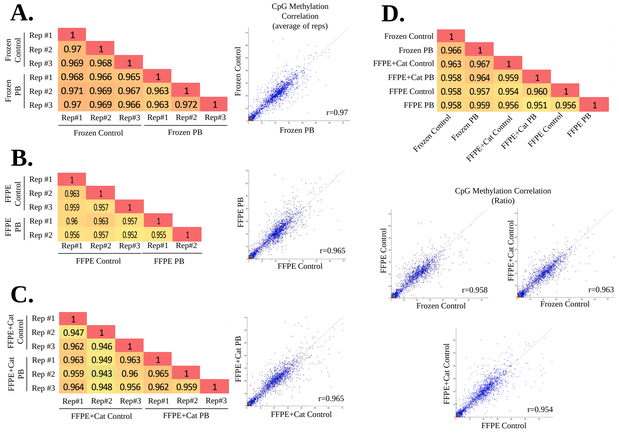


Figure 1. Pearson's correlation (r) of CpG methylation at matched sites for different sample types and treatment. Data represents mapping to CpG islands as an example. (A) Frozen control and PB sample reps, (B) FFPE control and sample reps, (C) FFPE+Cat control and sample reps, and (D) representative samples of all sample types and treatments.

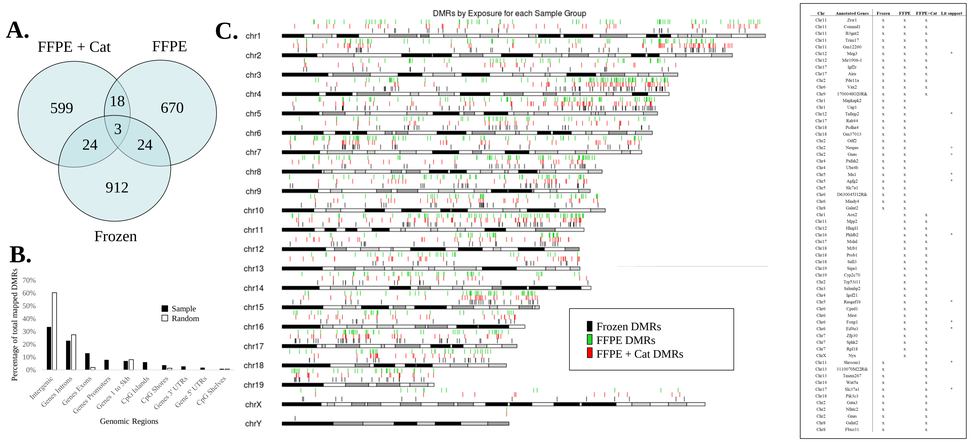


Figure 2. Differentially Methylated Regions (DMRs) due to PB exposure. (A) Common and distinct PB-induced DMRs among sample types. (B) Location of DMRs across genomic features compared to randomly picked regions. (C) Chromosomal locations of all DMRs for each sample type.

Table 2. Subset of DMRs enriched in two or three sample types. x = significant PB-induced DMRs in sample type. * = PB-induced DEG over 1 - 91d time course (Lempiainen, et al. 2013). + = PB-induced linked gene at 1 - 91d exposure (Thomson et al. 2013).

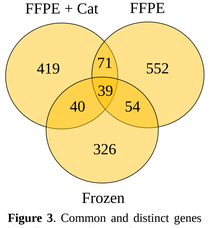


Figure 3. Common and distinct genes linked to DMRs by sample type.

A better understanding of epigenetic changes and adverse outcomes can be obtained by revisiting *archived toxicological studies* with new methods.

Summary and Future Directions

- FFPE-sourced samples (without biocatalyst treatment) exhibited nearly twice as many reads than frozen-sourced samples despite the same DNA input. Most of these reads translated to shallow (1 read) depth to unique CpGs, indicating damaged DNA inducing sequencing errors and mismatching to the genome.
- Biocatalyst treatment did improve these errors somewhat, however filtering mapped CpGs to those of 10 reads or more equalized the number of CpGs found between sample types. Variability was significantly higher in FFPE-sourced samples, regardless of biocatalyst treatment.
- Methylation levels of CpGs were highly correlated among reps and sample types, however PB-induced DMRs exhibited minimal overlap between sample types. Despite this, genes mapped to these regions did exhibit similarity among sample types and also reflected previously identified genes that were responsive to PB treatment in mouse liver.
- **Overall, the data indicates that the RRBS method can identify genes linked to differential DNA methylation due to chemical exposure in archived, poorer quality FFPE-sourced samples similar to better quality, frozen-sourced samples.**
- We plan to further optimize these results by augmenting the number of samples used and apply different bioinformatic strategies to reduce data variability and false-positive DMR calls observed with FFPE samples.
- Long-term, these methods will improve and refine early key events in adverse outcome pathways while providing a bridge between animal studies and cell-based assays.

References

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