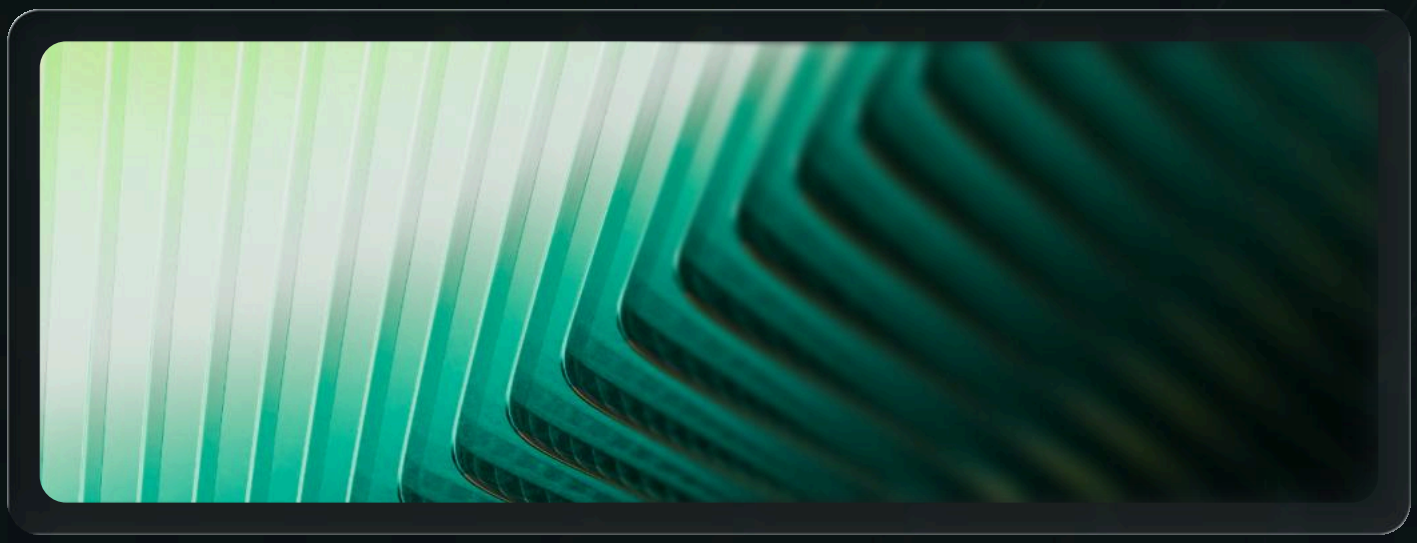
RENEW

Case Study



Renew Biotechnologies' Array Replacement

Direct Whole Methlyome Sequencing

Head-To-Head Comparison of Methylation Array vs. dWMS

Introduction

For decades, methylation arrays have been a staple in epigenetic research due to their accessibility and well-established workflows[1]. However, their reliance on predefined loci, bias toward pG islands, and dependence on bisulfite conversion introduce errors and limit scope[2,3]. These limitations have driven the need for holistic, unbiased approaches that offer higher sensitivity and deeper insights, particularly in clinical epigenetics[4]

Renew Biotechnologies' (Renew) Direct Whole Methylome Sequencing (dWMS) addresses this need by leveraging Oxford Nanopore Technologies' native long-read sequencing platform. This solution delivers robust genome-wide methylation data with extensive CpG coverage, eliminating the biases of bisulfite-based methods. Enhanced by the user-friendly ModSeqR toolkit, dWMS simplifies analysis and empowers researchers to explore clinical applications with precision and efficiency. Because dWMS uses the ONT platform, nanopore sequencing will be highlighted in this case study.

Challenges in Transitioning to Native Sequencing

Native DNA sequencing platforms, such as Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PacBio), have emerged as strong alternatives to traditional arrays because they sequence DNA in its native states. This capability allows direct detection of epigenetic modifications, including 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), without the chemical damage caused by bisulfite conversion or the synthetic biases of DNA amplification.

Although these platforms were once error-prone[5], recent advancements in ONT's Kit 14 chemistry, have achieved Q20+ accuracy (>99%)[6,7] with Q30 accuracy (99.9%) reported using duplex basecalling and super-accurate (SUP) workflows[8]. Despite increasing affordability compared to short-read and array technologies, the complexity of nanopore data analysis remains a barrier to widespread adoption.

This case study demonstrates how Renew's dWMS addresses the challenges of traditional bisulfite-based approaches, offering a robust and flexible solution for genome-wide methylation analysis. To further enhance accessibility and usability, ModSeqR, a proprietary Renew R package, simplifies dWMS analysis workflows. Together, these tools empower researchers to advance epigenetic research and explore clinical applications with greater precision and efficiency.

Direct Whole Methylome Sequencing

Renew's Direct Whole Methylome Sequencing (dWMS) offers a scalable solution to the challenges of traditional approaches, combining a proprietary shearing protocol, optimized library preparation with enhanced ONT chemistry, and direct native whole-genome sequencing on the Nanopore platform. By leveraging native reads and high accuracy, dWMS delivers superior sequencing performance with data-rich outputs for research, biomarker discovery, and clinical development.

dWMS provides extensive, unbiased coverage of over 27 million CpG sites—more than 30 times the coverage offered by traditional arrays (850,000 CpG sites). This bisulfite conversion- and PCR-free approach eliminates common biases, offering a complete and accurate view of the methylation landscape.

Renew's dWMS Service Includes:

- Proprietary Shearing and Library Prep
- ONT Whole Genome Sequencing
- Accessible Bioinformatics Toolkit

Renew's Bioinformatics Toolkit: ModSeqR

The large volume of data generated by dWMS can pose challenges in storage and analysis. Renew Biotechnologies addresses this with two innovative solutions: CH3, a file format that reduces data size by up to 95% (from 20 GB to 1 GB), and ModSeqR, a user-friendly bioinformatics toolkit. The ModSeqR R package allows researchers to extract summary data, perform differential methylation analyses, and visualize results from CH3 files with just a few lines of code, simplifying the transition from arrays to dWMS workflows.

With Renew's dWMS and ModSeqR toolkit, researchers can efficiently access native methylation data without extensive bioinformatics expertise. This integrated solution advances biomarker discovery, population epigenetics, and clinical diagnostics, paving the way for expedited insights into disease mechanisms, the development of predictive biomarkers, and the translation of epigenomic findings into clinical applications.

Methylation Array vs. Renew's dWMS
A Head-to-Head Comparison

Objective

To compare the efficacy of dWMS to traditional methylation array.

Methods

A comparative analysis was conducted to evaluate the performance of traditional methylation arrays and Renew's dWMS across two tissue types (n = 4 per tissue).

To assess traditional array, genomic DNA (gDNA) was extracted from Tissue 1 and Tissue 2, bisulfite converted, hybridized to Infinium HumanMethylation450 BeadChip microarrays (Illumina) and analyzed according to the manufacturer's protocols. To assess Renew's dWMS approach, gDNA was similarly extracted from Tissue 1 and Tissue 2, processed, and sequenced according to the dWMS protocol. Sequencing reads were aligned to the human genome (GRCh38) using the minimap2 software package with the default settings for Nanopore sequencing reads with base modifications identified using the Dorado basecaller.

Expanded CpG Island Coverage

Although the human genome contains approximately 28,890 CpG islands (CGIs) excluding repeat sequences[9], traditional methylation arrays typically cover only around 26,000 CGIs[10]. In our comparative analysis of Tissue 1 and Tissue 2, the EPIC array identified 4,854 differentially methylated CGIs, 92% of which (4,469 CGIs), were also captured by dWMS (Figure 1). dWMS captured an additional 6,166 differentially methylated CGIs, a 2.4-fold increase compared to the array.

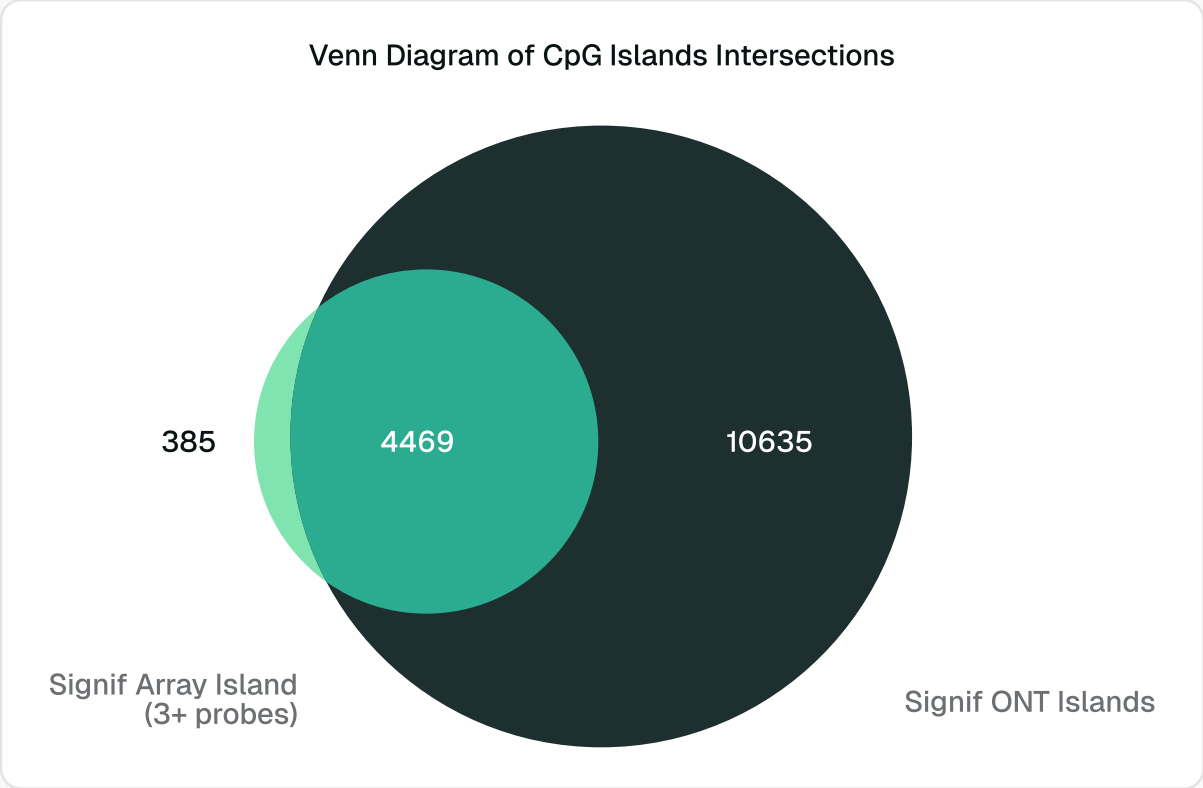


Figure 1: In a comparison of two tissue types, dWMS identifies significantly more differentially methylated CpG islands (CGIs) than traditional microarrays. DNA was extracted from Tissue 1 (n = 4) and Tissue 2 (n = 4) and analyzed with both methylation array and dWMS. The dWMS method captured 2.4 times more CGIs with significant differential methylation, including 92% of those detected by the array.

While the potential clinical significance of the 385 differentially methylated CGIs detected by array but not dWMS and the 6,166 CGIs identified by dWMS but not array are uncertain, the approximate 2.4-fold increase in differentially methylated CGI coverage with dWMS highlights its enhanced potential for biomarker discovery and epigenetic research. We suspect that the remaining approximate 8% (385) CGIs not called by the array may be due to biases introduced during DNA treatment and PCR amplification. To further explore this hypothesis, we are currently conducting additional validation studies.

Minimal Batch Effects

Methylation arrays are known to be highly susceptible to batch effects[11], which can significantly impact downstream analyses and conclusions[12], potentially leading to systemic false positives[13].

To assess batch effects, dWMS was performed on the two tissues at two different physical locations with different PromethION machines. The analysis displayed high correlation within each tissue group (Figure 2A, average r > 0.97) and clear differentiation between Tissue 1 and Tissue 2, indicating minimal batch effects (Figure 2). These findings support dWMS as a reliable option for scalable epigenetic research and clinical assay development.

Figure 2. Renew's dWMS demonstrates high reproducibility with minimal inter-sample variance within each group. DNA from Tissue 1 (n = 4) and Tissue 2 (n = 4) were sequenced on two different PromethION machines at two different sites. A correlation matrix (A) and principal component analysis (PCA) plot display high similarity within Tissue 1 and Tissue 2 groups (average correlation r > 0.97), highlighting minimal variation between the two locations and, therefore, batch effects.

Tissue of Origin

DNA methylation is increasingly investigated in cell-of-origin studies across oncology and other fields to trace the origins of circulating DNA[14]. In a comparative analysis of methylation data for tissue-of-origin studies, we evaluated Illumina's EPIC array against Renew's dWMS using the same DNA regions (CpG islands covered by the array). As expected, dWMS captured significantly more CpG sites, enabling a more detailed analysis of cell-type-specific signatures.

In the representative 15 kb region shown in Figure 3, the EPIC array assessed 10 CpG sites, whereas dWMS captured approximately 15 times more. For example, in the 1.5 kb region around position 127,401 kb, the EPIC array identified six CpG sites, showing predominantly methylated sites in Tissue 1 and largely unmethylated sites in Tissue 2. dWMS not only confirmed these findings but also captured a significantly higher number of CpG sites within this region, demonstrating its superior resolution and suitability for complex tissue-of-origin studies.

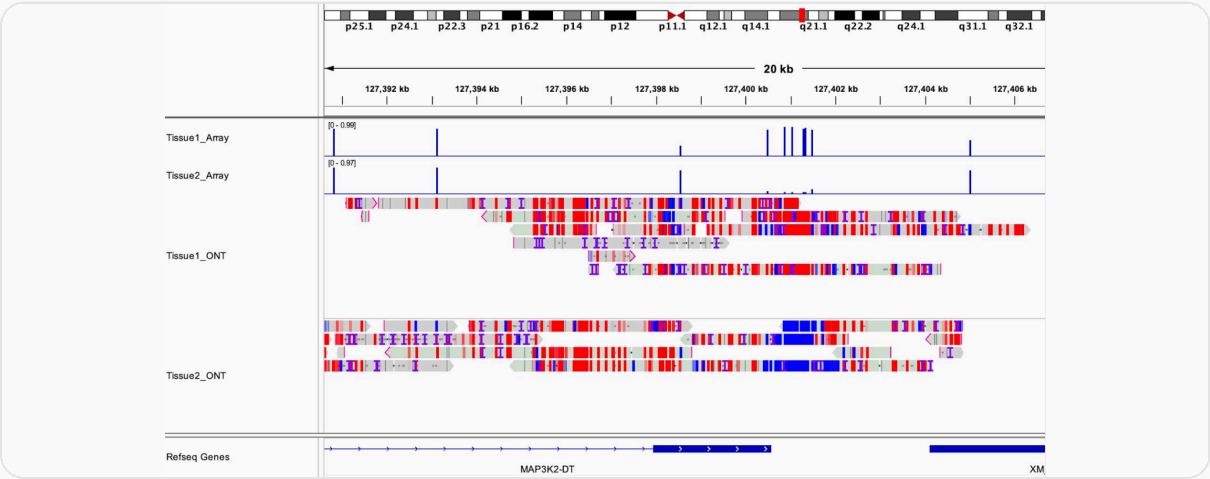


Figure 3: Superior CpG detection with Renew's dWMS for regional methylation and cell-of-origin analyses. Renew's dWMS significantly outperforms the EPIC array in CpG site detection within a representative 15 kb region. The EPIC array (top two tracks) identifies 10 CpG sites, while dWMS (bottom tracks) detects ~15 times more CpG sites. In the array data, blue bar height represents methylation levels. In the dWMS tracks, each line represents a single read, with red indicating methylated sites and blue indicating unmethylated sites. This expanded coverage highlights dWMS's superior resolution and suitability for complex methylation and tissue-of-origin studies.

The enhanced sequencing quality of dWMS not only matches the data quality and coverage of traditional arrays at array-specific sites but also significantly expands CpG coverage across the genome. With single-molecule resolution, dWMS enables precise analysis of complex methylation patterns that may hold clinical relevance. This capability supports advanced applications such as cell-of-origin studies, disease and aging-specific methylation pattern analyses, early cancer detection through liquid biopsy, and tracking methylation changes in response to environmental factors. By providing comprehensive coverage and single-molecule resolution, Renew's dWMS unlocks new possibilities for exploring the epigenome with unmatched detail.

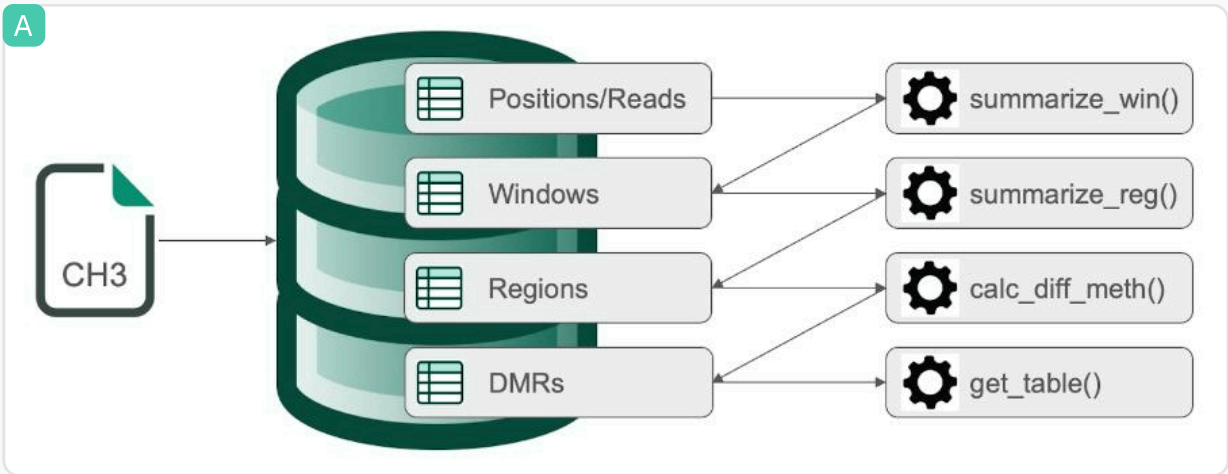
Efficiency and Usability

Renew's dWMS is both powerful and accessible, designed to optimize data storage, transfer, and analysis. The CH3 file format reduces data size by up to 95%—from 20 GB modkit outputs to just 1 GB. This memory-efficient format stores data in an on-disk database, enabling larger-than-memory tasks. The analysis pipeline is fast and user friendly, performing differential methylation analyses across 27+ million CpGs in approximately 80 seconds on a standard laptop and sliding window calculations (about 3 billion) in roughly 15 minutes.

To support researchers familiar with array workflows, Renew developed the ModSeqR R package, which converts ONT methylation data into array-like formats compatible with tools such as minfi, DMRcate, and chAMP. This solution empowers researchers to efficiently leverage dWMS data within familiar frameworks, facilitating advanced epigenetic insights.

ModSeqR Capabilities

- Methylation summaries by base position
- CpG Island coverage
- Sliding window analysis
- Differential methylation analysis by region, read, or position
- Quality control functions



B

	sample_name	chrom	ref_position	cov	c_counts	mh_counts	mh_frac
1	barcode81	chr1	184864	6	0	6	1.0000000
2	barcode81	chr1	185073	6	0	6	1.0000000
3	barcode81	chr1	197311	6	0	6	1.0000000
4	barcode81	chr1	188004	4	0	4	1.0000000
5	barcode81	chr1	191525	4	0	4	1.0000000
6	barcode81	chr1	199451	2	2	0	0.0000000
7	barcode81	chr1	199749	2	2	0	0.0000000
8	barcode81	chr1	193779	2	0	2	1.0000000
9	barcode81	chr1	196498	4	2	2	0.5000000
10	barcode81	chr1	199509	1	1	0	0.0000000
11	barcode81	chr1	200610	2	2	0	0.0000000
12	barcode81	chr1	200833	1	1	0	0.0000000
13	barcode81	chr1	203444	2	0	2	1.0000000
14	barcode81	chr1	502408	3	0	3	1.0000000
15	barcode81	chr1	503115	1	0	1	1.0000000
16	barcode81	chr1	507273	1	0	1	1.0000000
17	barcode81	chr1	532333	1	0	1	1.0000000
18	barcode81	chr1	533388	2	0	2	1.0000000
19	barcode81	chr1	587849	2	0	2	1.0000000
20	barcode81	chr1	587934	2	0	2	1.0000000

Positional Summary

C

sample_name	region_name	mean_mh_frac	mean_cov
barcode81	chr1_933387_937410	0.26126126	1.860335
barcode81	chr1_963795_964507	0.99107143	2.604651
barcode81	chr1_975496_980305	0.26987448	1.943089
barcode81	chr1_981068_981400	1.00000000	2.500000
barcode81	chr1_982152_982511	0.89583333	2.526316
barcode81	chr1_990274_990694	0.95238095	2.470588
barcode81	chr1_1228780_1230163	0.99074074	2.347826
barcode81	chr1_1247918_1248493	0.92857143	1.750000
barcode81	chr1_1254322_1254548	1.00000000	1.312500
barcode81	chr1_1259643_1260635	0.97368421	1.900000
barcode81	chr1_1267435_1268986	1.00000000	1.387097
barcode81	chr1_1271425_1272074	0.53571429	2.153846
barcode81	chr1_1289707_1291126	0.98412698	2.333333
barcode81	chr1_1534331_1536136	0.96428571	1.647059
barcode81	chr1_1550453_1552015	0.98000000	5.000000
barcode81	chr1_1558587_1559359	0.95454545	1.294118
barcode81	chr1_1564421_1570006	0.85558583	2.463087
barcode81	chr1_1580377_1580595	1.00000000	1.333333

Summaries by Region

*Outputs can be made for m calls, h calls, or (as shown here) both combined

Figure 4: ModSeqR commands to extract positional, regional, and differential methylation summaries from CH3 files (A) and representative examples of outputs by Positional (B) and Regional (C) Summaries.

Implications and Future Directions

Renew's dWMS significantly outperforms traditional methylation arrays, covering over 27 million CpG sites compared to the ~850,000 typically captured by arrays. This capability allows for the unbiased acquisition of sequence, methylation, and hydroxymethylation data in a single run, delivering more detailed and accurate insights.

With extensive CpG coverage, dWMS enables the discovery of novel epigenetic biomarkers linked to disease and other biological processes. In population epigenetics, it offers a powerful tool for investigating epigenetic variations across cohorts, advancing our understanding of how genetics, lifestyle, and environmental factors shape epigenetic patterns. This precision in capturing the epigenetic landscape opens opportunities for more accurate diagnostics and targeted therapies for improved patient outcomes.

Conclusion

Renew's dWMS represents a transformative advancement in methylation research, leveraging the strengths of nanopore sequencing—rapid throughput, high accuracy, affordability, and portability[15]. More than just an alternative, dWMS is a foundational technology poised to redefine epigenomic research and accelerate clinical adoption. Its ability to deliver quality, comprehensive data in a user-friendly format empowers researchers to uncover deeper epigenetic insights and explore the epigenome's critical role in health and disease.

Partnering with Renew Biotechnologies

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