**Development of a single cell methylation summarization tool and application to lineage analysis of glioma-related macrophages**

**Masters Thesis**

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# Declaration

**This Thesis was written at the Deutsches Krebsforschungszentrum in the period from 02/06/2021 to 02/12/2021 under the supervision of Dr. Reka Toth**

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**I herewith declare that I wrote this Masters Thesis independently, under supervision, and that I used no other sources and aids than those indicated throughout the thesis.**

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| 02/12/2021 |  | **A pair of glasses  Description automatically generated with medium confidence** |
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# Table of Contents

[Declaration 1](#_Toc88049338)

[Table of Contents 2](#_Toc88049339)

[List of figures 3](#_Toc88049340)

[List of tables 3](#_Toc88049341)

[List of abbreviations 3](#_Toc88049342)

[1. Abstract 5](#_Toc88049343)

[2. Introduction 5](#_Toc88049344)

[2.1. Epigenetics 5](#_Toc88049345)

[2.1.1. Methylation 5](#_Toc88049346)

[2.1.2. Gene regulation 5](#_Toc88049347)

[2.1.3. Differentiation and development 6](#_Toc88049348)

[2.1.4. Epimutations 6](#_Toc88049349)

[2.2. Implications to disease 7](#_Toc88049350)

[2.2.1. Cancer 7](#_Toc88049351)

[2.2.2. Glioma 8](#_Toc88049352)

[2.2.3. Tumor-associated macrophages 8](#_Toc88049353)

[2.2.4. Potential for treatments 8](#_Toc88049354)

[2.3. Quantifying methylation 10](#_Toc88049355)

[2.3.1. Methylation arrays 10](#_Toc88049356)

[2.3.2. Whole genome bisulfite sequencing 10](#_Toc88049357)

[2.3.3. Single cell bisulfite sequencing 10](#_Toc88049358)

[2.4. Project aim 11](#_Toc88049359)

[3. Methods 11](#_Toc88049360)

[3.1. scMethrix 11](#_Toc88049361)

[3.1.1. Overview 11](#_Toc88049362)

[3.1.2. Object structure 12](#_Toc88049363)

[3.1.3. Data handling 12](#_Toc88049364)

[3.1.4. Analysis 13](#_Toc88049365)

[3.1.5. Benchmarking 17](#_Toc88049366)

[3.2. Data collection 17](#_Toc88049367)

[3.2.1. Raw data 17](#_Toc88049368)

[3.2.2. Cell deconvolution 17](#_Toc88049369)

[4. Results 18](#_Toc88049370)

[4.1. Reference methylomes 18](#_Toc88049371)

[4.1.1. Array-based 18](#_Toc88049372)

[5. Discussion 18](#_Toc88049373)

[6. Future Outlook 18](#_Toc88049374)

[7. Conclusion 18](#_Toc88049375)

[8. Data Availability 18](#_Toc88049376)

[9. Supplementary Data 18](#_Toc88049377)

[10. References 19](#_Toc88049378)

# List of figures

[**Figure 1. Package Overview of scMethrix** 10](#_Toc87454340)

[**Figure 2. Structure of the scMethrix class.** 11](#_Toc87454341)

[**Figure 3. Workflow for analyzing single-cell data with scMethrix.** 13](#_Toc87454342)

[**Supplementary Figure 1. Runtime of single-threaded and parallel processing of the workflow in Supplementary Method 1.** 15](#_Toc87454816)

# List of tables

**No table of figures entries found.**

# List of abbreviations

|  |  |
| --- | --- |
|  |  |
| 5mC | 5-methylcytosine |
| BED, BedGraph | Browser extensible data format |
| CpG, CG | cytosine - phosphate - guanine |
| CGI | CpG Island |
| CTCF | CCCTC-binding factor |
| DNAme | DNA methylation |
| DNMT | DNA methyltransferase protein |
| GBM | glioblastoma |
| GEO | gene expression omnibus |
| HDF, HDF5 | hierarchical data format |
| iPCA | iterative principal component analysis |
| IDH | isocitrate dehydrogenase |
| IL | interleukin |
| kNN | k-nearest neighbour |
| mCpG | methylated CpG |
| NA | not applicable |
| NK | natural killer |
| PCA | principal component analysis |
| R | R programming language |
| RF | random forest |
| RRBS | reduced representation bisulfite sequencing |
| scBS-seq | single cell bisulfite sequencing |
| SNP | single nucleotide polymorphism |
| TAM | tumor-associated macrophage |
| TCGA | The Cancer Genome Atlas program |
| TET | ten-eleven translocation protein |
| TF | transcription factor |
| TSG | tumor-suppressing genes |
| tSNE | t-distributed stochastic neighbor embedding |
| UMAP | uniform manifold approximation and projection |
| WGBS | whole genome bisulfite sequencing |

# Abstract

# Introduction

## Epigenetics

Epigenetics is the study of heritable and reversible changes of the genome that do not change the underlying DNA sequence1. These changes are fundamental for controlling gene expression in all organisms, and can occur through intrinsic methods (e.g., X-chromosome inactivation in females2) or external causes (e.g., exposure to ultra-violet radiation3). The molecular marks of epigenetics span several levels from overall chromatin structure, such as histone modifications or nucleosome positioning, down to changes of individual bases, primarily with methylation1.

### Methylation

Methylation is the most studied epigenetic modification. The nucleotide bases adenine and cytosine both readily accept methylation on N4and C4/C5, respectively4. The bulky methyl groups provide a physical block to binding of transcription factors and RNA polymerase5. As such, methylation is a major factor in gene expression in most organisms. Methylated adenine is weakly studied but widely known in bacteria and lower eukaryotes6,7, but is not considered to have any effect or significant presence in mammalian DNA8,9, where instead cytosine modifications are more common. 5-methyl-cytosine (5mC) is the dominant epigenetic mark in humans, with about 28 million sites present in the genome10. These sites, known as CpG sites for the cytosine-phosphate-guanine bridge, are not randomly distributed but instead highly localized around regulatory motifs: 70% of promoters show high density of CpG sites11. This density is called a CpG island (CGI)5. Due to the high presence, and their significance for gene regulation, mechanisms for adding and removing methylation to CpG sites must be strictly regulated.

These epigenetic marks can be added *de novo* by DNA Methyltransferase proteins (DNMT3a/b)12,13 or removed by ten-eleven translocation methylcytosine dioxygenases (TET family)14, so these modifications are considered reversible. Recruitment of DNMT3A/B is not well understood, but is regulated by mechanisms including chromatin modifications, transcription levels, and the presence of non-coding RNAs or DNA-binding factors12. DNMT3A/B also aids in maintaining methylation state15, as demethylation can occur passively during errors in replication, or actively by numerous demethylation pathways16, including via TET proteins. As with DNMT3, there are many mechanisms for TET recruitment or inhibition, including distinct metabolites, cofactors, and post-translational modifications14. TET does not directly remove 5mC, but instead catalyzes hydroxylation into 5-hydroxymethylcytosine, which can later be removed by the base excision repair pathway. Free of the obstructing methyl group, transcription can proceed4.

### Gene regulation

Transcription of genes can be affected directly or indirectly by methylation1. As CpG islands are dense in promoter regions, transcription can be directly blocked via steric hinderance, as transcription factor (TF) binding sites are blocked by the bulky methyl molecules5. The major TF families E2F17, NF-κB18, and Myc19 all show methylation-sensitivity. As well, methylated CpGs (mCpGs) can indirectly repress transcription by recruiting methyl-binding proteins to compete for TF binding sites. Other chromatin remodeling proteins (e.g., histone deacetylases) are also recruited and can further impede transcription. Conversely, TFs have been found that preferentially bind to mCpGs *in vivo* (e.g., ARNT2, DIDO1, MEF2A and HOXA920), so methylation is not limited to only gene repression. However, *in vitro* experiments show contradictory evidence of pro-methylation TF binding, so it is unknown whether increased gene expression by mCpGs is a widespread effect21.

Gene products can also be affected by methylation via alternative splicing, as transcription factors also bind to exons and can similarly be blocked as in promoters4. In the human CD45 gene, CCCTC-binding factor (CTCF) binds to exon 5 and reduces the elongation rate of RNA pol II, thus favoring the inclusion of the exon into mature mRNA, but when CTCF binding motifs are methylated, exon 5 is typically excluded22. Though CD45 isoforms have shown to strongly influence leukocyte function, these mechanisms and effect of alternate exons are still unclear23. Alternative splicing can also occur via recruitment of splicing factors. Heterochromatin protein 1 can create a protein bridge with mCpGs, and is known enhancer and silencer for splicing alternative exons24.

### Differentiation

Differentiation of cells is strongly influenced by epigenetic factors. *Dnmt3*−/− embryonic stem (ES) cells that are terminally unmethylated (0.6% of normal) are still viable and can self-renew, but unable to initiate differentiation25. If differentiation is induced in hypomethylated *Dnmt1*−/− ES cells, apoptosis will occur25. Conversely, TET1−/− neural progenitor cells (NPCs) show impaired self-renewal but maintaining the differentiation capacity26. TET2−/− hematopoietic stem cells (HSCs) have increased self-renewal but bias differentiation towards myelomonocytic lineage27, and TET2−/− mice universally develop highly aggressive myeloid leukemia28.

Among non-mutant cells, there is a global trend towards hypermethylation during differentiation, including gamete-specific and pluripotency-associated genes29. However, specific loci remain hypomethylated in a cell-type-dependent manner30. Terminally differentiated cells generally maintain their methylation state29, and may even resist aberrant or artificially-induced demethylation, though with some shift in phenotype31. Depending on cell type, they may still proliferate and pass on their epigenetic identity32.

Methylation patterns are maintained through cell division, so they are considered heritable33. During DNA replication, proliferating cell nuclear antigen (PCNA) molecules associate with DNA polymerases at 3’ end of the replication fork. The PCNA molecules recruit NP95, which preferentially binds to the hemi-methylated regions resulting from semi-conservative replication. NP95 aids in localization of DNMT1 to methylate the newly replicated, unmethylated strand. This imparts an epigenetic memory into cells, and the lineage of a cell can be traced via their specific methylation patterns30,34,35. This also means that aberrant methylation can be passed to daughter cells as well.

### Epimutations

Epigenetic mutations, or epimutations, are aberrant chromatin states that can be caused by hypo- and hypermethylation of CpG sites. These can arise spontaneously, as like DNA replication, methyl transfer by DNMTs is not error-proof35. Spontaneous epimutations occur at a rate of 10-100x that of mutations during cell division, and can be caused by impairment of methylation machinery or by dilution of the classical methyl donor, S-adenosyl methionine36. While a single epimutation alone is unlikely to cause drastic phenotype changes, it can act as a traceable cell tag. This has previously been observed in continuously differentiating colon crypt cells32 as well as multiple types of leukocytes37. During replication, about 0.3-1.3 epimutations are expected to randomly occur36, allowing lineage mapping from hematopoietic stem cell to terminal differentiation34.

External factors can also cause aberrant methylation, such as ultraviolet (UV) radiation3. While methylation marks typically block polymerases, UV radiation can induce pyrimidine dimers preferentially at mCpGs38. These dimers do not block polymerases, allowing for error-free bypass. UV radiation has also shown to cause global hypomethylation as well as localized hypermethylation of many genes3. Environmental disasters may also drive epimutations. Nutrition has been linked to methylation39, and it has been suggested that past famines have disrupted methylation patterns, causing low birth weight and metabolic defects in the grandchildren of famine survivors40. Heavy metals are known to induce epimutations through both hypo- and hypermethylation of numerous loci, and similar effects were seen with persistent organic pollutants (POPs) like dichlorodiphenyltrichloroethan (DDT) and polychlorinated biphenyls (PCBs)41. This is further problematic since, as stated above, epimutations are heritable during cell replication, but are also heritable transgenerationally from parent to offspring as well. Diethylstilbestrol, a formerly used nonsteroidal estrogen medication prescribed to pregnant women but now known to induce aberrant methylation42, has shown links to breast cancer in their children43 and ADHD in their grandchildren44. Aberrant epimutations have significant, and likely understated, contribution to human disease.

## Implications to disease

There are numerous diseases solely attributed to epigenetic abnormalities. These often happen via genomic imprinting, where genes are differentially expressed based on which parent the gene was inherited from45. For instance, insulin-like growth factor 2 is only expressed from the paternal allele46. The end result is similar to X-chromosome inactivation, though this happens via an unrelated process2. If a gene is incorrectly imprinted, disease can result45. However, disease is not limited to direct epigenetic defects. Immune cells show epigenetic reprogramming during development, activation, and differentiation30,47, so epimutations may indirectly affect the outcomes of all diseases.

### Cancer

Aberrant methylation is ubiquitous in cancer, typically showing global hypomethylation and localized hypermethylation in CpG islands48. Mutations in DNMT proteins have been found in multiple cancers (e.g., DNMT3A in 25% of acute myeloid leukemia cases and DNMT1 in 12% of uterine cancer cases49), and TET mutations are also common (e.g., up to 42% of chronic myelomonocytic leukemia cases50). This is problematic with pro-tumor and tumor-suppressing genes (TSGs), as single epimutations can have significant effect: hypermethylation of single CpGs is found in TSGs at 10x the rate in breast cancer patients versus controls51.

Genetic imprinting has a known link to cancer45

Epigenetics can also drastically change the phenotype of cells and induce a pro- or anti-cancer state.

### Glioma

Glioma is a devastating, rapidly progressing disease with one of the poorest outcomes of any cancer52. Each year, about 250 000 individuals are diagnosed with glioma (1.4% of new cancer diagnosis)53. The disease progresses from mild episodic effects like headaches and seizures, to severe functional decline including motor dysfunction and neurocognitive failure52. Epigenetics has shown to play a key role in glioma, from enabling subtype classification to causing key driver mutations54. Epigenetic imprinting has shown links to some cancers45, but this is less clear for glioblastoma55.

Glioma tumor microenvironment is distinct from other types of tumors. The brain is physically protected from the rest of the body due to the blood-brain barrier, which can selectively prevent circulating molecules and cells from passing through56. As well, the immune cell landscape is unique, containing cells not found elsewhere in the body57. Macrophages are particularly interesting, as up to 50% of the tumor bulk in glioblastoma is made up of these tumor-associated macrophages (TAMs)58.

### Tumor-associated macrophages

Microglia are the resident macrophages unique to the CNS. The differentiate only during embryogenesis, but survive with long lifespans and local proliferation57. However, in response to brain lesions caused by glioma or other diseases, circulating lymphocyte antigen 6hi C-C chemokine receptor type 2+ (Ly‑6ChiCCR2+) monocytes are preferentially recruited into the brain, which then differentiate into microglia-like cells59,60. These non-native microglia show functional differences to their native counterparts, and thus are an important factor for disease progression. For instance, in Alzheimer's disease, only microglia that differentiated from infiltrating monocytes are able to phagocytose the amyloid plaques that contribute to disease onset, whereas native microglia are unable to do so61. The mechanism behind this is not clear, but transgenic overexpression of interleukin-1β (IL-1β) have shown as a key signal for plaque phagocytosis62. This activation effect could have relevance for cancer progression, as glioblastoma has shown to aberrantly express IL-1β63. If similar mechanisms exist, infiltrating monocytes may have an increased tumor fighting response to glioblastoma compared to the native microglia, which could drastically affect patient outcome. Identifying the types of macrophages involved and their functional capacities could lead to improved treatment.

Traditionally, macrophages have been divided into two types: the classically activated, pro-inflammatory, anti-tumor M1 macrophages and the alternatively activated, anti-inflammatory, pro-tumor M2 macrophages64. However, *in situ*, macrophages (including microglia) show high plasticity with molecular characteristics and functions of both. Phenotypic markers exist to differentiate them (e.g., CD11b(+)CD209(+) for M2), but external stimulus can cause reprogramming between M-types65. For instance, in cytokine-deficient medium, M1 macrophages can transition to M2 type and start expressing CD11b(+)CD209(+) markers66. Thus, immunophenotypic markers are weakly reliable in classifying macrophages *in situ*. Transcriptomes can be used to identify M-type *in vivo*65, but their inherent plasticity *in vitro* leaves a complex web of transcriptional and pathway differences that makes identification difficult. Epigenetic phenotype has shown to be a strong method in differentiating closely related cells67. Lineage can also be observed, such as monocyte-to-macrophage differentiation, as there are highly localized DNAme changes68.

### Potential for treatments

As mentioned previously, methylation can influence CD45 isoforms, and may be a target for treatment. In HIV, CD45 isoforms have shown significant effect on viral replication69. Targeting antibodies for one specific isoform, CD45RO, virtually stopped RO-microglial proliferation as well as strongly inhibiting overall HIV-1 production70. With glioma, CD45 isoforms haven’t been extensively studied, but it is still a key marker protein for resident (CD45low) and infiltrating microglia (CD45high)71. More research into glioma is necessary to discover the contributory effects of CD45 isoforms caused by aberrant methylation, and the epigenetic cell lineage of the TAMs that present them. This could allow indirect treatment of glioma by identifying and targeting of pre-tumorigenic macrophages, or direct treatment by antibodies against TAMs.

After ischemic stroke, native microglia show compromised cell progression and largely adopt a pro-inflammatory phenotype compare to infiltrating monocytes72.

## Quantifying methylation

DNAme has several advantages over other types of genetic analysis: (1) It is cell type specific, unlike normal DNA. (2) It is more robust against transient variation than RNA expression. (3) A CpG site is either methylated or unmethylated, so there is no ambiguity, in contrast to expression counts. (4) Special handling is not required, as is necessary with RNA. (5) Assays for DNAme easily integrated into a clinical setting as they are similar to normal DNA sequencing. NP95 and DNMT1 are not present during polymerase chain reaction (PCR) or other types of synthetic replication, so there is no direct passing of epigenetic marks, but additional treatments allow detection. There are many methods of DNAme quantification73, but two show the majority of use: microarray- and sequencing-based.

### Methylation arrays

Microarrays are attractive for many assays due to low cost, low DNA requirement, and low sample processing time73. This allows for high throughput of large numbers of clinical samples. The major disadvantage to this technique is the limited number of probes available. Only about 850K CpG sites can be profiled via the premier Illumina MethylationEPIC array, compared to the approximately 28M CpG sites in the genome. However, it does cover 99% of human genes, 95% of CpG islands, reliable between runs, and is generally consistent with the previous Illumina’s previous 450k and 27k probe arrays74. For complex regulatory systems, this may not be sufficient though, as single CpG sites can drastically affect gene expression, so a technique with a wider genomic range may be necessary.

### Whole genome bisulfite sequencing

Whole genome bisulfite sequencing (WGBS) is the gold standard sequencing-based technique for studying the epigenomic landscape since its development in 199273. In this technique, sodium bisulfite deaminates unmethylated cytosines into uracil, whereas the 5mC bases are bisulfite conversion. Subsequent PCR converts the newly generated uracils into thymines, and by comparing pre- and post-treatment sequencing, the resulting single nucleotide polymorphisms (SNPs) allow identification of the 5mC bases. This is highly reliable (>99% accuracy) and reproducible75. However, the treatment is very harsh; bisulfite treatment randomly induces single strand nicks, so up to 95% of the CpG sites lost during sequencing76, so a coverage of at least 30x is recommended.

Unfortunately, normal WGBS suffers some drawbacks due to its bulk processing. First, low population samples cannot give suitable coverage. If the cells cannot be grown, like some bacteria which are viable but not culturable, or if it may be dangerous to allow cell growth, such as cancer cells in a patient, this technique will not work. Secondly, it can be difficult to differentiate between heterogenous cells. Rare or aberrant cell types can have significant effect on mediating immune responses or ever the pathogenesis of cancer77. This cell might differ in only a few CpG sites, and thus lost as noise during WGBS. As mentioned previously, single CpG hypermethylation is implicated in breast cancer51. Single cell DNAme quantification would allow this level of analysis.

### Single cell bisulfite sequencing

Whole genome single cell bisulfite sequencing (scBS-seq) has more recently been developed (2014)78, and serves to address the major drawbacks of bulk WGBS. In contrast, scBS-seq typically involves lysing of single cells, treatment with bisulfite, and the resulting DNA fragments are primed with tagged adapters. The cells can be then combined, and the PCR amplification will use indexed primers corresponding to the tagged adapters to generate multiple single-cell libraries at once. This technique is suitable for low population samples as well as detecting rare or aberrant cell types and can allow for deeper analysis where cell-to-cell differences are necessary.

Lineage of cells can be weakly studied using bulk WGBS. Distinct cell states can be observed, but does not have the resolution necessary for the mapping the trajectory of intermediate cell types79. Cells retain a strong epigenetic memory, as mentioned above30,34,35, so can be used to determine lineage with better precision than other single-cell based techniques (e.g., scRNA-seq).

While scBS-seq can allow for high resolution analysis, it some drawbacks. Data sparsity is a significant challenge, as a vast majority of CpGs will be undetected in each cell. Grouping of homogenous cells is a common technique, and this can be achieved by methylation-specific clustering methods, such as Epiclomal software package80, and allow discovery of hidden subpopulations where bulk WGBS is unable to do so.

As scBS-seq is a relatively new technique to the mainstream, few tools adequately support it. Existing bulk WGBS tools are unable of handle sparsity and the resulting high proportion of missing values. Statistical analysis is complicated by missing data and may require specialized algorithms. As well, there is high memory requirement if stored in a dense matrix, as most also require. As more scBS-seq experiments are performed, new software tools will be necessary to handle single cell data. Therefore, we have developed a new tool to address these problems and allow a pipeline similar to traditional WGBS tools.

## Project aim

The aim of this project is two-fold. First, a new tool is to be developed for handling single cell methylome data, as the challenges of data sparsity and incompatibility with established downstream methylation analysis techniques remain poorly fulfilled by existing software tools. The software package will be modelled after its bulk WGBS predecessor, the methrix R package81, but will give additionally functionality for imputation, clustering, binning, and more. Our intention is to integrate it within the Bioconductor ecosystem to maximize compatibility and audience. Secondly, to help validate the tool, we will look at the relationship between glioma and macrophage methylation. Using epigenetic lineage, we hypothesize that methylation can be used to identify and help determine the origins of TAMs implicated in gliomagenesis, whether they be native microglia or differentiated from infiltrating monocytes. This will involve three steps: (1) Generate a methylation signature matrix for the major cells in the tumor microenvironment, (2) Classify sub-types of monocytes, macrophages, and microglia by epigenetic lineage, (3) Classify glioma and glioblastoma sub-types using known reference tools, and (4) Use these new signatures to analyze bulk tumor cell samples for macrophage presence. With this knowledge, it may be possible to generate prognostic outcomes based on diversity of TAMs or their characteristic methylation profiles. This may improve patient-specific treatment and aid in identifying further avenues of research for fighting this devastating disease.

# Methods

## scMethrix

### Overview

We introduce scMethrix, an R-based data storage and manipulation tool that is ideal for single cell methylation data. A complete overview of the package structure is show in Figure 1. A condensed workflow is provided in Supplemental Method 1, as well as a comprehensive vignette that outlines the specifics of each function via a sample workflow using publicly available single cell methylation data (see [Data Availability](#_Data_Availability) section).

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| Figure 1. Overview of the scMethrix package.  Single cell bisulfite data stored in BedGraph-based files can be imported via read\_beds() to be stored as a summarized experiment within the scMethrix object. Numerous functions are available for quality control, filtering, and visualization. Multiple export formats are supported for further downstream analysis. |

### Object structure

The scMethrix experiment object extends the SingleCellExperiment82 container and will interface with many other packages in the Bioconductor ecosystem. It largely uses the data.table83 and DelayedMatrixStats84 R packages for quick and efficient operations.

As this is an S4 object85, multiple components are encapsulated into a single instance. This includes genomic regions of features, data from inputted or transformed assays, additional sample or feature information, and reduced dimensionality data (Figure 2).

The scMethrix class has functions to collapse samples (e.g., homogenous cells from the same patient, or clustering of broad cell types) and bin CpGs (e.g., into CGIs or promoter regions). Subsequent operations are unaffected, as the general structure of the object does not change. For the purposes of this section, a CpG or sample can refer to one or more features or cells, respectively.

### Data handling

**Data input:** scMethrix imports BedGraph-based file formats via the read\_beds() function. Samples are contained in columns and CpGs are represented in rows. Data points with an NA value can be included. Pre-configured settings are available for many commonly used methylation calling tools, including Bismark86, MethylDackel87, methylCtools88, BisSNP89, and BS-Seeker290. Other calling tools can be supported via manual input of column indexes. Reference CpGs can optionally be inputted for alignment or exclusion of CpG sites before importing, or the CpG sites can be generated from the input files themselves. Providing coverage data is optional, as scBS-seq data typically has one or two reads per CpG per chromatid per cell, depending on strandedness. However, the coverage matrix is necessary for certain analysis functions (e.g., mask\_by\_coverage()) and export formats (e.g., export\_bsseq()). Experiment objects can also be built using subsets of files and later merged.

**Data storage:** Experimental data can be stored either in-memory or stored on the hard-disk as an hierarchical data format object (HDF5; via HDF5Array R package91), and can be converted between each other, depending on system resources. Most external functions cannot interface with HDF5 data, so it may be cast as a matrix before processing. Genomic coordinates are handled via GenomicRanges92, and can be stored as stranded or unstranded, with the option to collapse the strands during read\_beds(). Metadata for the experiment (e.g., additional sample IDs, machines used, relevant dates, CpG annotation) can be stored as either rowData() for CpGs, colData() for samples, or metadata() for overall experiment information. Named assays can be stored in the object using assays(), as well as dimensionality reduction data using reducedDim(). The experiment object can be serialized and stored on-disk for ease of transfer or later analysis.

**Data exporting:** Multiple common sequencing file types can be output with this package, including full compatibility with BedGraph, metilene, bigWig, and BSSeq. There is partial compatibility with Seurat, but as it is designed more for coverage data from RNA-seq experiments, there is limited functionality available for downstream analysis of scMethrix objects. The scMethrix object can also be used directly with any package that supports SingleCellExperiment objects (e.g., scater93 or scran94, though like Seurat, these are designed primarily for RNA-seq data).

### Analysis

**Workflow:** A interactive HTML summary report can be generated for an initial verification of successful file import (see Supplemental Method 1). Then, numerous quality control and analysis modules are available to (1) verify data quality, (2) select relevant features, (3) reduce data, and (4) generate and visualize relationships between samples (Figure 3).

**Quality control:** As described above, sparsity is a key challenge for single cell methylome data. Low coverage CpG sites may be unreliable and can be masked by sample count or by coverage (if provided). Low variance of CpG beta values can indicate homogeneous methylation, which may not be useful for downstream analysis, and can be masked as well. The mask\_scMethrix() function is flexible for many different types of statistical filtering by either samples or CpGs. Masked sites can be removed via remove\_uncovered().

**Feature selection:** Descriptive statistics can be generated to help identify usefulfeatures and samples by get\_region\_summary(), get\_rowdata\_stats(), and get\_coldata\_stats(). Genomic regions can be subset by sample, chromosome, or region. The GenomicRanges datatype allows easy generation of genomic windows as well as range-based set operations to isolate the target features.

**Binning:** There are multiple options to customize binning windows. Binning can be done as whole genome or by regions, and within these, windows can be specified by number of base pairs or CpG sites. By default, the binning will calculate the mean of the region, except for the coverage matrix, in which the sum count will be taken. The transformation used in binning is user customizable by assay. The list of CpGs binned in each region can optionally be stored in rowData().

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| figure2 |
| Figure 2. Structure of the scMethrix class.  Multiple containers are present in the scMethrix object to store data from single cell methylation experiments. Genomic loci for CpGs are stored as GenomicRanges in rowData(). The rows and columns of each assay() represent each CpG and sample, respectively. Sample information is stored as a data.frame in colData(), as is the dimensionality reduction data stored in reducedDims(). The green and blue colored bands among each component indicates CpGs and samples, respectively. For synchronicity, each CpG and sample in assays must have a corresponding match among the row and column metadata. |

**Collapsing:** Samples can be collapsed via a specified colData() column. Typically, this column would be given by the user during read\_beds() from some type of external sorting (e.g., from a cell identification assay) or after clustering (see below). Like binning, mean or sum is used by default for calculations, but an arbitrary function can also be used. As well, colData() can capture the names of samples in each collapsed group.

**Imputation:** Due to the sparsity of single cell data, imputation is typically used to fill the gaps after binning or collapsing. This package contains three methods of imputation: k-nearest-neighbor (kNN; via impute95), iterative principal component analysis (iPCA; via missMDA96), and random forest (RF; via missForest97). Other imputation functions can be used as well via an arbitrary input function. Imputation can be performed either by whole genome or by regions, but this should be done with the assumption of quasi-independence of those regions.

**Clustering:** Before clustering, multiple distance metrics are available to create the distance matrix: Euclidean, Manhattan, Canberra, binary, and Minkowski (via base R), as well as Pearson, Spearman, and Kendall (via biodist98). Using this metric, clusters can be generated via hierarchy or partitions (via base R), as well as model-based clustering (via mclust99). Other clustering algorithms and distance metrics can be used via arbitrary function.

**Dimensionality reduction:** For plotting and clustering visualization, dimensionality must be reduced. Using the dim\_red\_scMethrix() function, reduction can be done by PCA (stats), uniform manifold approximation and projection (UMAP; via umap100), or t-distributed stochastic neighbor embedding (tSNE; via tsne101). The number of CpGs to use can be either by highest variance or randomly chosen. This data is stored in the experiment object for later plotting under reduced\_dims().

**Transformation:** Further operations to each data point can be done directly on assays. A new assay will be generated via some arbitrary transformation function, such as the included binarize() function, and stored in the experiment object. Unneeded assays, excluding the score matrix, can easily be removed from the scMethrix object.

**Visualization:** Using ggplot2102, dataset characteristics can be plotted, including β-value/coverage (plot\_violin(), plot\_density(), plot\_coverage()) and quality control (plot\_sparsity(), plot\_stats()) as well as dimensionality reduction results, if performed previously (plot\_dim\_red()). Shape or color of data points can be specified using colData() derived from clustering or previous user input.

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| figure3 |
| Figure 3. Workflow for analyzing single-cell data with scMethrix.  This describe the basic workflow progress |

### Benchmarking

Batch processing:

**Parallelization:** Many of the functions used in scMethrix can utilize multiple cores. This can provide a substantial increase in performance at the cost of memory usage. Through the parallel103 and doParallel R packages104, both Windows and Unix-based systems are supported. See sample runtimes in Supplemental Method 1.

## Data collection

### Raw data

**Datasets:** Methylationdata files were obtained from the Gene Expression Omnibus (GEO). This includes immune cell types, including monocyte, neutrophil, natural killer (NK) cells, eosinophils (GSE35069105, GSE49618106, GSE49667107, GSE88824108, GSE166844109); dendritic cells (GSE8345831); brain-specific cells, glia and neuron (GSE66351110), microglia67 and microglia-like (GSE121483111), and glioma-related immune cells (GSE151506112). Neoplastic cells were also obtained, including glioma (GSE152035113, GSE137845114) and glioblastoma (IDH-WT and IDH-mut; GSE151506112). For lineage analysis, precursors to immune cells were also obtained, including hematopoietic stem cells (HSC; from bone and blood), multipotent progenitors (MPP; from bone and blood), common lymphoid progenitors (CMP), granulocyte-monocyte progenitors (GMP), common myeloid progenitors (CMP), and common monocyte progenitors (cMOP) from GSE49618106, GSE63409115, GSE8719630, GSE121483111. Data for microglia and extended data for GSE151506 and was obtained directly from the respective authors. For datasets where cell types are identified only by protein markers, archetypal cell type was used instead (e.g., CD45+ representing NK cells116). As well, for studies investigating methylation and disease (e.g., GSE66351 with Alzheimer’s disease), only the data from control subjects was taken. See Supplementary Table S1 for full breakdown of cell types and sample sizes.

**Annotation:** Identification data for Illumina 450k/EPIC methylation array probes was obtained via Bioconductor117,118, then converted to genomic positions via Minfi119. Probes that have previously shown to be cross-reactive, low-quality genomic mapping, or target polymorphic CpGs were removed120. Array data and bisulfite sequencing show correlation coefficients ranging from 0.95 to 0.9776, so are suitable for comparison. For data sourced from mouse models (GSE121483), only the 19,420 probes conserved with humans were kept121, reasoning that CpG methylation for neuronal cells is highly conserved (> 85%) between mice and humans122. All genome assemblies for sample data and array probes were translated to Genome Reference Consortium Human Build 38 (hg38123) by the liftOver124.

### Data processing

**Data importation:** For array-based data, datasets were generally provided as raw 450K/EPIC Illumina Methylation array (.idat) files or scraped from Series Matrix Files or SOFT family files associated with the GEO accession. This data was processed, quality control (QC) checked, and normalized by the single sample noob function in Minfi125, then converted into 450k arrays. Some datasets were previously processed and used directly (see Supplementary Table S1 for processing details).

**Data filtering:** All array-based samples were subset to include only 450k probes using 6 bp windows around each probe to account for differences due to 0- and 1-based genomic coordinates, strand sense offset, and conversion between hg19 and hg38 reference genomes. Probes common to less than 5% cells or showed homogenous methylation (beta value SD < 0.05) were removed. Samples with less than 50,000 identified CpGs were filtered out (excluding RRBS samples). RRBS tumor samples in GSE151506 were collapsed by patient into average methylation per CpG. To bridge RRBS and array-based data, a window of 1000 bp was defined for each probe, and overlapping windows were combined and tiled into a minimum number of 1000 bp windows. This resulted in 207,623 disjoint windows, and sample methylation values were calculated from the mean of all overlapping sites in each widow.

### Cell deconvolution

To verify annotation accuracy, most samples were checked against known reference samples

**Immune cells:** For .idat files,immune cell type in blood-derived samples were pre-screened by regression calibration during importation via Minfi::estimateCellCounts().

**Neuronal cells:** For cells collected from the brain, neuronal and non-neuronal cells were separated by regression calibration via Minfi::estimateCellCounts(). Samples that did not align to their expected cell type were excluded.

**Tumor cell separation:** Glioma cells were identified by glioma-specific CpG probes taken from by a previous TCGA bulk DNA methylation study126. A 1000 bp window was generated for each probe, described above. Classification of tumor subtypes was performed using DKFZ classifier calibrated scores127.

**Signature Matrix:** A reference tumor microenvironment signature matrix for the brain was generated using sixteen different cell types (n = 232; Supplementary Table S1): B‑cells, CD4+ T‑cells, CD8+ T‑cells, dendritic cells, endothelial cells, eosinophils, glial cells, glioma tumor cells, granulocytes, microglia, monocytes, neurons, neutrophils, NK cells, Treg cells, and whole blood. The missing values in the 1000 bp windows, described above, were imputed by kNN with k = 10. Then, to find cell-specific signatures, methylCIBERSORT was used for deconvolution128. To identify features, a pair-wise comparison was done between each cell type using a modified version of methylCIBERSORT::FeatureSelect.V4 to select the 100 top pairwise features using a median β-value difference of 0.2 and false discovery rate of 0.01. This yielded XXX features.

**Feature Analysis:** For each discovered feature, the genes overlapping with the respective feature window were collected. The molecular function for each gene was determined by cross-referencing the Gene Ontology (GO) project129.

For feature selection, we used a modified function of FeatureSelect.V4 as described in Williamson et al.

This was generated using the 100 top pairwise features

### Lineage reconstruction

Immune, tumor, and brain cells were

**Signature Validation:** Deconvolution was verified against known proportions of immune (GSE110554, GSE112618) and neuronal cells (GSE41826) .

Immune cells 18 mixed genomic samples of known quantities (GSE110554, GSE112618).

Neuron and glial cells

# Results

## Reference methylomes

### Array-based

Methylation arrays showed

Dendritic cells pretty much only in brain during neuroinflammation

# Discussion

# Future Outlook

# Conclusion

# Data Availability

Extended documentation and vignettes for *scMethrix* are also available on Github (<https://github.com/CompEpigen/scMethrix/>). The datasets presented in this study can be found in online open-source repositories. The names of the repository/repositories and accession number(s) can be found in the [Methods](#_Data_collection) section or in Supplementary Table S1. Scripts for data import and analysis are available on Github (<https://github.com/knacko/monobrainDNAme>).

# Supplementary Data

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| **Supplementary Figure 1. Runtime of select functions in single- and multi-threaded configuration.**  The workflow is described in Supplementary Method 1. The workstation was equipped with a 2.9 GHz AMD Ryzen 7 4800H processor, 2x16GB of PC4 25600 RAM, and HDF5 data was stored on a 150,000 IOPS solid state drive. |

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| Supplementary Table 1. Cell types obtained from the Gene Expression Omnibus (GEO) accessions included in the study. |

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| **Supplementary Method 1. Condensed workflow for scMethrix.**  This workflow uses data from GEO accession GSE |

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