Workshop 6: DNA Methylation Analysis using Bisulfite Sequencing

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Workshop 6 Outline

Day 1:

Introduction to DNA methylation & WGBS
Quick review of linux, Hoffman2 and high-throughput sequencing glossary.
Aligning WGBS reads using bwa-meth

Day 2:

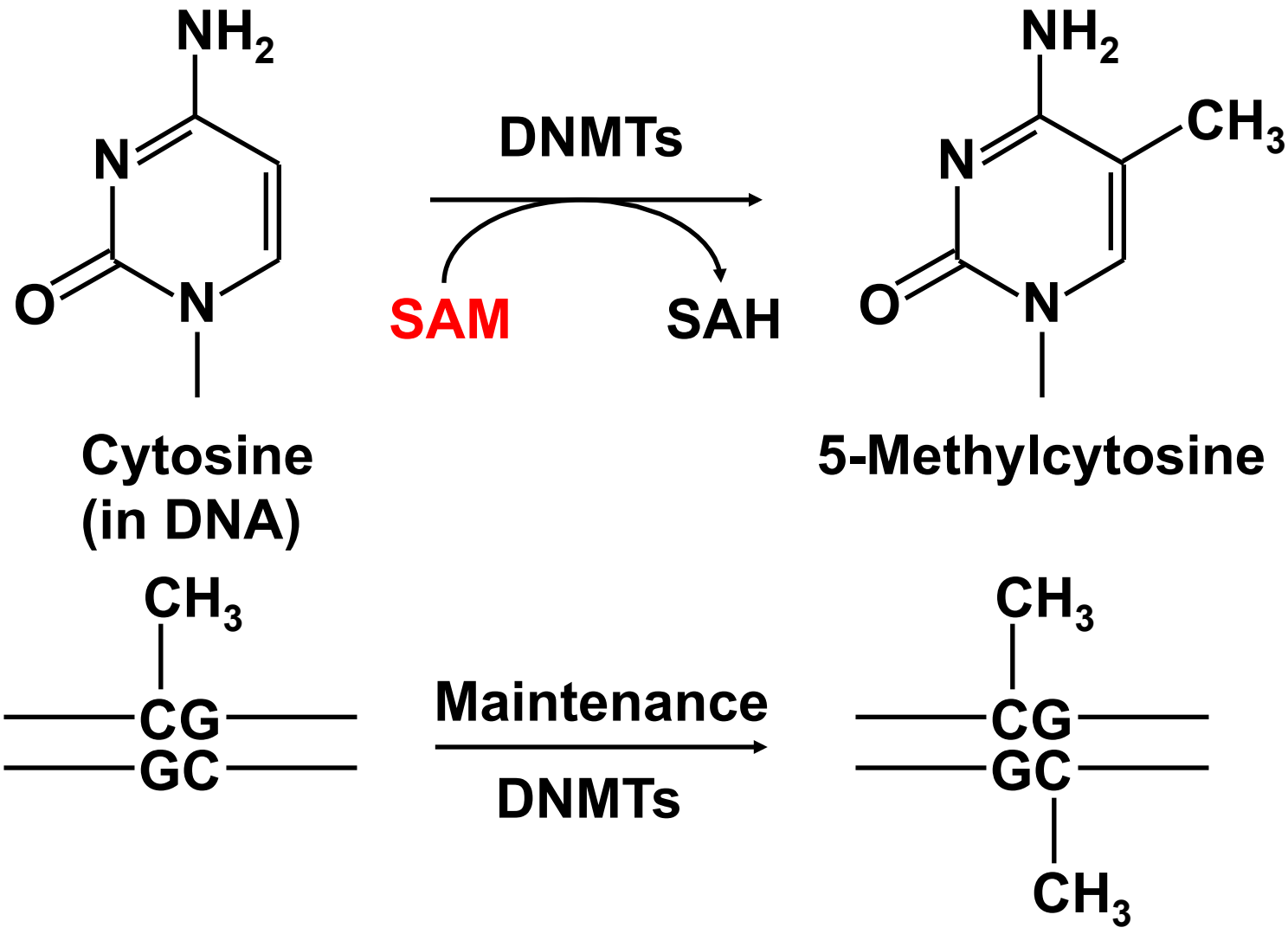
DNA methylation calling using Bis-SNP
Analysis of differentially methylated regions (DMRs) using metilene

Day 3:

Visualization of DNA methylation data
WGBS analysis using BS-Seeker2

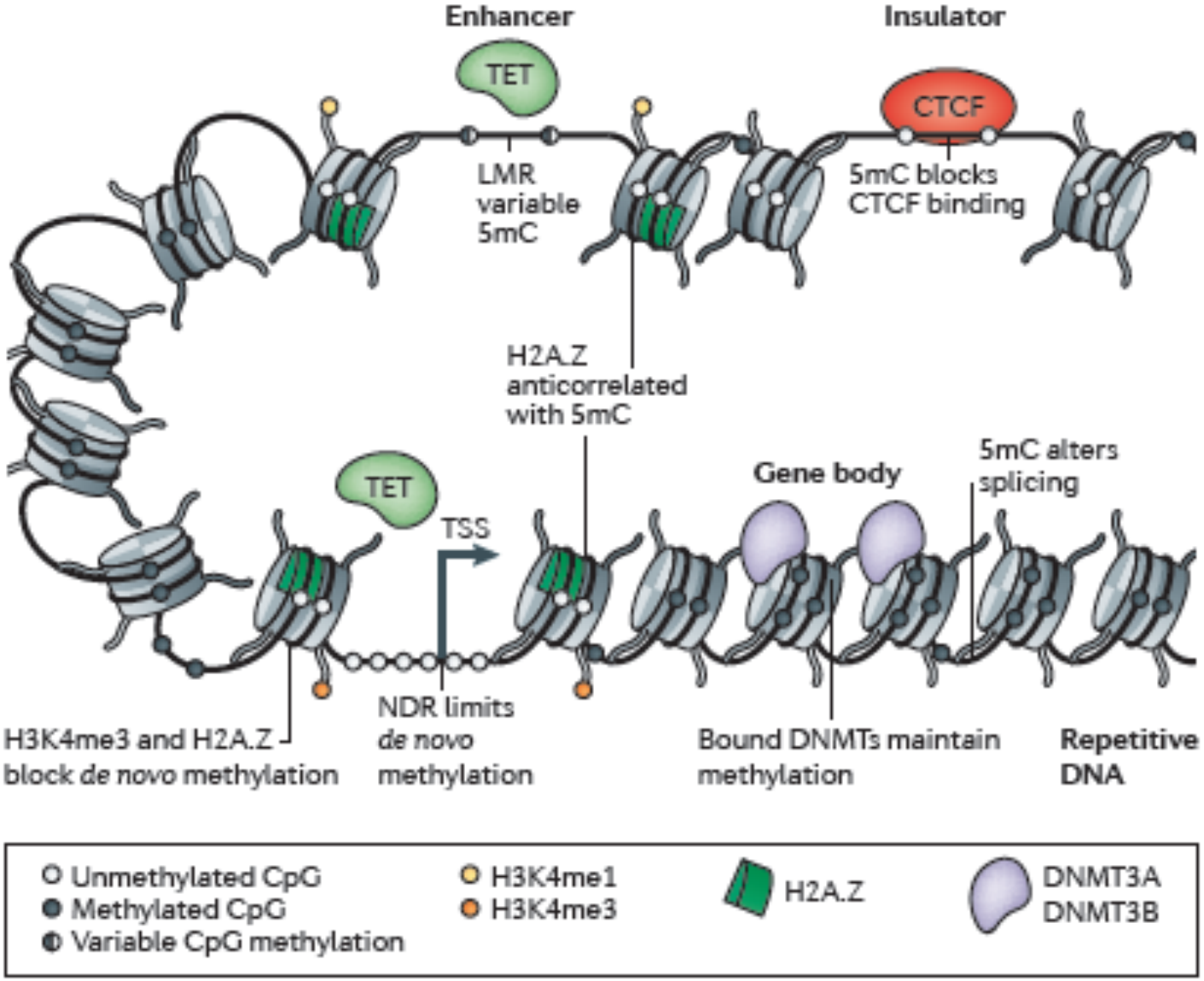
Day 1

DNA Methylation



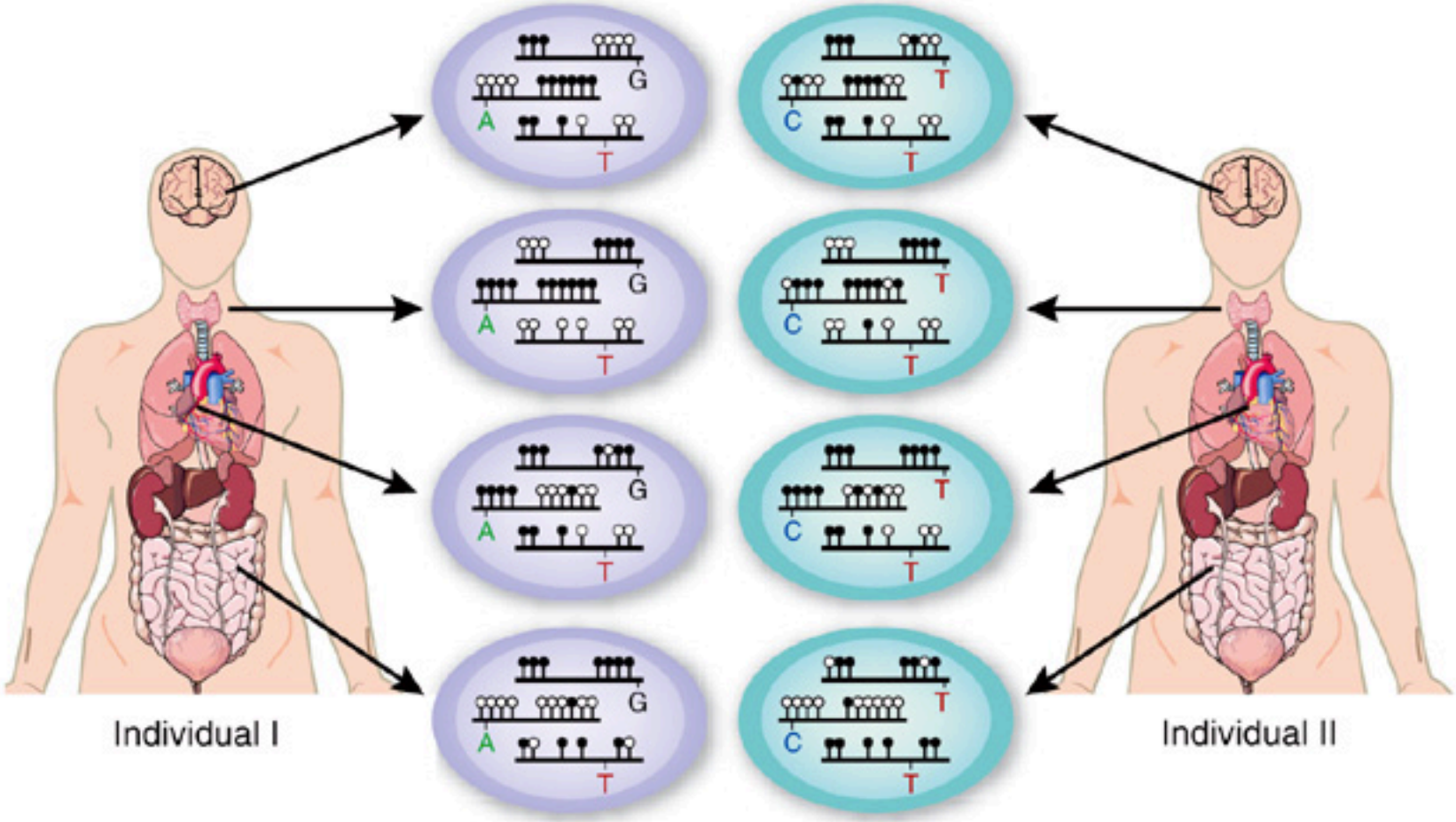
Why Study DNA Methylation?

DNA Methylation: A Component of Epigenetic Mechanisms



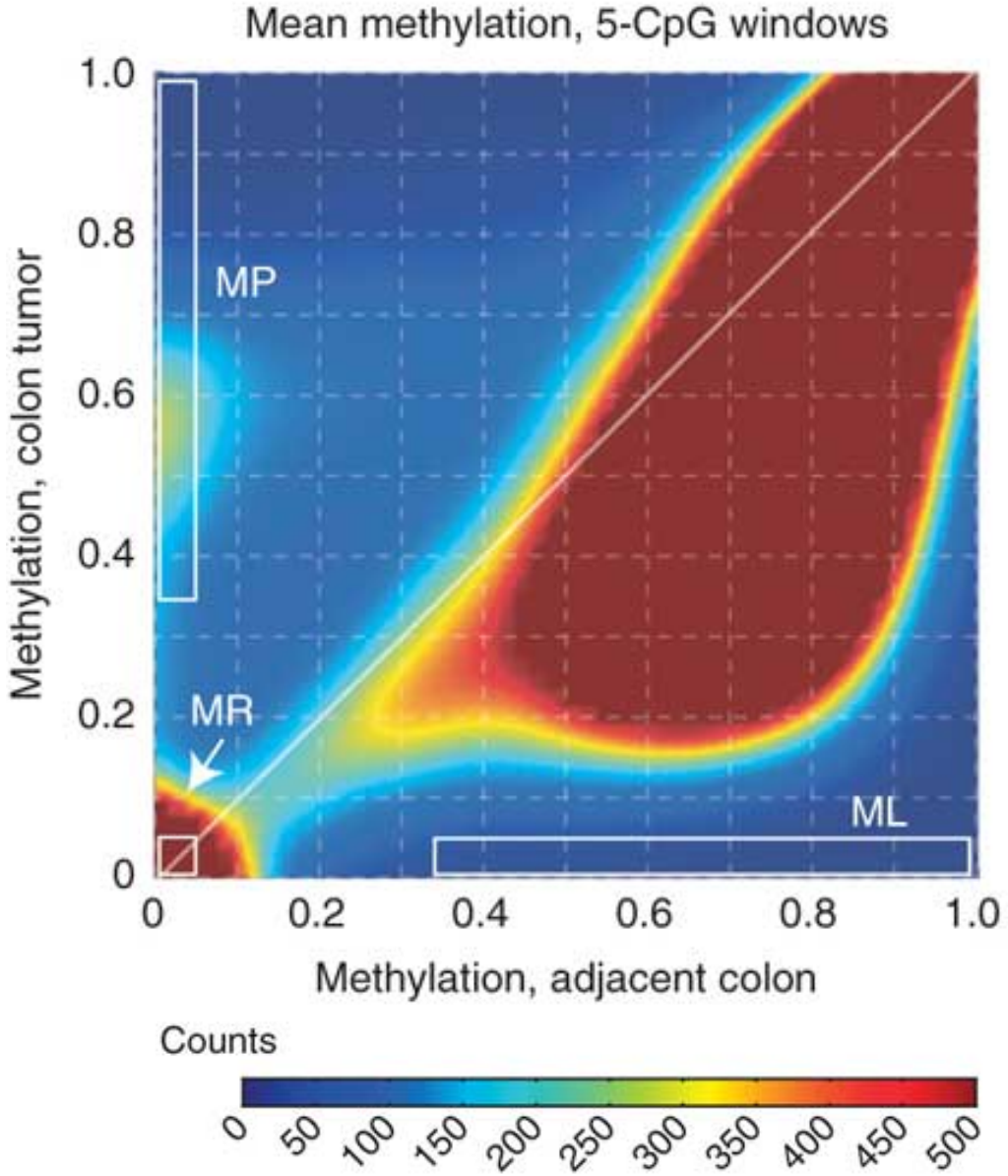
Jones, 2012

DNA Methylation Patterns are Unique to Each Cell Type and May Vary Across Individuals



Caesar, K, 2006

DNA Methylation Alteration is a Feature of Cancer Cells



Berman BP et al, 2012

What are the functions of DNA Methylation?

REVIEWS

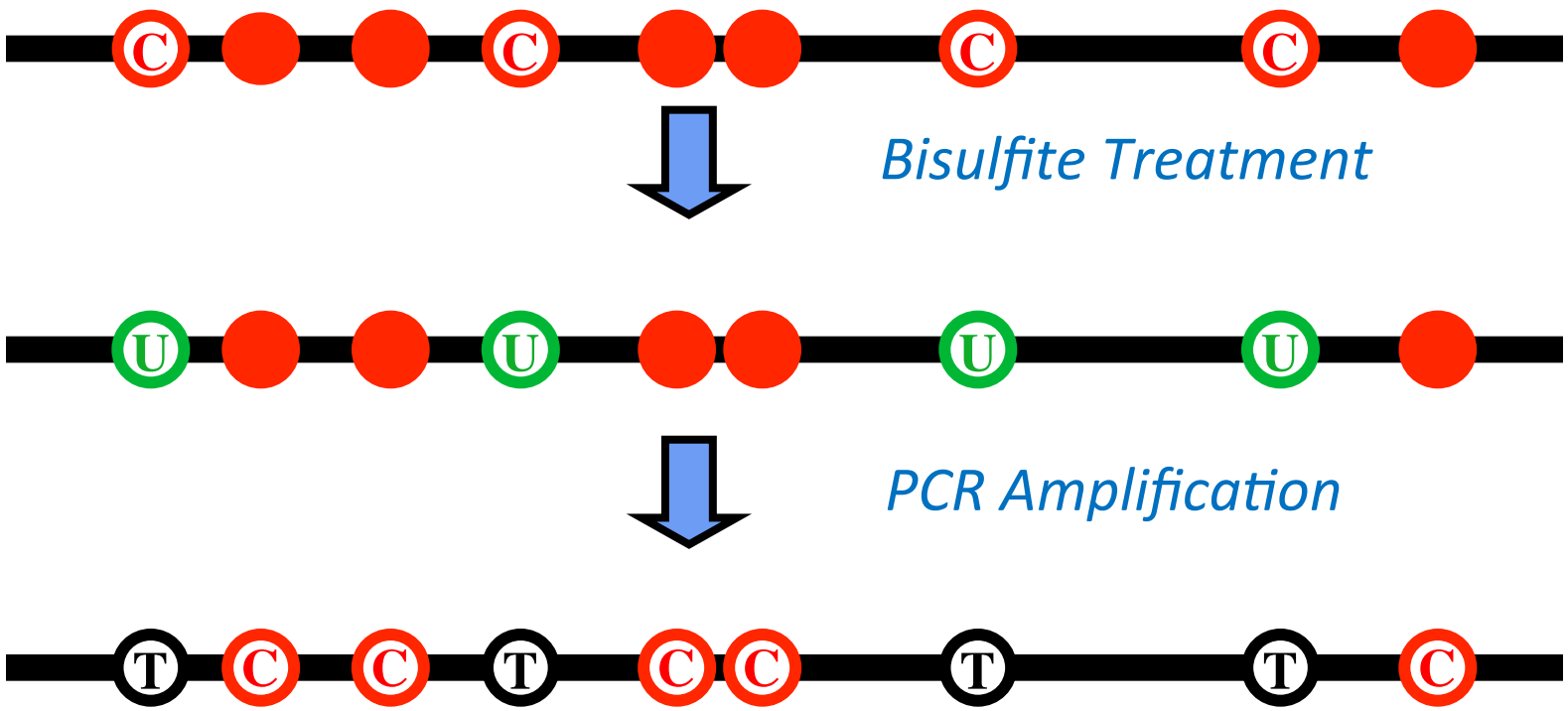
Functions of DNA methylation: islands, start sites, gene bodies and beyond

Peter A. Jones

Abstract | DNA methylation is frequently described as a 'silencing' epigenetic mark, and indeed this function of 5-methylcytosine was originally proposed in the 1970s. Now, thanks to improved genome-scale mapping of methylation, we can evaluate DNA methylation in different genomic contexts: transcriptional start sites with or without CpG islands, in gene bodies, at regulatory elements and at repeat sequences. The emerging picture is that the function of DNA methylation seems to vary with context, and the relationship between DNA methylation and transcription is more nuanced than we realized at first. Improving our understanding of the functions of DNA methylation is necessary for interpreting changes in this mark that are observed in diseases such as cancer.

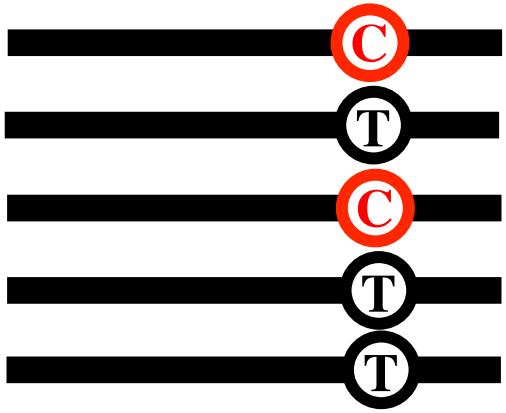
Jones, 2012

Bisulfite Sequencing



Measuring DNA Methylation

READS/ AMPLICONS



C Methylated (M)

T Unmethylated (U)

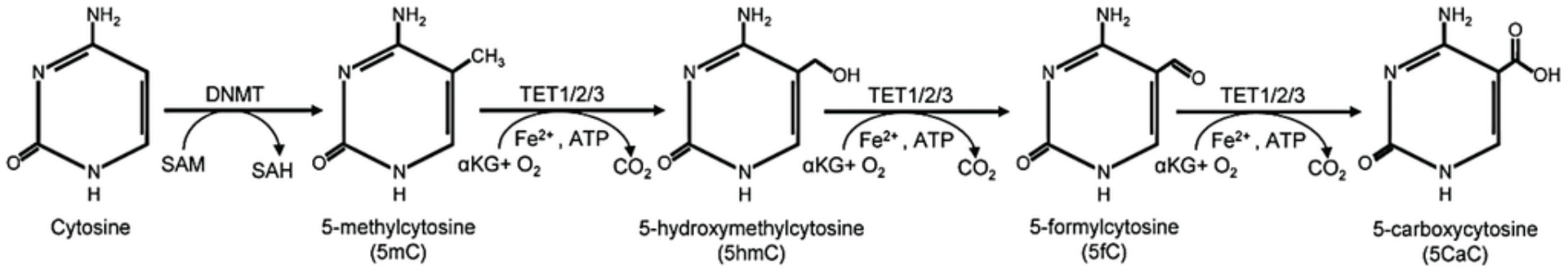
$$\%\text{Methylation} = \frac{M}{M + U} \times 100$$

In the above example, the methylation level of the locus is 40%

Bisulfite Sequencing-Based Assays

- **Whole-genome Bisulfite Sequencing (WGBS)**
  - ✧Unbiased (ie. whole genome)
  - ✧Undigested template
- **Reduced Representation Bisulfite Sequencing (RRBS)**
  - ✧Enrich for CpG islands area (CGI) which accounts for ~1% of the genome
  - ✧Digestion with methylation insensitive restriction enzyme (ie.MspI)
- **Nucleosome Occupancy and Methylome Sequencing (NOMe-seq)**
  - ✧Dual measurement of nucleosome occupancy and DNA methylation levels
  - ✧Treated with GpC Methyltransferase to footprint nucleosome occupancy
- **Chromatin Immunoprecipitation Bisulfite Sequencing (ChIP-BS-seq)**
  - ✧ Measurement of DNA methylation at regions enriched for specific protein or histone marks.

Oxidized Derivatives of 5-MethylCytosine

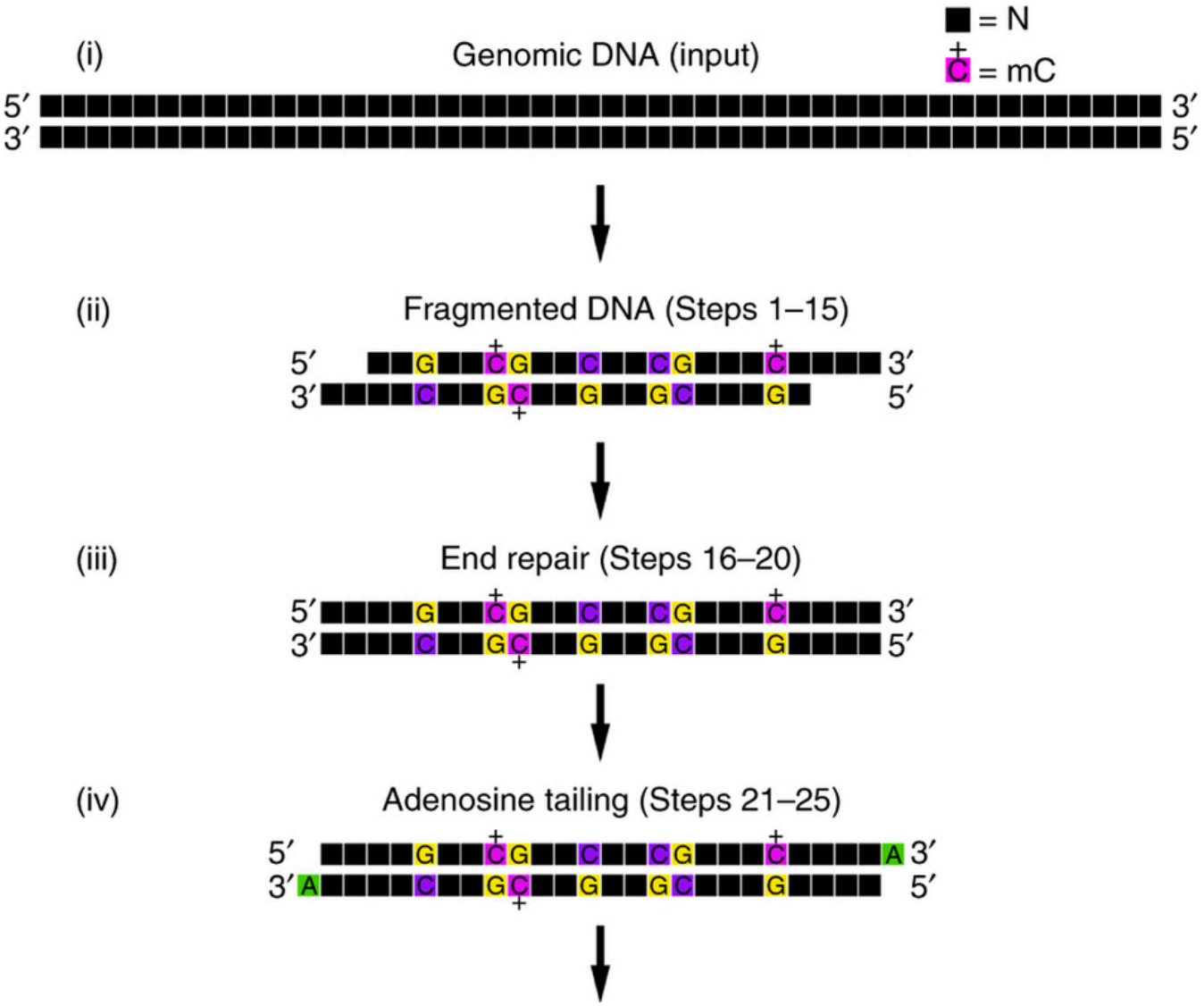


Advances in the profiling of DNA modifications: cytosine methylation and beyond

Nongluk Plongthongkum, Dinh H. Diep* and Kun Zhang*

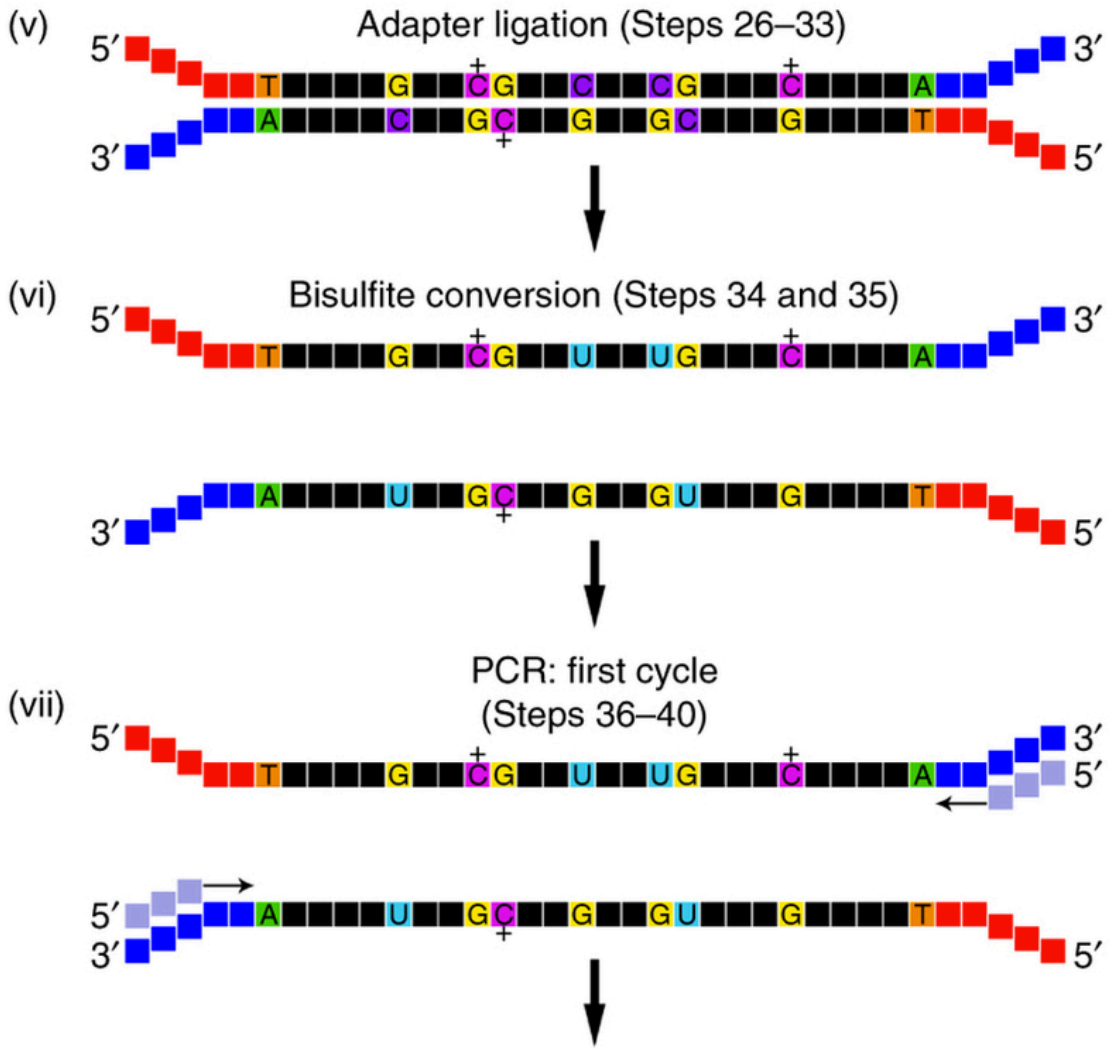
Abstract | Chemical modifications of DNA have been recognized as key epigenetic mechanisms for maintenance of the cellular state and memory. Such DNA modifications include canonical 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC). Recent advances in detection and quantification of DNA modifications have enabled epigenetic variation to be connected to phenotypic consequences on an unprecedented scale. These methods may use chemical or enzymatic DNA treatment, may be targeted or non-targeted and may utilize array-based hybridization or sequencing. Key considerations in the choice of assay are cost, minimum sample input requirements, accuracy and throughput. This Review discusses the principles behind recently developed techniques, compares their respective strengths and limitations and provides general guidelines for selecting appropriate methods for specific experimental contexts.

WGBS Library Preparation



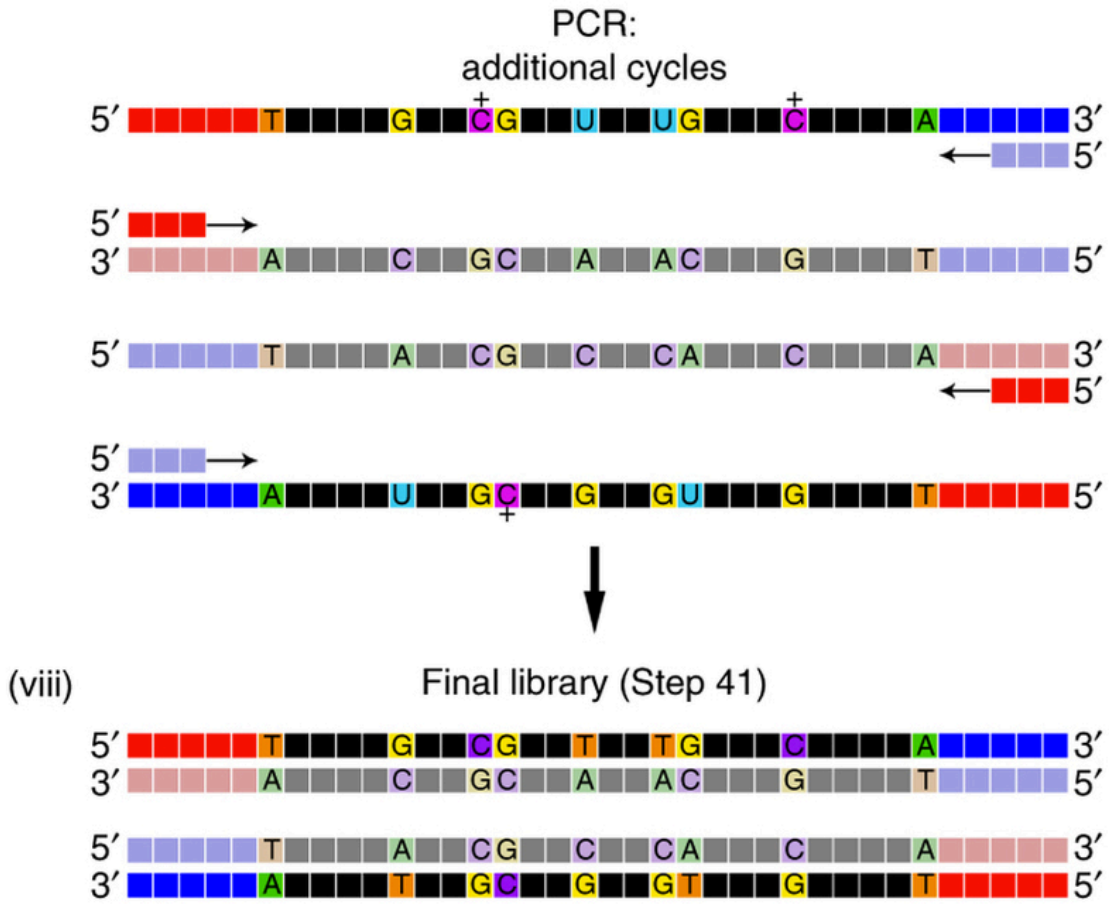
Urich et al, Nature Protocols 2015

WGBS Library Preparation



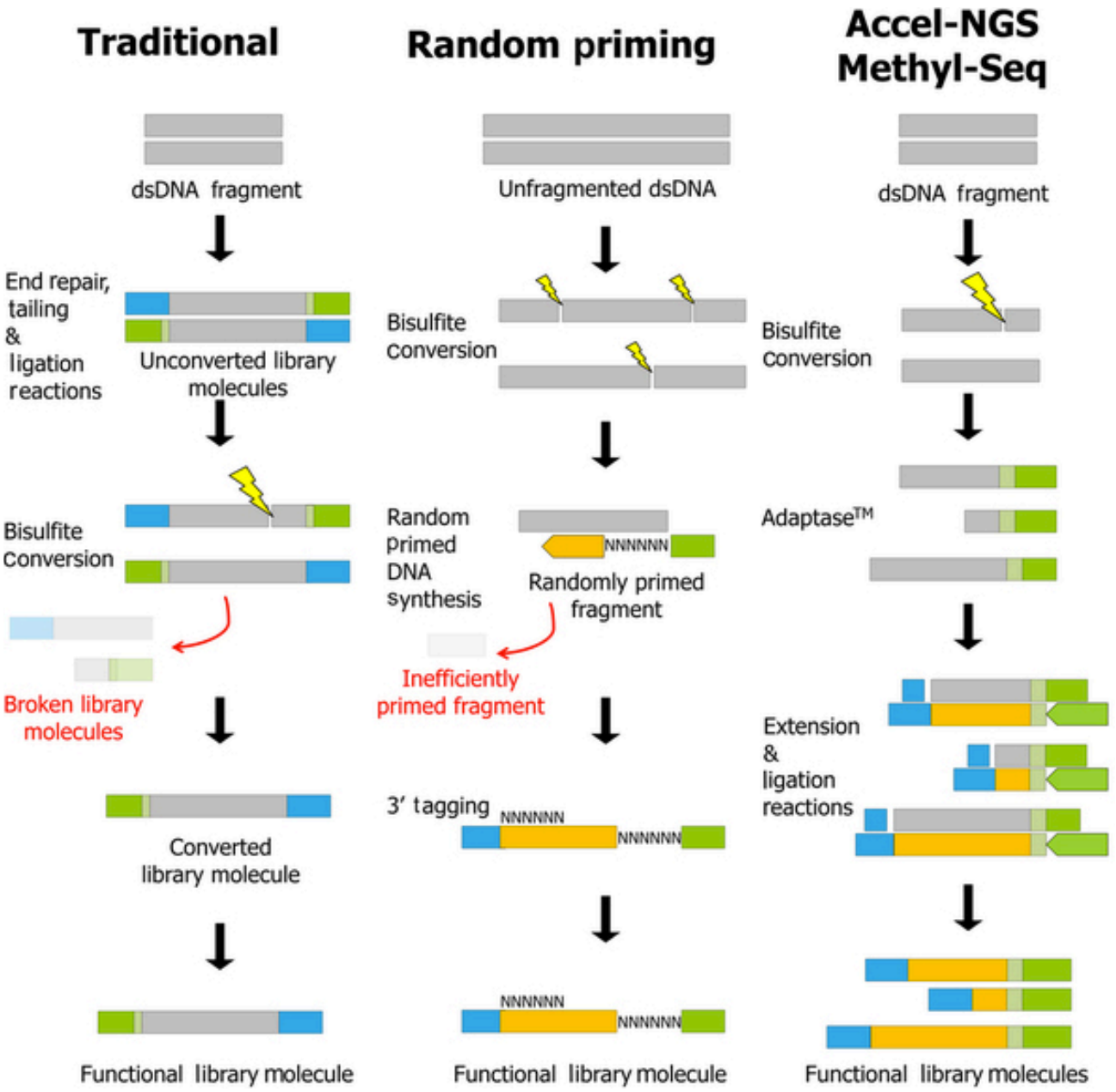
Urich et al, Nature Protocols 2015

WGBS Library Preparation



Urich et al, Nature Protocols 2015

Post-Bisulfite Adapter Tagging



Study Design

What coverage do you need?

- Generally depends on the magnitude of methylation difference to detect: the larger the difference, the lower coverage needed.
- REMC recommendation: 30x (~800M reads, 100PE)
- Data driven: 5-15x (<400M reads,

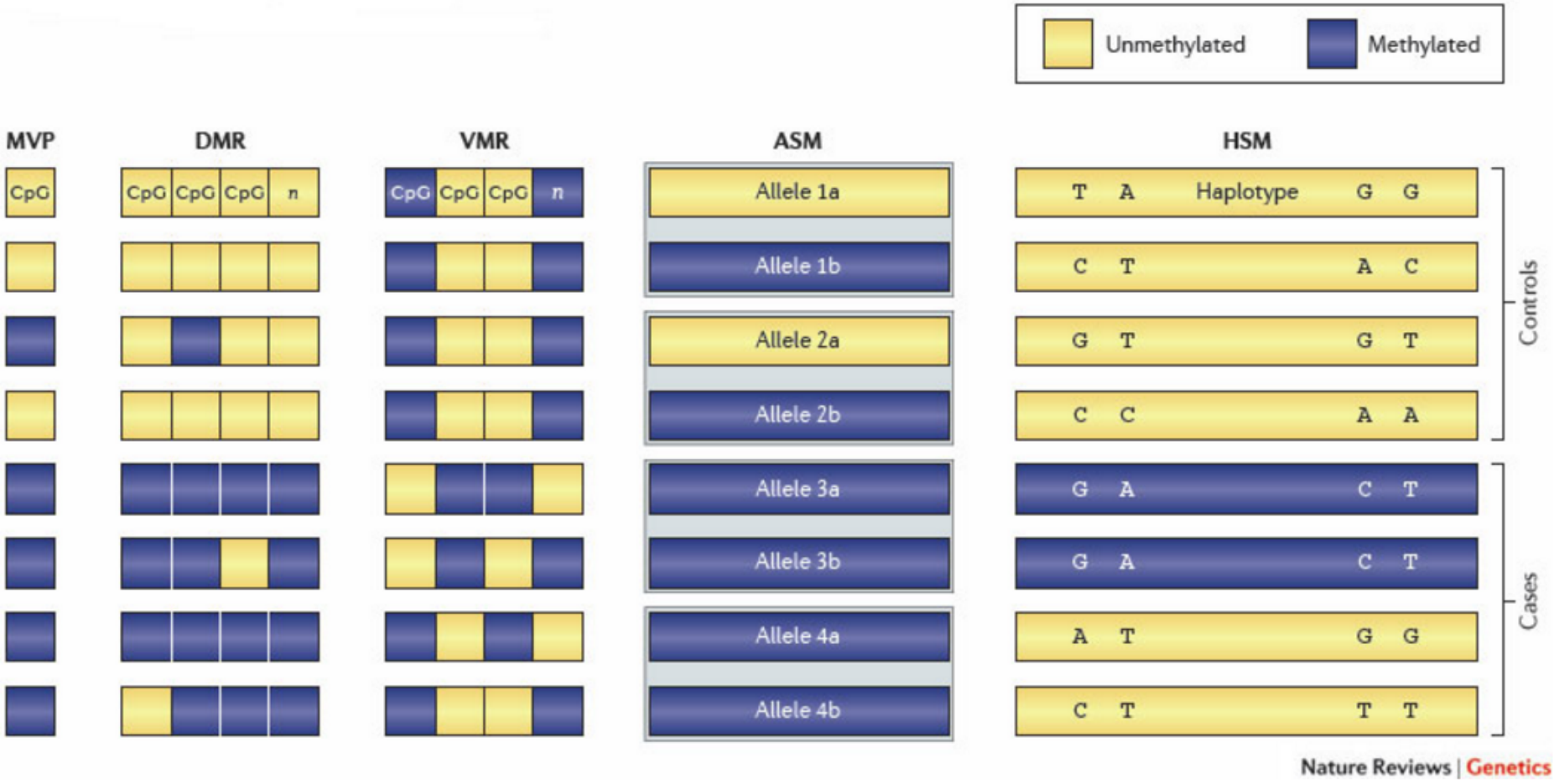
How many replicates?

- REMC: 2 biological reps
- Data driven: 3 biological reps (2 acceptable as minimal drop off in sensitivity)

REMC 2011

Ziller et al Nature Methods 2015

Features and Variation of DNA Methylation



Rakyan et al 2011

Features and Variation of DNA Methylation

Methylation variable position (MVP). A CpG site that shows differential methylation — for example, between different disease states, as illustrated in the figure. Given recent findings on non-CpG methylation, potentially all Cs could be MVPs.

Differentially methylated region (DMR). A region of the genome at which multiple adjacent CpG sites show differential methylation. DMRs can occur in many different contexts, such as:

- iDMR — imprinting-specific differentially methylated region
- tDMR — tissue-specific differentially methylated region
- rDMR — reprogramming-specific differentially methylated region
- cDMR — cancer-specific differentially methylated region
- aDMR — ageing-specific differentially methylated region.

Variably methylated region (VMR). These regions are defined by increased variability rather than gain or loss of DNAm.

Allele-specific methylation (ASM). These are positions or regions that vary in DNAm depending on the parent-of-origin, the presence of a polymorphism or as a result of a stochastic event.

Haplotype-specific methylation (HSM). This is a differentially methylated region that is defined by a set of co-inherited SNPs (a haplotype).

CpG islands (CGIs). These are regions enriched for CpG sites. Most CGIs are unmethylated in all cell types.

CGI shores. These are regions immediately adjacent to CGIs and display higher variation in DNAm than CGIs despite their lower density of CpG sites.

The figure shows different types of DNAm variation that can be identified with epigenome-wide association studies. The notation *n* is used to indicate the variable size of the regions shown. For the purpose of this simplified illustration, the cases and controls are assumed to have methylated or unmethylated CpG states only. Real samples will contain populations of different cells and hence display much more heterogeneous methylation levels across the full dynamic range between 0% and 100%.

Rakyan et al 2011

Various File Format: Covered in Workshop 2

- Fastq: text-based format containing sequencing reads and quality scores

```
[flay@login2 raw]$ head K562_R1.fastq
@DDLZ38V1:356:C33YCACXX:2:1101:1168:2186 1:N:0:GCGCTA
TTTTGTGAATATTGTTATATAATAAGATTAAATTTTAAGTTTATTTTTTGATTTTTTATTTTTATAGTTATGAAG
+
@@@DDDAB<D4CF<FH@G>IICI>>@@HHIC<FHEH4:9EFI4<FHIIIGBFHGGEI;CHCGH=G@=DGCE@=CA
@DDLZ38V1:356:C33YCACXX:2:1101:1359:2177 1:N:0:GCGCTA
AAATTTTGTTTTTATTAAAAATATAAAAATTAGATGGGTTTGGTGGTAAGTGTTTGTAATTTTAGTTATTTGGGA
+
CC@FFFFFHHHHHJJJJJJJIIJJJJIIHIJHIHGIJJFGHII:DHFHHHIJJJIJHHHGIIIGIHICGHJIGHE
@DDLZ38V1:356:C33YCACXX:2:1101:1894:2198 1:N:0:GCGCTA
AGGTTTTTTTGAGATATTTGGTAATAATGATTAAAATGTTTTTTTTTTAAAGGAGGTTTATAATTGATTTATTTA
```

- SAM/BAM: binary format of aligned reads

Various File Format: Covered in Workshop 2

- VCF: Variant Call Format, text file containing meta-information lines, header lines and data lines containing genotype info at each position

```
##fileformat=VCFv4.2
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=file:///seq/references/1000GenomesPilot-NCBI36.fasta
##contig=<ID=20,length=62435964,assembly=B36,md5=f126cdf8a6e0c7f379d618ff66beb2da,species="Homo sapiens",taxonomy=x>
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Total Depth">
##INFO=<ID=AF,Number=A,Type=Float,Description="Allele Frequency">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=DB,Number=0,Type=Flag,Description="dbSNP membership, build 129">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FILTER=<ID=q10,Description="Quality below 10">
##FILTER=<ID=s50,Description="Less than 50% of samples have data">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
#CHROM POS     ID        REF    ALT     QUAL FILTER INFO                              FORMAT      NA00001        NA00002        NA00003
20     14370   rs6054257 G      A       29   PASS   NS=3;DP=14;AF=0.5;DB;H2           GT:GQ:DP:HQ 0|0:48:1:51,51 1|0:48:8:51,51 1/1:43:5:.,.
20     17330   .         T      A       3    q10    NS=3;DP=11;AF=0.017               GT:GQ:DP:HQ 0|0:49:3:58,50 0|1:3:5:65,3   0/0:41:3
20     1110696 rs6040355 A      G,T     67   PASS   NS=2;DP=10;AF=0.333,0.667;AA=T;DB GT:GQ:DP:HQ 1|2:21:6:23,27 2|1:2:0:18,2   2/2:35:4
20     1230237 .         T      .       47   PASS   NS=3;DP=13;AA=T                   GT:GQ:DP:HQ 0|0:54:7:56,60 0|0:48:4:51,51 0/0:61:2
20     1234567 microsat1 GTC    G,GTCT  50   PASS   NS=3;DP=9;AA=G                    GT:GQ:DP    0/1:35:4       0/2:17:2       1/1:40:3
```

Various File Format: Covered in Workshop 2

- Bed: tab-delimited text format containing chrNum, chrStart, chrEnd (required), name, score, strand, etc (optional)

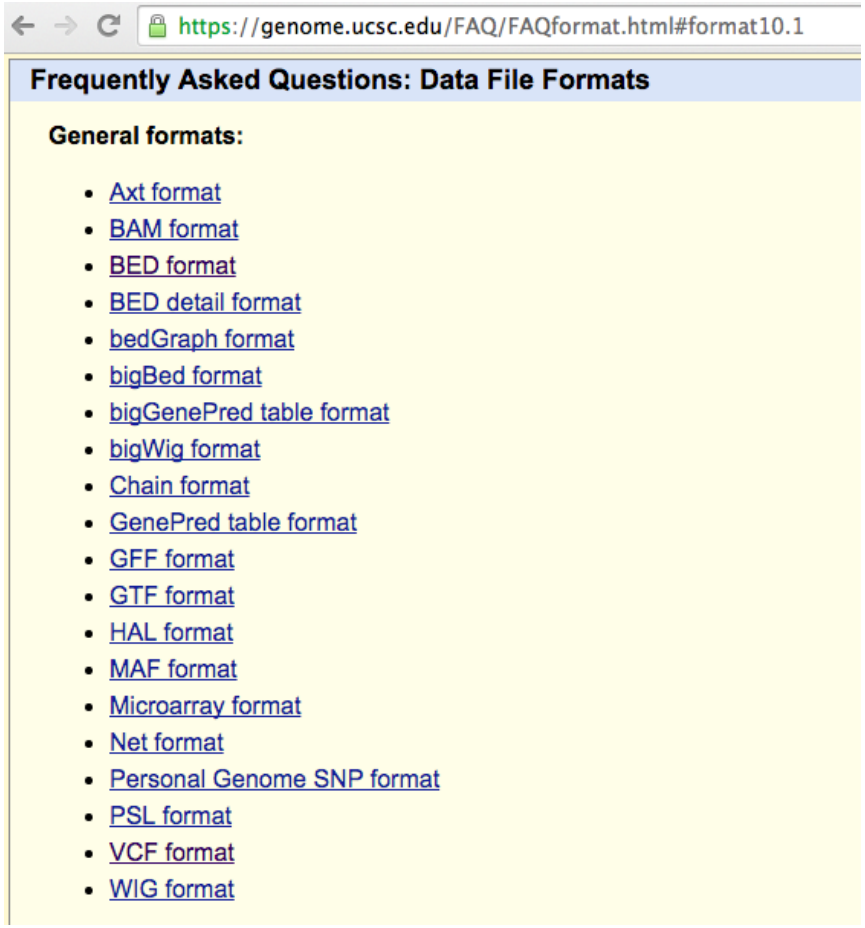
```
[flay@hpc-uec flay]$ head fdft1.bed
chr8    11666017        11666018        cg13963446      0       *
chr8    11665962        11665963        cg11598005      0       *
chr8    11665969        11665970        cg01402994      0       *
```

- Bedgraph: tab-delimited text format containing chrNum, chrStart, chrEnd and dataValue (required)

```
[flay@login2 bwameth]$ head test.bedgraph
chr1    847784  847784  0.0
chr1    1433528 1433528 100.0
chr1    1433539 1433539 100.0
chr1    1433574 1433574 100.0
chr1    1433638 1433638 100.0
chr1    1730072 1730072 100.0
chr1    1730163 1730163 100.0
chr1    1730204 1730204 100.0
chr1    1433677 1433677 100.0
chr1    3041726 3041726 0.0
```

Various File Format: Covered in Workshop 2

- Wig: a text file format defining continuous data track
- bigWig: condensed data track format for graphing and visualization



Useful UNIX Commands: Covered in Workshop 1 & 2

• Where am I?	pwd
• Change directory	cd cd ~/data
• Move up one level	cd ..
• List files in folder	ls
• Look at a file	less fileName
• Copy a file	cp ~/data/file ~/otherdir/
• Delete a file	rm fileName
• Delete a directory	rmdir ~/dirName/
• Move a file	mv ~/data/file ~/otherdir/file
• Secure copy	scp user@host1:dir/file user@host2:dir/file
• Compress a file	gzip –c file > file.gz
• Uncompress a file	gunzip file.gz
• Make a new folder	mkdir data2
• Current directory	./
• Home directory	~/
• Count lines in a file	wc –l fileName

Questions/In Doubt/Lost?

- Unix manual: man functionX
- Google is your best friend!
- Any of your friendly QCB fellows

General WGBS Workflow

Pre-alignment Processing (covered in Workshop 2&4)

- Demultiplexing
- Raw sequencing QC (FastQC)
- Adapter Trimming (TrimGalore)

Alignment

- Align FastQ files to reference genome and output .bam files
- Various tools: BS-Seeker2, BSMAP, Bismark, bwa-meth, etc

Post-alignment

- Methylation Calling (Bis-SNP, Bsmooth, etc)
- DMR identification(metilene, bisseq)
- Visualization (IGV, UCSC, IGB etc)

Publicly Available WGBS Aligners

Program	Version	Aligner type	Aligner description	Language	Alignment engine	Paired end	Color space	Non directional	Multithread	Reference
BatMeth	1.04b	Three letter	FM index of the C-to-T converted genome. Filtering step for low complexity reads with Shannon's entropy.	C, Perl	None	Yes	Yes	Yes	Yes	[11]
Bismark	v0.14.2	Three letter	FM index of the C-to-T converted genome. Mapping step taken into account basecall qualities.	Perl	Bowtie, Bowtie2	Yes	No	Yes	Yes	[12]
Bisulfighter	1.3	Wild-card	Spaced suffix array index of the original (i.e. unconverted) reference genome.	C/C++, Python, R	LAST	Yes	No	Yes	No	[13]
BRAT-BW	2.0.1	Three letter	FM index of the C-to-T converted genome. Multi-seeding starting from different positions within reads.	C++	None	Yes	No	Yes	Yes	[14]
BSMAP	2.74	Wild-card	Hash table of the original genome. Based on SOAP alignment algorithm.	C++, Python	None	Yes	No	Yes	Yes	[15]
BSmooth	0.8.1	Wild-card	FM-index, nucleotide base Y for C and T matches. Support for color-space read mapping.	Perl	Bowtie 2, Merman	Yes (with Bowtie2)	Yes (with Merman)	Yes	Yes	[16]
BS-Seeker	–	Three letter	FM index of the C-to-T converted genome. Only accepts reads in fixed length in FASTQ.	Python	Bowtie	No	No	Yes	No	[17]
BS-Seeker2	v2.0.9	Three letter	FM index of the C-to-T converted genome. Filtering step for the reads with incomplete bisulfite conversion.	Python	Bowtie, Bowtie2, SOAP, RMAP	Yes	No	Yes	Yes	[18]
B-SOLANA	1.0	Color-space	FM-index of the C-to-T converted genome. Support for color-space read mapping.	Python	Bowtie	No	Yes	No	Yes	[19]
GSNAP	2014-01-21	Wild-card	Hash table of the C-to-T converted genome. Uses wild-card letter Y for Cs and Ts in reads.	C, Perl	None	Yes	No	Yes	Yes	[20]
ERNE-BS5	2.1	Wild-card	Hashing the reference genome with 5-letter, A, T, G, Cu, Cm.	C++	None	Yes	Yes	No	Yes	[21]
LAST	548	Wild-card	Spaced suffix array index of the original genome. Modified score matrix for C-to-T and G-to-A matches.	C/C++, Python	None	Yes	No	Yes	Yes (requires GNU parallel)	[22]
MAQ	v0.6.6	Wild-card	Multiple hash tables of reads. Nonunique reads assigned randomly to one of the best-matching positions.	C/C++, Perl	None	Yes	Yes	No	No	[23]

Tsuji & Weng, 2015

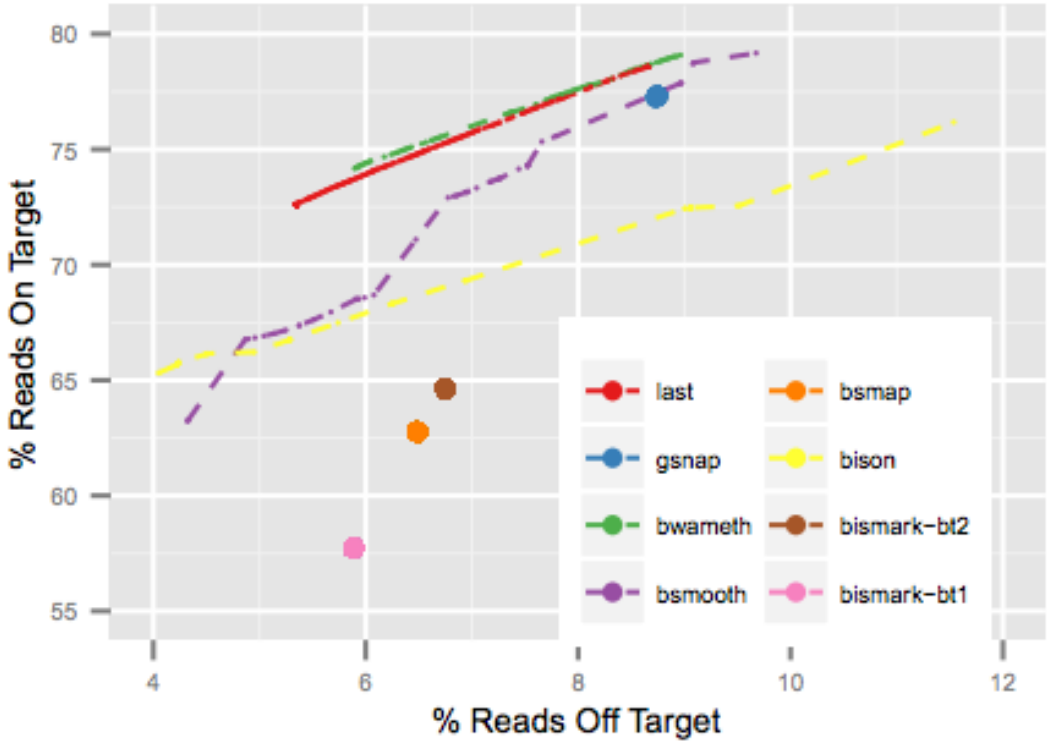
bwa-meth

Fast and accurate alignment of long bisulfite-seq reads

Brent S. Pedersen [1,*] Kenneth Eyring [1], Subhajyoti De [1,2], Ivana V. Yang [1] and David A. Schwartz [1]

[1]Department of Medicine, University of Colorado Denver, School of Medicine, Denver, Colorado, USA. 80045

[2]University of Colorado Cancer Center, Molecular Oncology Program, Aurora, Colorado, USA



bwa-meth

Advantages:

- Simplicity and ease of use: a single script for fastq alignment and methylation calling; wraps bwa mem option
- Speed: efficient bwa parallelization
- Memory usage: compressed or uncompressed input files, reads streamed directly into aligner and not written to disk, so lower memory requirement
- Useful output: sorted & indexed .bam files containing map-q score, read-group & alignment flags, compatible with picard tools and gatk for downstream processing
- Strand-specificity: increase accuracy
- Accuracy without trimming: due to improved bwa local alignment

Disadvantages:

- Only works for directional library preparation protocol

Logging into Hoffman2

ssh flay@hoffman2.idre.ucla.edu

change to your user ID

Logging into Hoffman2

flay@login3:~ — ssh — 139×38

```
Fides-Lays-MacBook-Pro:~ fideslay$ ssh flay@hoffman2.idre.ucla.edu
flay@hoffman2.idre.ucla.edu's password:
Last login: Wed Mar  2 10:03:31 2016 from 164.67.160.102
Welcome to the Hoffman2 Cluster!

Hoffman2 Home Page:     http://www.hoffman2.idre.ucla.edu
Consulting:             https://support.idre.ucla.edu/helpdesk

All login nodes should be accessed via "hoffman2.idre.ucla.edu".

Please do NOT compute on the login nodes.

Processes running on the login nodes which seriously degrade others'
use of the system may be terminated without warning.  Use qrsh to obtain
an interactive shell on a compute node for CPU or I/O intensive tasks.

The following news items are currently posted:

   Globus access changes February 13, 2016
   Mathematica upgraded to 10.3
   IDRE Winter 2016 HPC Classes
   News Archive On Web Site

Enter shownews to read the full text of a news item.
[flay@login3 ~]$
```

Useful Tools on Hoffman2

module available

```
[flay@login3 ~]$ module available

------------------------------------------------ /u/local/Modules/modulefiles ------------------------------------------------
ATS                                             gcc/4.3.5                                       netbeans/7.4
R/2.12.0                                        gcc/4.4(default)                                netbeans/8.0.2(default)
R/2.12.1                                        gcc/4.7.2                                       netcdf/4.1.3(default)
R/2.12.2                                        gcc/4.9.3                                       netcdf/4.1.3=shared
R/2.13.2                                        gcta/0.93.9                                     netcdf/4.1.3_1
R/2.15.1                                        gdal/1.9.2                                      netcdf/4.2.3=c
R/2.9.1                                         gdc/0.30                                        netcdf/4.4.2=fortran
R/3.0.1(default)                                gdc/4.9.3(default)                              ngsplot/2.47
R/3.1.1                                         geant4/10.00.p02_wGDML                          nwchem/6.5(default)
R/3.2.1                                         geant4/4.9.3.p02(default)                       octave/3.6.1
R/3.2.3                                         geant4/4.9.4.p02                                octave/3.6.4(default)
Rstudio/0.98(default)                           geant4/4.9.5                                    openbabel/2.3.1(default)
Rstudio/R-2.15.1                                geant4/9.6.2                                    openmpi/1.6_gcc=4.4
abaqus/6.11                                     geant4/9.6.p02_wGDML                            opensees/2.4.1
abaqus/6.12                                     geos/3.4.2(default)                             opensees/2.4.3
abaqus/6.13                                     gftp/2.0.19                                     opensees/2.4.4(default)
abaqus/6.14(default)                            ghc/7.6.3                                       opensees/2.4.4_parallel
activeperl/5.16(default)                        gmp/4.3.2                                       paraview/3.6.2(default)
affymetrix/apt-1.14.3(default)                  gnuplot/4.2.3                                   perl/5.10.1(default)
amber/14                                        gnuplot/4.4.4(default)                          pgi_compiler/15.3
amber-intel11.1-mvapich1.9a2/12                 gotoblas2/1.13_multi_threaded                   pgsql/9.1
annovar/v2011Jun18                              gotoblas2/1.13_single_threaded                  plink/1.08
annovar/v2011Oct02                              gpac/0.5.1                                      pop-c++/1.3(default)
annovar/v2014Jul14(default)                     grads/2.0.1(default)                            preseq/1.0.2
ansys/14.0                                      graphviz/2.28.0(default)                        proj/4.8.0
armadillo/4.600.4                               gromacs/4.6.5                                   pypy/1.9
bamtools/2.1.0(default)                         handbrake/0.10.0(default)                       pypy3/2.4.0
bcftools/1.2                                    handbrake/0.9.8                                 python/2.6(default)
bedtools/2.13.3                                 haskell/2013.2.0.0                              python/2.7
bedtools/2.17.0(default)                        hdf5/1.8.11_intel13.cs                          python/2.7.3
bedtools/2.23.0                                 hdf5/1.8.11_intel13.cs_intelmpi4.1.1            python/3.1
blat/35                                         hdf5/1.8.14_intel-13.1.1_intelmpi-4.1.1         python/3.4
boost/1_55_0(default)                           homer/4.7                                       qcachegrind/0.7.4
boost/1_59_0                                    hyperworks/11.0                                 qchem/3201s
bowtie/0.12.7                                   hyperworks/12.0(default)                        qchem/3202mpich
bowtie/0.12.8(default)                          idl/6.3                                         qchem/3202s
bowtie2/2.1.0                                   idl/8.1(default)                                qchem/4.3
bowtie2/2.2.5(default)                          idr/2010-10                                     qchem/4.3mpich(default)
bwa/0.6.2                                       impute/2.3.0(default)                           qchem/4002mpich
bwa/0.7.7(default)                              intel/11.1                                      qchem/4002s
castep/5.5(default)                             intel/12.0                                      qchem/4101
cern_root/5.26.00                               intel/12.1                                      qchem/4101mpich
cern_root/5.30.00(default)                      intel/13.0                                      qiime/1.8.0
cern_root/5.32.01                               intel/13.cs(default)                            qiime/1.9.0(default)
cern_root/5.34.18                               intel/14.cs                                     relion/1.3
cernlib/2006(default)                           intelmpi/4.1.1                                  ruby/1.9.2(default)
clhep/2.1.0.1(default)                          intelmpi/4.1.3                                  samtools/0.1.17
clhep/2.1.1.0                                   intelmpi/5.0.0                                  samtools/0.1.18
clhep/2.1.3.1                                   jags/3.3.0                                      samtools/0.1.19(default)
cmake/2.8.7                                     jags/3.4.0(default)                             samtools/1.2
cmake/3.0.2(default)                            java/1.6.0_23(default)                          seqlogo/2.8.2
comsol/4.3a                                     java/1.7.0_45                                   shapeit/1
comsol/4.3b                                     julia/0.3.11                                    solar/4.3.1(default)
comsol/4.4                                      lammps/10Feb15-intel14.0-impi5.0(default)       soxr/0.1.0
comsol/5.0                                      lammps/22Feb13-intel11.1-openmpi1.4.5           splicetrap/0.90.5
comsol/5.2(default)                             lammps/28Jun14-intel13.1-impi4.1                stata/11
cuda/4.0(default)                               lynx/2.8.7(default)                             stata/13
cuda/4.1                                        maple/16                                        stata/14(default)
cuda/4.2                                        maple/17(default)                               svn/1.6(default)
cuda/5.0                                        maq/0.7.1(default)                              tcltk/8.4_thread-disabled(default)
cuda/5.5                                        mathematica/10.0                                tecplot360/2014
cuda/7.0                                        mathematica/10.3(default)                       tecplot360/2015(default)
cufflinks/2.0.2                                 mathematica/8.0.4                               texlive/2012(default)
cufflinks/2.1.1(default)                        mathematica/9.0                                 tmux/1.3(default)
cufflinks/2.2.1                                 matlab/7.11                                     tophat/1.3.3
ddd/3.3.12(default)                             matlab/7.14                                     tophat/2.0.14(default)
ddplot/2.5                                      matlab/7.7                                      tophat/2.0.4
ddplot/4.0(default)                             matlab/8.2                                      tophat/2.0.9
eclipse/3.6                                     matlab/8.4                                      treemix/1.12
eclipse/4.3(default)                            matlab/8.6(default)                             trinity/2013-08-14
espresso/5.0.3                                  mats/3.0.7                                      vcftools/0.1.12a
fastx_toolkit/0.0.13.2                          necab/0.996                                     vcftools/0.1.14(default)
ffmpeg/0.11.1                                   mono/2.8(default)                               vcftools/0.1.9
ffmpeg/1.1.1(default)                           mopac/2012-cpu                                  vegas/0.8.27
freebayes/17Feb12                               mpc/0.8.1                                       vmd/1.8.7(default)
freetds/0.91                                    mpfr/2.4.2                                      vtk/5.8(default)
fribidi/0.19.6                                  mplayer                                         x264/000(default)
gatk/1.0.4                                      nag/5.3                                         x264/20141209-2245
gatk/2.7.2                                      ncl/5.2.1(default)                              yasm/1.2.0(default)
gatk/3.1.1                                      nco/4.0.6(default)                              zlib/1.2.8
gatk/3.3.0(default)                             nco/4.4.4
```

Useful Tools on Hoffman2

ls /u/local/apps/

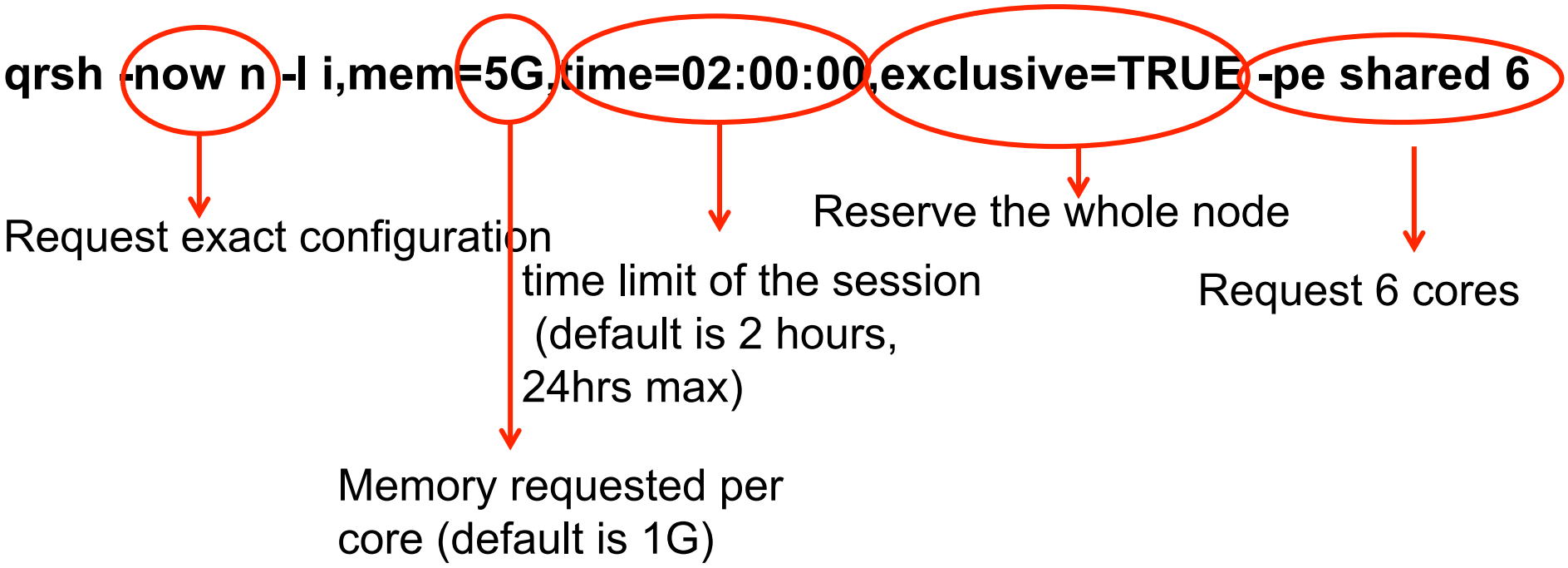
```
[flay@login3 ~]$
[flay@login3 ~]$ ls /u/local/apps/
abaqus            blcr            emacs           grads           libgtextutils   mumps           picard-tools    scilab          tvmet
abaqusdocs        blitz           espresso        graphicsmagick  libtool         muscle          plink           scons           uclust
accelrys          bmapuclatools   fastphase       graphviz        libxc           mygroup         pop-c++         sentaurus       udunits
Accelrys          boost           fasttree        gromacs         lmdb            mysql           povray          shapeit         usearch
activeperl        boost-jam       fastx_toolkit   gsl             loni_pipeline   namd            pplcer          shapelib        usr_lib_GLoverride
ActiveTcl         bowtie          fbat            hadoop          lumerical       ncl             preseq          skampi          valgrind
adina             bowtie2         fe-safe         handbrake       lynx            nco             proj.4          slots           vasp
Adobe             bwa             ffmpeg          harminv         mach            netbeans        protobuf        snappy          vcftools
affymetrix        caffe           fftw2           haskell         mafft           netcdf          pypy            solar           vegas
AFNI              casava          fftw3           hdf             manorm          newbler         pypy3           sortmerna       ViennaRNA
aida              cdbtools        flex            hdf5            maple           ngsplot         python          soxr            vim
amber             cd-hit          fltk            homer           maq             nlopt           qcachegrind     splicetrap      visit
ampliconnoise     cernlib         freebayes       hyperworks      maqview         numpy           qchem           spm             vmd
anaconda          cern_root       freesurfer      iaida           mathematica     nwchem          qgfe            stata           votca
annovar           clearcut        freetds         idl             matio           ocaml           qhull           stressapptest   vtk
ansys_inc         clhep           fribidi         idr             matlab          octave          qiime           structure       wannier90
ant               cln             fsl             igraph          mats            omssa           qiime_data      subversion      weblogo
antlr             cmake           gamess          ilog            mecab           oommf           qrupdate        suitesparse     wolfram
armadillo         common          gatk            IM-IMA          meep            openbabel       qsub            sundials        x264
arpack            comsol          gatk-queue      impute          meld            opencv          qt              superlu_dist    x86_open64
atlas             conan           gaussian        imsl            merlin          openfoam        quantiSNP       swarm           xerces-c
atomeye           condor          gaussview       infernal        metis           openmotif       queue.logs      sysstat         xfdtd
atompaw           consed          gcta            inspect         mfold           opensees        R               szip            xfig_OLD
autoconf          cp2k            gdal            installation    microbiomeutil  osiris          raxml           t500            xilinx-vivado
automake          cpmd            gdc             intel           migrate         osu-micro-benchmarks rdp_classifier tau             xmd
bamtools          ctffind         geant4          isight          minirosetta     papi            relion          tcl             xmedcon
bcftools          cufflinks       genetorrent     jags            mira            paraview        remcom          tcsh            xpdf_OLD
bcl2qfastq        cytoscape       geos            jam             molcas          parmetis        repeatmasker    tecplot360      yaml
beagle            ddd             gflags          java            molden          parsec          RepeatModeler   texlive         yasm
beaglecall        ddplot          gftp            jaxodraw        molpro          parsinsert      resmap          tmux            zlib
beagle_utilities  dejagnu         ginac           jmol            mono            pcre            rosetta         tophat
bedtools          dirac           git_OLD         julia           mopac           pdt             rstudio         toscastructure
bfast             diskusage       globalarrays    kepler          mothur          penncnv         rsync           totalview
bioscope          dl_poly         glog            lammps          mpc             perl_modules    rtax            trans-ABySS
blas              dx              gmp             lapack          mpfr            petsc           ruby            treemix
blast             eclipse         gnuplot         lapack++        mpiblast        pgsql           sage            trilinos
blast+            eigen           gpac            ldope           mplayer         phantompeakqualtools samtools       trinity
blat              eigensoft       grace           leveldb         mrt             phase           scalapack       tt
[flay@login3 ~]$
```

Alignment Using bwa-meth: On Hoffman2 Interactively

#grab a Hoffman2 session:

qrsh

or



bwa-meth

ls /u/home/galaxy/collaboratory/apps

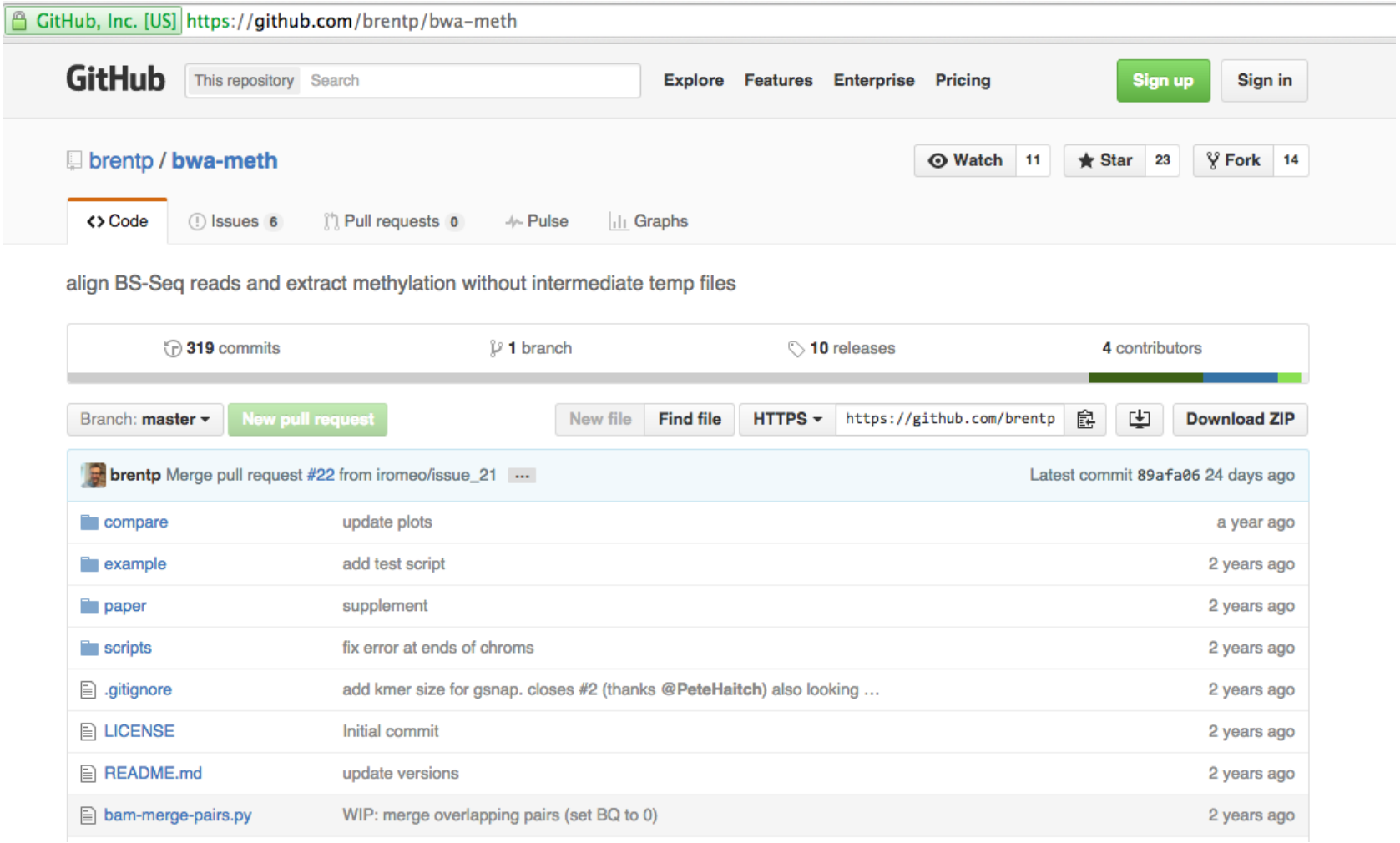
```
[flay@login3 apps]$ ls /u/home/galaxy/collaboratory/apps
bedops_v2.4.20.v2  FastQC      HiC-Pro        samtools-1.1
bin                fit-hi-c    hicup_v0.5.8   sratoolkit.2.7.0-centos_linux64
biscuit-release    gatk        MACS           v0.10.tar.gz
bwa-meth           HiCPlotter  picard.jar
bwa-meth-0.10      hic-pro     release.zip
```

ls /u/home/galaxy/collaboratory/apps/bwa-meth-0.10

```
[flay@login3 apps]$ ls /u/home/galaxy/collaboratory/apps/bwa-meth-0.10/
bias-plot.py  compare  ez_setup.py  paper         requirements.txt  setup.py
bwameth.py    example  LICENSE      README.md     scripts
```

bwa-meth

#For documentation, go to https://github.com/brentp/bwa-meth



bwa-meth: Dependencies

#load all the dependencies prior to running your program

```
module load bwa
module load python/2.7.3
module load samtools/1.2
```

#install toolshed and seaborn for python

```
pip install toolshed --user
```

```
[flay@login2 bwa-meth]$ module load python/2.7.3
[flay@login2 bwa-meth]$ pip install toolshed --user
Downloading/unpacking toolshed
  Downloading toolshed-0.4.5.tar.gz
  Running setup.py (path:/work/tmp/pip_build_flay/toolshed/setup.py) egg_info for package toolshed

Installing collected packages: toolshed
  Running setup.py install for toolshed

    Installing toolshed script to /u/home/f/flay/.local/bin
Successfully installed toolshed
Cleaning up...
[flay@login2 bwa-meth]$
```

```
pip install seaborn --user
```

##this is optional

```
[flay@login2 bwa-meth]$ pip install seaborn --user
Downloading/unpacking seaborn
  Downloading seaborn-0.7.0.tar.gz (154kB): 154kB downloaded
  Running setup.py (path:/work/tmp/pip_build_flay/seaborn/setup.py) egg_info for package seaborn

Downloading/unpacking pandas (from seaborn)
  Downloading pandas-0.17.1.tar.gz (6.7MB): 6.7MB downloaded
  Running setup.py (path:/work/tmp/pip_build_flay/pandas/setup.py) egg_info for package pandas
```

bwa-meth

python /u/home/galaxy/collaboratory/apps/bwa-meth-0.10/bwameth.py

```
[flay@n2066 ~]$ python /u/home/galaxy/collaboratory/apps/bwa-meth-0.10/bwameth.py --h
usage:
map bisulfite converted reads to an insilico converted genome using bwa mem.
A command to this program like:

    python bwameth.py --reference ref.fa A.fq B.fq

Gets converted to:

    bwa mem -pCMR ref.fa.bwameth.c2t '<python bwameth.py c2t A.fq B.fq'

So that A.fq has C's converted to T's and B.fq has G's converted to A's
and both are streamed directly to the aligner without a temporary file.
The output is a corrected, sorted, indexed BAM.

       [-h] --reference REFERENCE [-t THREADS] [-p PREFIX] [--calmd]
       [--read-group READ_GROUP] [--set-as-failed {f,r}]
       fastqs [fastqs ...]

positional arguments:
  fastqs                bs-seq fastqs to align. Runmultiple sets separated by
                        commas, e.g. ... a_R1.fastq,b_R1.fastq
                        a_R2.fastq,b_R2.fastq note that the order must be
                        maintained.

optional arguments:
  -h, --help            show this help message and exit
  --reference REFERENCE
                        reference fasta
  -t THREADS, --threads THREADS
  -p PREFIX, --prefix PREFIX
  --calmd
  --read-group READ_GROUP
                        read-group to add to bam in same format as to bwa:
                        '@RG\tID:foo\tSM:bar'
  --set-as-failed {f,r}
                        flag alignments to this strand as not passing QC
                        (0x200). Targetted BS-Seq libraries are often to a
                        single strand, so we can flag them as QC failures.
                        Note f == OT, r == OB. Likely, this will be 'f' as we
                        will expect reads to align to the original-bottom (OB)
                        strand and will flag as failed those aligning to the
                        forward, or original top (OT).
```

Alignment Using bwa-meth: Copy Files

#copy all the files you need to scratch folder

```
cd /u/scratch/f/flay/
```

change to your ID

```
cp /u/scratch/f/flay/workshop6.tar.gz .
```

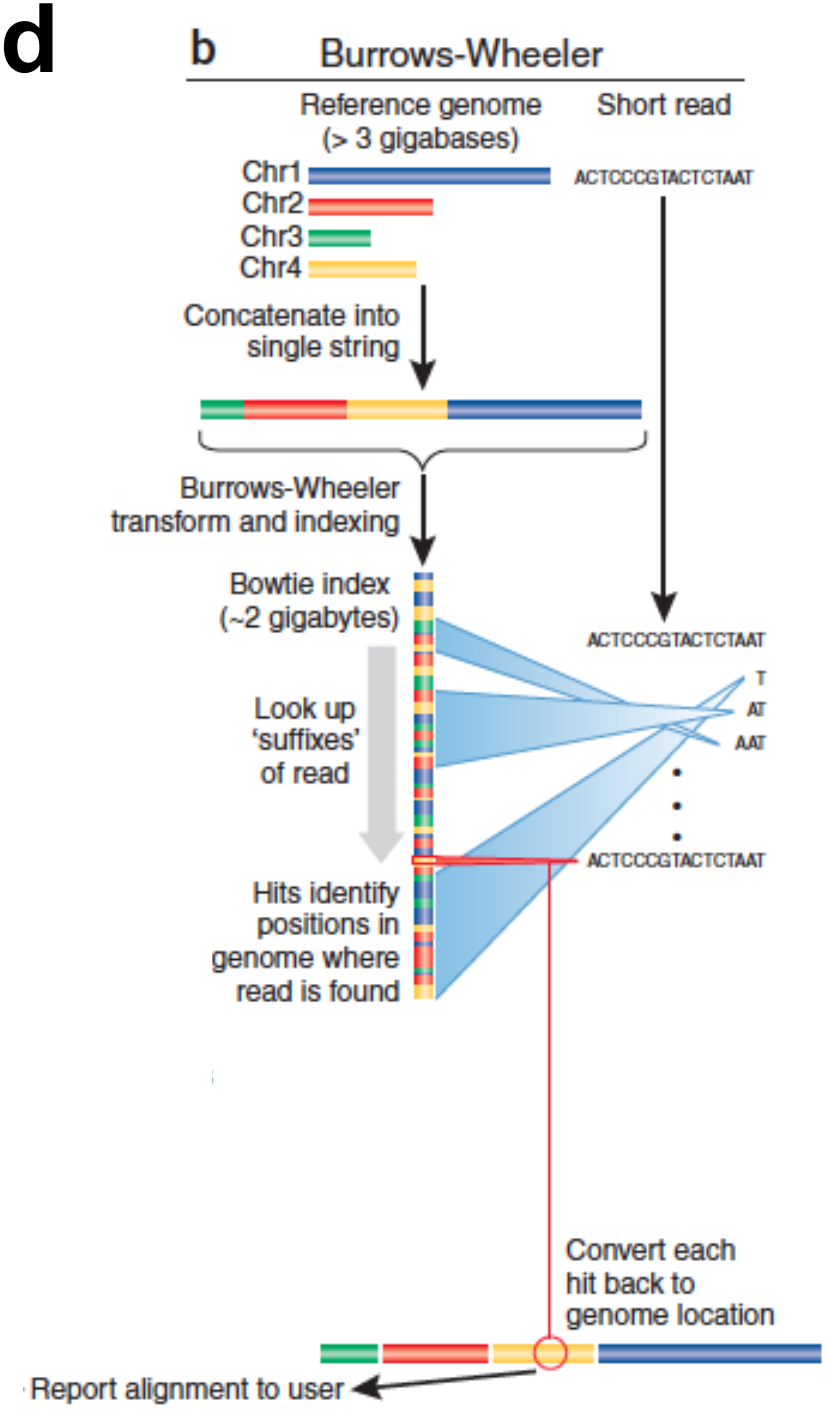
Indicates current directory

```
tar -xvzf workshop6.tar.gz

cd workshop6

ls
```

Bwa Alignment and Indexing



Alignment Using bwa-meth: Indexing the Reference Genome

#Use chr1 as an example
#Change directory to where reference genome fasta is

cd genome/chr1
python /u/home/galaxy/collaboratory/apps/bwa-meth-0.10/
bwameth.py index chr1.fa

Alignment Using bwa-meth: Indexing the Reference Genome

```
[flay@n2066 chr1]$ python /u/home/galaxy/collaboratory/apps/bwa-meth-0.10/bwameth.py index chr1.fa
converting c2t in chr1.fa to chr1.fa.bwameth.c2t
indexing: chr1.fa.bwameth.c2t
[bwa_index] Pack FASTA... 5.88 sec
[bwa_index] Construct BWT for the packed sequence...
[BWTIncCreate] textLength=788781728, availableWord=67501160
[BWTIncConstructFromPacked] 10 iterations done. 100000000 characters processed.
[BWTIncConstructFromPacked] 20 iterations done. 195555488 characters processed.
[BWTIncConstructFromPacked] 30 iterations done. 280551552 characters processed.
[BWTIncConstructFromPacked] 40 iterations done. 356092224 characters processed.
[BWTIncConstructFromPacked] 50 iterations done. 423228976 characters processed.
[BWTIncConstructFromPacked] 60 iterations done. 482896288 characters processed.
[BWTIncConstructFromPacked] 70 iterations done. 535924720 characters processed.
[BWTIncConstructFromPacked] 80 iterations done. 583052480 characters processed.
[BWTIncConstructFromPacked] 90 iterations done. 624935760 characters processed.
[BWTIncConstructFromPacked] 100 iterations done. 662157696 characters processed.
[BWTIncConstructFromPacked] 110 iterations done. 695236640 characters processed.
[BWTIncConstructFromPacked] 120 iterations done. 724633216 characters processed.
[BWTIncConstructFromPacked] 130 iterations done. 750756992 characters processed.
[BWTIncConstructFromPacked] 140 iterations done. 773971824 characters processed.
[bwa_index] 348.12 seconds elapse.
[bwa_index] Update BWT... 3.95 sec
[bwa_index] Pack forward-only FASTA... 4.10 sec
[bwa_index] Construct SA from BWT and Occ... 117.57 sec
[main] Version: 0.7.7-r441
[main] CMD: bwa index -a bwtsw chr1.fa.bwameth.c2t
[main] Real time: 491.483 sec; CPU: 479.627 sec
```

Alignment Using bwa-meth: Indexing the Reference Genome

#Output files after indexing

```
[flay@n2192 mm9]$ ls -lh *chr1*
-rw-r--r-- 1 flay matteop 192M Mar  1 17:42 chr1.fa
-rw-r--r-- 1 flay matteop 380M Mar  4 22:17 chr1.fa.bwameth.c2t
-rw-r--r-- 1 flay matteop 1.2K Mar  4 22:24 chr1.fa.bwameth.c2t.amb
-rw-r--r-- 1 flay matteop   83 Mar  4 22:24 chr1.fa.bwameth.c2t.ann
-rw-r--r-- 1 flay matteop 377M Mar  4 22:24 chr1.fa.bwameth.c2t.bwt
-rw-r--r-- 1 flay matteop  95M Mar  4 22:24 chr1.fa.bwameth.c2t.pac
-rw-r--r-- 1 flay matteop 189M Mar  4 22:26 chr1.fa.bwameth.c2t.sa
```

Alignment Using bwa-meth: Understanding Commands and Options

```
[flay@n2066 chr1]$ python /u/home/galaxy/collaboratory/apps/bwa-meth-0.10/bwameth.py
usage:
map bisulfite converted reads to an insilico converted genome using bwa mem.
A command to this program like:

    python bwameth.py --reference ref.fa A.fq B.fq

Gets converted to:

    bwa mem -pCMR ref.fa.bwameth.c2t '<python bwameth.py c2t A.fq B.fq'

So that A.fq has C's converted to T's and B.fq has G's converted to A's
and both are streamed directly to the aligner without a temporary file.
The output is a corrected, sorted, indexed BAM.

       [-h] --reference REFERENCE [-t THREADS] [-p PREFIX] [--calmd]
       [--read-group READ_GROUP] [--set-as-failed {f,r}]
       fastqs [fastqs ...]

map bisulfite converted reads to an insilico converted genome using bwa mem.
A command to this program like:

    python bwameth.py --reference ref.fa A.fq B.fq

Gets converted to:

    bwa mem -pCMR ref.fa.bwameth.c2t '<python bwameth.py c2t A.fq B.fq'

So that A.fq has C's converted to T's and B.fq has G's converted to A's
and both are streamed directly to the aligner without a temporary file.
The output is a corrected, sorted, indexed BAM.
```

Alignment Using bwa-meth: Align Fastq Files to the Reference Genome

#aligned paired-end reads to the reference genome (hg19)

cd /u/scratch/f/flay/workshop6/data/raw

python /u/home/galaxy/collaboratory/apps/bwa-meth-0.10/bwameth.py --threads 5 --prefix N25 --reference /u/scratch/f/flay/workshop6/genome/hg19_rCRSchrm.fa N25_R1.fastq N25_R2.fastq

Alignment Using bwa-meth: Align Fastq Files to the Reference Genome

```
[flay@n2066 raw]$ python /u/home/galaxy/collaboratory/apps/bwa-meth-0.10/bwameth.py --threads 5 --prefix N25 --re
ference /u/scratch/f/flay/workshop6/genome/hg19_rCRSchrm.fa N25_R1.fastq N25_R2.fastq
running: bwa mem -T 40 -B 2 -L 10 -CM -U 100 -p -R '@RG ID:N25_R        SM:N25_R' -t 5  /u/scratch/f/flay/worksho
p6/genome/hg19_rCRSchrm.fa.bwameth.c2t '</u/local/apps/python/2.7.3/bin/python /u/home/galaxy/collaboratory/apps/
bwa-meth-0.10/bwameth.py c2t N25_R1.fastq N25_R2.fastq'
writing to:
samtools view -bS - | samtools sort - N25
converting reads in N25_R1.fastq,N25_R2.fastq
WARNING: 12500 reads with length < 80
       : this program is designed for long reads
[M::main_mem] read 12500 sequences (950000 bp)...
[M::mem_pestat] # candidate unique pairs for (FF, FR, RF, RR): (0, 3755, 0, 0)
[M::mem_pestat] skip orientation FF as there are not enough pairs
[M::mem_pestat] analyzing insert size distribution for orientation FR...
[M::mem_pestat] (25, 50, 75) percentile: (138, 152, 172)
[M::mem_pestat] low and high boundaries for computing mean and std.dev: (70, 240)
[M::mem_pestat] mean and std.dev: (155.65, 25.45)
[M::mem_pestat] low and high boundaries for proper pairs: (36, 274)
[M::mem_pestat] skip orientation RF as there are not enough pairs
[M::mem_pestat] skip orientation RR as there are not enough pairs
[M::mem_process_seqs] Processed 12500 reads in 18.257 CPU sec, 5.553 real sec
[main] Version: 0.7.7-r441
[main] CMD: bwa mem -T 40 -B 2 -L 10 -CM -U 100 -p -R @RG       ID:N25_R        SM:N25_R -t 5 /u/scratch/f/flay/w
orkshop6/genome/hg19_rCRSchrm.fa.bwameth.c2t </u/local/apps/python/2.7.3/bin/python /u/home/galaxy/collaboratory/
apps/bwa-meth-0.10/bwameth.py c2t N25_R1.fastq N25_R2.fastq
[main] Real time: 157.097 sec; CPU: 74.820 sec
running: samtools index N25.bam
```

Alignment Using bwa-meth: Output Files

```
[flay@n2176 raw]$ ls -lh
total 6.3M
-rw-r--r-- 1 flay matteop 906K Jun 16 22:17 N25.bam
-rw-r--r-- 1 flay matteop 1.6M Jun 16 22:17 N25.bam.bai
-rw-r--r-- 1 flay matteop 1.3M Jun 16 22:04 N25_R1.fastq
-rw-r--r-- 1 flay matteop 1.3M Jun 16 22:04 N25_R2.fastq
```

Assessing Library Quality: Alignment and Pairing Rate

#use samtools calculated statistics to look at alignment rate
samtools flagstat fileName.bam

```
[flay@n2176 raw]$ samtools flagstat N25.bam
12197 + 306 in total (QC-passed reads + QC-failed reads)
3 + 0 secondary
0 + 0 supplementary
0 + 0 duplicates
11878 + 306 mapped (97.38%:100.00%)
12194 + 306 paired in sequencing
6097 + 153 read1
6097 + 153 read2
11180 + 0 properly paired (91.68%:0.00%)
11830 + 306 with itself and mate mapped
45 + 0 singletons (0.37%:0.00%)
474 + 0 with mate mapped to a different chr
212 + 0 with mate mapped to a different chr (mapQ>=5)
```

Submitting Alignment Jobs on Hoffman2

#make a bash script containing bwameth commands.
#For example:

vi bwameth_align.sh
i

```
#!/bin/sh
bwameth.py --threads 6 --prefix fileName --reference /u/scratch/f/
flay/genome/hg19_rCRSchrm.fa /u/scratch/f/flay/test/
fileName_R1.fastq /u/scratch/f/flay/test/fileName_R2.fastq
```

qsub -cwd -V -N bwameth -l h_data=4G,exclusive -l h_rt=20:00:00
-pe shared 8 bwameth.sh

Change to current working directory

Job name

Export the environment you are in