Differential Methylation Analysis

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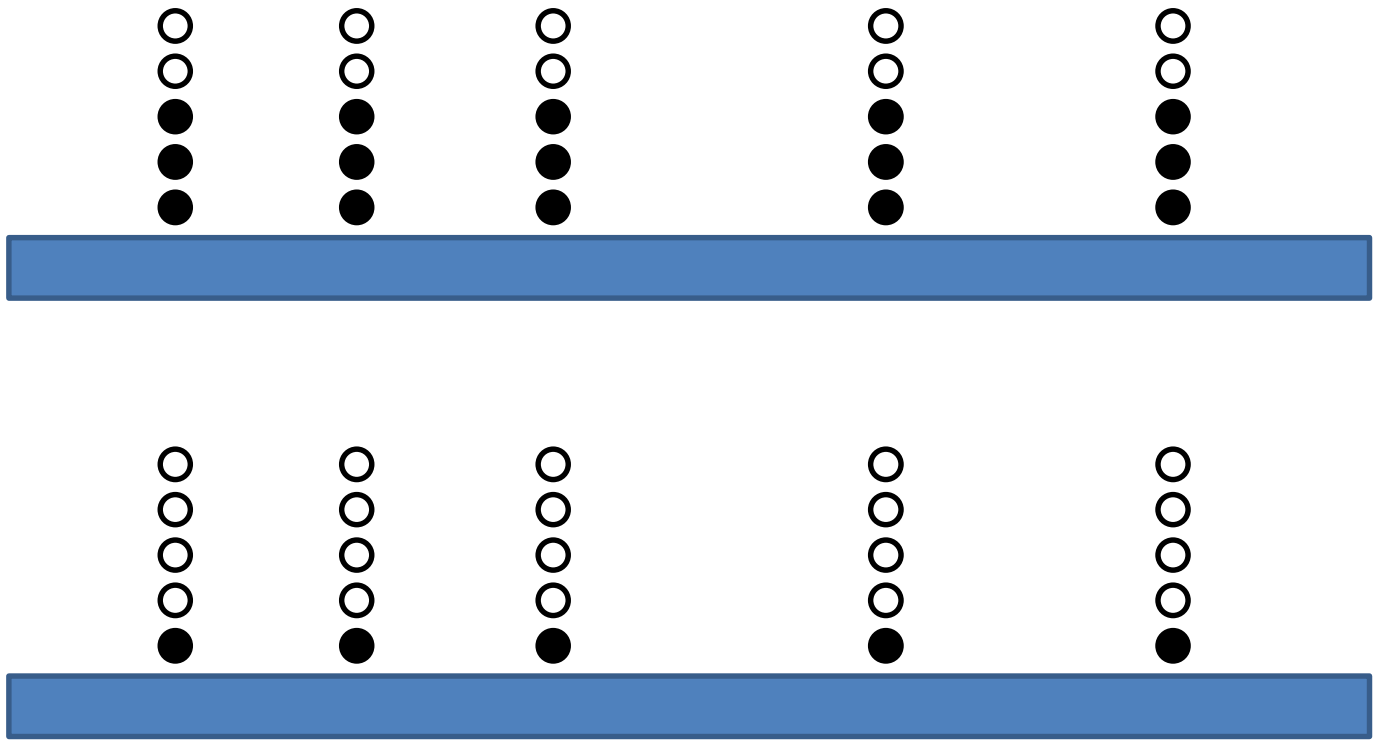
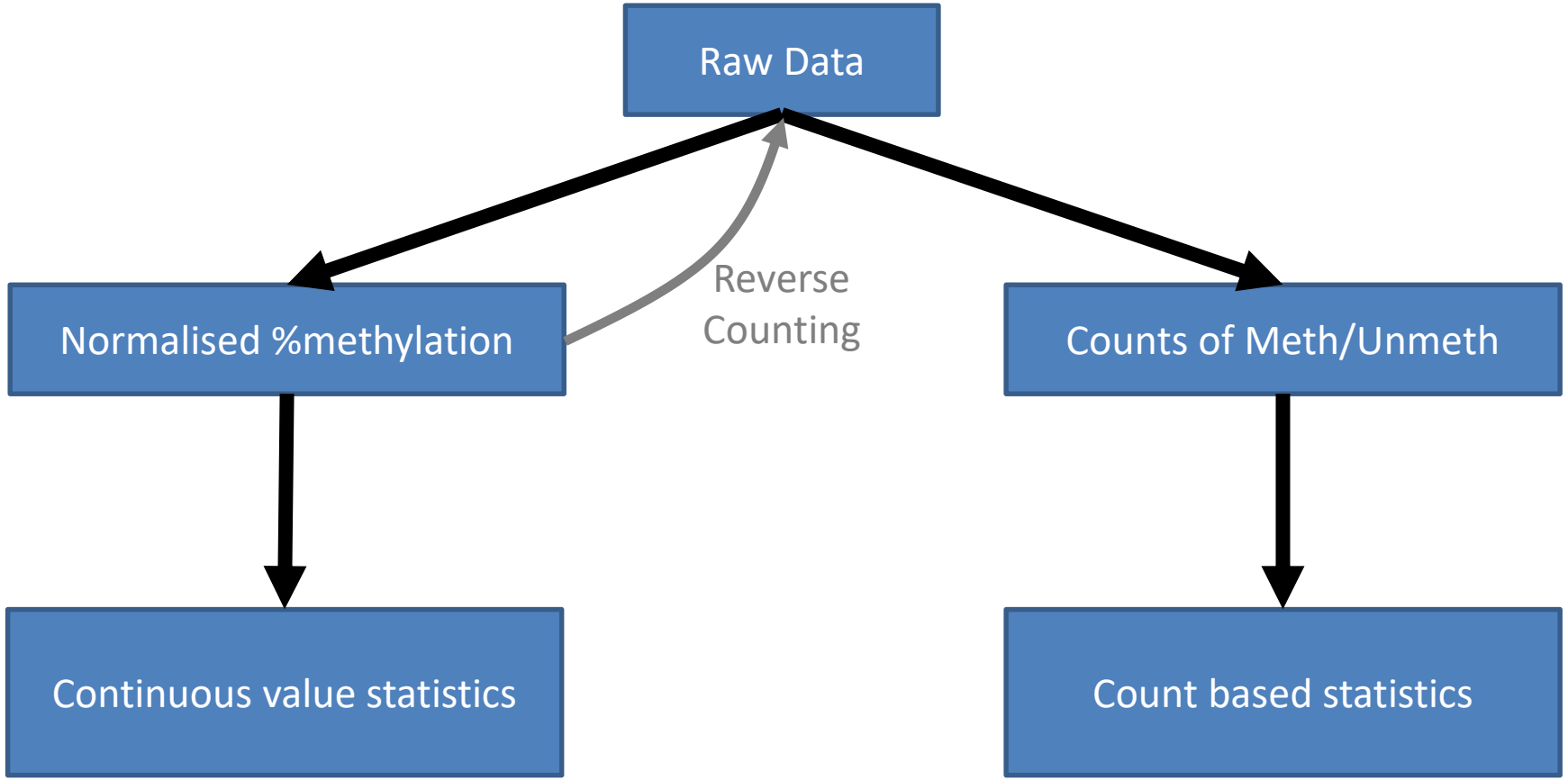
A basic question…



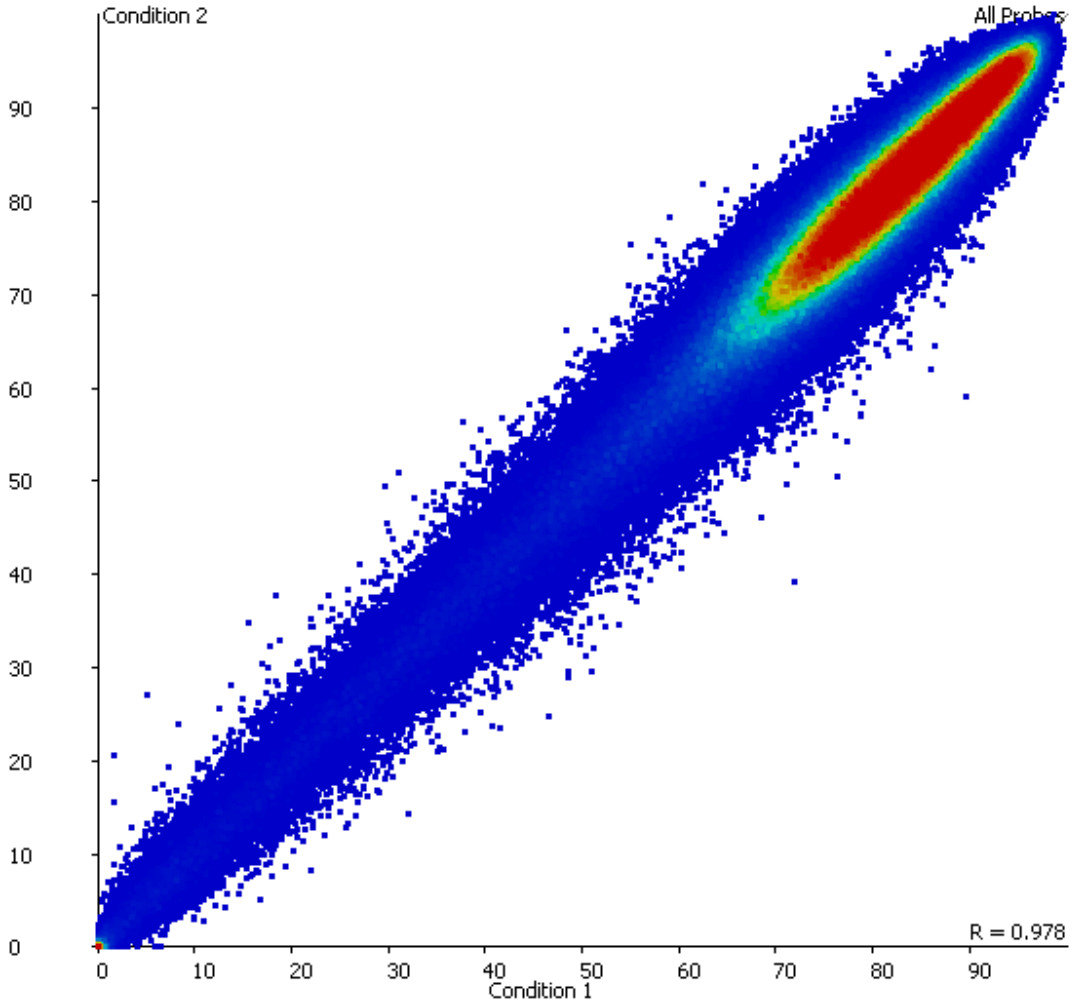
Two Strategies



Factors to consider

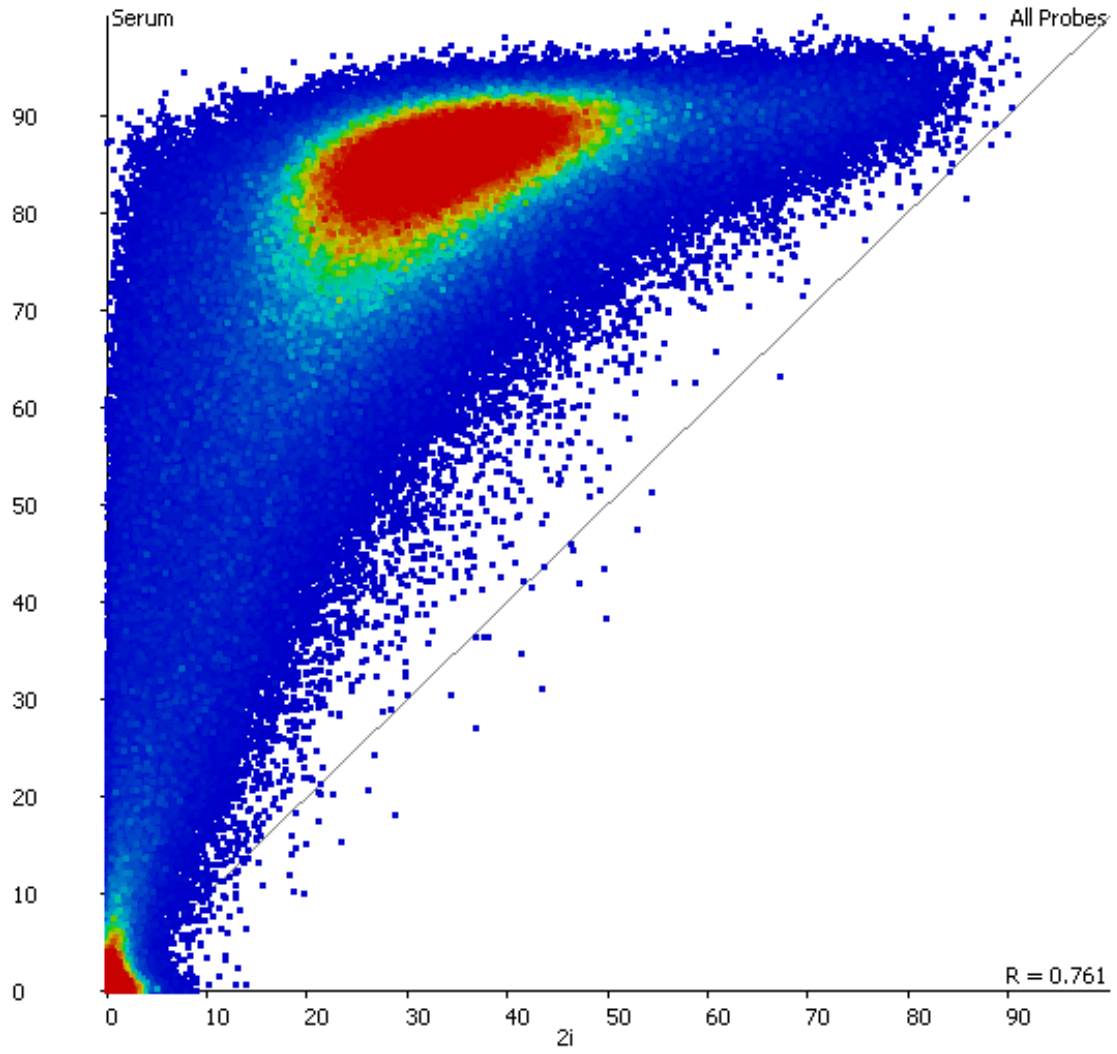
- Formulating a sensible question
- Applying corrections if needed
- Assessing statistical power
- Relating hits to biology

Question



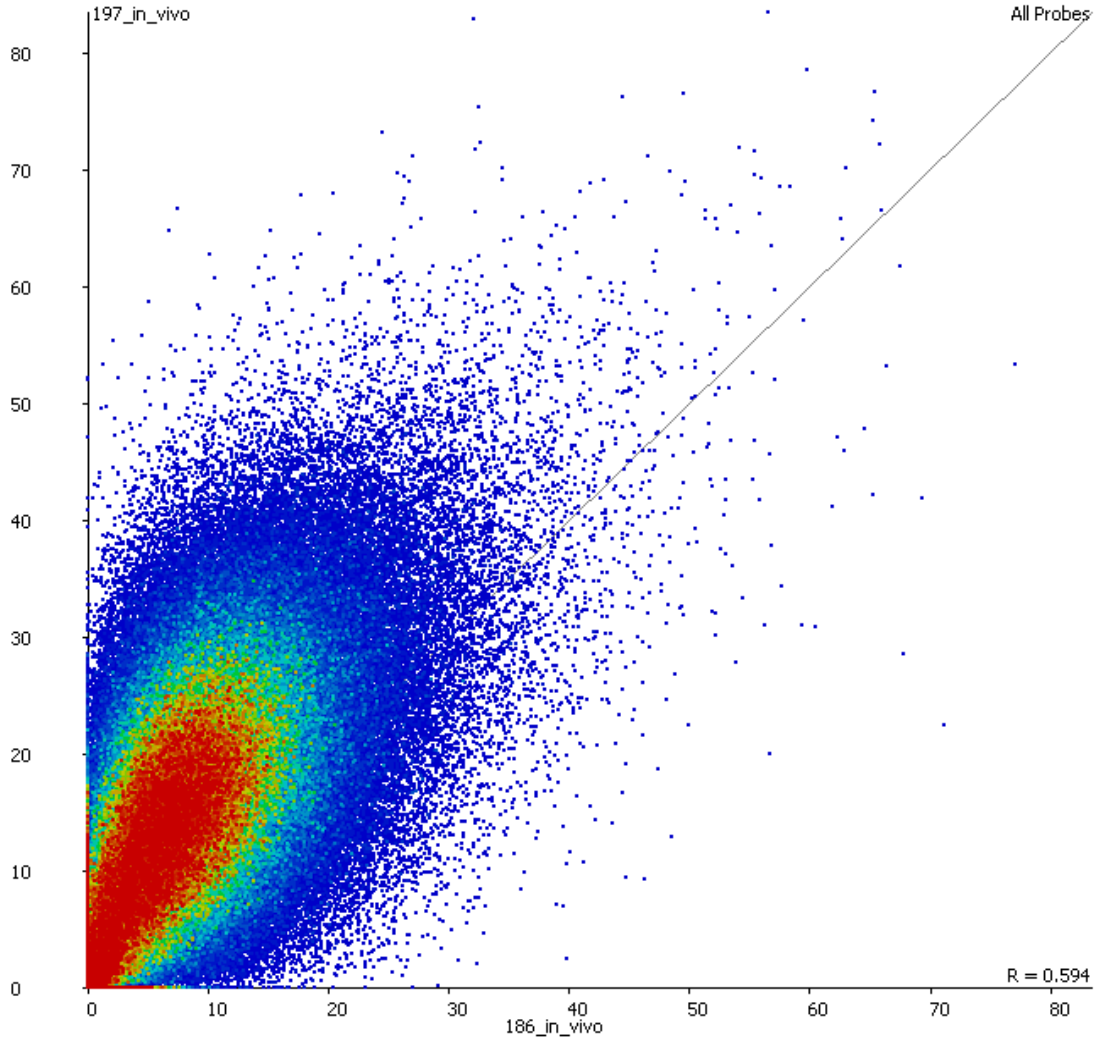
Which areas show a significant change in methylation level between the two conditions?

Question



Which areas show a change in methylation which is larger or smaller than the global change in the samples overall?

Question



Which areas show a change in methylation after correcting for the small global differences?

Count based statistics

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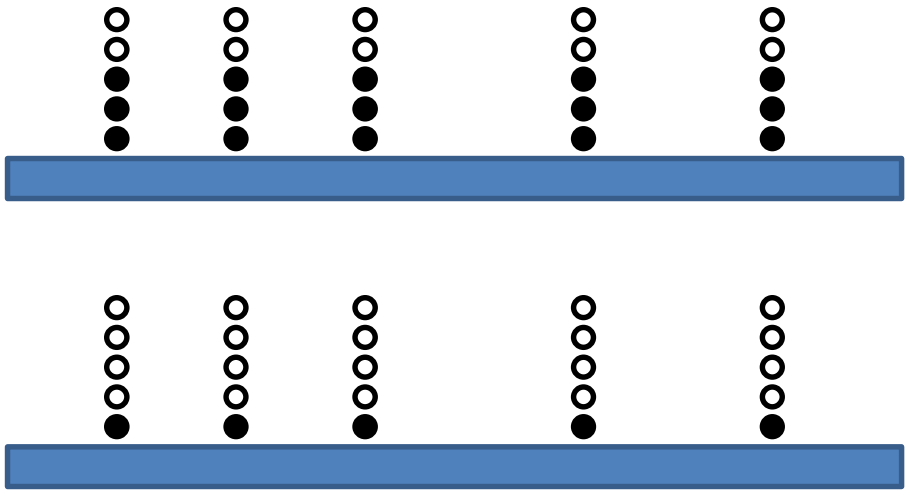
Count Data



	Meth	Unmeth
Sample 1	18	10
Sample 2	5	20

Is the difference in ratios significant **given the observation levels** of the samples

The problem of power…

- Ideally want to cover every Cytosine (CpG)
- Should correct for the number of tests

- It’s unlikely you’ll collect enough data to analyse each C and have p-values which survive multiple testing correction

- Generally need to analyse in windows

Window sizes

Small ← Effect size → Large

- Good resolution
- Specific biological effects
- High MTC burden
- Small observations
- High p-values

- Lots of data
- High statistical power
- Low MTC burden
- Low p-values
- Effect averaging

Power Analysis

(Assuming a human genome with $p<0.05$ and power of detection of 0.8)

Window Size (# CpG cytosines)

Absolute methylation change (from 80%)

	1	10	25	50	100	200	500
1	158805	14212	5419	2609	1254	602	228
5	6794	608	232	112	54	26	10
10	1825	164	63	30	15	7	3
20	509	46	18	9	5	2	1
50	94	9	4	2	1	1	1

Required Fold Genome Coverage

Without Multiple Testing Correction

	1	10	25	50	100	200	500
1	25583	2559	1024	512	256	128	52
5	1094	110	44	22	11	6	3
10	294	30	12	6	3	2	1
20	82	9	4	2	1	1	1
50	15	2	1	1	1	1	1

Applicable Statistics

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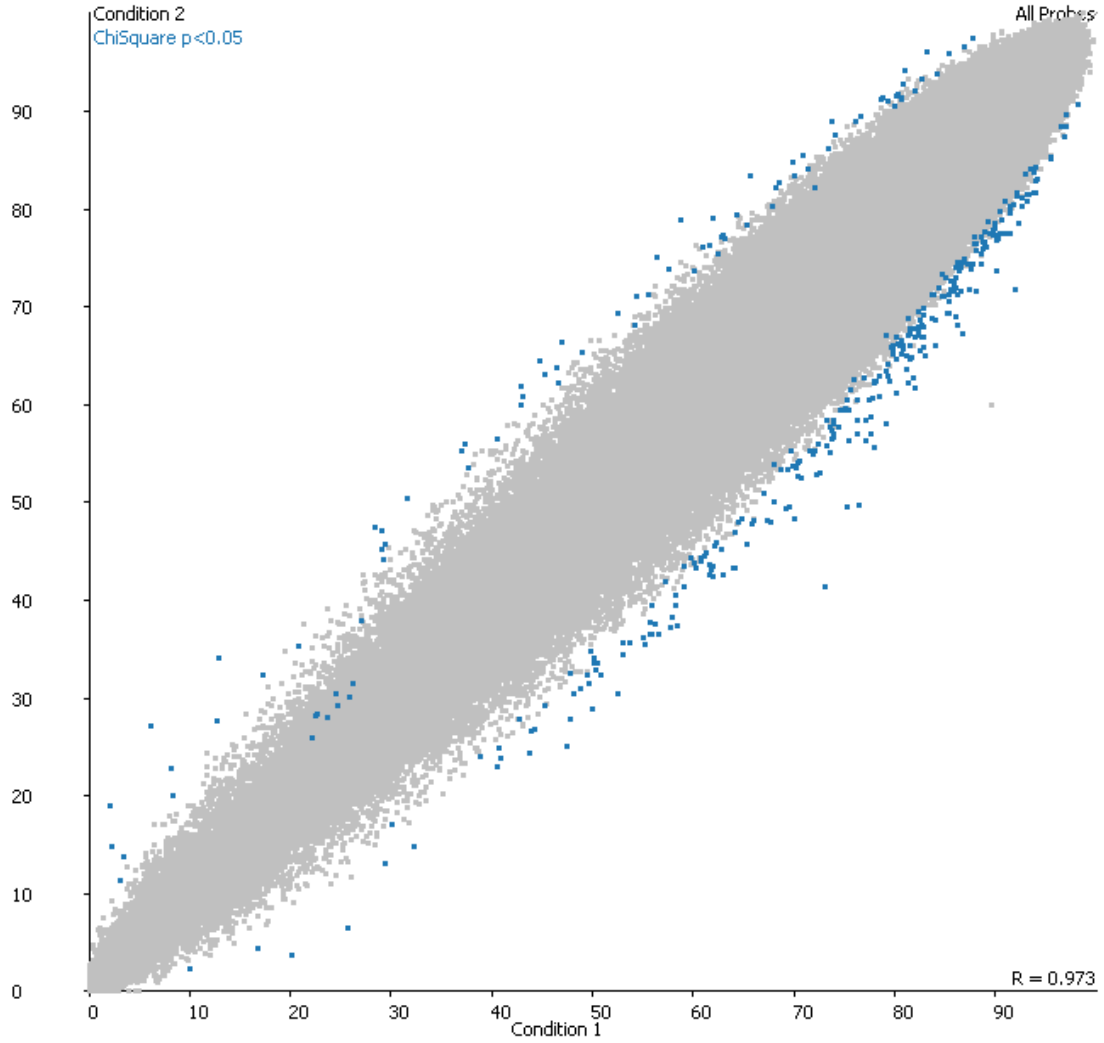
Contingency Statistics are simple to use for differential methylation in well behaved data

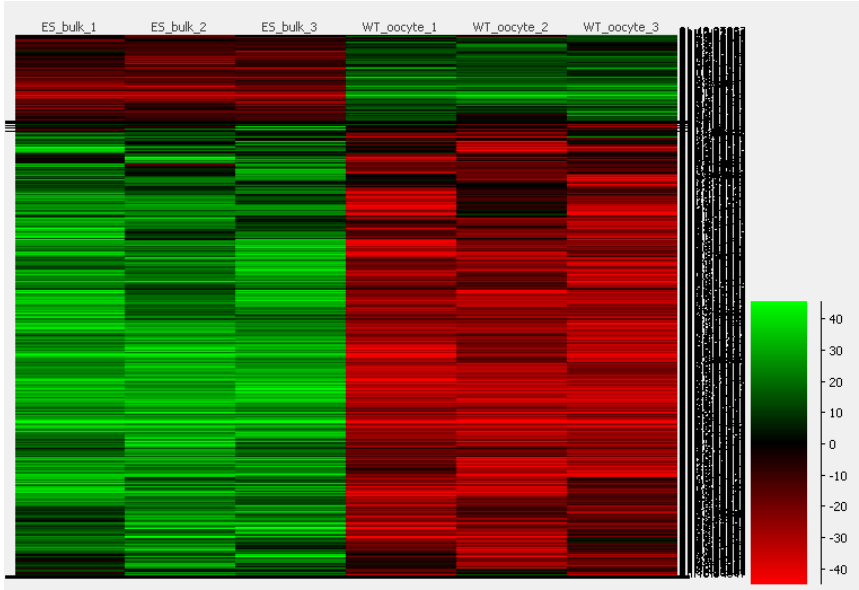
- Unreplicated
  - Chi-Square
  - Fisher's Exact



Contingency Statistics are simple to use for differential methylation in well behaved data

- Replicated Contingency
  - Logistic Regression

- Linear Modelling of counts
  - EdgeR



F1000Research 2017, 6:2055 Last updated: 20 APR 2018

Check for updates

METHOD ARTICLE

Differential methylation analysis of reduced representation bisulfite sequencing experiments using edgeR [version 1; referees: 2 approved, 1 approved with reservations]

Yunshun Chen[1,2], Bhupinder Pal[1,2], Jane E. Visvader[1,2], Gordon K. Smyth[2,3]

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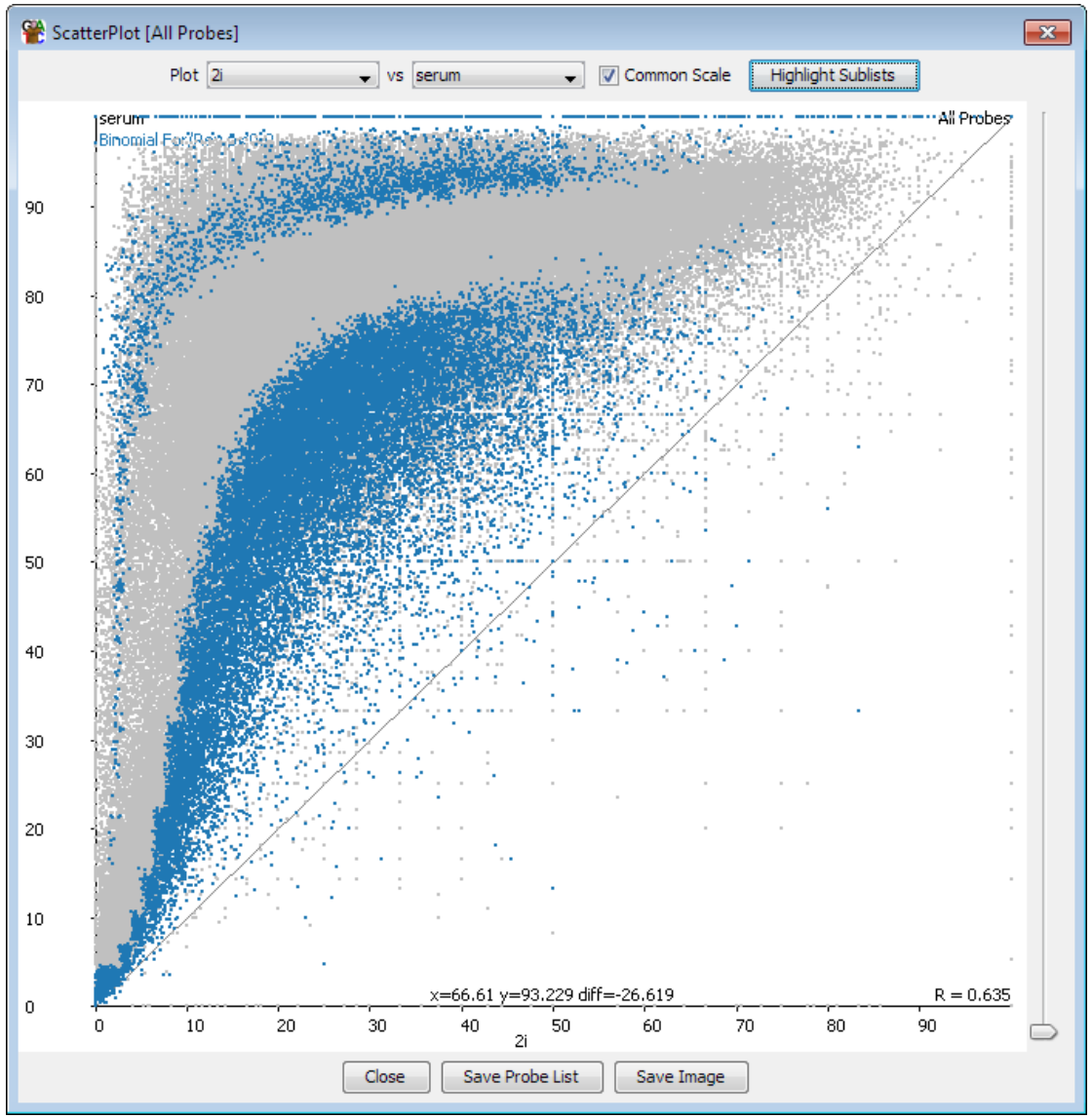
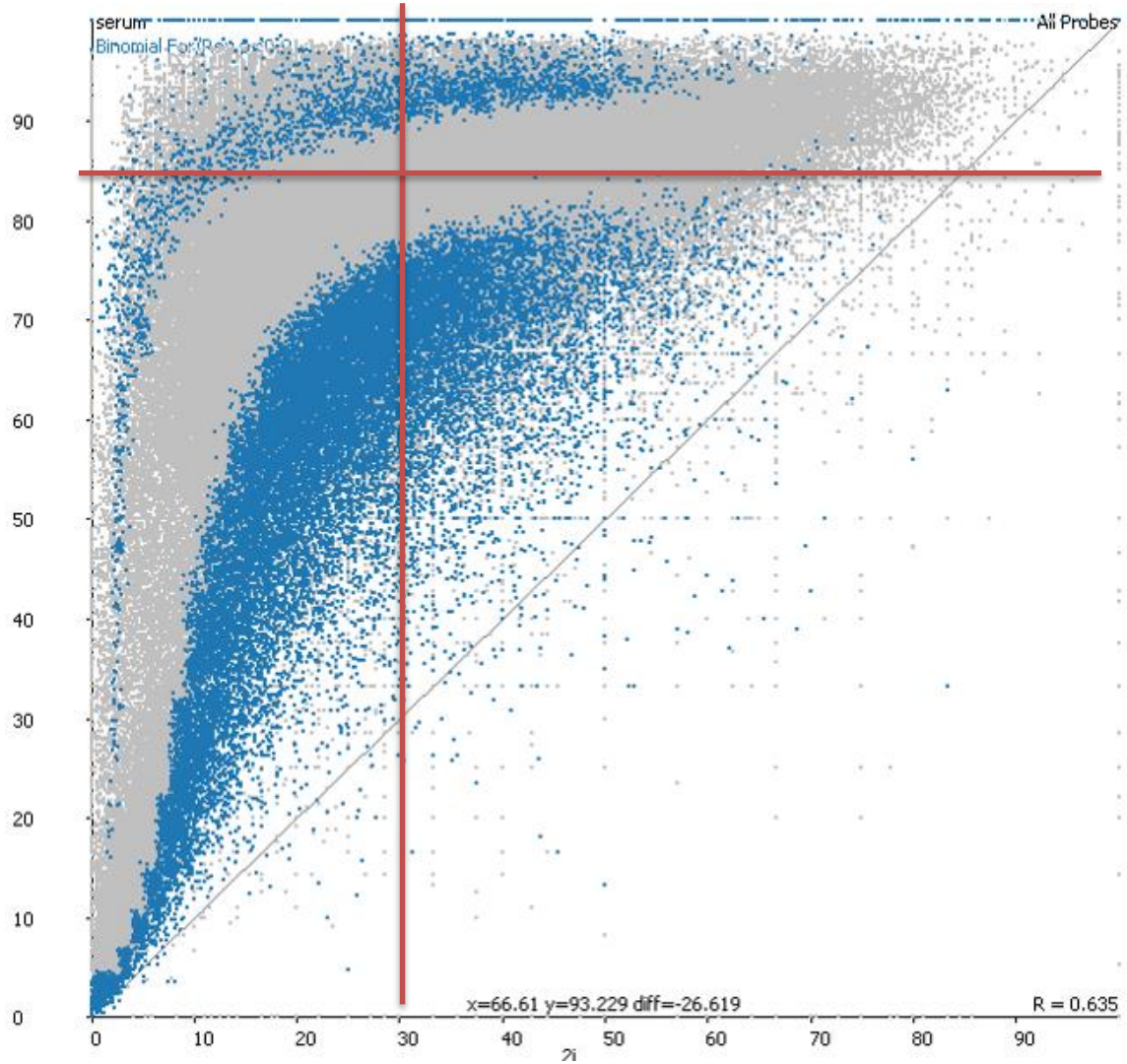
[3]School of Mathematics and Statistics, The University of Melbourne, Melbourne, VIC, 3010, Australia

Binomial statistics can find interesting points in globally changing datasets



- Changes the default expectation
- Find average difference for each starting point
- Select points which exhibit unusual change

Globally changing example



Starting level = 30%

Observations = 14 meth 6 unmeth

Expected End level = 85%

Binomial test, p=0.85, trials=20, successes=14

Raw p=0.106

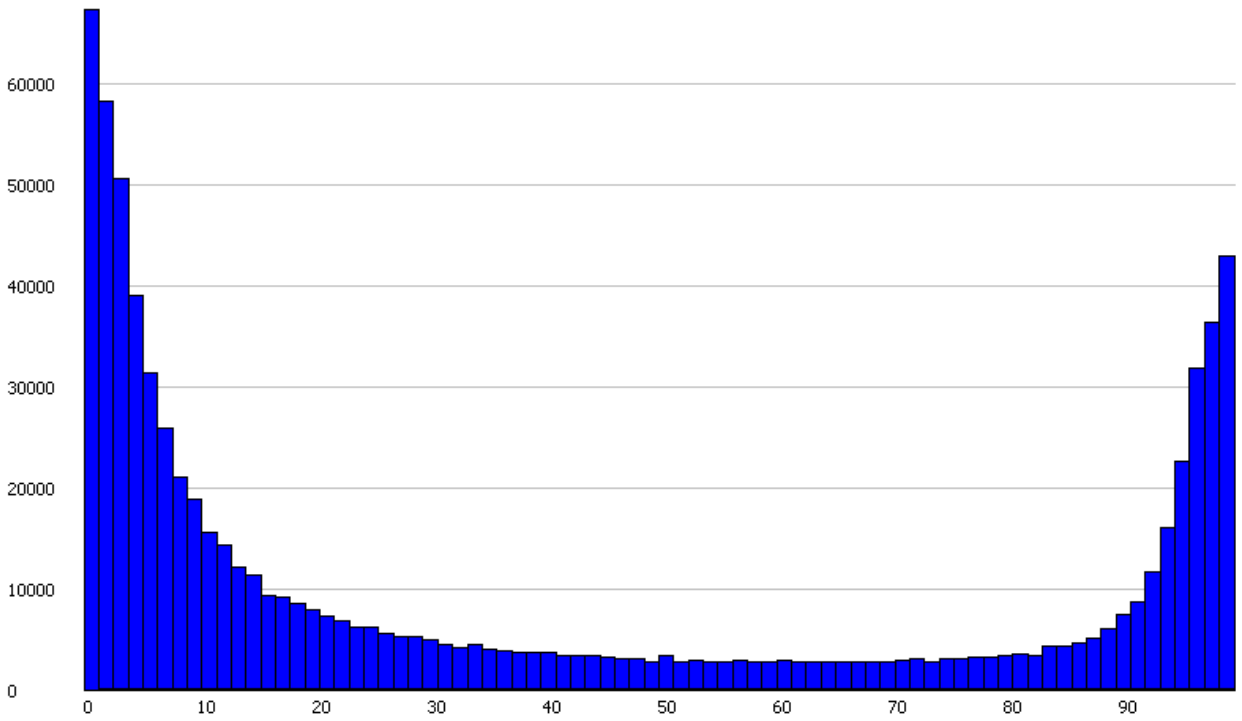
Beta Binomial Models

- What is the probability distribution for the true methylation level?
- Simple model: Binomial stats to estimate confidence
- Can we do better?

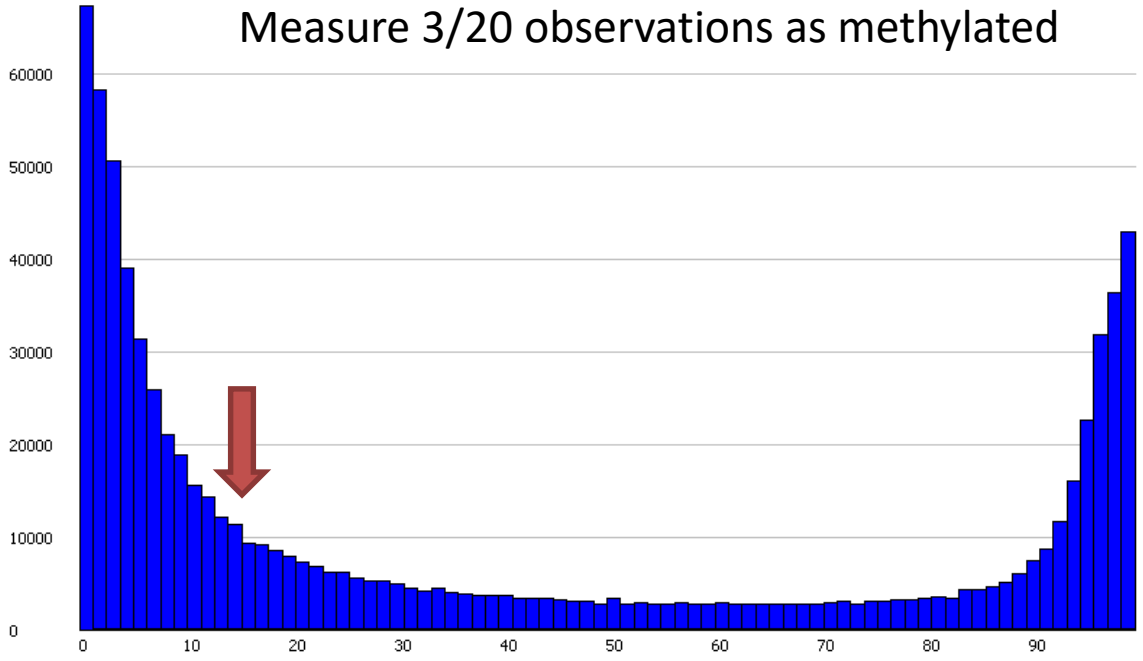
Genome-wide methylation profile.

All levels are not equally likely

Can inform the construction of a
Custom beta binomial distribution



Beta-binomial model



The binomial distribution would be defined by the mean and observations

Using the whole genome prior a beta-binomial model would upweight the lower methylation levels, since these are more common.

Provides increased power in comparisons between major groups

Often computationally intensive

Limitations of count based stats

- No subdivision of calls – all calls are equal even when coverage isn't
  - Supplement with differences based on better quantitation

- Potential biased by power
  - Can alleviate with CpG window based analysis
  - Easy to bias data otherwise
  - Problem of interpretation, not statistics

Methylation Level Statistics

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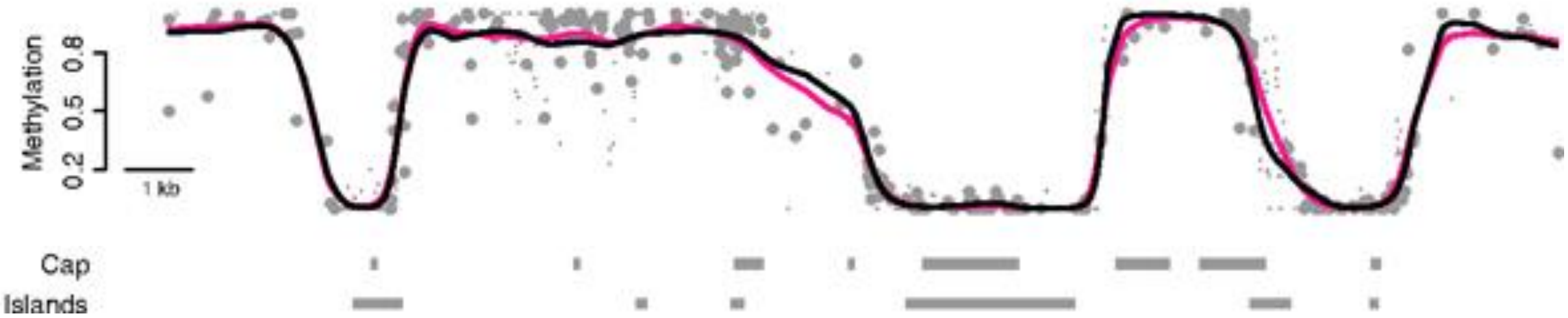
BSmooth algorithm for methylation correction



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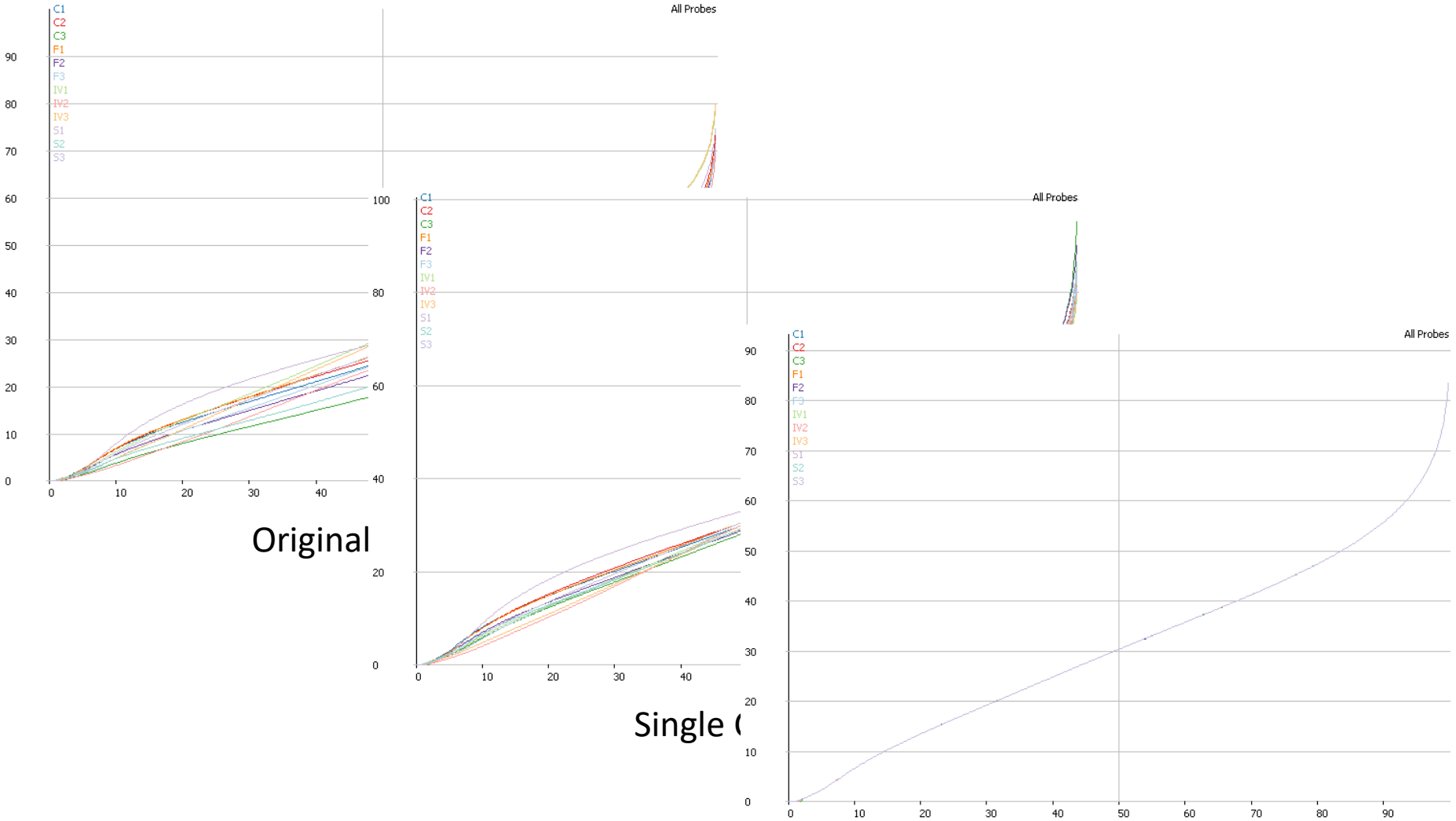
Normalisation for methylation levels



Original

Single (

Quantile Normalisation

Statistics

- Standard continuous statistics
  - T-Test
  - ANOVA

- Information sharing continuous stats
  - LIMMA

- Reduced power – one value per replicate

Reverse counting

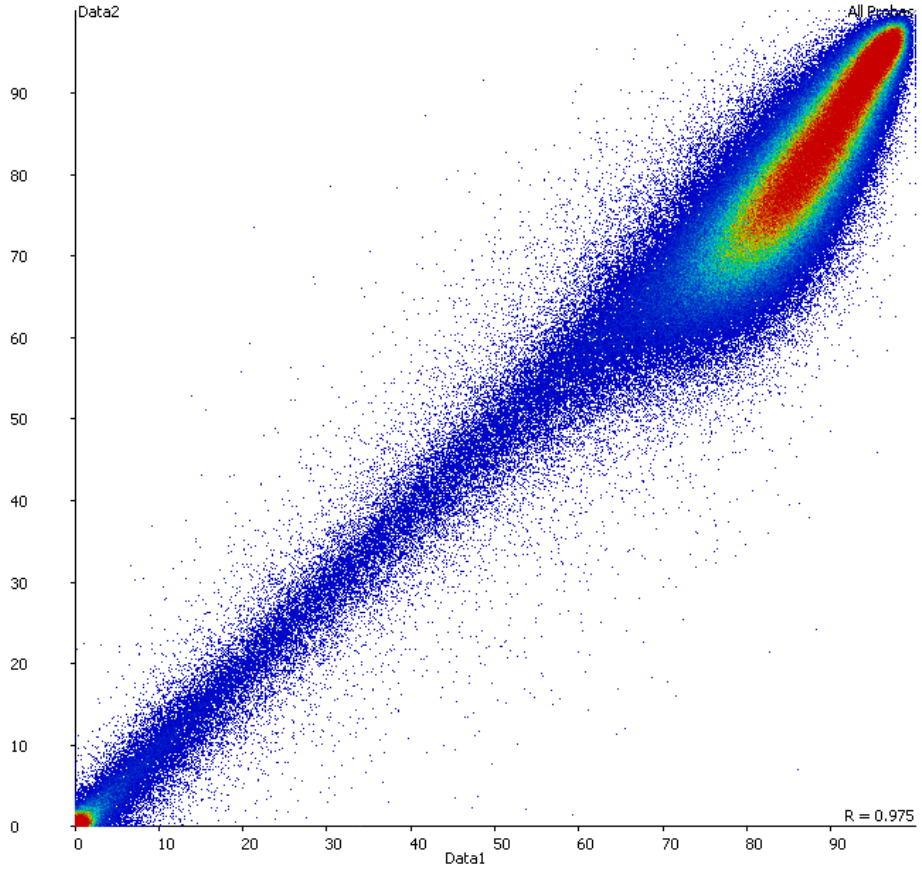
- Some packages offer a conversion from normalised methylation back to counts

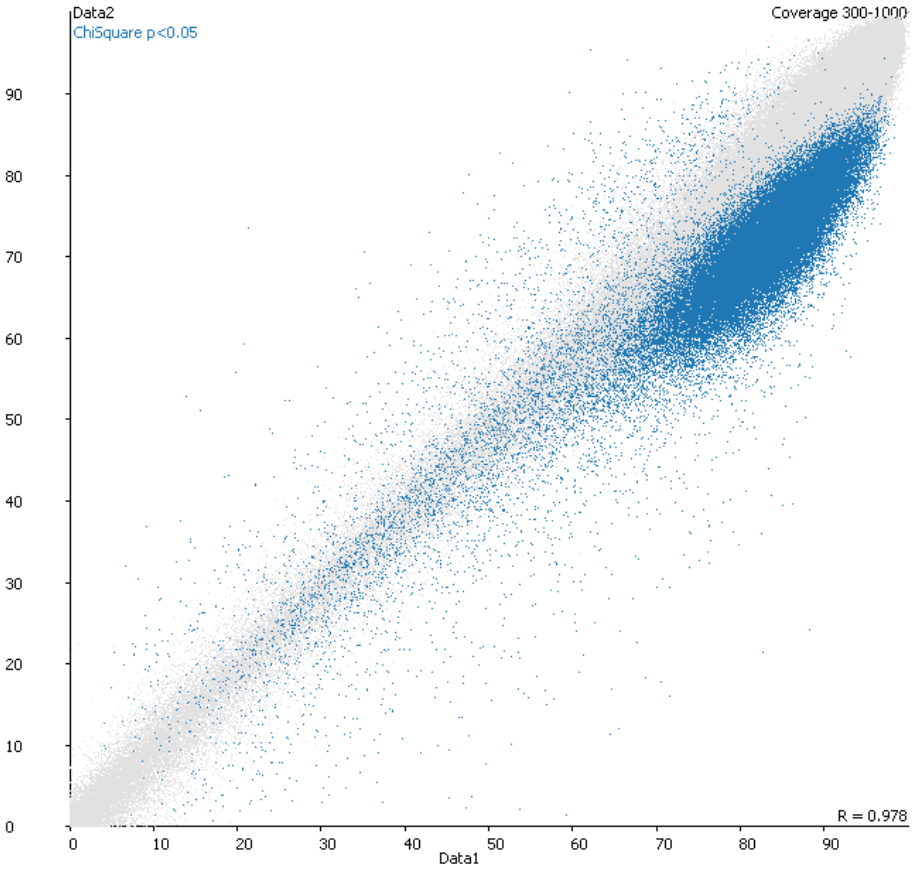
  True observations: Meth=20 Umeth=30 (40% meth)
  Corrected % methylation = 50%
  Reversed counts: Meth=25 Unmeth=25

- Allows count based statistics – regains the lost power from normalisation

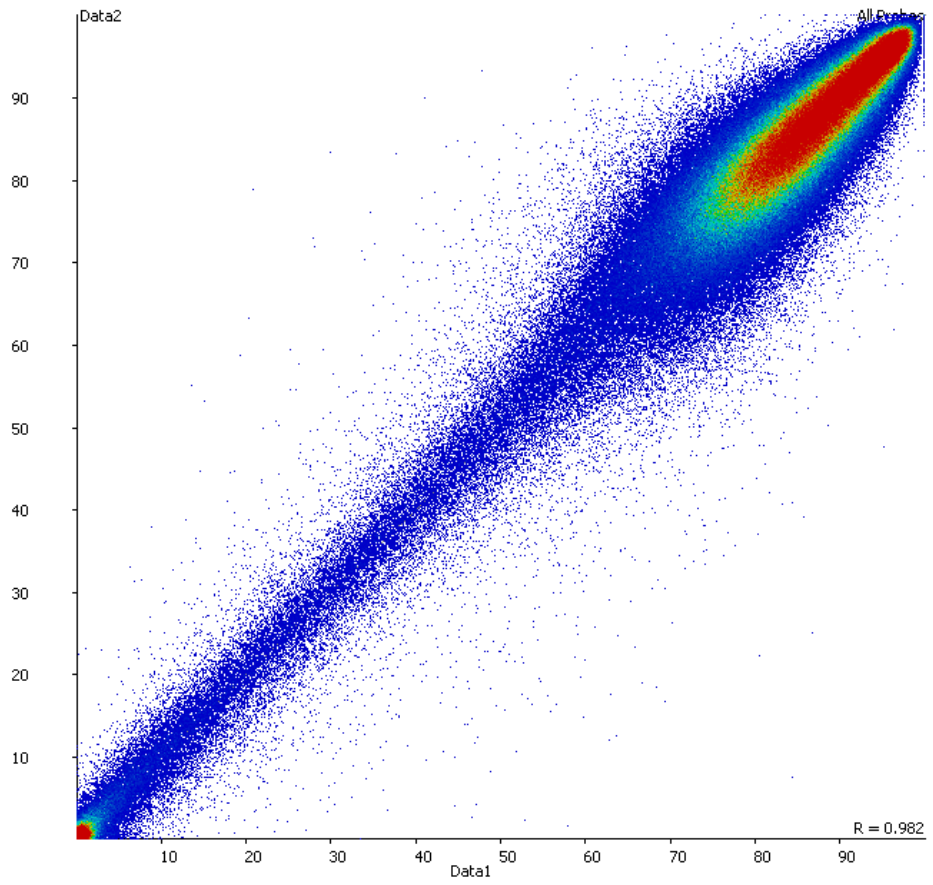
- Retains information about noise from the true observation level

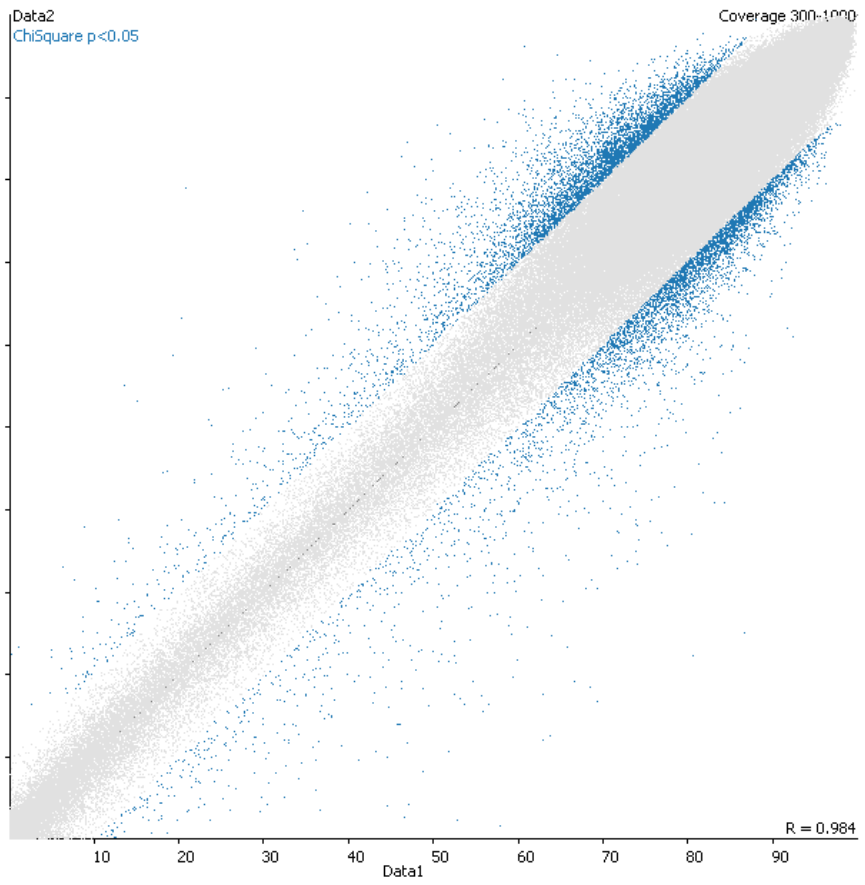
Reverse counting of normalised data can give very different results





Reverse counting of normalised data can give very different results





Reviewing Hits

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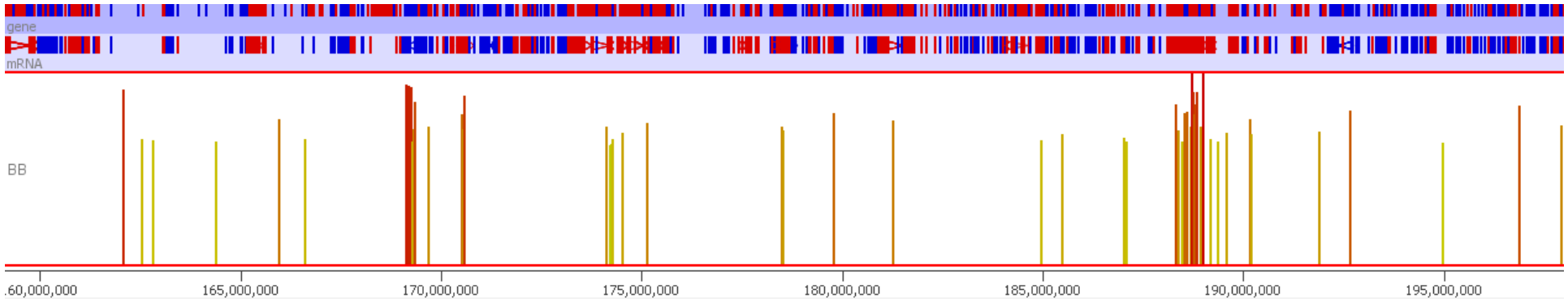
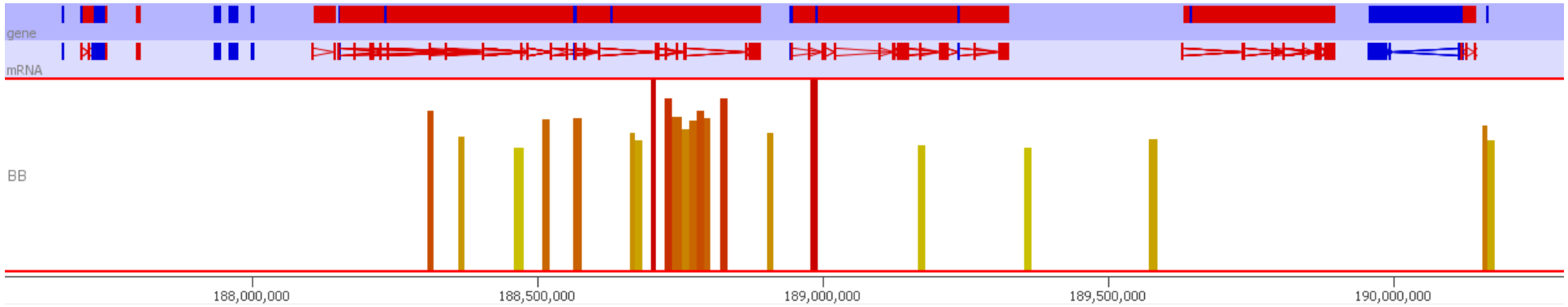
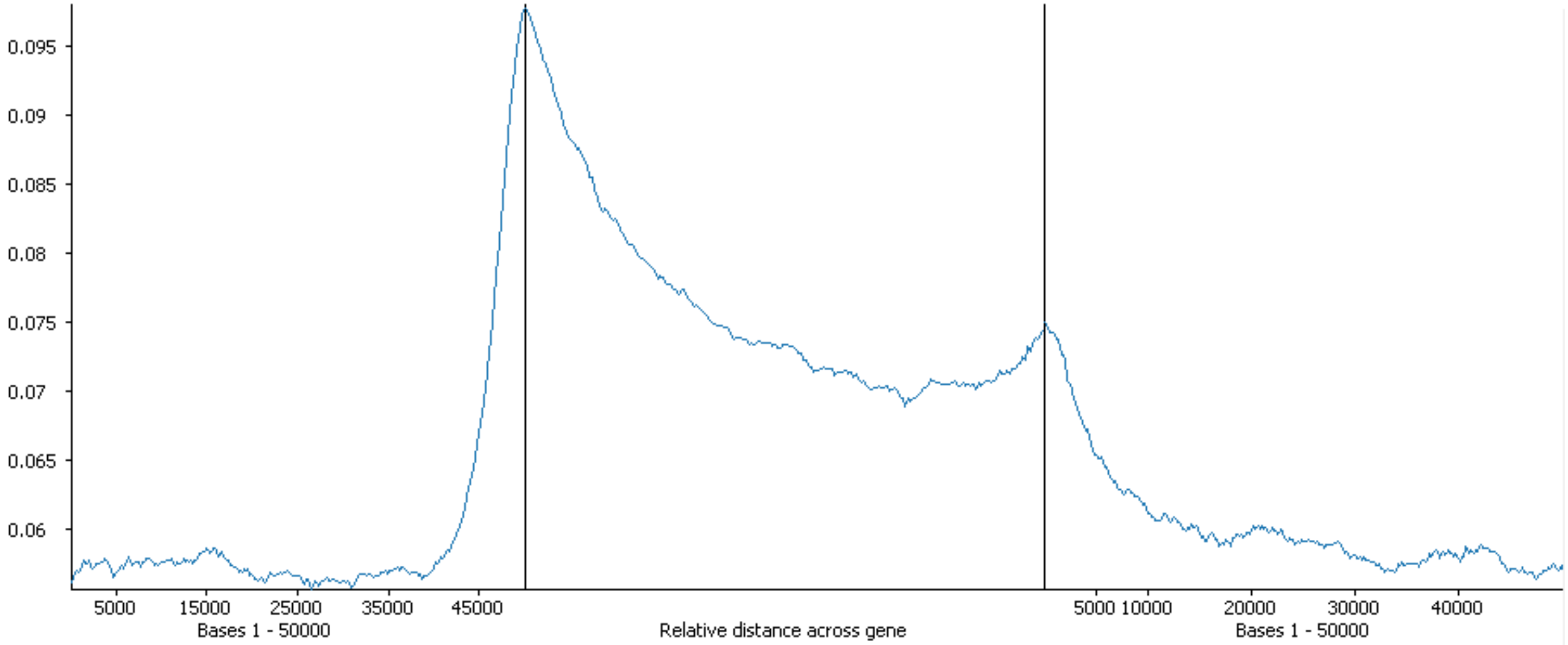
Look for hit clusters



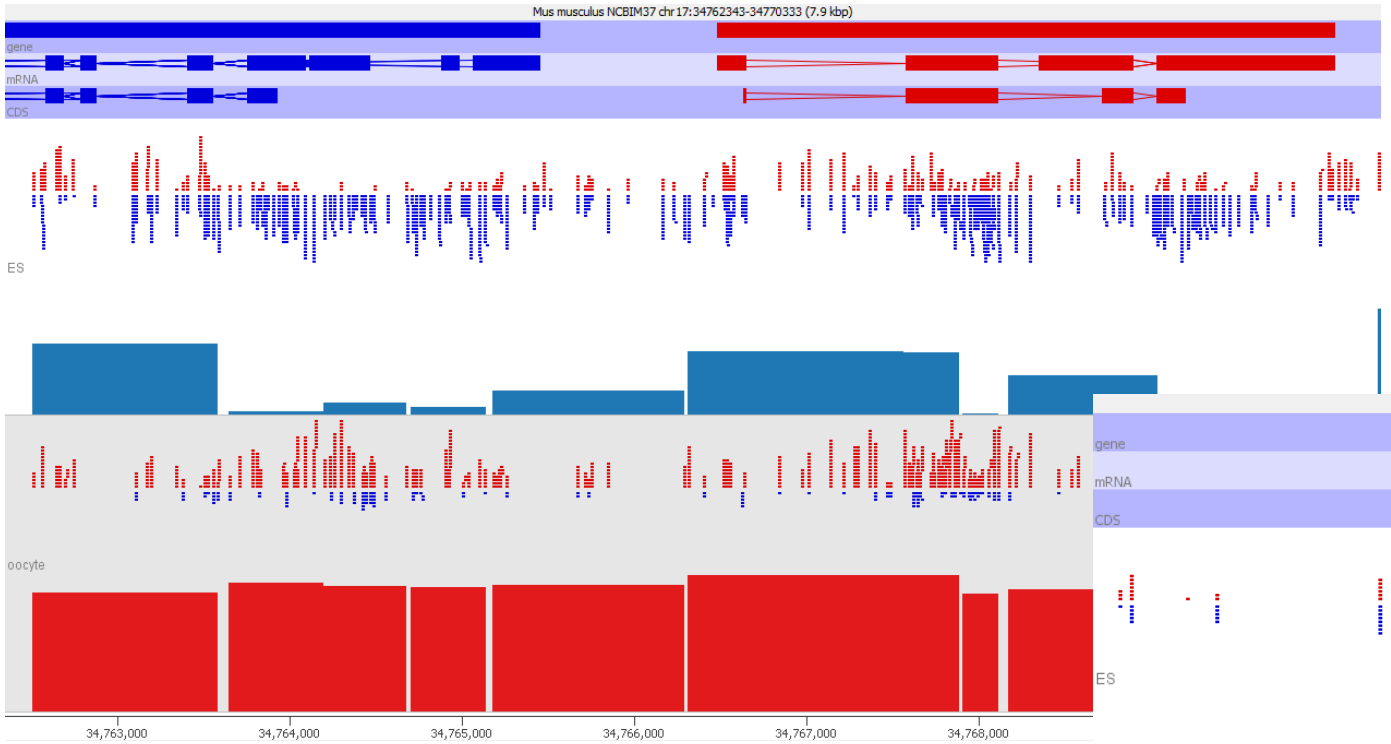


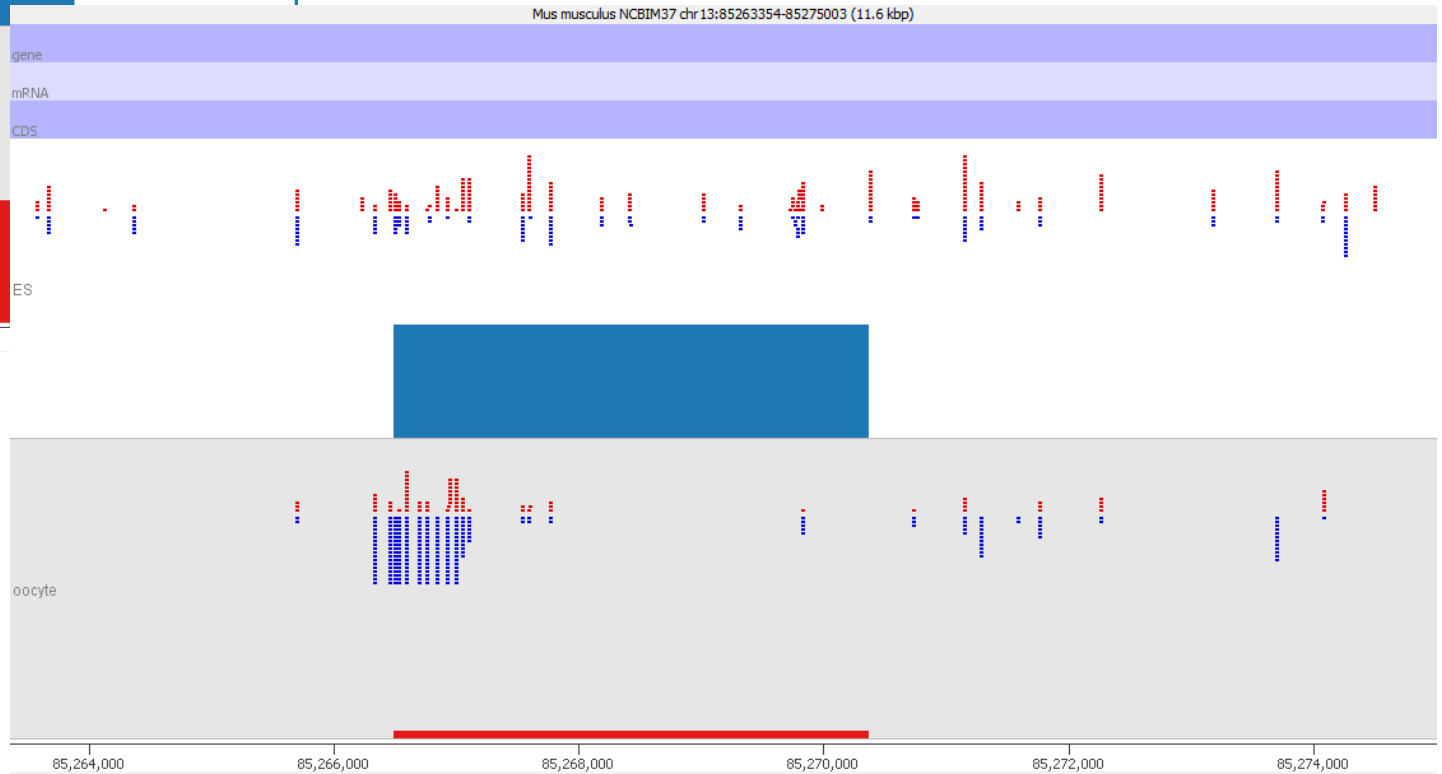
- Grouping to create larger candidate regions
- Check intermediate regions for consistency

Patterning of hits may suggest more specific ways to quantitate and analyse.



Look at underlying data for artefacts





Biological considerations

- Minimum relevant effect size?
  - Balance power vs change
  - What makes biological sense
  - (what would you follow up?)
- Position relative to features
- Consistent change over adjacent regions

Methylation statistics packages

- **SeqMonk** (Graphical Analysis Package)
  - Flexible measurement based on fixed windows, fixed calls or features. Complex corrected methylation calculation and several optional post-calculation normalization options. Chi-Square with optional resampling for unreplicated data, logistic regression with optional resampling for replicated data.

- **EdgeR** (R-package by Gordon Smyth)
  - Originally designed for count data (RNA-Seq mostly), there is now a mode which models paired counts for meth/unmeth to provide differential methylation statistics. Stats are based around negative binomial linear models.

- **methylKit** (R-package by A. Akalin et al.)
  - Sliding window, Fisher's exact test or logistic regression. Adjusts p-values to q-values using SLIM method.

- **bsseq** (R/Bioconductor by K.D. Hansen)
  - Implements the BSmooth smoothing algorithm. Numerous CpG-wise t-tests and p-value cutoff to define DMRs. Outperforms Fisher's exact test. Requires biological replicates for DMR detection

- **BiSeq** (R/Bioconductor by K. Hebestreit et al.)
  - Beta regression model, impractical for very large data other than RRBS or targeted BS-Seq

- **MOABS** (C++ command line tool by D. Sun et al.)
  - Beta binomial hierarchical model to capture sampling and biological variation, Credible Methylation Difference (CDIF) single metric that combines biological and statistical significance