Differential Methylation Analysis

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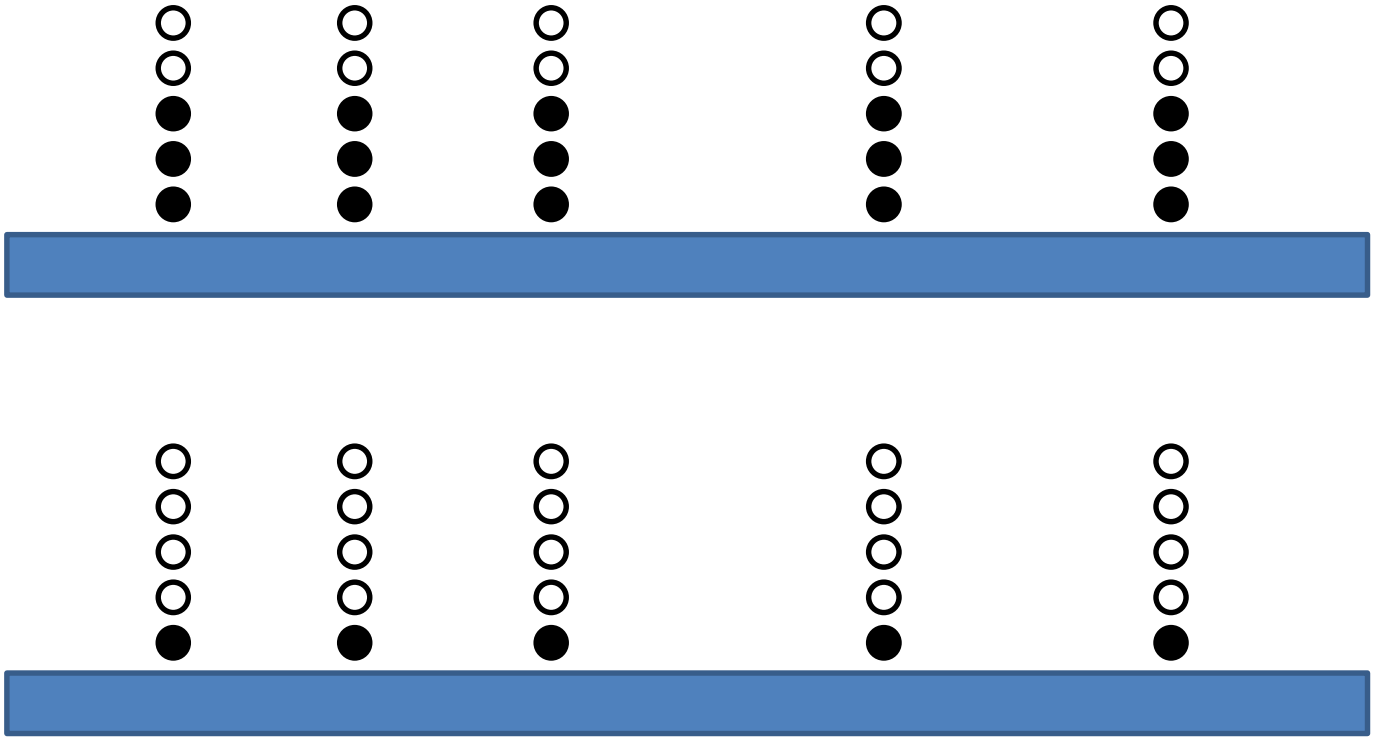
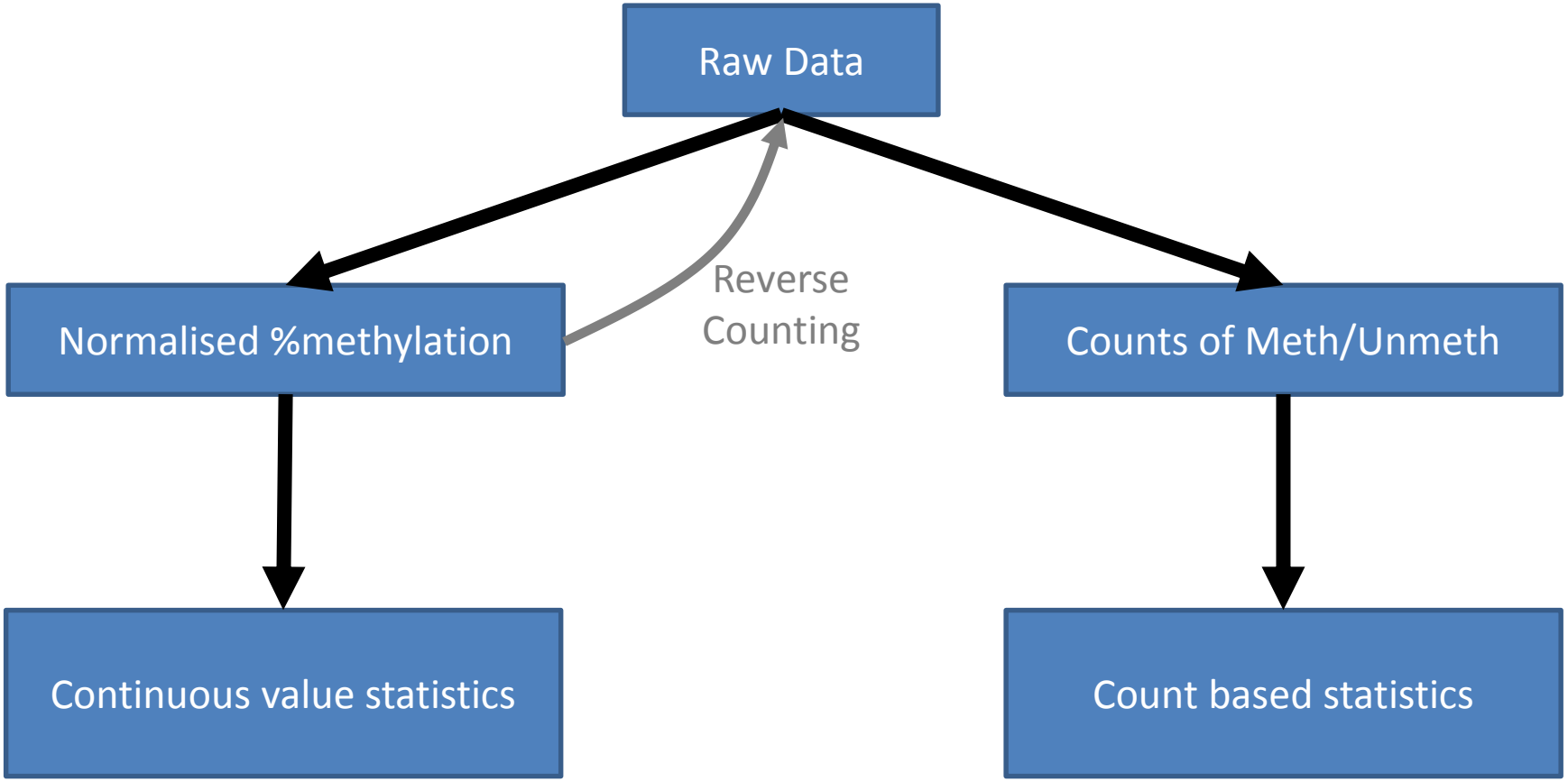
A basic question…



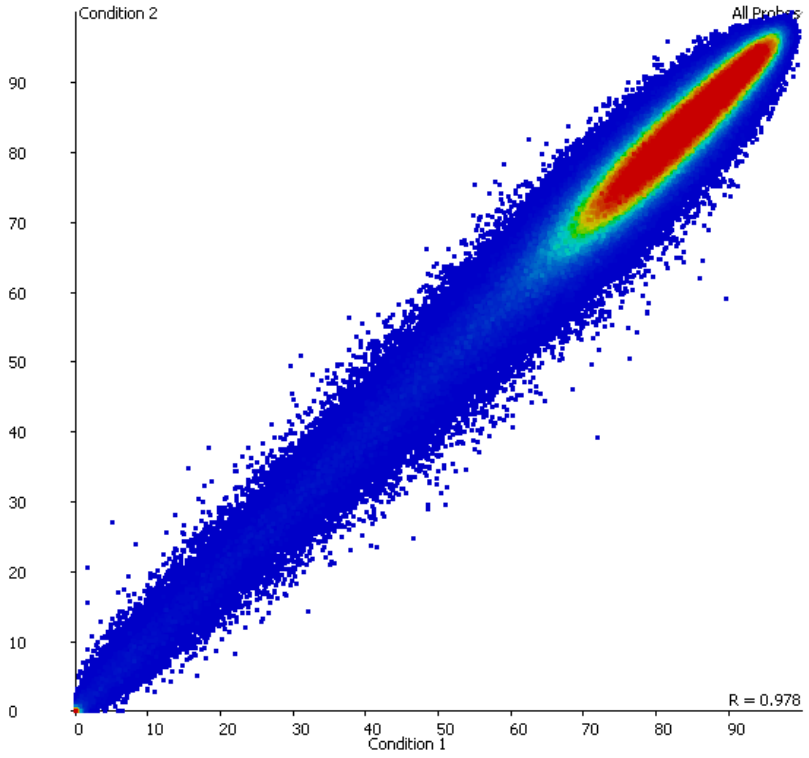
Two Strategies



Factors to consider

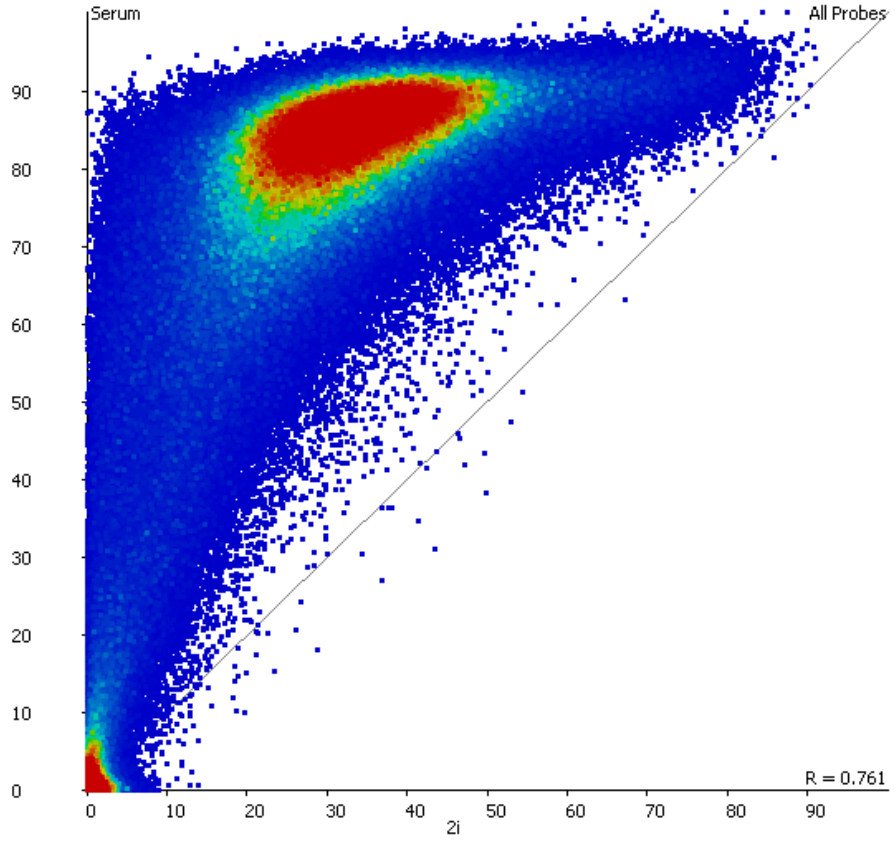
- Formulating a sensible question
- Applying corrections if needed
- Assessing statistical power
- Relating hits to biology

Question



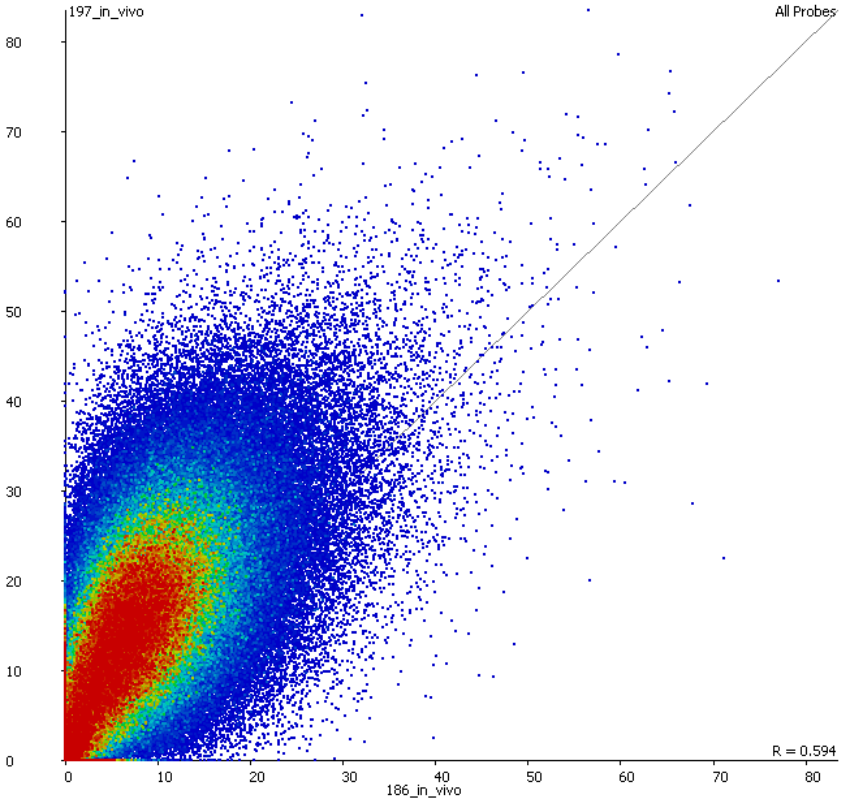
Which areas show a significant change in methylation level between the two conditions?

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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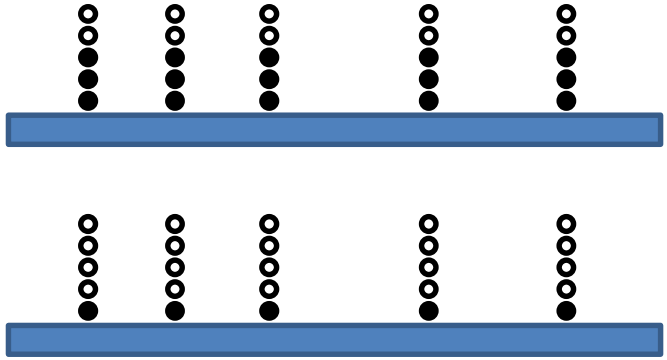
Count Data



	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

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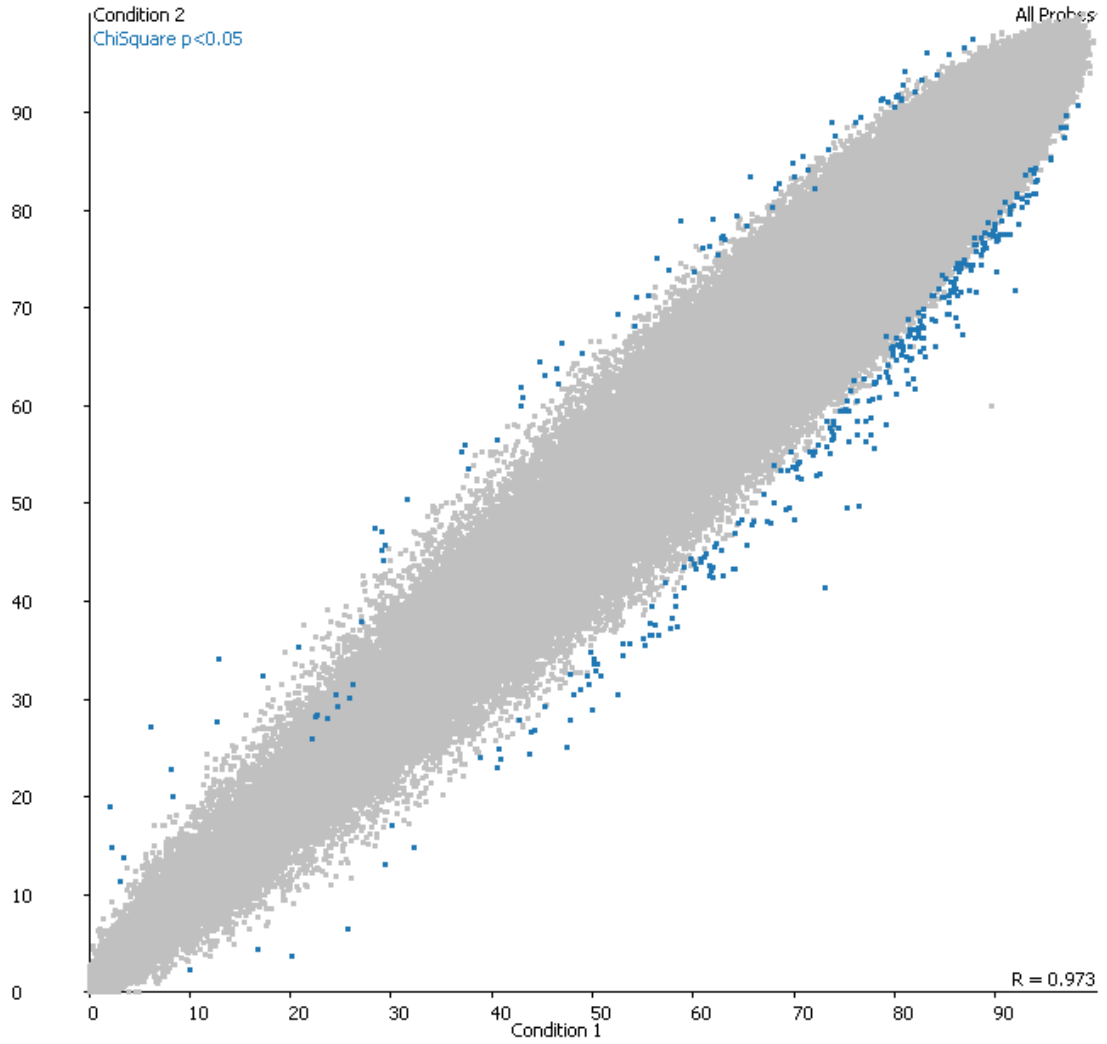
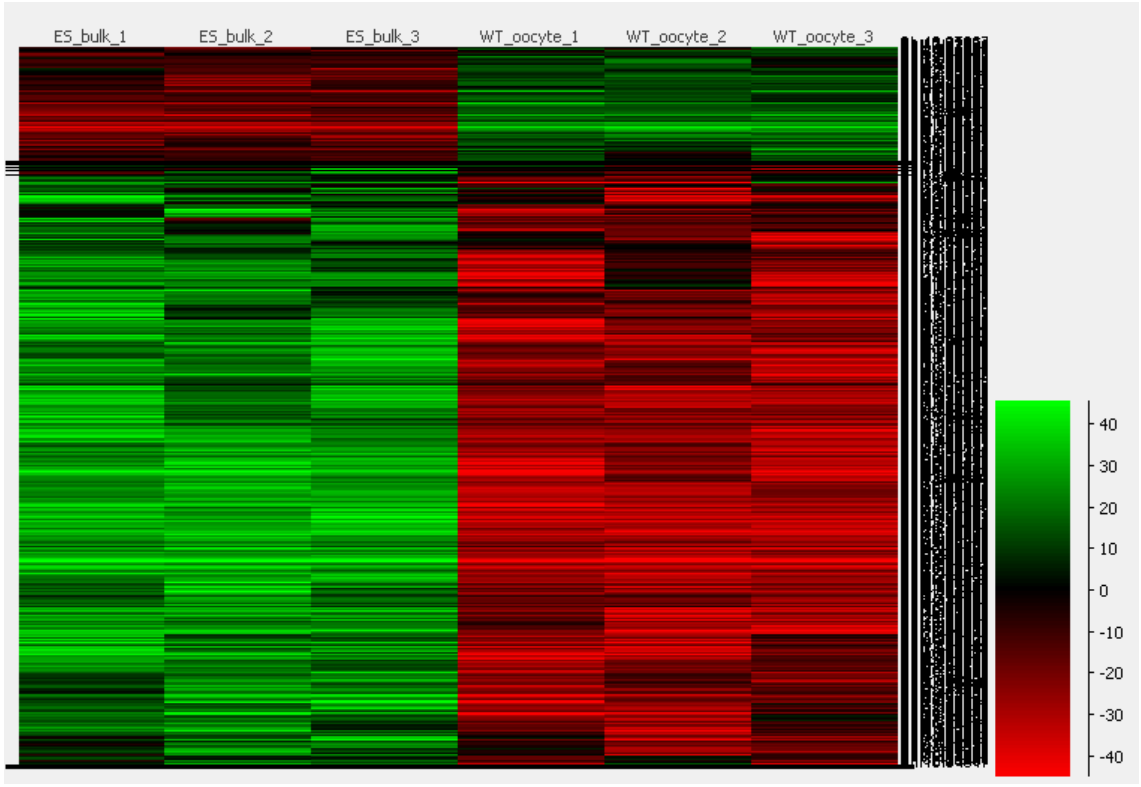
Contingency Statistics are simple to use for differential methylation in well behaved data

- Unreplicated
  - Chi-Square
  - Fisher's Exact

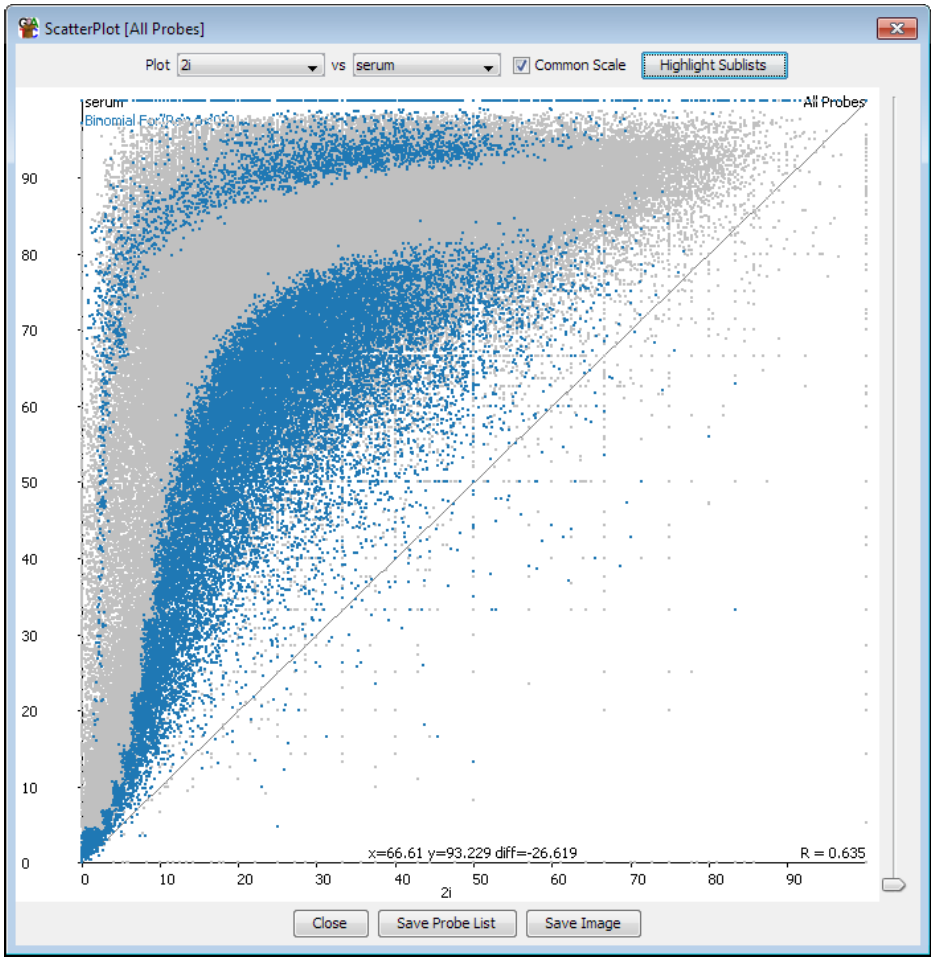


Contingency Statistics are simple to use for differential methylation in well behaved data

- Replicated
- Logistic Regression

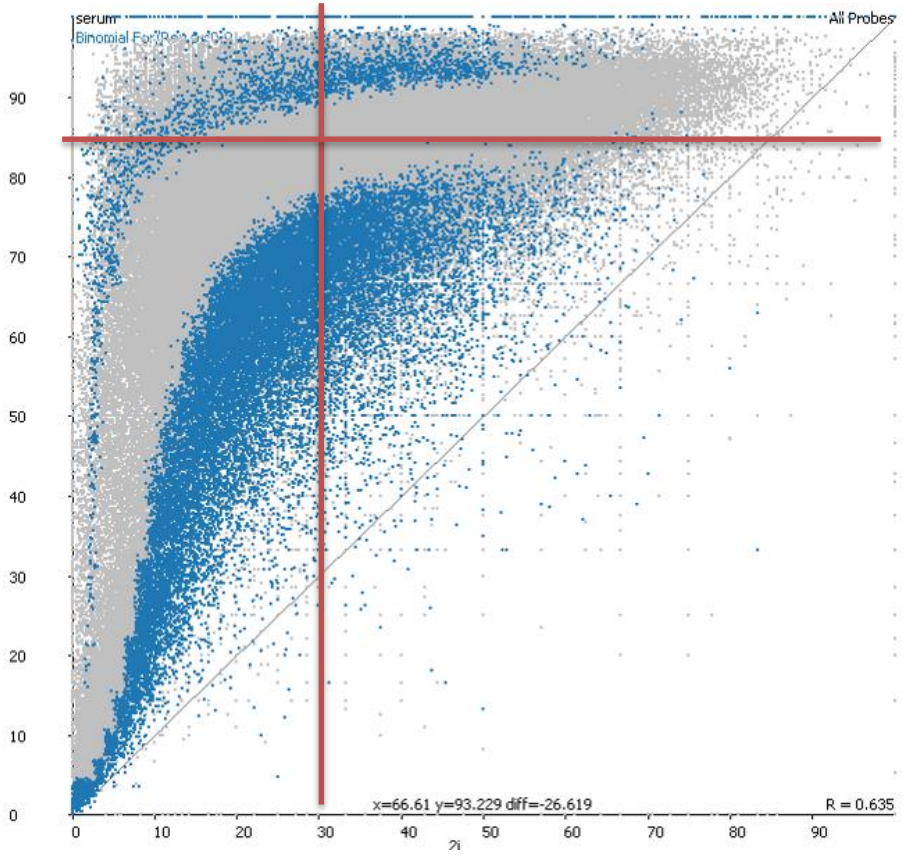


Binomial statistics can find interesting points in globally changing datasets



- 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

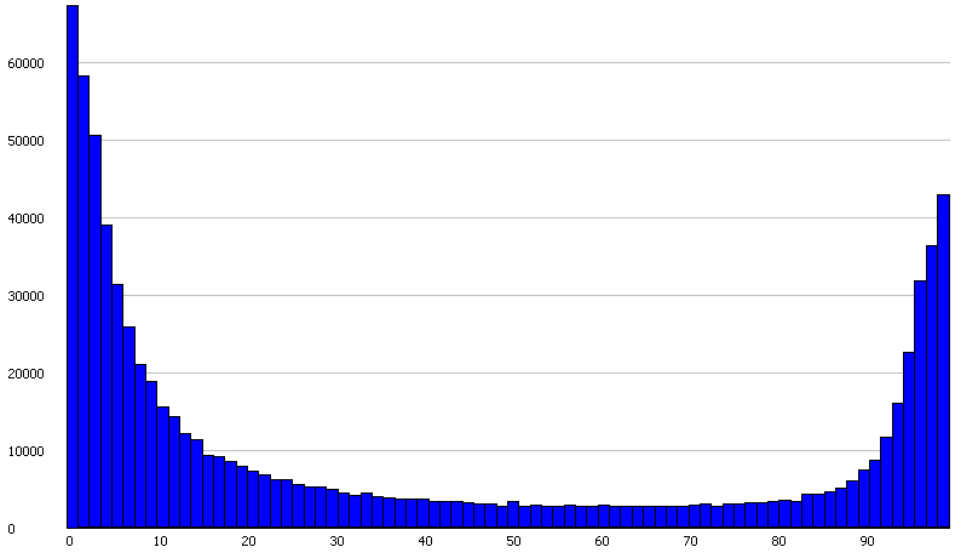
Beta Binomial Models

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

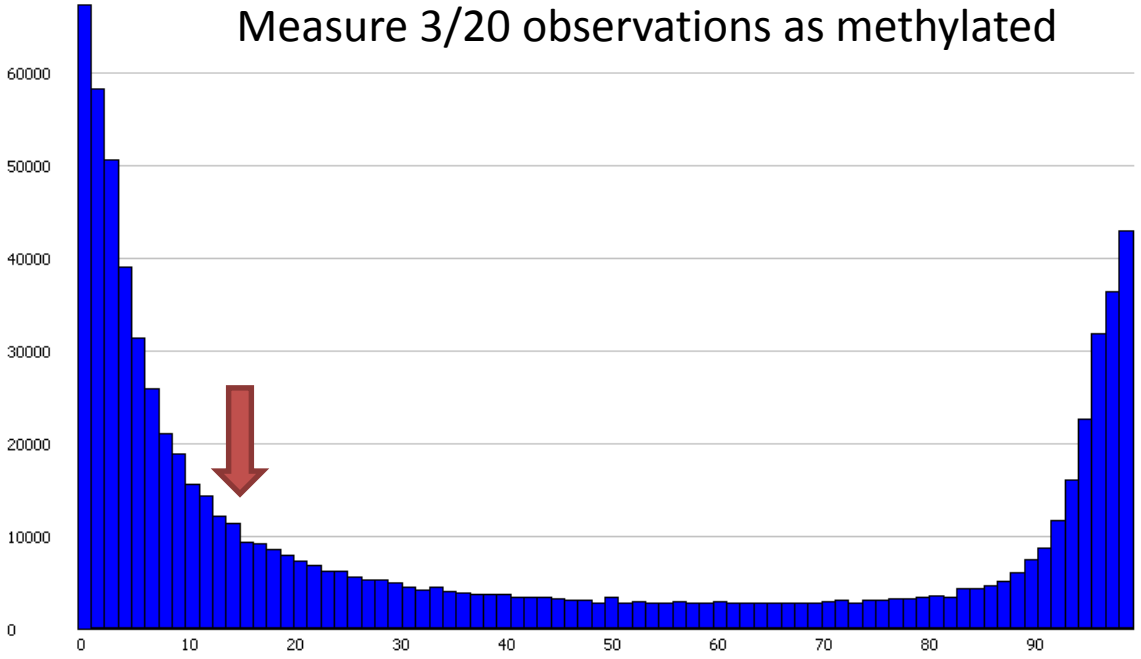
Genome-wide methylation profile.

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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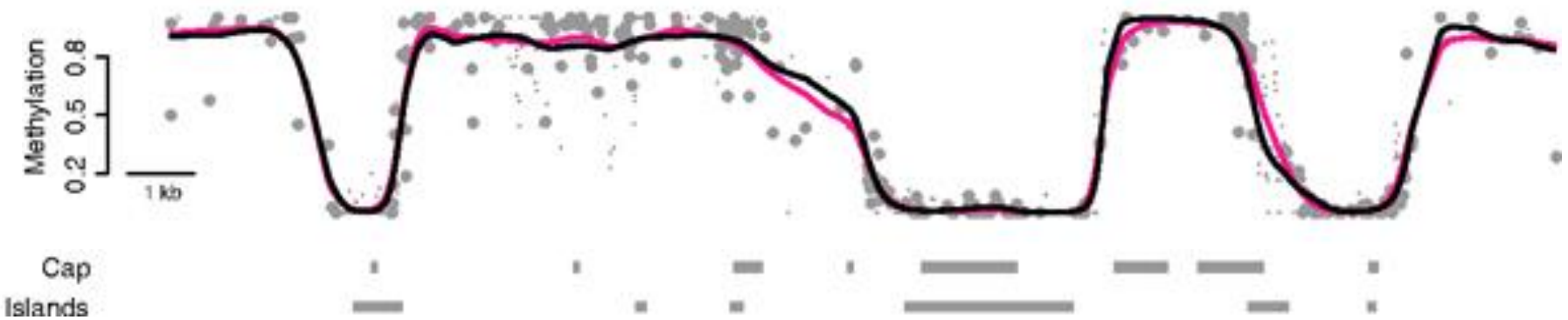
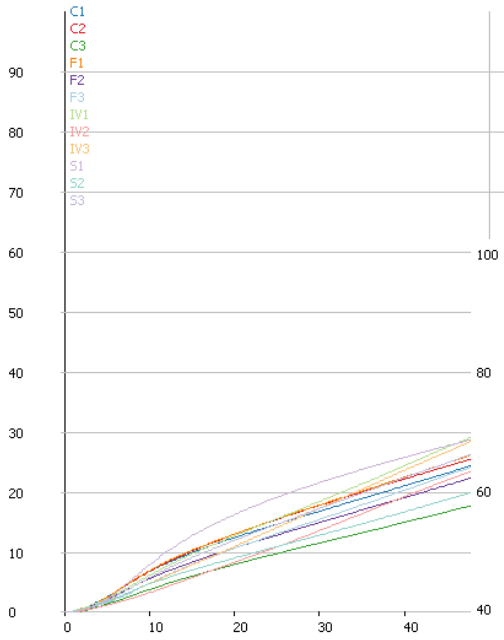
BSmooth algorithm for methylation correction



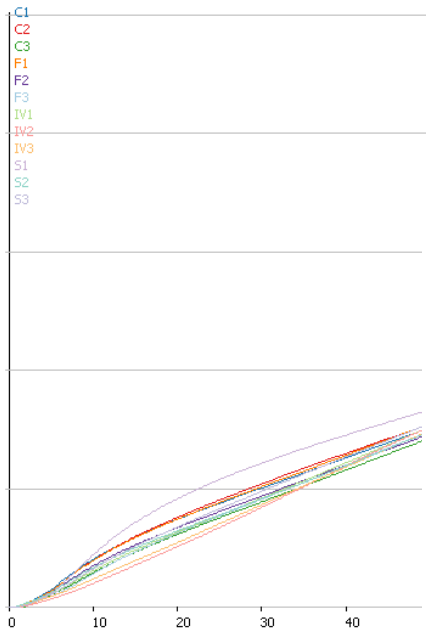
black: 25x (Lister)

pink: 4x (Lister)

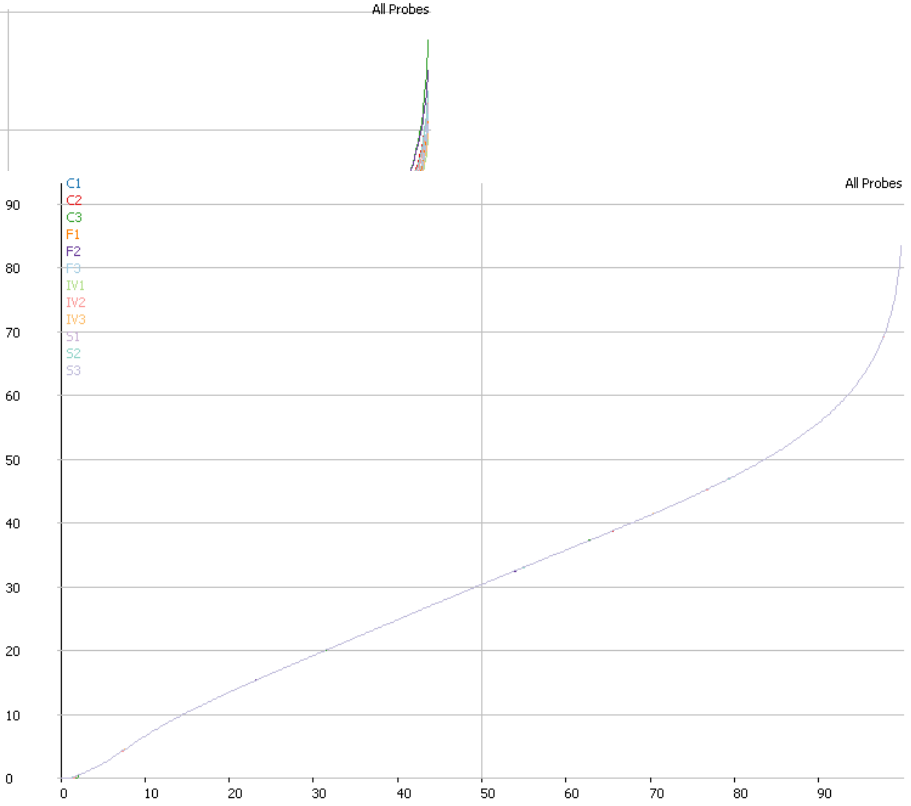
Normalisation for methylation levels



Original



Single (



Quantile Normalisation

Statistics

- Standard continuous statistics
  - T-Test
  - ANOVA
- Information sharing continuous stats
  - LIMMA
- Reduced power – one value per replicate

Reverse counting

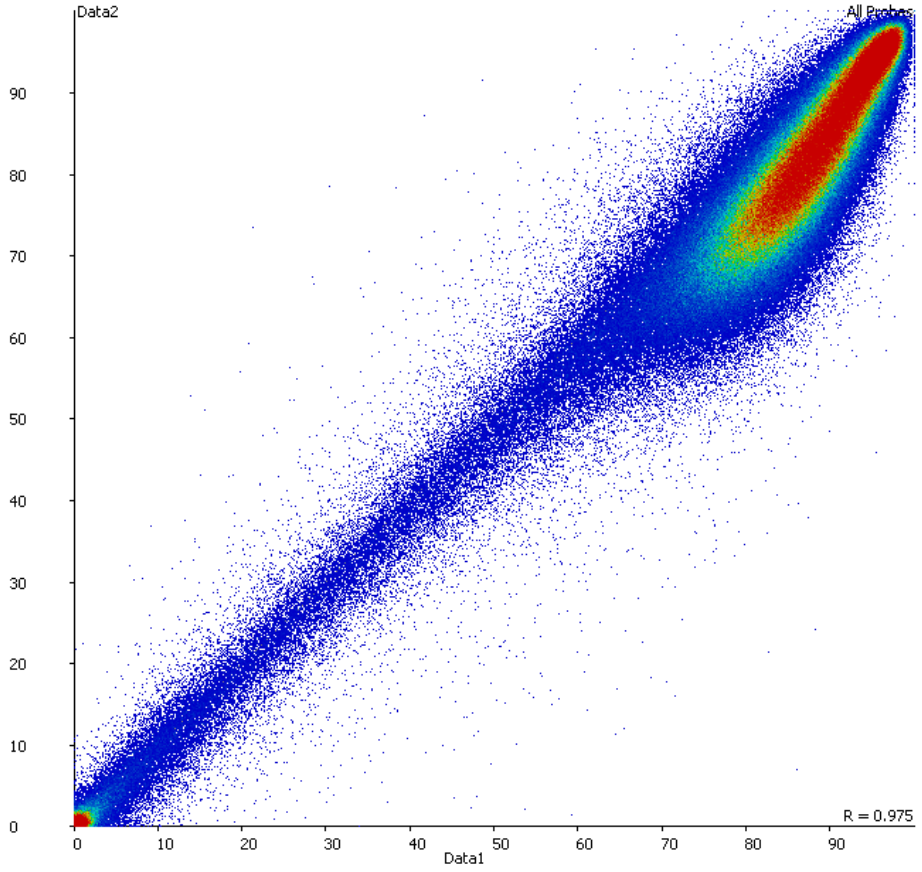
- Some packages offer a conversion from normalised methylation back to counts

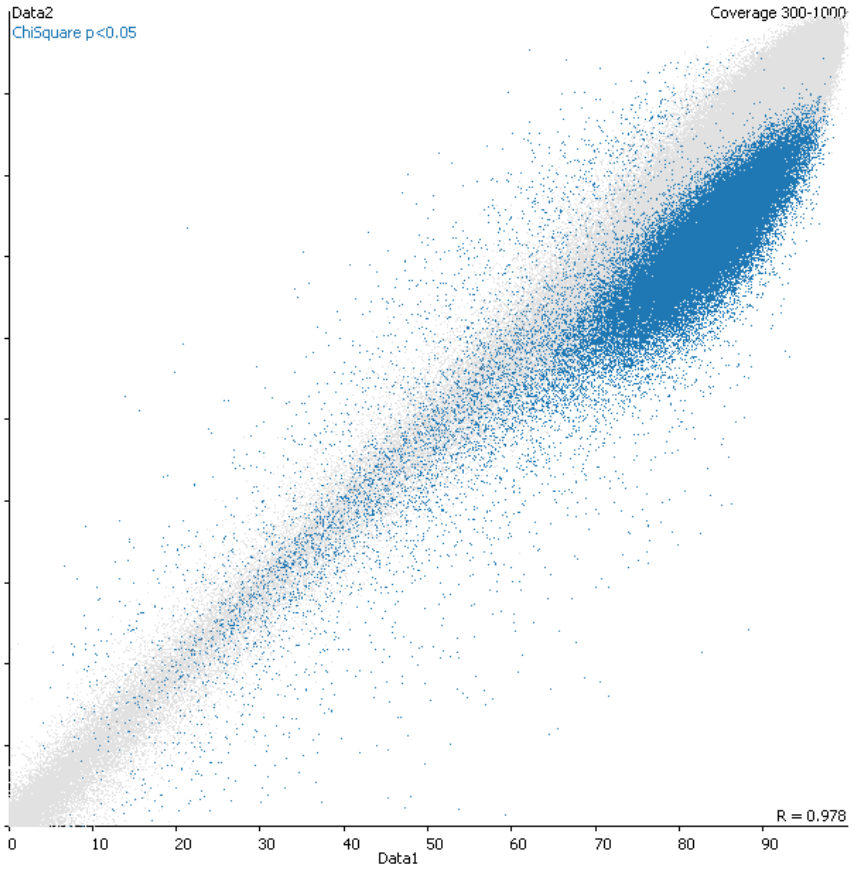
  True observations: Meth=20 Umeth=30 (40% meth)
  Corrected % methylation = 50%
  Reversed counts: Meth=25 Unmeth=25

- 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





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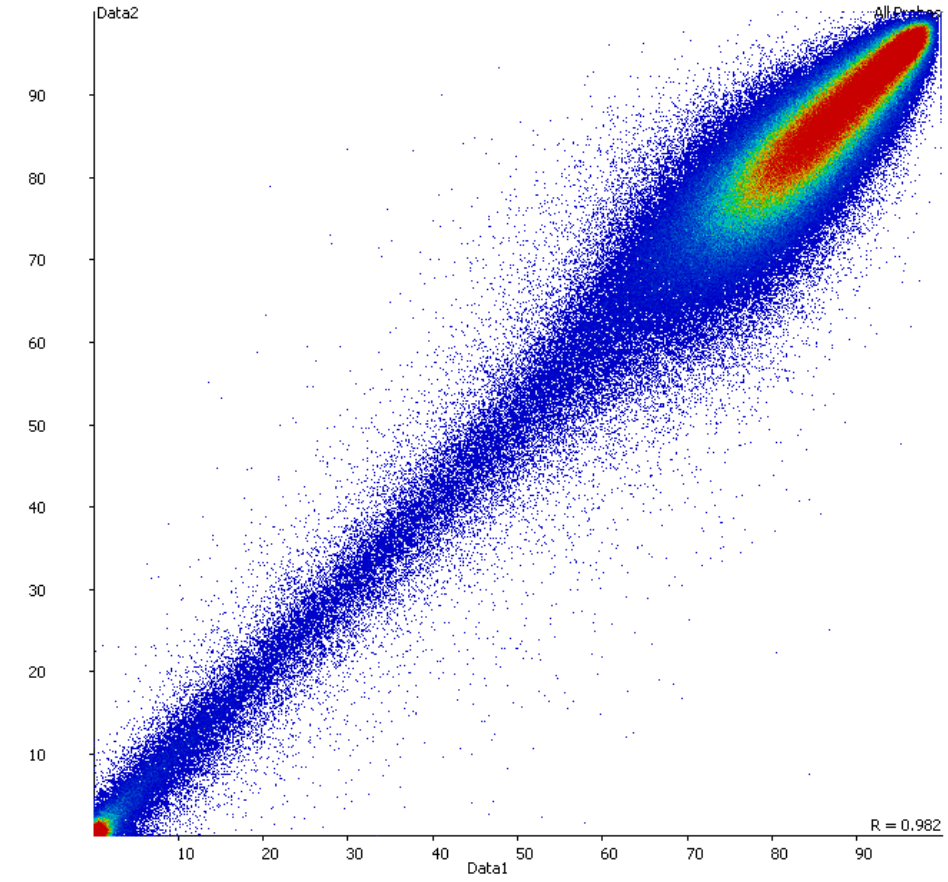
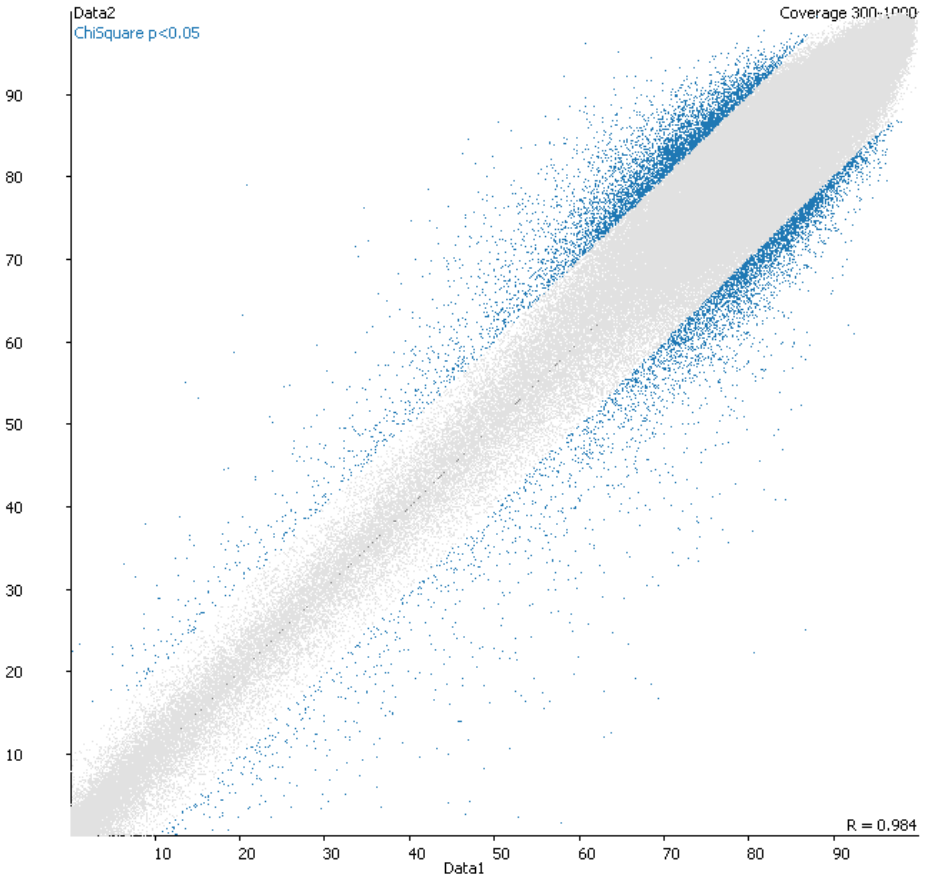
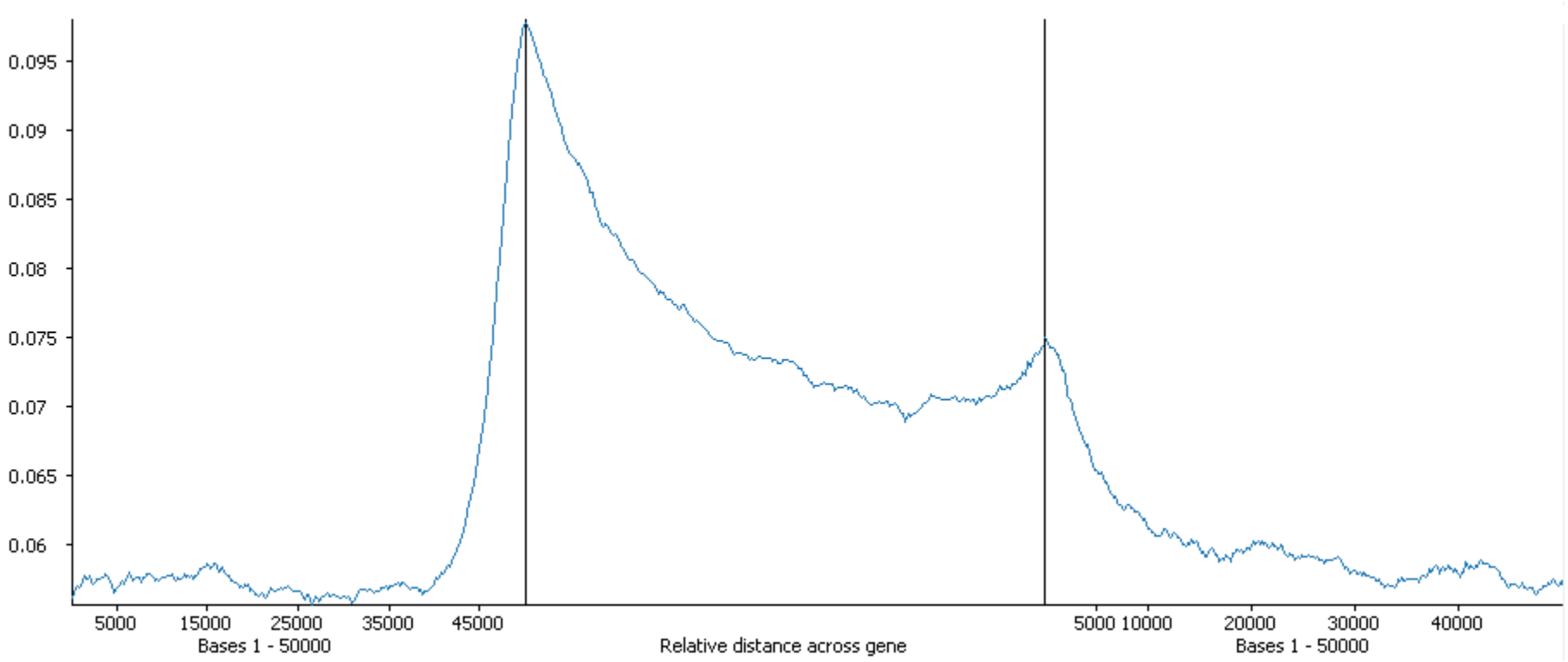




Patterning of hits may suggest more specific ways to quantitate and analyse.



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.

- **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