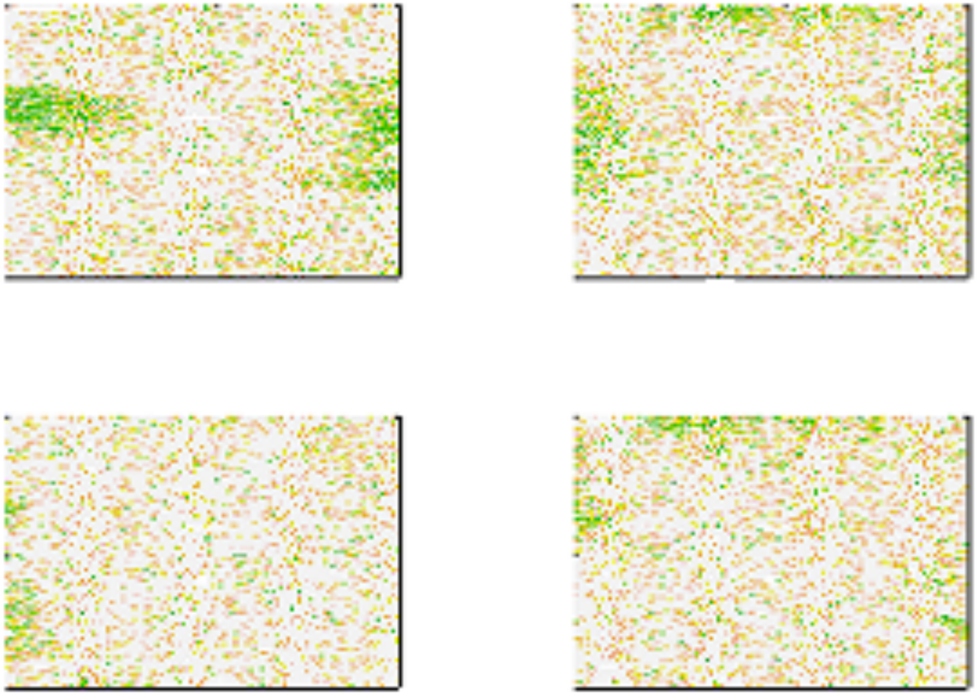
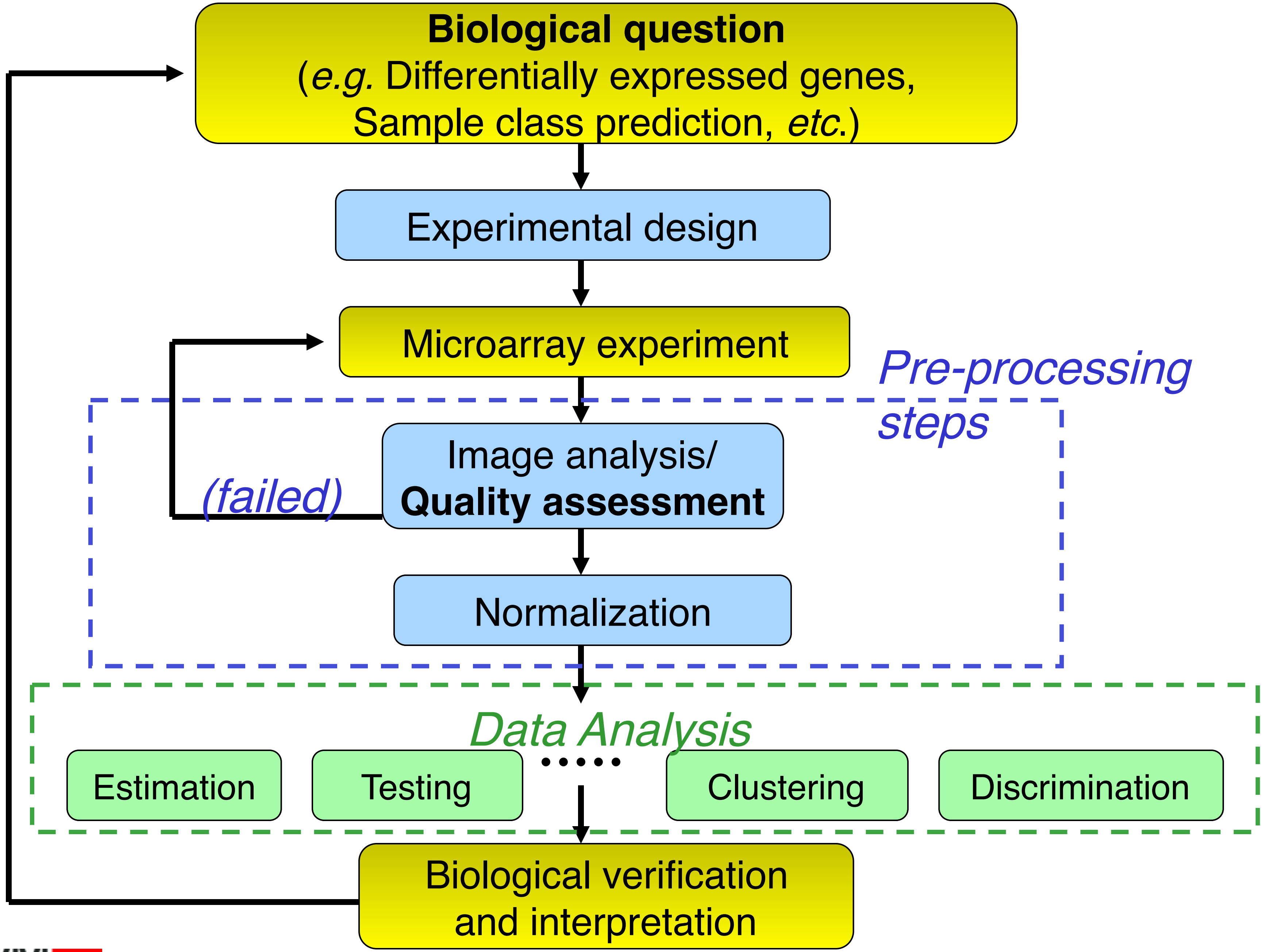
Statistics for Genomic Data Analysis

Affymetrix QA/QC ; Robust regression



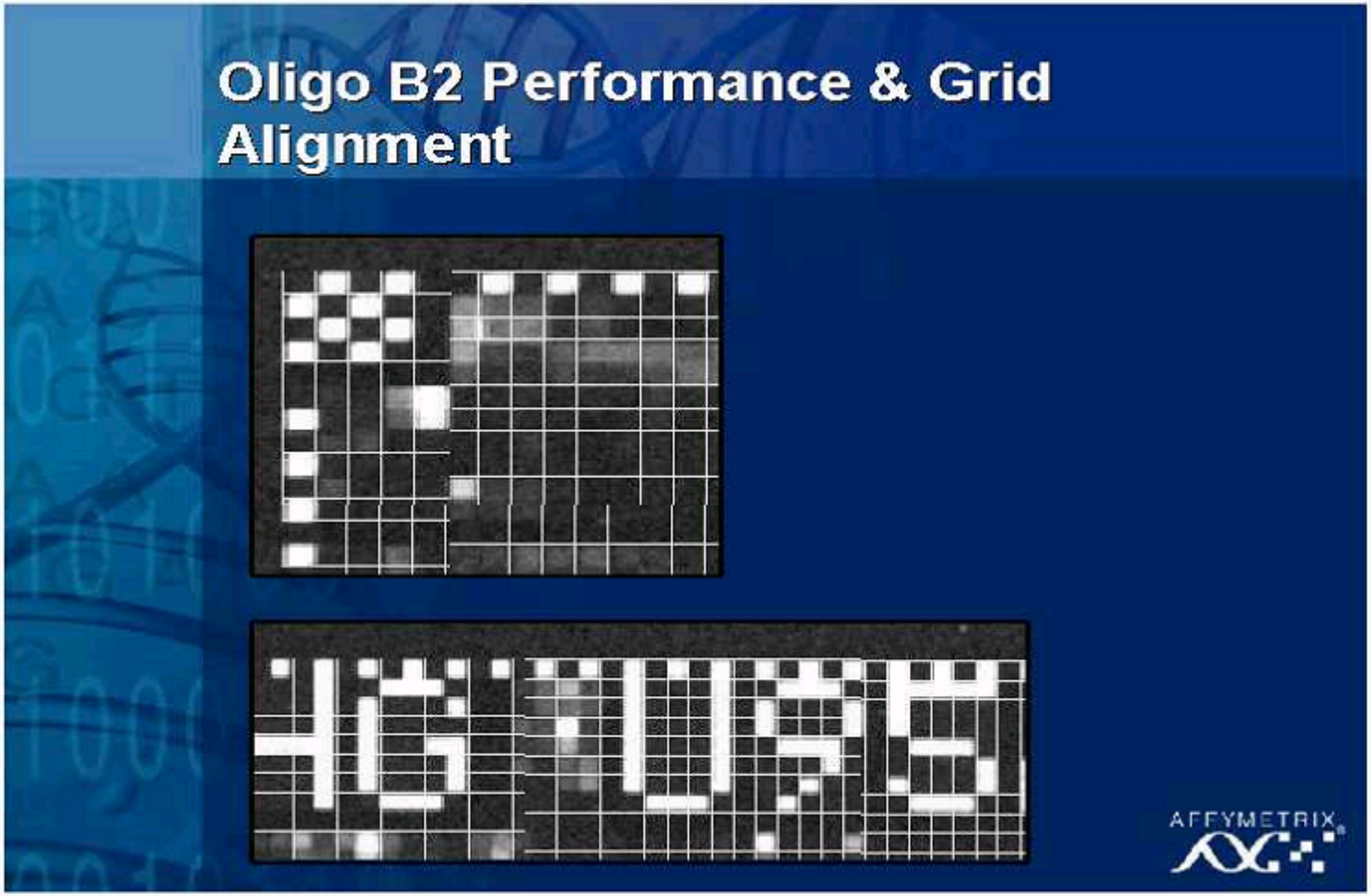
http://moodle.epfl.ch/course/view.php?id=15271


Biological question
(*e.g.* Differentially expressed genes,
Sample class prediction, *etc.*)
Experimental design
Microarray experiment
Pre-processing steps
Image analysis/
Quality assessment
(failed)
Normalization
Data Analysis
Estimation
Testing
……
Clustering
Discrimination
Biological verification
and interpretation

Affymetrix recommended QC

- Sample prep QC
  - pre-hyb QC
  - bioanalyzer profiles
  - preempt hybing poor quality
- Data QC
  - post-hyb QC
  - visual inspection of image, oligo b2, grid alignment
  - metrics in rpt file

Oligo B2 Performance



Oligo B2 Performance & Grid Alignment

Spike-ins and controls

- *Unlabelled poly-A controls* : *dap, lys, phe, thr, tryp* ; used to monitor wet lab work
- *Hybridization controls* : *bioB, bioC, bioD, cre*
- *Housekeeping/control genes* : actin, gapdh
  - 3' to 5' signal intensity ratios of control probe sets

Control Spikes

```
Spike Controls:
Probe Set         Sig(5')     Det(5')     Sig(M')     Det(M')     Sig(3')     Det(3')
      Sig(all)    Sig(3'/5')
BIOB        60.8  M     63.7  P     63.9  A     62.81 1.05
BIOC        134.7 P                 75.1  P     104.91      0.56
BIODN       105.0 P                 677.7 P     391.35      6.46
CREX        907.2 P                 1486.7      P     1196.97     1.64
DAPX        14.6  A     8.5   A     1.8   A     8.30  0.12
LYSX        1.4   A     8.4   A     11.0  A     6.92  8.09
PHEX        3.7   A     1.8   A     5.3   A     3.60  1.46
THRX        1.4   A     4.0   A     3.3   A     2.91  2.39
TRPNX       4.2   A     4.3   A     1.7   A     3.42  0.40
______________________________________________________________________
```

- BioB should be P ~ 70% of the time
- BioC, BioD, cre should always be P

Internal control genes

```
Housekeeping Controls:
Probe Set          Sig(5')     Det(5')     Sig(M')     Det(M')     Sig(3')     Det(3')
      Sig(all)     Sig(3'/5')
HUMISGF3A/M97935   26.4   P    149.6 M     272.6 P     149.54      10.31
HUMRGE/M10098      3.1    A    5.0    A    10.7   A    6.26   3.49
HUMGAPDH/M33197    3300.4      P     3005.6      P     3221.6      P     3175.87     0.98
HSAC07/X00351      7532.9      P     8839.1      P     6645.4      P     7672.49     0.88
M27830             65.3   P    35.7   A    144.4 A     81.81 2.21
_____________________________________________________________________________
```

- actin, gapdh should have all P
- 3’/5’ ratio < 3

Quality metrics in Affy rpt file

- % Present call: *20-50%* ; consistency
- Scaling Factor:
  - Target/(2% trimmed mean signal values) ; consistency
- P/A calls, SF : measure how much is PM > MM
- Background: *under 100* ; consistency
  - Average signal in lowest 2%
- Noise (RawQ): *1.5-3* is ok
  - Pixel-to-pixel variation among probe cells used to calculate the background

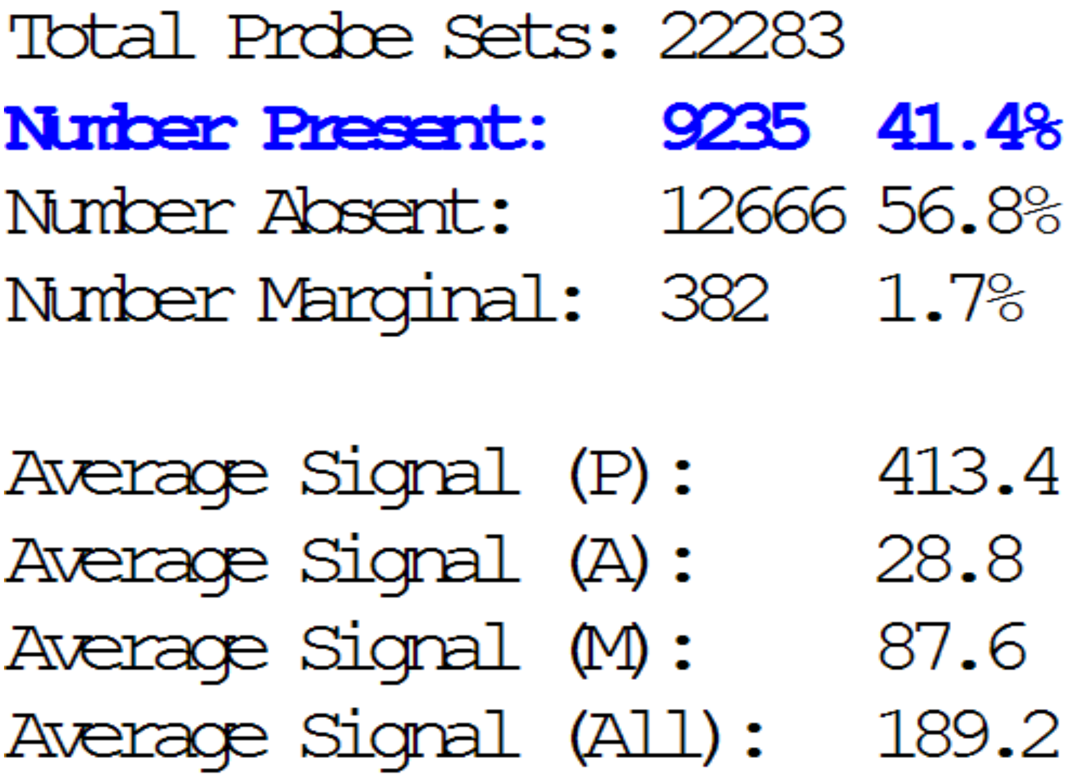
MAS 5 algorithms

- *Present calls* : *p*-value from Wilcoxon signed rank test based on $R_i = (PM_i - MM_i)/(PM_i + MM_i)$
  - *H:* median $(R_i - \tau) = 0$ vs. *A:* median $(R_i - \tau) > 0$
  - τ small (=0.015)
  - P = `present’: $p < 0.04$ ; A = `absent’: $p \geq 0.06$; M = `marginal’: $0.04 < p < 0.06$
- *Signal* : $\log_2(S) = \Sigma_i \, w_i \log_2 (PM_i - MM_i^*)$,

  with w_i Tukey biweight from initial fit
- Tukey biweight: $w_i = (1 - (r_i / c^2)^2$ if $| \, r_i \, | \leq c$;

  $= 0$ otherwise

% Present

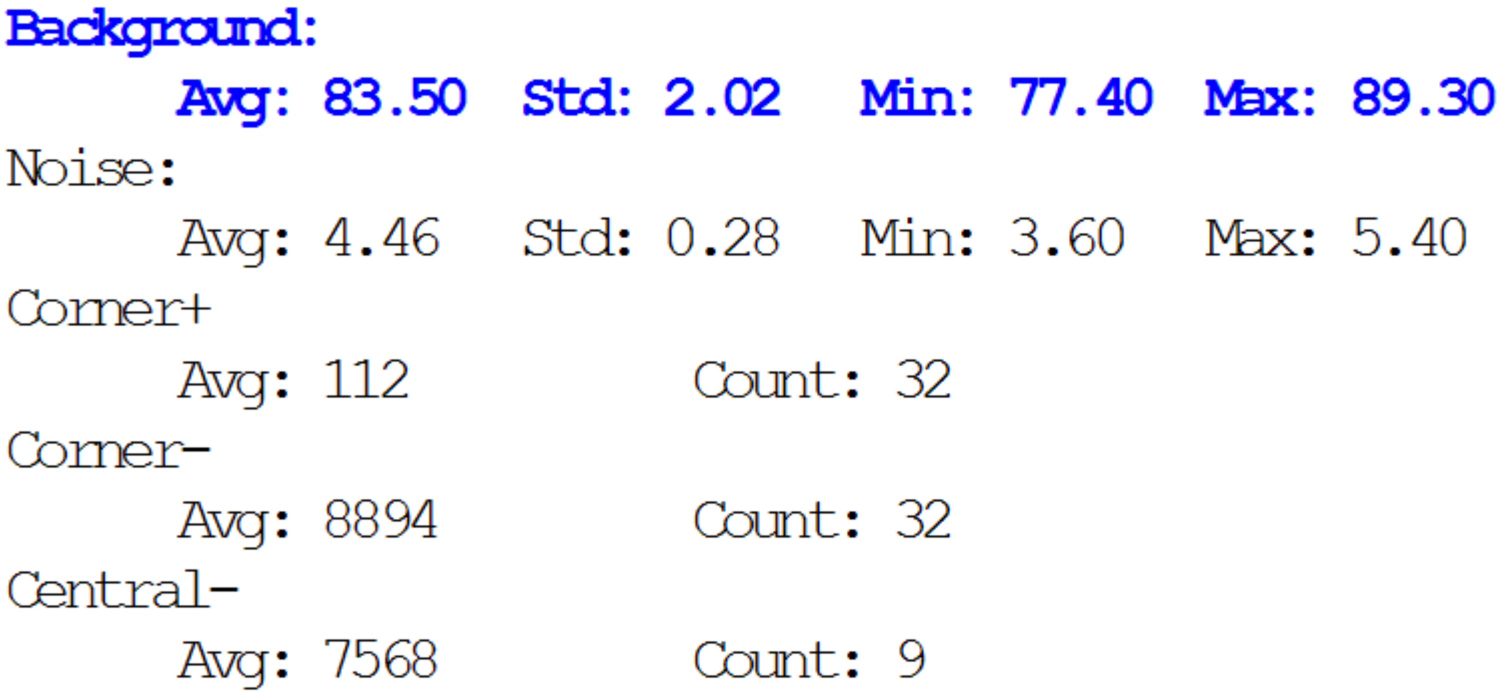


```
Total Probe Sets: 22283
Number Present:    9235   41.4%
Number Absent:     12666 56.8%
Number Marginal:   382    1.7%

Average Signal (P):     413.4
Average Signal (A):     28.8
Average Signal (M):     87.6
Average Signal (All):   189.2
```

- % P ~ 20 - 50%
- ‘good indicator of assay performance’
- similar values across replicates (also SF, RawQ)

Background



```
Background:
        Avg: 83.50   Std: 2.02    Min: 77.40   Max: 89.30
Noise:
        Avg: 4.46    Std: 0.28    Min: 3.60    Max: 5.40
Corner+
        Avg: 112           Count: 32
Corner-
        Avg: 8894          Count: 32
Central-
        Avg: 7568          Count: 9
```

- Should be under 100
- similar values across replicates

Problems with these measures

- Relate to the experimental process, *not* directly to the end result (gene expression)
- Quality of spike-in data may not be representative of whole chip quality
- In general, thought, inferences (DE, clustering, etc.) are based on ME
- *Single chip* measures, which do not put each chip in the context of the others
- *By-products of RMA calculation (robust regression) can also provide quality info*

What is ‘quality’?

- It is useful to distinguish between the various facets of the general term ‘*quality*’
- In chronological order:
  - condition of the starting RNA (*RNA integrity*)
  - caliber of the experimental process and resulting hybridization (*noise*)
  - acceptability of the resulting expression measures:
    - *array adjustment*
    - *outlier identification*

New quality measures – RMA-QC

- Aims:
  - To use QA/QC measures directly based on expression summaries and that can be used in a routine way
  - To examine whether chips are different in a way that affects expression summaries
- Focus on *weights* and *residuals* from fits in probe intensity models

RMA - Additive model for gene expression based on probe intensity data

- *Probe-level model* for gene expression:

  $\log_2(PM^*_{ij}) = c_i + p_j + \varepsilon_{ij}$

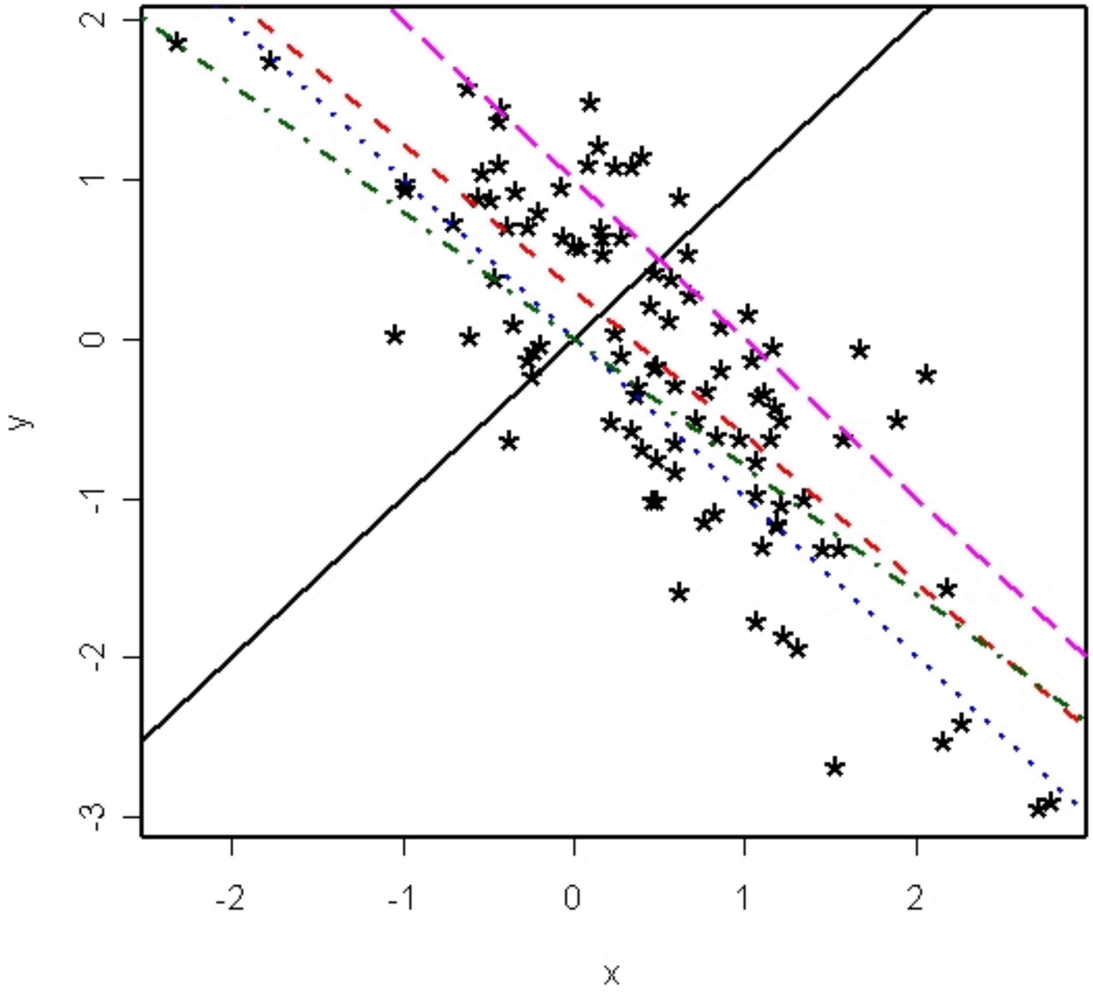
  - c_i = $\log_2$ scale expression level for chip j
  - p_j = probe affinity effect
  - ε_{ij} = iid error term
- For *identifiability*, fit with constraint $\Sigma_j \, p_j=0$
- Model fit (separately) for *each probe set*

RMA: Summary

- Chips analysed in *sets* (e.g. an entire experiment)
- *Use only PM*, ignore MM
- *Background* correct PM on raw intensity scale
- *Quantile Normalization* of $\log_2(PM^*)$
- Assume additive model (on $\log_2$ scale) for each probeset: $\log_2 \text{normalized}(PM_{ij}^*) = c_i + p_j + e_{ij}$
- Parameters c_i provide measure of gene expression for each chip
- Estimate parameters using a *robust* method
  - median polish – quick
  - robust linear model – yields quality diagnostics

Simple linear modeling: which line?

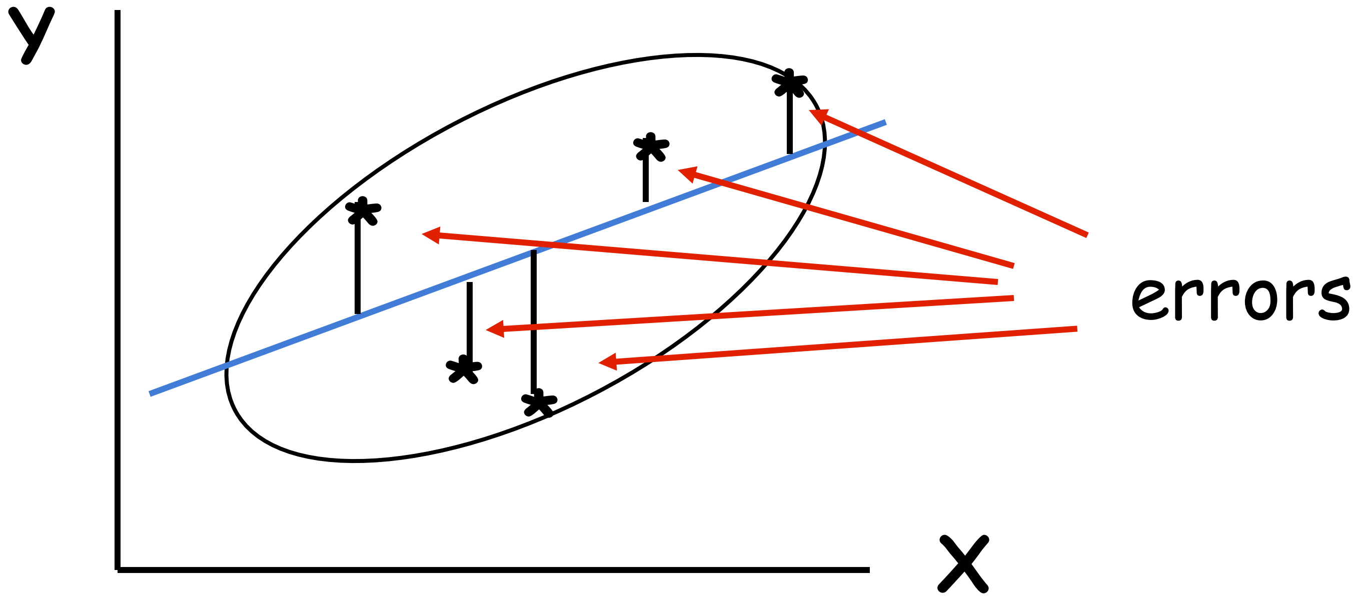
- There are *many possible lines* that could be drawn through the cloud of points in the scatterplot ...
- How to choose?



Least Squares

- *Q* : Where does the regression equation come from?

  A: It is the line that is 'best' in the sense that it *minimizes* the sum of the *squared* errors (residuals) in the vertical (*Y*) direction



What is robustness?

- The term *robustness* is used to mean several possible things:
  - Lack of sensitivity to *distributional assumptions* (especially normality)
  - Lack of sensitivity to *outliers*
  - Small sets of the data *don't have a strong influence*

Why robust (vs. LS)?

- Want fitting procedure to produce good estimates in the presence of various types of outliers:
  - *probe outliers* : *e.g.* probes that `don't work'
  - *chip outliers* : chips that are unusual
  - Image artifacts
- Want procedure to assess quality
- Distinguish between approach based on *outlier identification /exclusion* and approach based on *modeling / quality weights*

Median polish algorithm

$$\begin{array}{cccc|c} y_{11} & \cdots & y_{1J} & 0 \\ \vdots & \ddots & \vdots & \vdots \\ y_{I1} & \cdots & y_{IJ} & 0 \\ \hline 0 & \cdots & 0 & 0 \end{array}$$

Sweep Rows

Sweep Columns

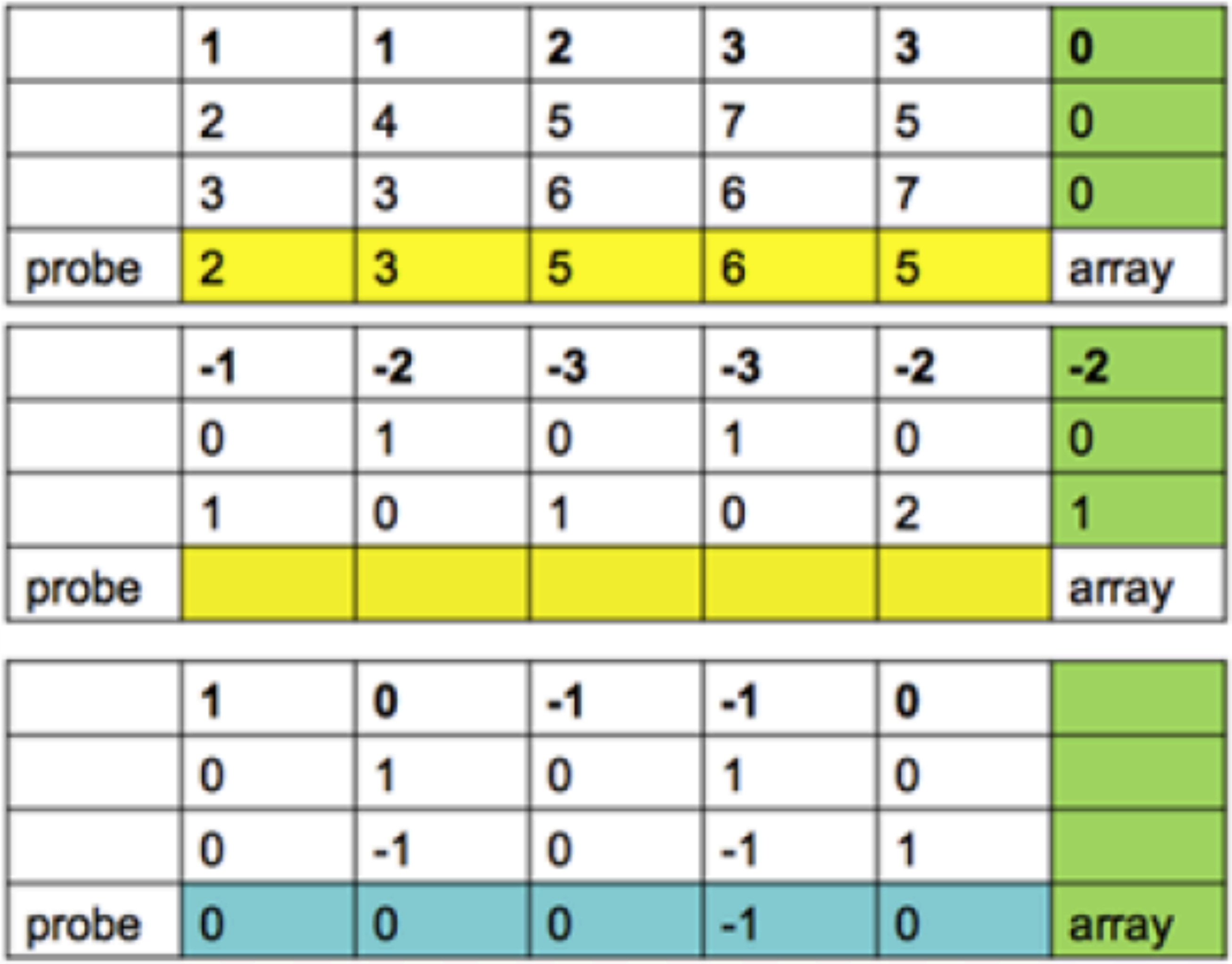
Iterate

Imposes Constraints

$$\text{median}_i\, e_{ij} = \text{median}_j\, e_{ij} = 0$$

$$\begin{array}{ccc|c} \hat{\varepsilon}_{11} & \cdots & \hat{\varepsilon}_{1J} & \hat{\alpha}_1 \\ \vdots & \ddots & \vdots & \vdots \\ \hat{\varepsilon}_{I1} & \cdots & \hat{\varepsilon}_{IJ} & \hat{\alpha}_I \\ \hline \hat{\beta}_1 & \cdots & \hat{\beta}_J & \hat{m} \end{array}$$

Median polish - example



	1	1	2	3	3	0
	2	4	5	7	5	0
	3	3	6	6	7	0
probe	2	3	5	6	5	array

	-1	-2	-3	-3	-2	-2
	0	1	0	1	0	0
	1	0	1	0	2	1
probe						array

	1	0	-1	-1	0	
	0	1	0	1	0	
	0	-1	0	-1	1	
probe	0	0	0	-1	0	array

(BREAK)

Robust regression

- Idea: *downweight* observations that produce large residuals
- More *computationally intensive* than least squares regression (which gives equal weight to each observation)
- Use *maximum likelihood* if can assume specific error distribution
- When not, use *M-estimators*

Robust regression in microarray analysis

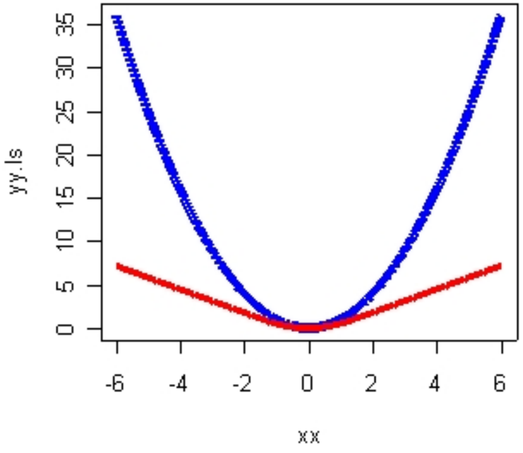
- There are many ways that robust regression can be/is used in analysis of microarray data
- We will use it in two ways:
  - for *quantifying gene expression* measured with Affymetrix GeneChips (like we saw with RMA)
  - for *assessing quality* of Affymetrix GeneChip gene expression measures (coming up next)

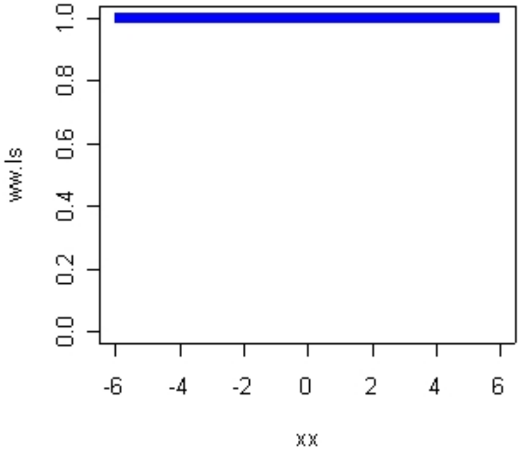
Loss, weight functions

- Least squares: ‘lose’ square of vertical error
- Here, squared error = *loss function*
- Each observation has *equal weight*
- Problem: *outliers* can have strong effect on estimates (slope, intercept of line; model parameters more generally)
- Solution: could use *other loss/weight functions*

Examples of Loss, Weight Functions

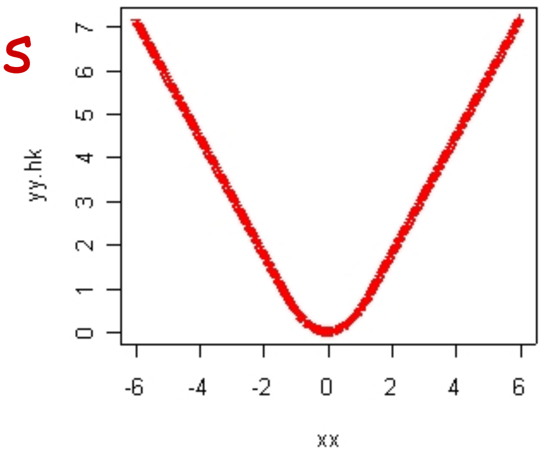
Squared error loss

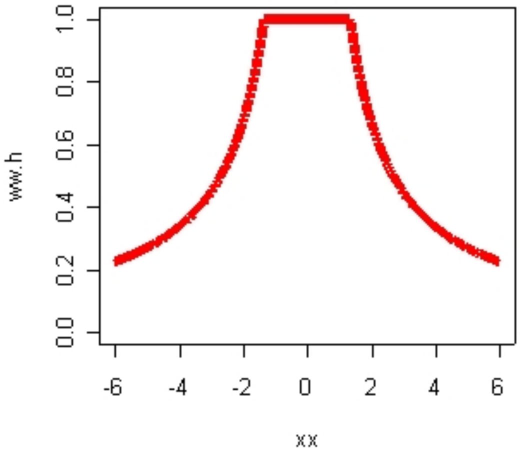




Equal weight

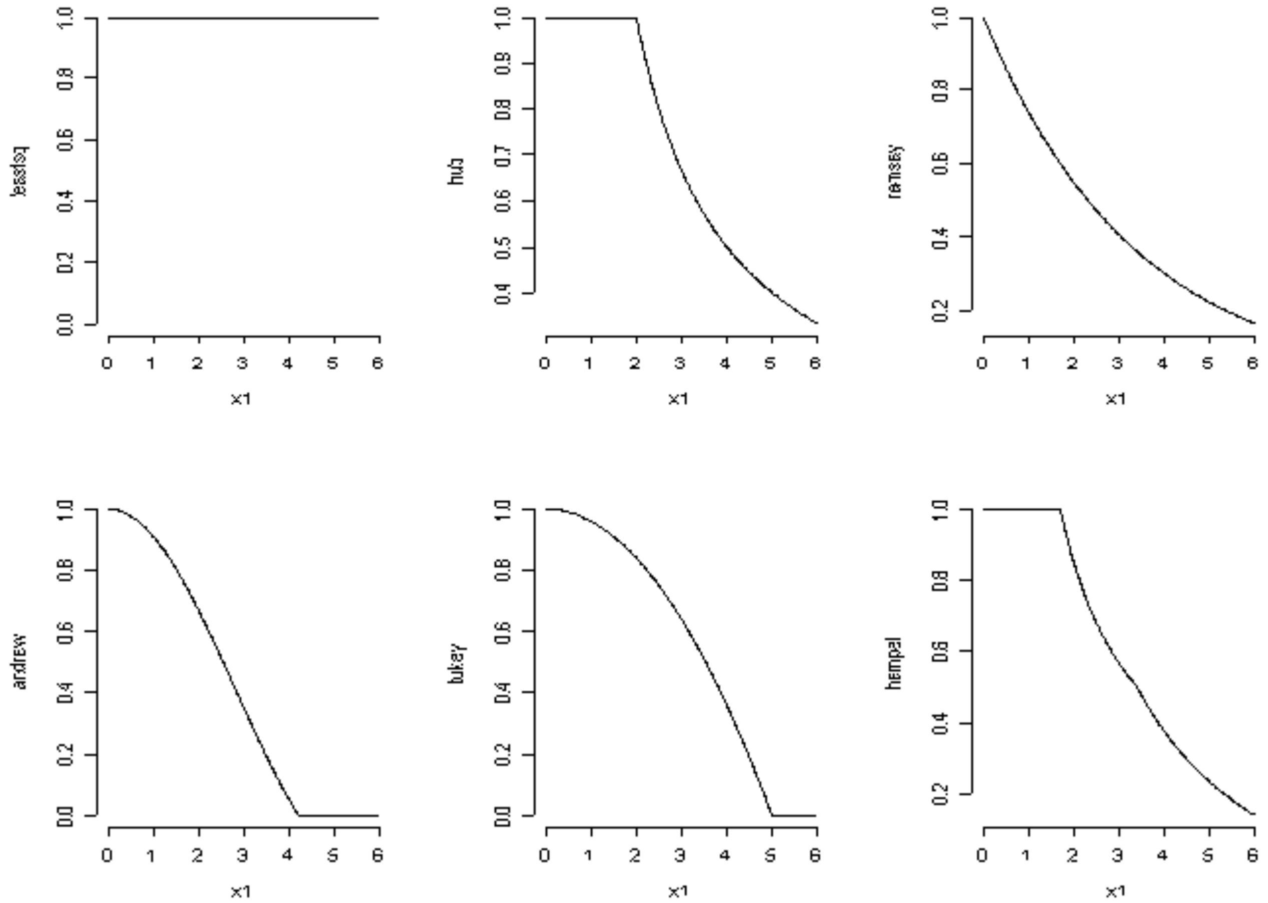
Huber loss





Huber weights

More weight functions



Robust regression estimation

- Robust procedures *perform well* under a range of possible models
- Facilitates *outlier detection*
- Good estimates even if some bad data points
- Can identify `bad' probe behavior:
  - some probes may cross hybridize to non-target fragments
  - some may not bind at all to target fragment

M-estimators

- ‘Maximum likelihood type’ estimators
- Assume independent errors with distribution f(ε)
- Parameter estimates solutions to

$$\min_{p_i,c_j}\sum_{i,j}\rho\left(\frac{Y_{ij}-p_i-c_j}{\hat{\sigma}}\right)=\min_{p_i,c_j}\sum_{i,j}\rho(u_{ij})$$

- ρ(x) is a (bounded for robustness) positive, symmetric function increasing more slowly than x
- $\hat{\sigma}$ is an estimate of scale (*eg.* MAD)
- eg, ρ(u) = u^2 corresponds to minimizing the sum of squares

M-estimation procedure

- To minimize $\Sigma_i \rho\{(Y_i - \underline{x}_i' \underline{\beta})/s\}$ wrt the β's, take derivatives and equate to 0 (`normal equations')
- Resulting equations *do not have an explicit solution* in general
- Solve by *iteratively reweighted least squares* (IRLS) with weights

$$w_{ij} = \rho'(u_{ij})/u_{ij} = \psi(u_{ij})$$

- Acts like *automatic outlier rejector*, since large residual values lead to very *small weights*

IRLS algorithm

- *Weights* at each iteration are calculated by applying the loss function to the residuals obtained from the previous iteration
- The weight function gives *lower weight* to points that do not fit well (‘outliers’)
- The results are *less sensitive* to outliers in the data (compared to OLS)

Robust fit by IRLS for each probe set

- Use *Huber* loss function ρ:
  - $\rho(e) = e^2/2$ for $|e| \leq k$; $k|e| - k^2/2$ for $|u| > k$
- Starting with robust (or LS) fit, at each iteration:
  - $r_{ij} = y_{ij}$ - *current est*(p_j) - *current est*(c_i)
  - $S = \text{mad}(r_{ij})\cdot c$ – robust est. of scale of σ
  - $u_{ij} = r_{ij}/S$ – rescaled residuals
  - $w_{ij} = \psi(|u_{ij}|)/|u_{ij}|$ – weights used in next fit (for Huber loss, $w = 1$ if $|u| \leq k$; $k/|u|$ if $|u| > k$)
- Next step estimates obtained by (weighted) LS

Quality Assessment using PLM

- PLM = Probe Level Model
- PLM quantities useful for assessing *chip quality (expression measure)*
  - Weights
  - Residuals
  - Standard Errors (NUSE)
- Expression values relative to (virtual) ‘median’ chip

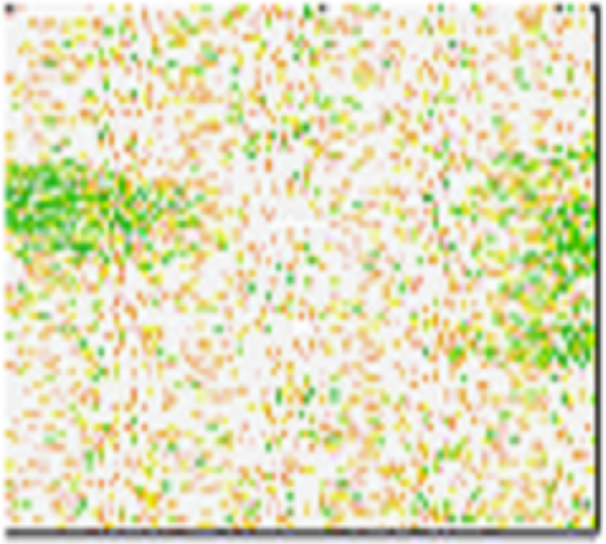
  (RLE = Relative Log Expression)

Role of model components in QA/QC

- Residuals, weights – now >200K per array
  - *summarize* to produce a chip index of quality
  - view as chip *image*, analyse spatial patterns.
  - scale of residuals for probe set models can be *compared* between experiments
- Chip effects > 20K (probe sets) per array
  - can examine distribution of relative expressions across arrays
- Probe effects > 200K per model (HG_U133A)
  - can be compared across fitting sets

Chip weight pseudo-images

- Image indicates the (robust regression) *weight* associated with the probe
- Areas of *low weight* (outliers) are greener, *high weights* are light gray
- ‘More color’ ⇔ ‘worse chip’ (more of an outlier)

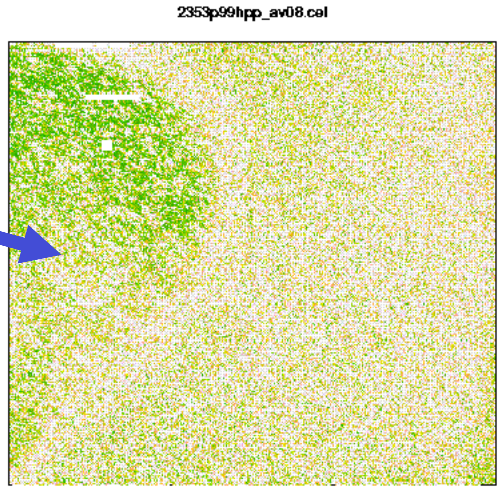


Using residuals from the fitting

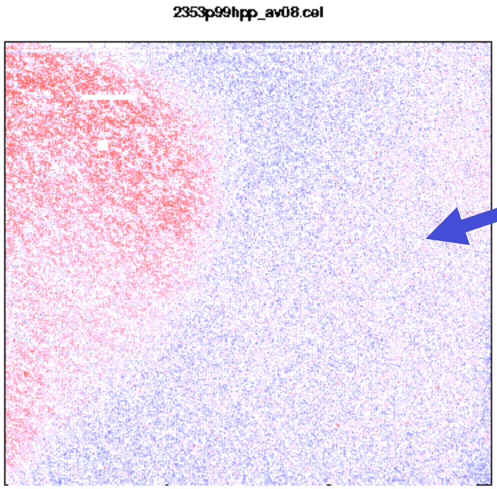
- Many types of *problems* will be reflected by *inflated residuals* from the fits to the probe + chip effect models
- *Summarizing the residuals* on a chip can provide good discrimination among chips producing data of varying quality

Pseudo-chip images

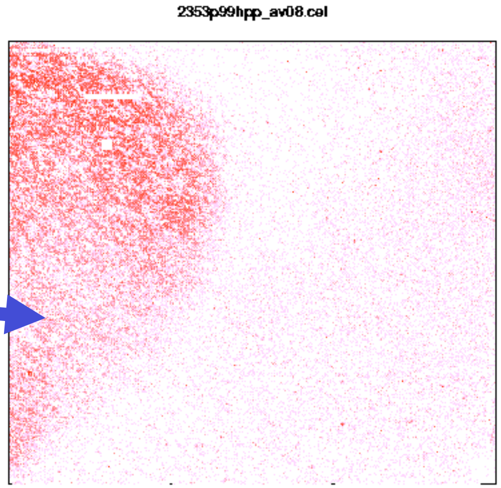
Weights



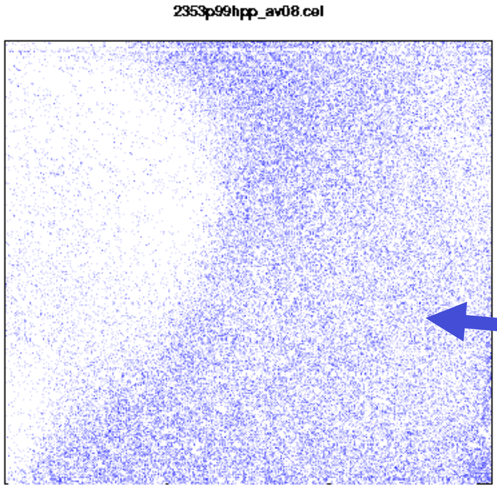
Residuals



Positive Residuals



Negative Residuals



Chip index of relative quality

- We assess gene expression index (eg, RMA value) variability for gene (probe set) *k* (=1, …, G genes) by its *unscaled SE* (*j* indexes *probes*):

$$\text{unscaled}\ \ \mathrm{SE}(\hat{c}_{\mathrm{ki}}) = 1 \Big/ \sqrt{\sum_j w_{kij}}$$

- We then *normalize* by dividing by the *median* unscaled SE over the chip set (*i*):

$$\mathrm{NUSE}(\hat{c}_{\mathrm{ki}}) = 1 \Big/ \sqrt{\sum_j w_{kij}} \Bigg/ median_i\left(1 \Big/ \sqrt{\sum_j w_{kij}}\right)$$

NUSE

- *NUSE* = ‘Normalized Unscaled SE’ – estimate SE(expression estimates), summarize at the chip level
- Each chip will have a NUSE for each probe set, which can be summarized by the *median*
- This provides one useful *summary* of the residual variability, and can be used to judge *quality* relative to other chips
- Median NUSE fluctuates around 1
- High values (> 1.05) indicate `worse' chips (unusual / outliers)

RLE

- How much are robust summaries affected?
- Can gauge reproducibility of expression measures by summarizing the distribution (across genes) of *relative log expressions*
- *RLE* $_i$ = RMA$_i$ – reference expression$_i$ (i = 1, …, p)
- For reference expression, can use *median expression value* for that gene in a set of chips
- This provides one useful summary of the residuals, and can be used to judge quality relative to other chips

RLE summaries

- IQR(RLE) measures variability
- Includes *Noise + DE* in biological replicates
- When biological replicates are similar (eg. RNA from same tissue type), can typically detect *processing effects* with IQR(RLE)
- Median(RLE) should be close to zero if

  # up-regulated genes ≈ down-regulated genes
- Can combine IQR(RLE)+|Median(RLE)| to give measure of chip expression measurement error

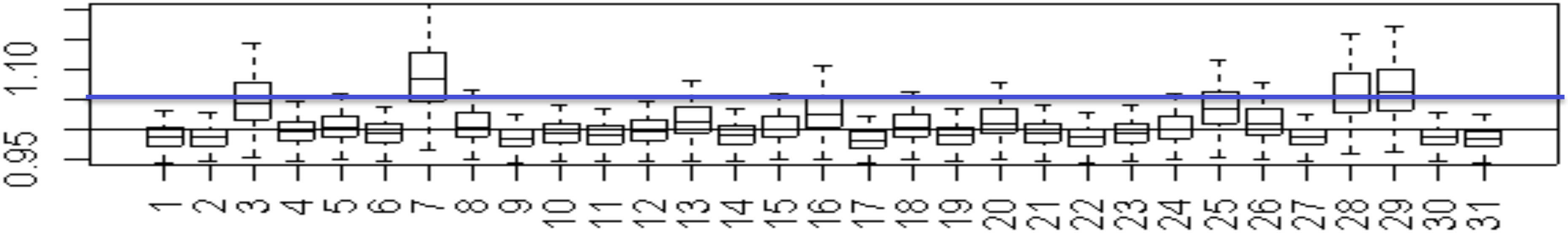
Example: HD

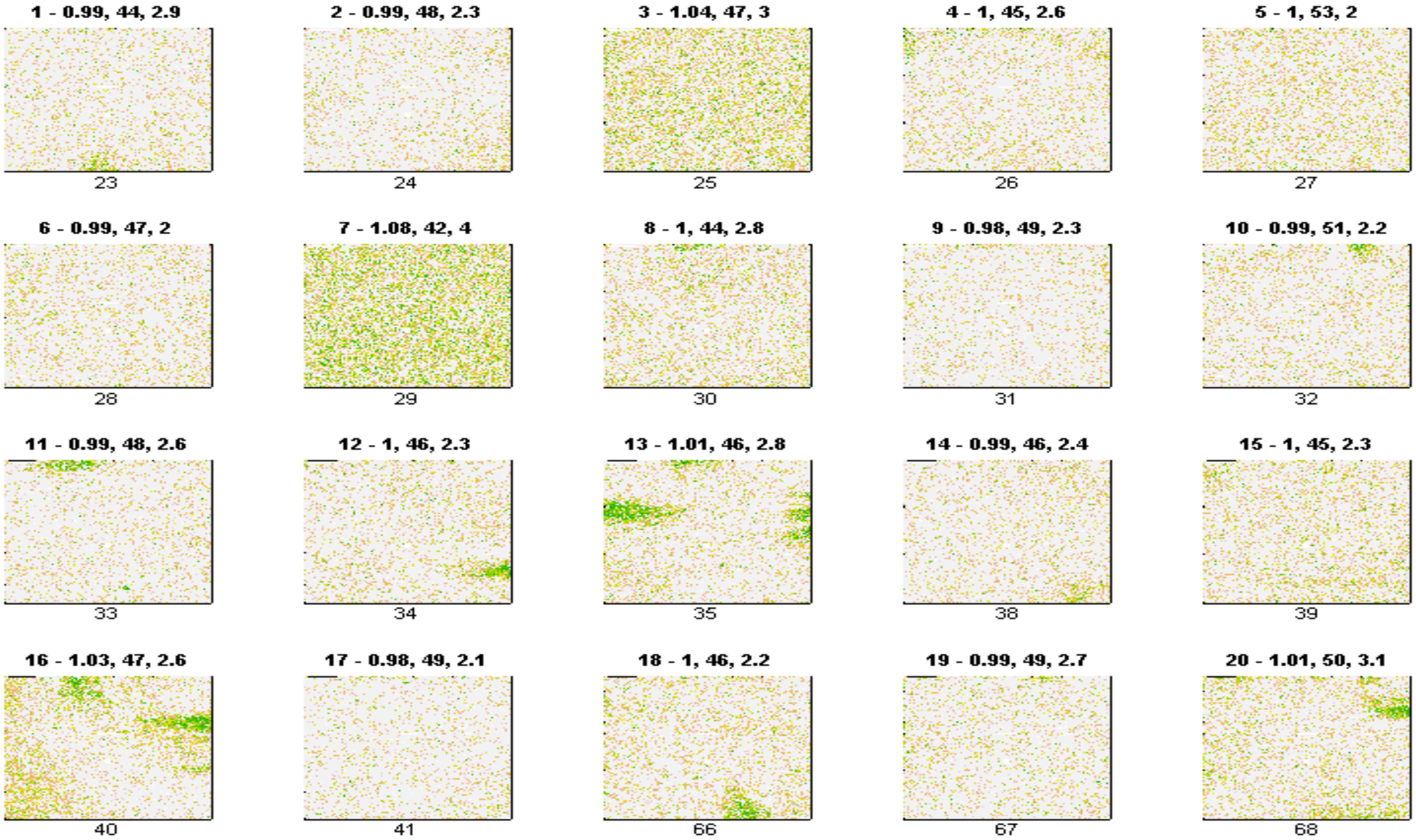
- About 70 individuals, U133A,B chips on each of 3 tissues
- Fitted RMA models
- Displays: NUSE plot, chip pseudo-image of residual weights

Title = Chip Number – Median NUSE, %P, SF

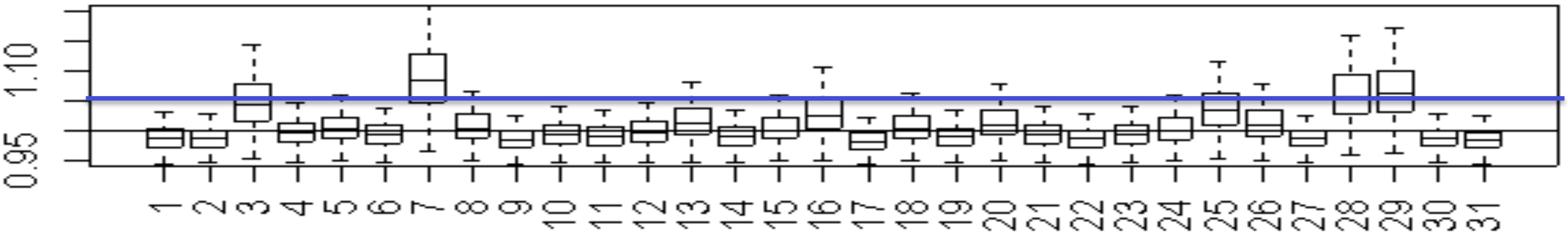
Subtitle = ChipId

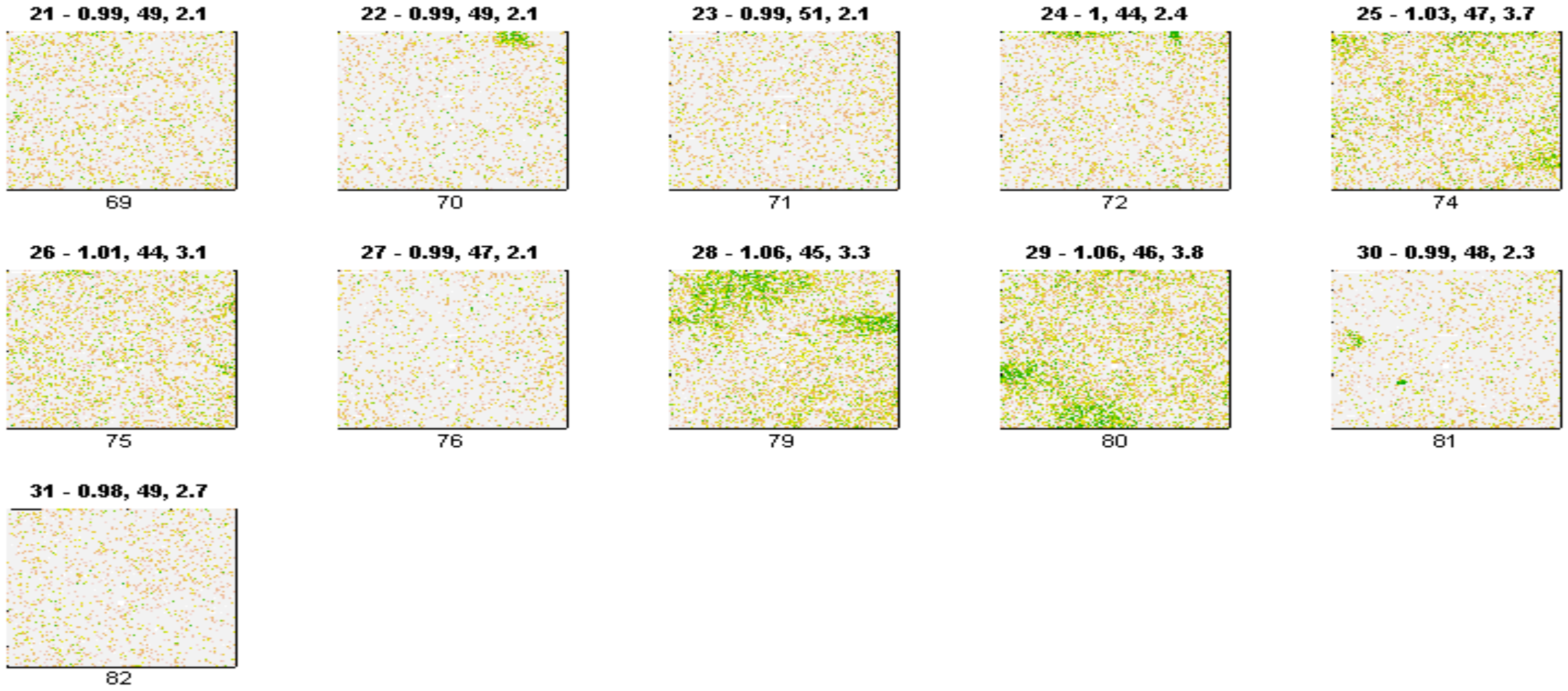
F. cerebellum.A.1 P. cerebellum.A.1 - Boxplots of NUSE values

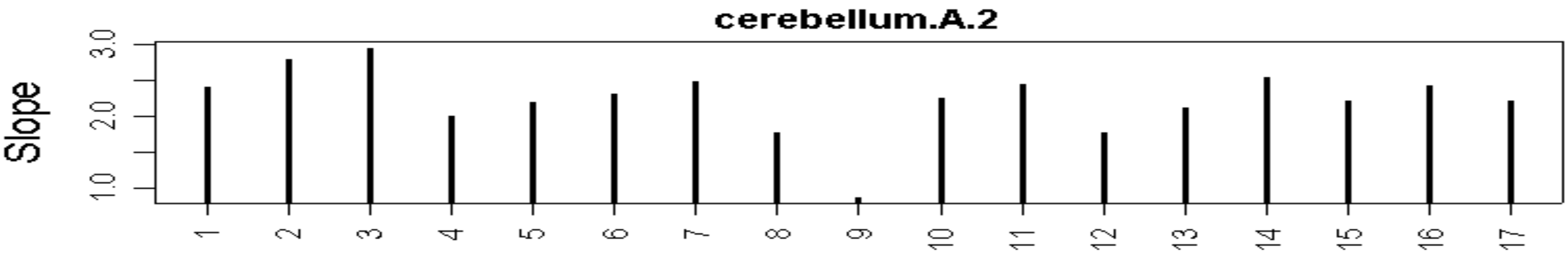


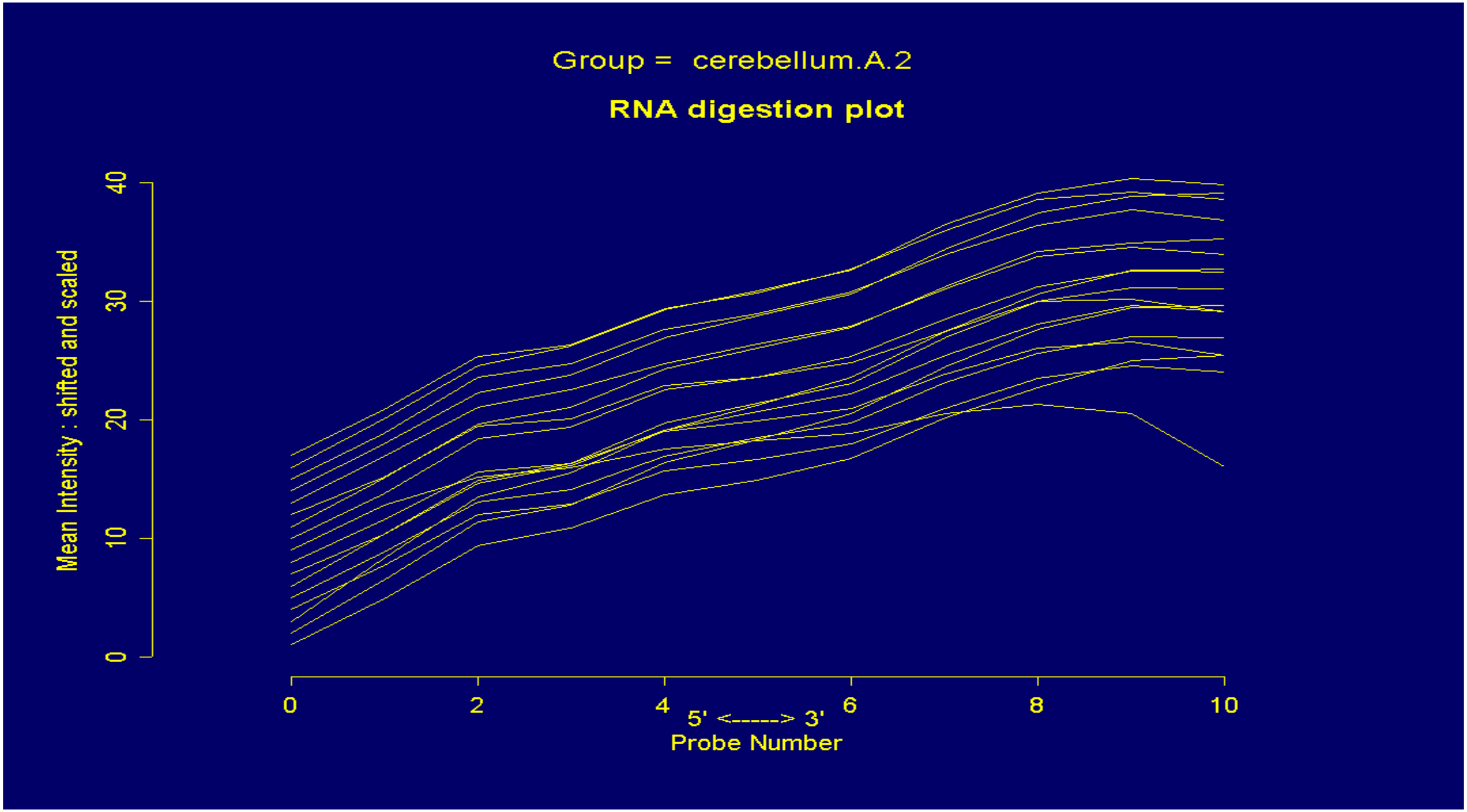


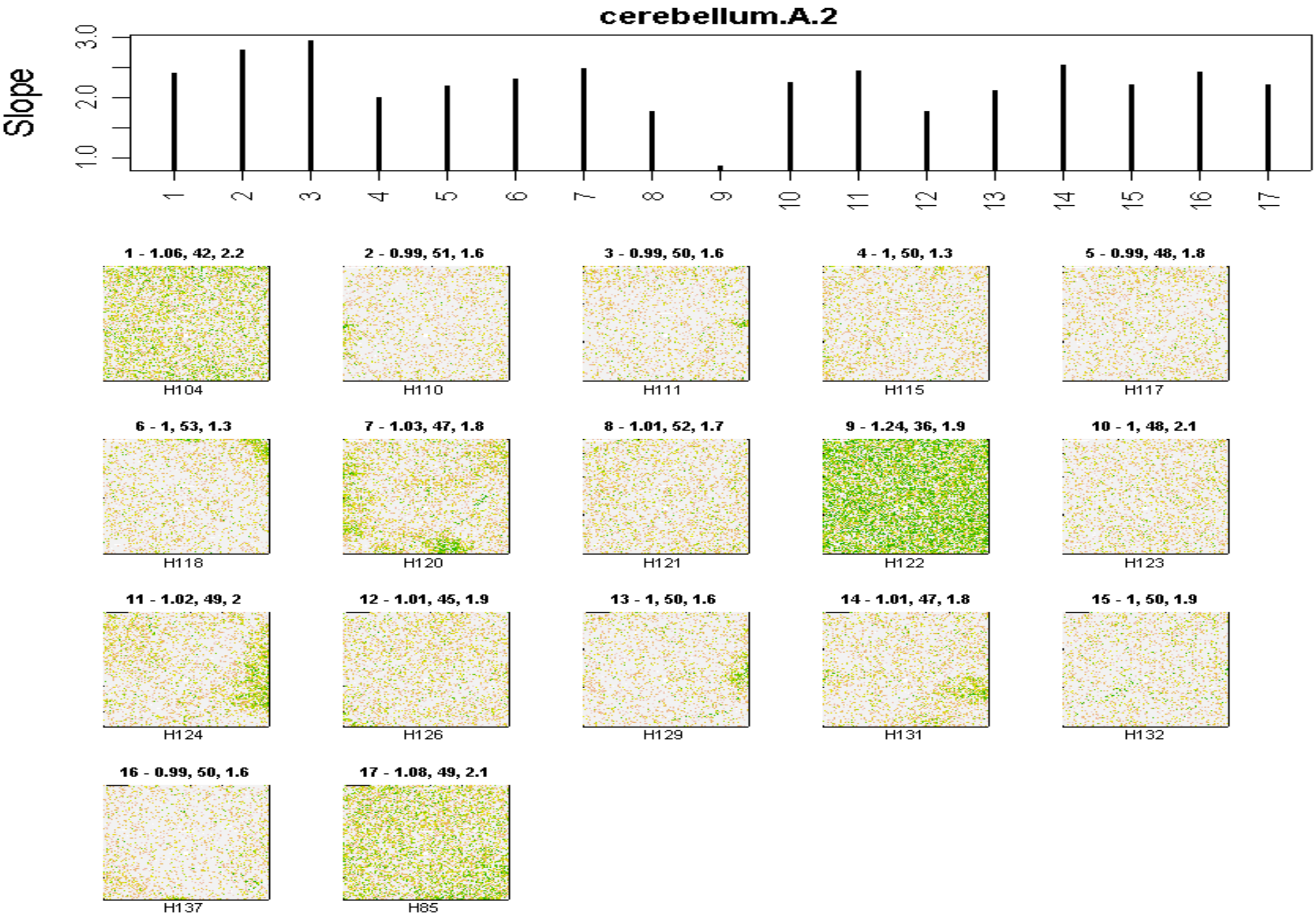
F. cerebellum.A.1 P. cerebellum.A.1 - Boxplots of NUSE values






cerebellum.A.2
Slope
1.0
2.0
3.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17


Group = cerebellum.A.2
RNA digestion plot
Mean Intensity : shifted and scaled
0
10
20
30
40
0
2
4
6
8
10
5' <-----> 3'
Probe Number


cerebellum.A.2
Slope
1.0
2.0
3.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
1 - 1.06, 42, 2.2
H104
2 - 0.99, 51, 1.6
H110
3 - 0.99, 50, 1.6
H111
4 - 1, 50, 1.3
H115
5 - 0.99, 48, 1.8
H117
6 - 1, 53, 1.3
H118
7 - 1.03, 47, 1.8
H120
8 - 1.01, 52, 1.7
H121
9 - 1.24, 36, 1.9
H122
10 - 1, 48, 2.1
H123
11 - 1.02, 49, 2
H124
12 - 1.01, 45, 1.9
H126
13 - 1, 50, 1.6
H129
14 - 1.01, 47, 1.8
H131
15 - 1, 50, 1.9
H132
16 - 0.99, 50, 1.6
H137
17 - 1.08, 49, 2.1
H85

Measuring quality

- Different measures view quality from different (but overlapping) perspectives
- Affymetrix measures (.rpt file) are most prominent in the *noise* and *integrity* aspects, but also touch on *array adjustment*
- RMA-QC measures dominate in *outlier identification*, but also include *array adjustment*

Conclusions

- PLM-based quality assessment appears to show good sensitivity to chip problems that impact *measures of expression*
- Provides useful basis for chip quality, inclusion/exclusion decisions
- RMA-QC measures implemented in the `affyPLM` package (BioConductor)
- `affyPLM` documentation gives more details of estimation procedure
- http://plmimagegallery.bmbolstad.com/

Exploratory data analysis/quality assessment

- PM signal intensity:
  - pseudo-images
  - histograms
  - boxplots
  - pairwise scatterplots (MA version)
- Pseudo-images of *weights* and *residuals*
- Boxplots of *NUSE values*
- Boxplots of *RLE values*
- Boxplots of normalized signal values (RMA)