

New methods for detailed genetic analysis

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Why test for genetic variants ?

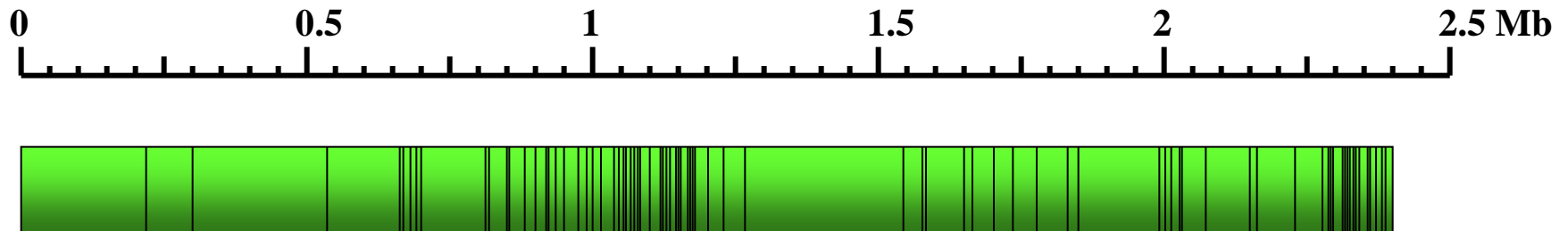
- Identifying the pathogenic variant (mutation) in dystrophin gene :
 - Confirms clinical diagnosis of DMD / BMD
 - Carrier testing for female relatives
 - Prenatal diagnosis for carriers
 - Preimplantation genetic diagnosis
 - Gene specific therapy – exon skipping, PTC trials, etc.

How do we test for variants ?

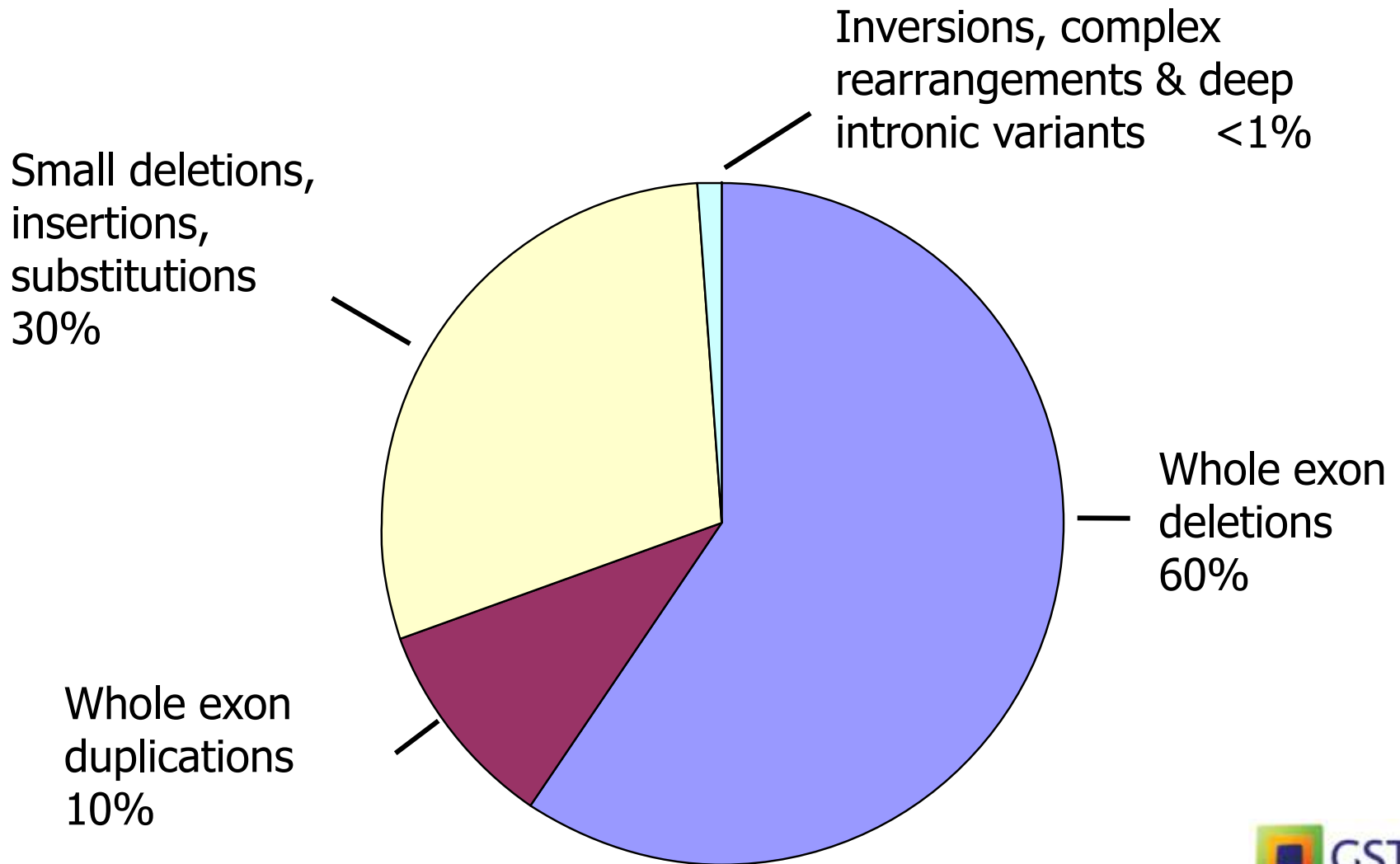
- Methodology depends on:
 1. gene structure
 2. types of variant (mutation spectrum)

Dystrophin gene

- 2.5 million bases
- 79 exons (14,000 bases)



Mutation spectrum in dystrophin gene



Dystrophin exon 45

ggagctttgtatttctttcttgccagtacaactgcatgtgtagcacactgtttaatcttt
ctcaaataaaaagacatggggcttcattttgtttgccttttggtatcttacag**GAAC**
TCCAGGATGGCATTGGGCAGCGGCAAACCTGTTG
TCAGAACATTGAATGCAACTGGGGAAGAAATAAT
TCAGCAATCCTCAAAAACAGATGCCAGTATTCTA
CAGGAAAAATTGGGAAGCCTGAATCTGCGGTGG
CAGGAGGTCTGCAAACAGCTGTCAGACAGAAAA
AAGAGgtagggcgacagatctaataaggaatgaaaacatttagcagacttttaag
ctttctttagaagaatattcatgagagattataagcagggtgaaaggcactaacattaa
agaacctatcaaccattaatcaacagcagtaaagaatttttatttcttttttcatatacta
aatatataacttgtggctagtta

Deletions

ctgacatatattctcaattgaactaactactgtatagg

Insertions / Duplications

ctgacatatattctcaattgaactactgtatagg

Methods to detect whole exon deletions/duplications

How many copies of each exon are present ?

Male

X Y

Female

X X

1 = normal

0 = deletion

2 = duplication

2 = normal

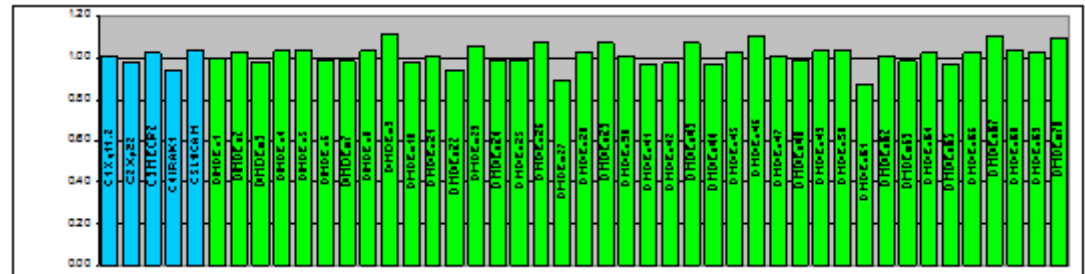
1 = deletion carrier

3 = duplication carrier

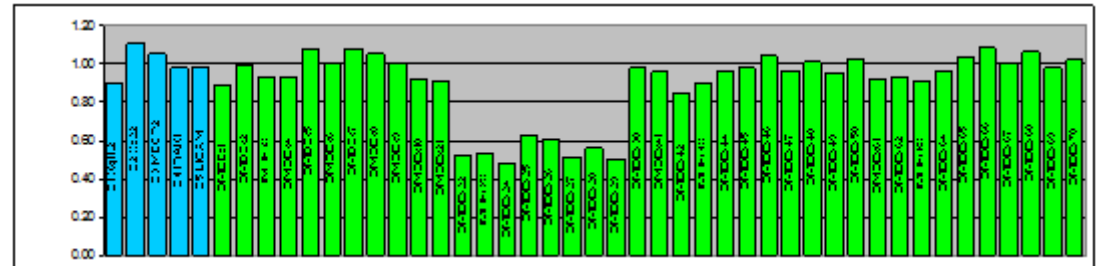
Detecting whole exon deletions / duplications

Multiplex Ligation-dependant Probe Amplification

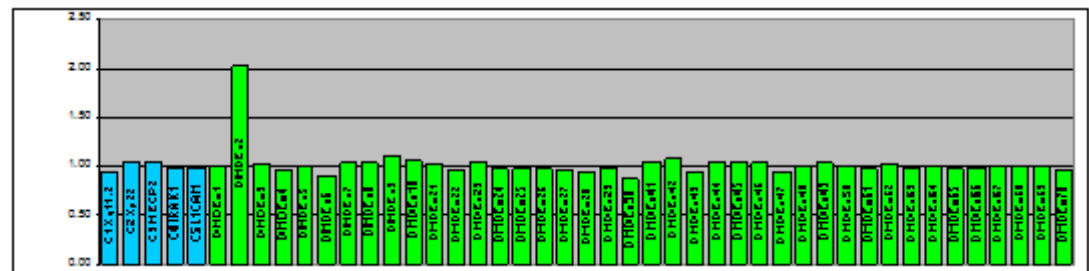
Normal control



Deletion carrier
Exons 22-29



Duplication, male
Exon 2



Substitutions

t

ctgacatatattctcaattgaactaactactgtatagg

Inversions

cat

ctgacatatattcttaattgaactaac tactgtatagg

Methods to detect small mutations

Deletions, duplications, insertions, substitutions

- of 1 or a few of the 2.5 million bases

Sequencing :

PCR amplify all 79 exons

Sequence individual exons

Compare sequence to normal reference sequence

Interpret differences

Mutation detection methods

- MLPA and Sequencing

Detect about 98% DMD mutations

Genetic diagnostic tests – success rates

Clinical referral	Gene	Confirm diagnosis
Developmental delay (fragile X)	FMR1	<1%
Spinal muscular atrophy	SMN1	<10%
X-linked Alport syndrome	COL4A5	~50%
Neuromuscular disease	several genes	~60%
Duchenne muscular dystrophy	DMD	~98%

Genetic diagnostic tests – explanations for “missing” mutations

1. Limitations in current methodology
2. Alternative diagnosis
3. Other causative genes not tested – known / unknown

Limitations of current methods

MLPA

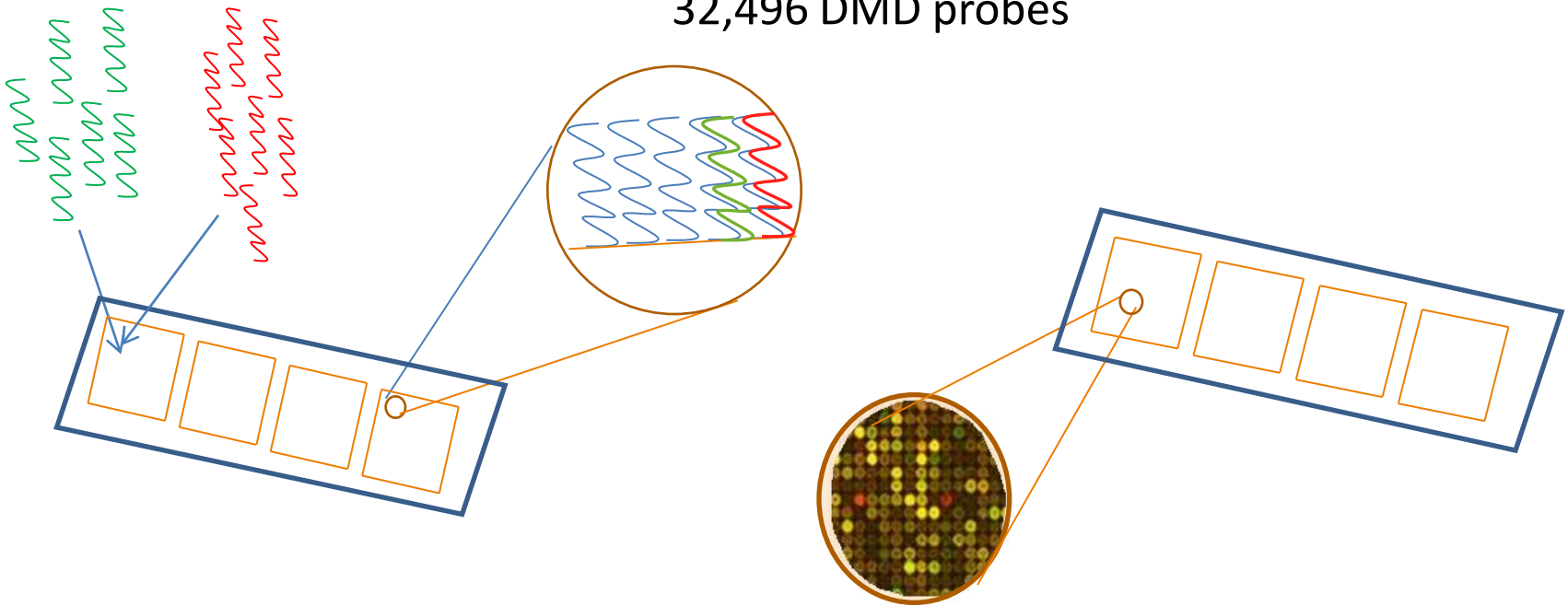
1. Only exons tested
2. Only one probe per exon
3. Breakpoints not located precisely
4. Deep intronic mutations, inversions, complex rearrangements not detected
5. Only tests 30 – 40 exons per assay
Generally only tests one gene at a time
6. Not available for all genes

New method - High density array Comparative Genomic Hybridisation

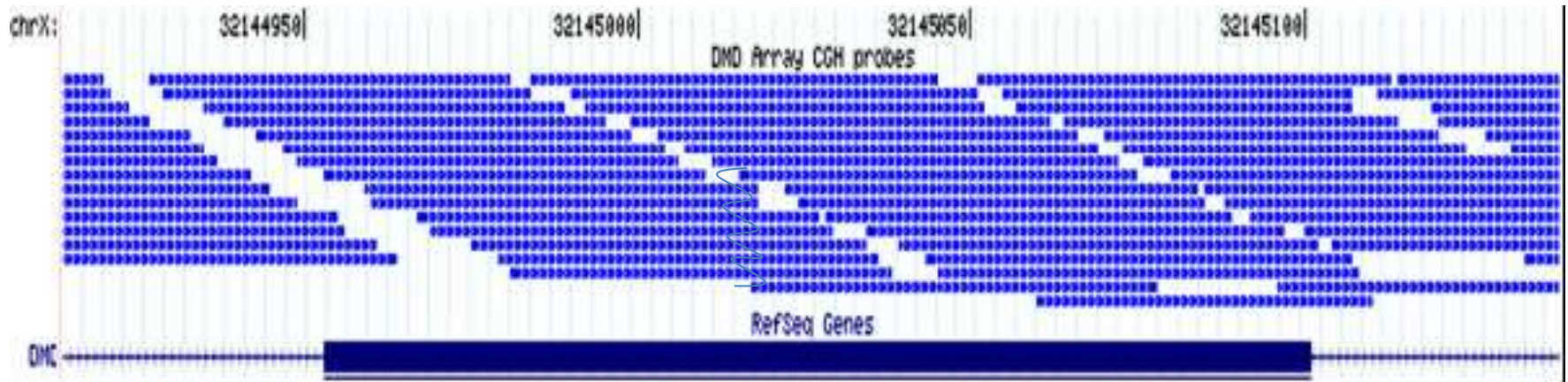
**Cy3 labelled
patient DNA**

**Cy5 labelled
control DNA**

44,000 probes
32,496 DMD probes



HD CGH array design (from Hegde et al, 2008)

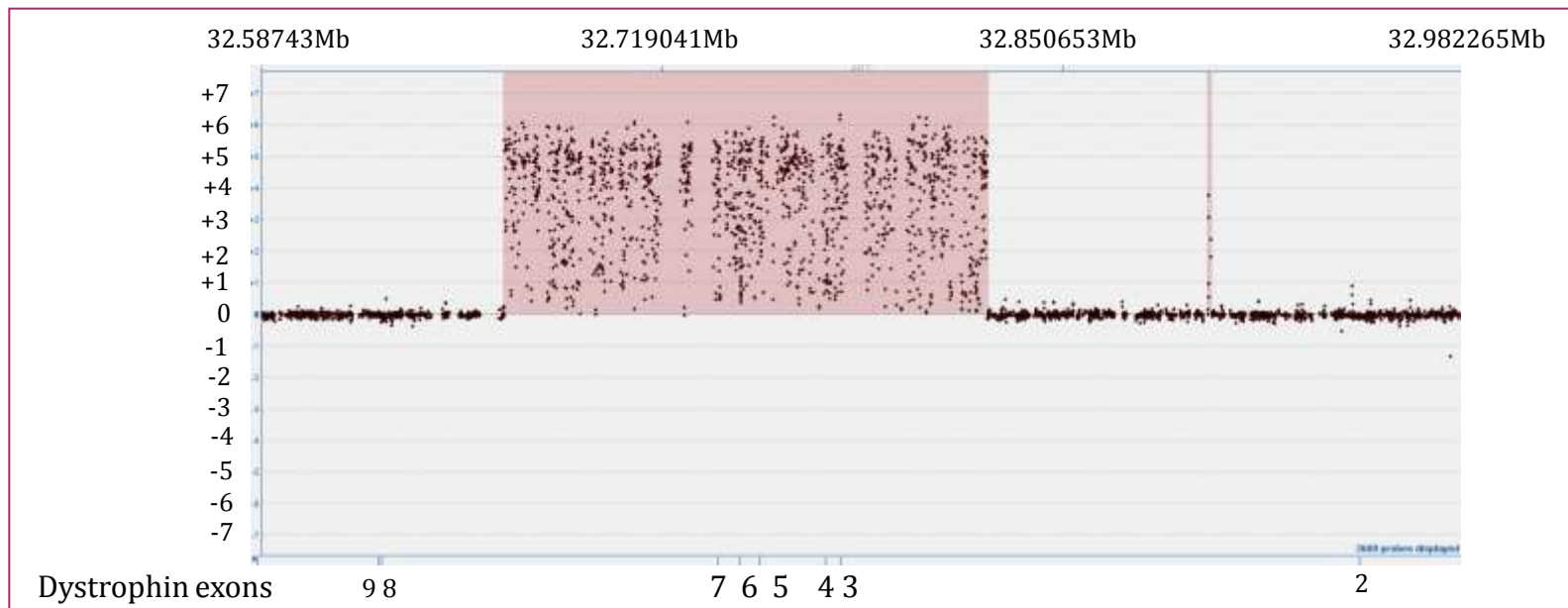


DMD Exon 44

probes 45-60 bases

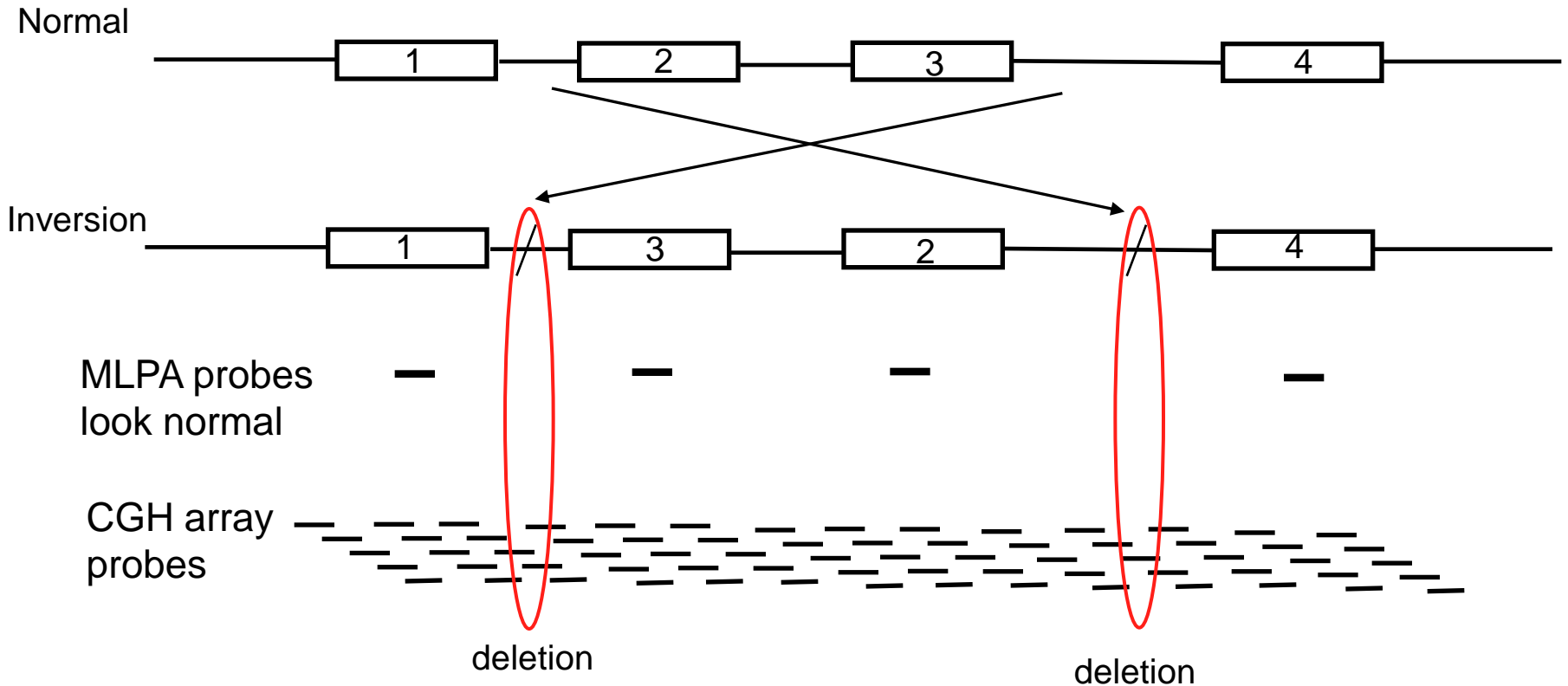
5 base spacing

Deletion of exons 3-7 in male

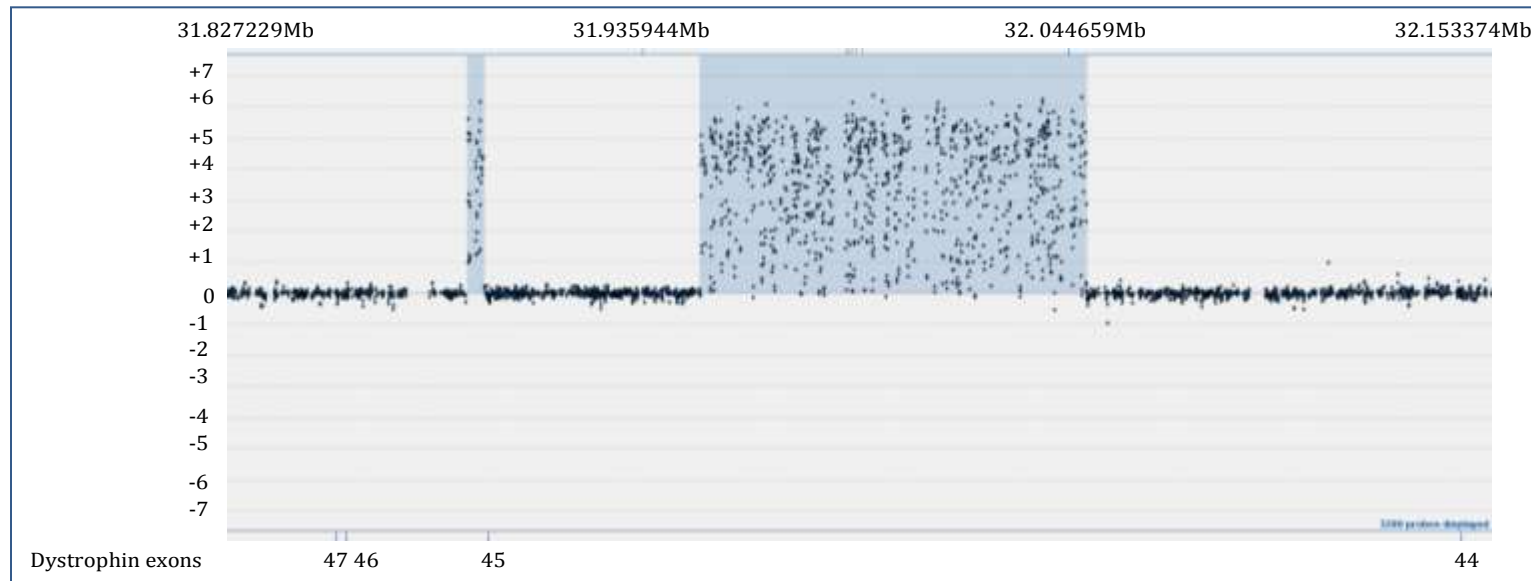


Inversion detection using CGH

Inversion of exons 2-3



Inversion Exon 45 c.6438+96064_6614+1540



Deletions in introns 44 and 45

Applications of dystrophin high density aCGH

Informing exon skipping trial targeting duplications

- **Difficulties in exon skipping for duplications**
 - Orientation
 - Structure
 - Position of breakpoints
- **Dystrophin aCGH study of 25 duplications**
 - Structure of duplications
 - Rapid breakpoint mapping
 - Understanding how dystrophin duplications arise

Duplication aCGH results

- aCGH results consistent with MLPA results
- Breakpoints localised and enabled sequencing
- In combination with RNA analysis able to show duplicated material lies in head to tail orientation

Conclusions on new genetic technologies

- New technology will increase mutation detection rates
- Number of patients with genetic confirmation of diagnosis will increase
- Additional tests to characterise mutations in more detail
 - Applicable to support design of targeted gene therapy

Acknowledgements

DNA Laboratory, Guy's Hospital

Joanna McCauley Vicky Cloke
Joo Wook Ahn Michael Yau

UCL, Gt Ormond St

Francesco Muntoni Jihee Kim

Emory University

Madhuri Hegde Ephrem Chin