MEDICAL GENETICS · LECTURE

Essential Genetic Testing Techniques

Karyotype · FISH · Chromosomal Microarray · Next-Generation Sequencing

Learning Objectives

* Expected exam Q: What is the most suitable testing platform (for a certain scenario)?

* important to know the utility & limitations for each test.

By the end of this session you should be able to:

1. **Compare** the resolution, sensitivity, and clinical utility of karyotyping, FISH, chromosomal microarray (CMA), and next-generation sequencing (NGS).
2. **Select** the most appropriate genetic test for common clinical scenarios — ID/DD, congenital anomalies, recurrent pregnancy loss, oncology, and prenatal diagnosis.
3. **Interpret** a basic genetic test report, including ACMG variant classification, mosaicism, and copy number variants (CNVs).
4. **Recognize** the limitations of each technique, including variants of uncertain significance (VUS) and findings each platform cannot detect.
5. **Counsel** patients on incidental/secondary findings and the ethical considerations of genome-scale testing.

Why Genetic Testing Matters in Clinical Practice

Modern medicine increasingly depends on molecular and cytogenetic diagnosis. Identifying the genetic basis of disease changes management in every specialty — from pediatrics to oncology to reproductive medicine.

~6,000+

Mendelian disorders catalogued in OMIM

"Online Mendelian Inheritance in Man"

1 in 20

Live births affected by a genetic or partly genetic condition

30–40%

Diagnostic yield of WES/WGS in undiagnosed pediatric cases

10%

Of all cancers attributable to an inherited predisposition

Five clinical impacts:

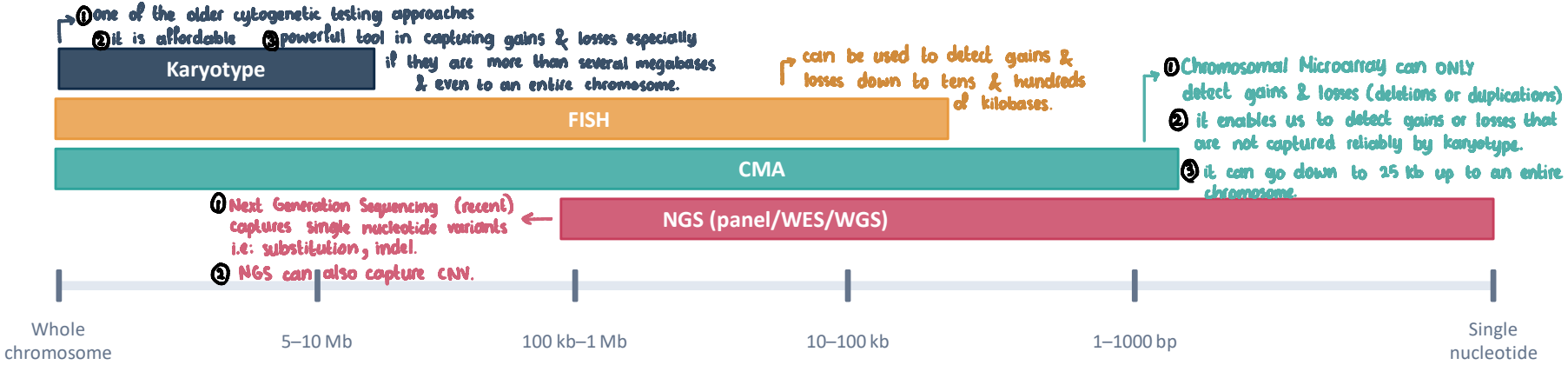
- Establishing a definitive diagnosis → provides guidance within ddx process.
- Tailoring therapy (pharmacogenomics, targeted oncology drugs)
  - ↳ there are genes which encode for enzymes & pkns with variable metabolic activity among individuals such as CYP21C9 which impacts response to drugs. + many treatments in oncology are nowadays based on the genetic profile.
- Predicting prognosis and disease course → Some mutations in oncology are known to have bad outcomes which may imply a more stringent treatment regimen.
- Cascade screening of at-risk family members
- Reproductive planning and prenatal diagnosis
  - ↳ genetic testing can be done during pregnancy due to abnormalities related to ultrasound abnormalities or advanced maternal & paternal age or abnormal pregnancy markers.

The Resolution Spectrum

*Note: ① karyotype & FISH can detect beyond gains & losses unlike CMA.

② usually, if the deletion or duplication were large (>1 kilobase) then it can be considered as Copy Number Variant (CNV)

What size of genetic change does each test detect?



← *LARGER changes (cytogenetic)*

smaller changes (molecular) →

Key teaching point

No single test detects every kind of genetic change. Match the test to the suspected lesion — karyotype for large structural rearrangements, FISH for known targeted regions, CMA for unbalanced submicroscopic CNVs, NGS for sequence-level variants. Tests are increasingly used in tiered or parallel fashion.

PART 1

Karyotyping

The original whole-genome view — chromosome morphology at light-microscope resolution

Karyotype: Principle

⇒ the cells must be alive to induce them to undergo cell cycle in order to capture the chromosomes in their condensed form.

Light-microscopic visualization of condensed metaphase chromosomes

1. **Sample** Peripheral blood lymphocytes, bone marrow, amniocytes, CVS, or solid tissue
2. **Culture** Cells stimulated to divide (e.g. phytohaemagglutinin for lymphocytes)
3. **Arrest** Colchicine arrests cells in metaphase, when chromosomes are most condensed
4. **Spread & stain** Hypotonic swelling, fixation, drop onto slide, G-banding with trypsin & Giemsa
5. **Analyze** Image, pair homologues, arrange by size & centromere position; ≥ 20 metaphases scored

↳ ideally 20 or more cells are needed to analyze in hereditary diseases.
(more are needed in somatic oncology).

Banding conventions

G-banding (Giemsa)
Standard; gives alternating light/dark bands. Resolution 400–550 bands; high-res up to 850.

ISCN nomenclature
Chromosome → arm (p/q) → region → band. Example: 7q31.2 = 7, long arm, region 3, band 1, sub-band 2.

Reporting
46,XX or 46,XY = normal female/male. Karyotype reads: total count, sex chromosomes, abnormalities.

Karyotype: What It Detects

Resolution ≈ 5–10 Mb (lesions smaller than a single band are invisible)

Aneuploidy

Trisomies 13, 18, 21; monosomy X; sex chromosome aneuploidies (47,XXY, 47,XYY, 47,XXX)

Translocations

↳ exchange of genetic material between non-homologous chromosomes.

Balanced reciprocal and Robertsonian; unbalanced derivative chromosomes — uniquely detected here

Large deletions / duplications

5p deletion — 4p deletion

Cri-du-chat (5p–), Wolf-Hirschhorn (4p–) — but only if > 5–10 Mb

(a deletion involving hundreds of kb CANNOT be seen with karyotyping)

Inversions

includes centromere — does NOT

Pericentric and paracentric; clinically relevant in infertility and recurrent miscarriage

Marker chromosomes

Small supernumerary chromosomes of unknown origin; need follow-up FISH/CMA

Mosaicism

Two or more cell lines (e.g. 45,X/46,XX); detectable if present in ≥ 10–20% of cells analyzed

i.e: an individual who is mosaic for Turner Syndrome, mixture of cells will be obtained & can be tested individually to calculate mosaicism.
(if mosaicism is ≥ 10-20%)

Karyotype: Clinical Use, Strengths & Limits

Indications

- Suspected aneuploidy (Down, Edwards, Patau, Turner, Klinefelter)
- Recurrent pregnancy loss (≥ 2 miscarriages) — parental karyotype
- Infertility (male and female) → i.e: AZF region in ch.Y.
- Disorders of sex development
- Hematologic malignancies (leukemia/lymphoma) — clonal rearrangements (e.g. t(9;22) BCR::ABL1)
- Family member of a known translocation carrier

→ balanced carriers might have partial or full monosomies or trisomies in their children.

Advantages

- Whole-genome overview in one test
- Detects balanced rearrangements (translocations, inversions) — unique among routine tests
- Identifies mosaicism if multiple cell lines present
- Well-established; standardized nomenclature (ISCN)
- Relatively inexpensive
- Useful when family history suggests structural rearrangement

Limitations

- Low resolution: misses changes < 5–10 Mb
- Requires viable, dividing cells → slow (5–14 days) → depends on the tissue cultured.
- Cannot identify the gene content of CNVs
- Labor-intensive and operator-dependent
- Cannot detect uniparental disomy or single-gene mutations
- Mosaicism below ~10% often missed

Karyotype in Practice

Clinical vignette

Clinical scenario

A 32-year-old G3P0 woman is referred after three first-trimester miscarriages. Her partner is healthy. Routine work-up for thrombophilia and uterine anatomy is unremarkable. The team orders parental karyotypes.

Result	Female partner: 46,XX,t(11;22)(q23;q11.2) — a balanced reciprocal translocation. Male partner: 46,XY (normal).
Interpretation	Balanced carriers are phenotypically normal but at risk of producing unbalanced gametes. Live-born offspring can have partial trisomy/monosomy (e.g. Emanuel syndrome).
Why karyotype, not CMA?	CMA reads dosage only; it cannot distinguish a balanced translocation from a normal genome. This rearrangement is invisible to microarray.

Q: Why not perform CMA?
bcz CMA is powerful for gains & losses but it does NOT detect balanced rearrangements.

PART 2

Fluorescence In Situ Hybridization

Targeted, fluorescent probes — fast answers about known regions

FISH: Principle

Fluorescently labelled DNA probes anneal to complementary chromosomal targets

A single-stranded DNA probe — labelled with a fluorophore — is denatured together with the patient's chromosomes (metaphase) or interphase nuclei. The probe hybridizes to its complementary genomic sequence; fluorescence microscopy reveals the location and copy number of the target.

probes can bind to the DNA whether it's diffuse or condensed

a probe is a sequence of DNA that is complimtary to a certain region in the genome.

1 Probe design
Labelled with fluorophore (e.g. SpectrumOrange/Green)

2 Denaturation
Both probe and target DNA → single-stranded

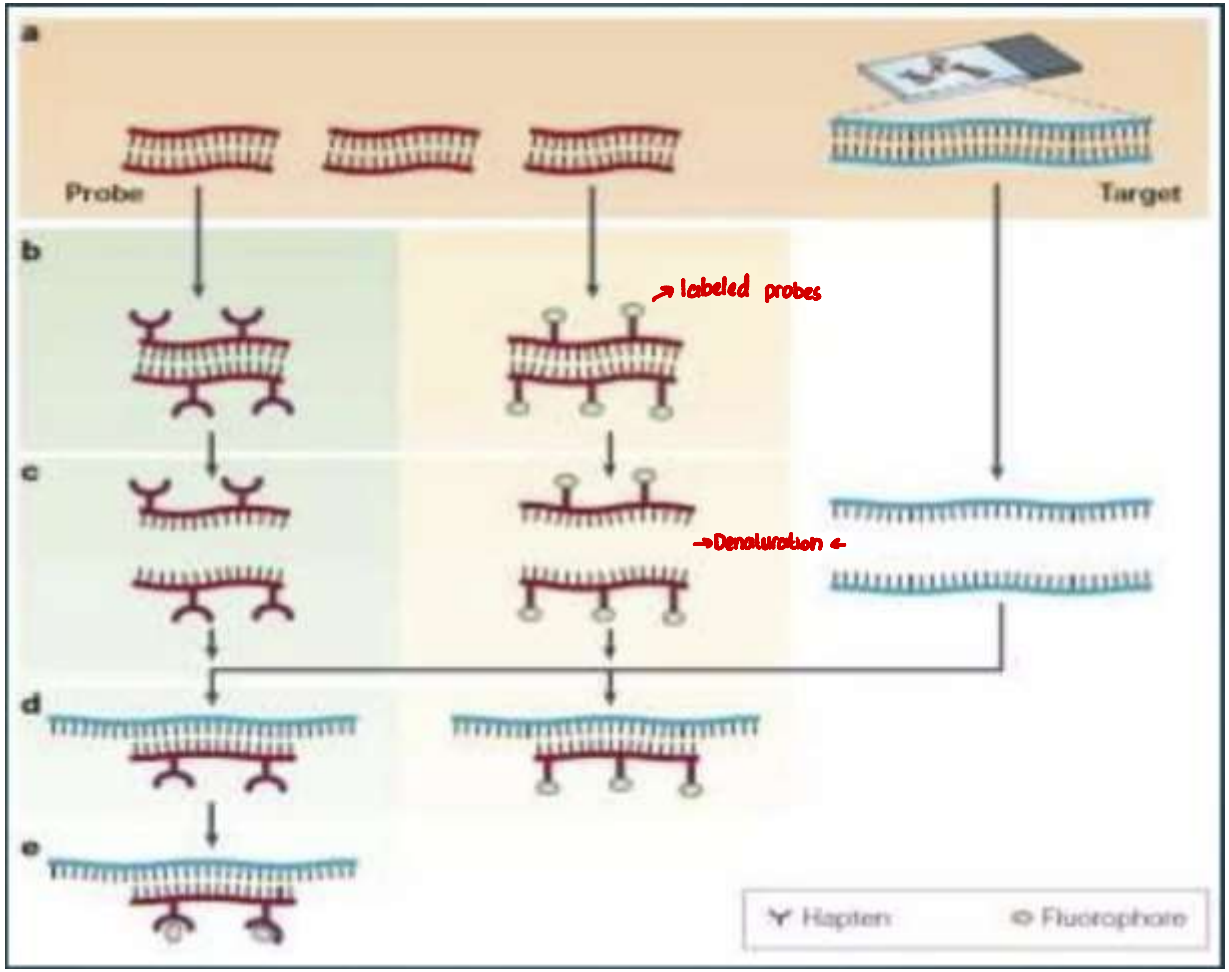
3 Hybridization
Overnight; complementary sequences anneal

4 Wash & DAPI
Remove non-specific binding; counterstain nuclei

5 Microscopy
Count fluorescent signals per nucleus or metaphase

Critical advantage: FISH works on **interphase nuclei** — no cell culture or division is required. This makes it the fastest cytogenetic test (results in 24–48 h), ideal for rapid prenatal aneuploidy screening on uncultured amniocytes or for HER2 status on a tumour section.

when biopsy is done, the tissue sample is preserved in Paraffin block then it is cut into small sections & stained → since these cells are dead, karyotype CANNOT be done (bcz we can't induce cell cycle) but FISH can be performed.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Probe
Target
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→ labeled probes
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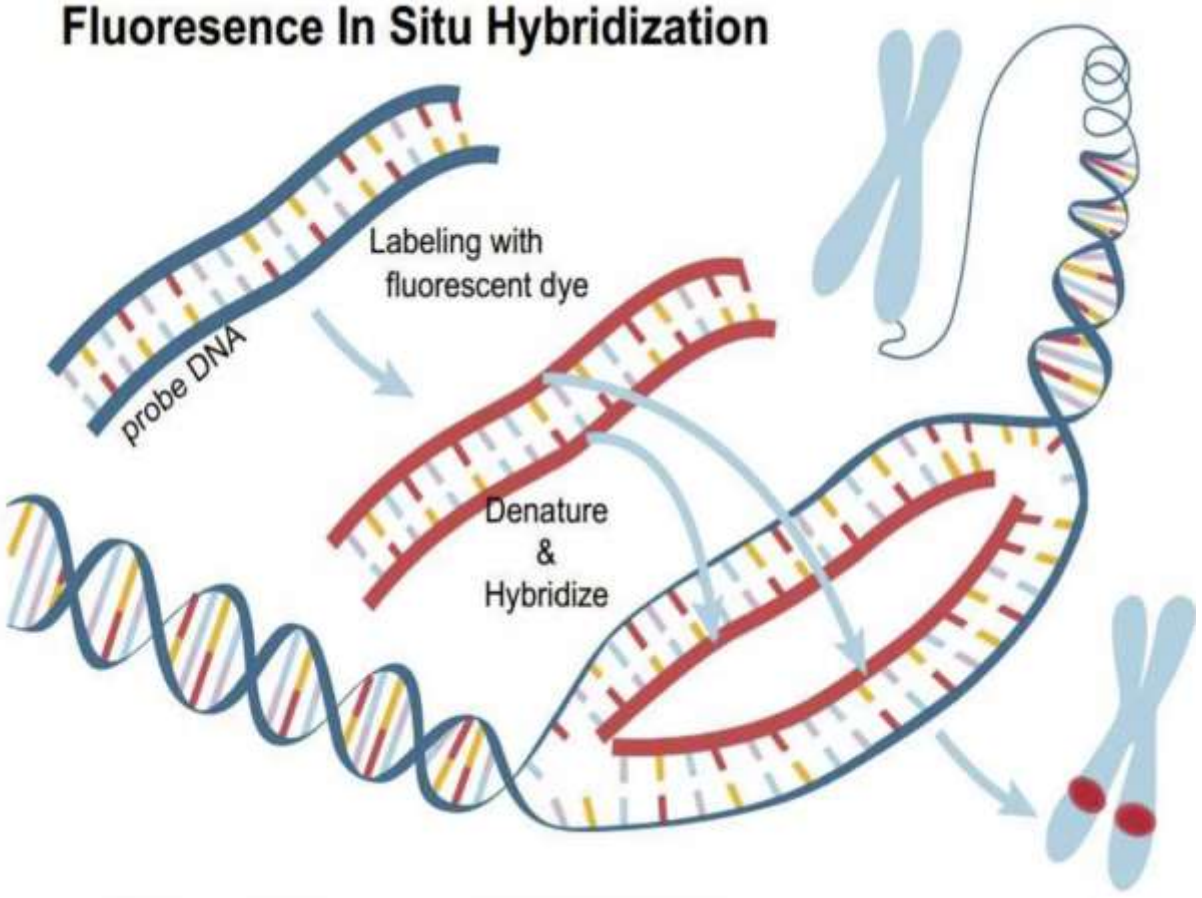

Fluoresence In Situ Hybridization
probe DNA
Labeling with
fluorescent dye
Denature
&
Hybridize

Diagnosing DiGeorge syndrome by fluorescence *in situ* hybridization (FISH)

DiGeorge syndrome in humans:

- Accounts for 5% of all congenital heart defects
- Affected people are heterozygous for a 22q11 deletion

↑ NOT big enough to be captured by karyotyping but can be captured by FISH.

condensed

FISH on human metaphase chromosomes

← Green dots; control probe for chromosome 22

it is probably a centromeric control probe or a probe to another region on ch.22 farther away from 22q11

Red dot; probe from 22q11 region

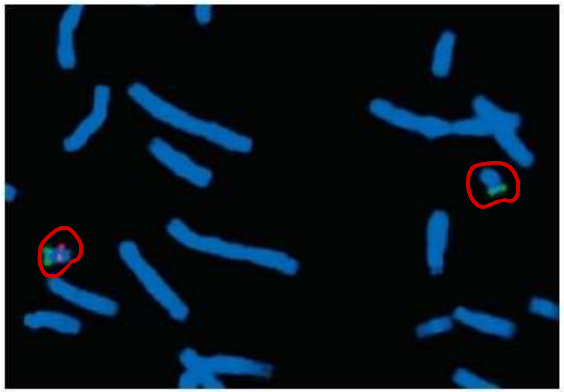


Fig. 13.10

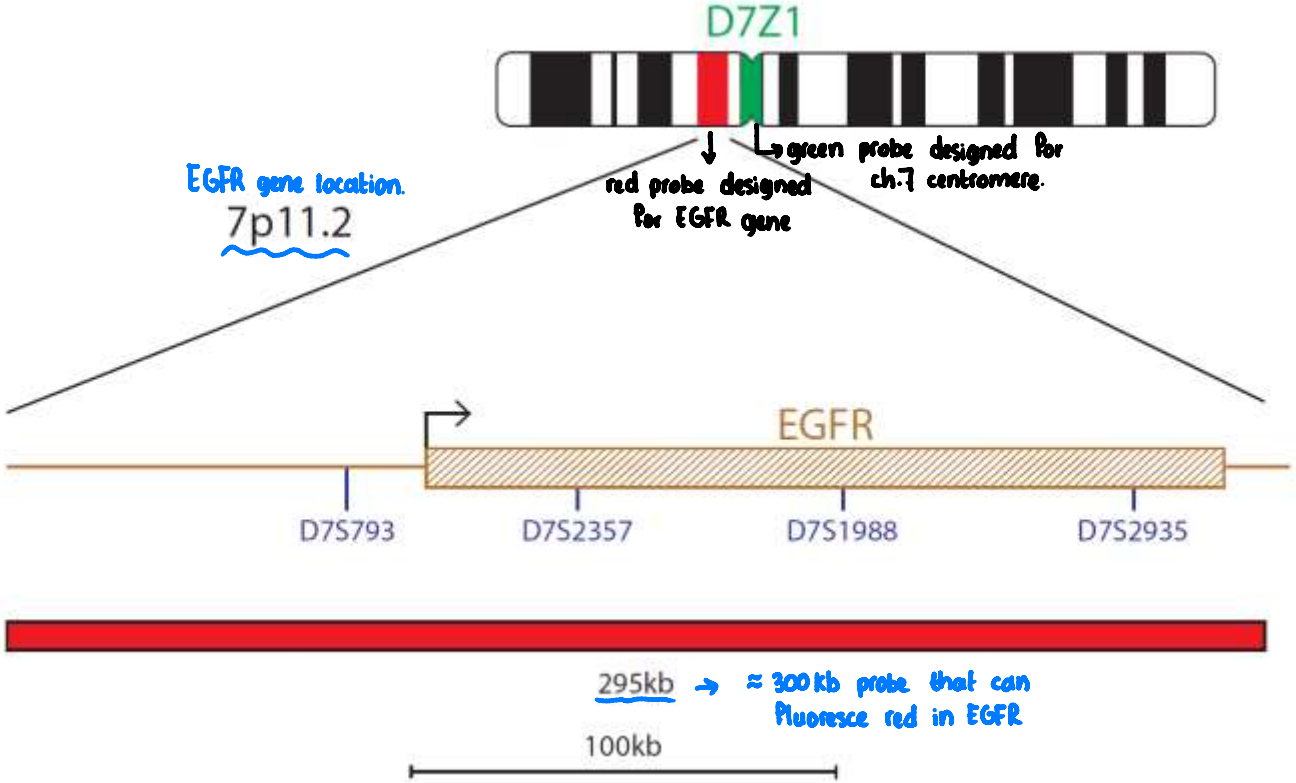
* Notice that:

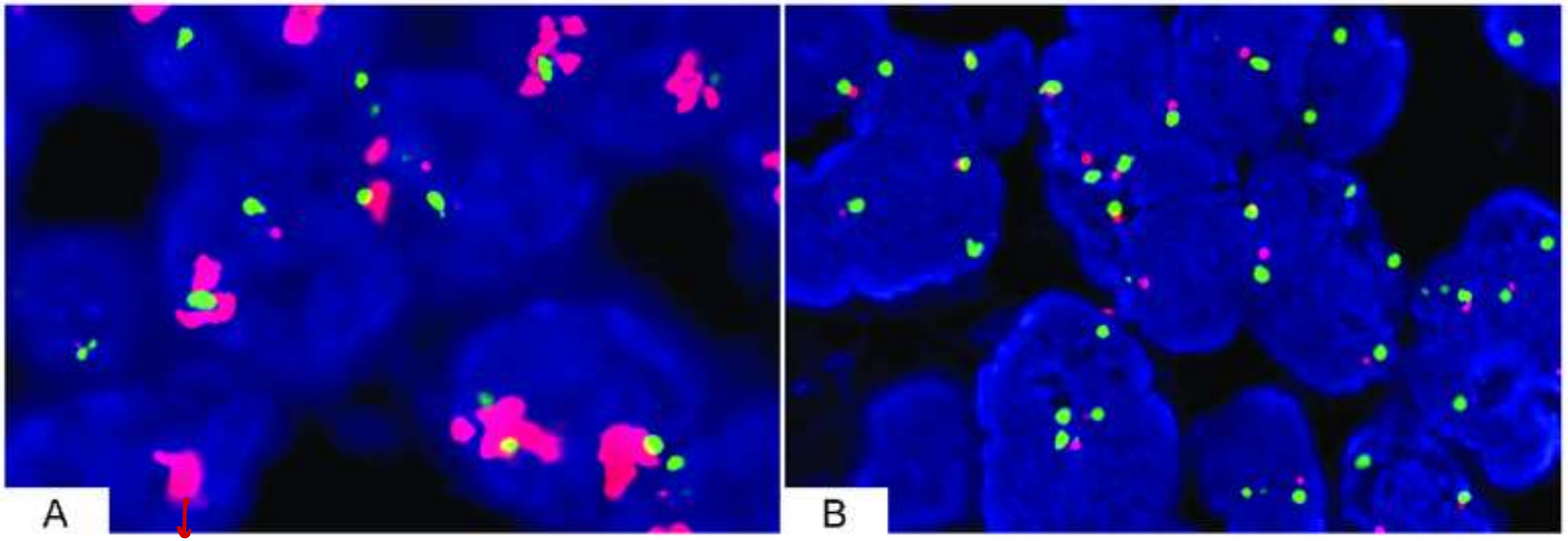
① all chromosomes are stained blue (DAPI stain)

② ch. 22 has green color & red color.

③ if a probe was designed to bind to 22q11 (labeled in red) & they appear in only one ch.22 & not the homologue (as in this image) then it means that 22q11 region is deleted in one of the homologues.

*Some cancers like glioblastoma & non-small cell lung carcinoma have EGFR amplification. (oncogene)





Analysis of EGFR gene amplification and mutations based on FISH and RT-PCR analysis. A: FISH positive (amplification), B: FISH negative (low trisomy),

Types of FISH Probes

Locus-specific (LSI)

Targets a single gene or chromosomal locus. Used for microdeletions (22q11.2/DiGeorge, 7q11.23/Williams), gene fusions (BCR::ABL1), or single-gene loci (ERBB2/HER2 in breast cancer).

one color → BCR
another color → ABL1
normally the colors are seperated (far) since BCR & ABL1 genes are on 2 different chromosomes
if there is fusion → colors next to each other.

Centromeric (alpha-satellite)

(green probe in the previous slide)

Targets repetitive alpha-satellite DNA at chromosome centromeres. Used for rapid aneuploidy detection (e.g. trisomy 21 on interphase amniocytes) and to enumerate chromosomes.

Telomeric / subtelomeric

Probes for the unique sequences just proximal to the telomeres. Detects cryptic subtelomeric rearrangements — a recognized cause of unexplained intellectual disability.

Whole-chromosome paint (WCP)

A mix of probes covering an entire chromosome. Identifies the origin of marker chromosomes and complex translocations seen on karyotype.

FISH: Common Clinical Applications

Setting	Indication	Probe / target
Prenatal	Rapid aneuploidy screen on amniocytes	13, 18, 21, X, Y centromeric probes
Pediatrics	Suspected DiGeorge / VCFS — heart defect + hypocalcaemia + immune defect	22q11.2 LSI probe (TUPLE1/HIRA)
Pediatrics	Suspected Williams syndrome — supravalvular aortic stenosis, elfin face	7q11.23 LSI probe (ELN)
Pediatrics	Prader-Willi / Angelman	15q11–q13 LSI (now often replaced by methylation testing)
Oncology	CML — confirm Philadelphia chromosome	BCR/ABL1 dual-fusion probe
Oncology	Breast cancer — assess HER2 status when IHC equivocal (2+)	ERBB2 (17q12) LSI probe
Oncology	MDS / AML — recurrent deletions	5q, 7q, 20q LSI probes

FISH: Strengths & Limitations

Advantages

- Fast turnaround — interphase results in 24–48 h
- Works on non-dividing cells (uncultured amniocytes, paraffin-embedded tumour)
- Detects submicroscopic deletions/duplications (down to ~100 kb)
- Can identify low-level mosaicism (analyze hundreds of cells)
- Useful adjunct when karyotype is ambiguous or impractical

Limitations

- Targeted — you only see what you ask about; cannot screen the whole genome
- Requires prior clinical hypothesis to pick the right probe
- Each probe is a separate test → cumulative cost
- Does not detect point mutations or single-base changes
- Cannot distinguish balanced from unbalanced changes in many cases

PART 3

Chromosomal Microarray

Genome-wide screen for copy-number variation — first-line for unexplained ID/DD/ASD/MCA

ID = intellectual disability
DD = developmental delay
ASD = Autism Spectrum Disorder
MCA = multiple congenital anomalies.

* the American Academy of Pediatrics (AAP) released updated guidlines last year in which NGS became 1st line for unexplained DD.

CMA: Principle

i.e: a popular chromosomal microarray platform is Affymetrix which use CytoScan HD for millions of probes
therefore if there was a duplication the signal would be stronger bcz there are more hybridized templates
(deletion = weaker signal)

CMA vs FISH
probe-based — probe-based
digital scanning ⇐ detected under the microscope

Quantitative, genome-wide detection of copy-number variants (CNVs)

Patient DNA is hybridized to thousands of probes spanning the genome on a single chip. By measuring fluorescence intensity at each probe — relative to a reference (aCGH) or to an expected baseline (SNP array) — the assay reveals whether each genomic region is present in normal, increased, or decreased copy number. → detects copy number change (deletion or duplication)

2 types of microarray (now they are combined)

Array-CGH

Comparative Genomic Hybridization

compare between patient & reference genomes

- Patient + reference DNA labelled with different fluorophores
- Co-hybridized; ratio of intensities → copy number
- Detects deletions and duplications only
- Cannot detect LOH or uniparental disomy (UPD)

SNP array

Single-Nucleotide Polymorphism array

- Only patient DNA; probes target known SNPs
- Provides genotype (AA, AB, BB) at each probe
- Detects deletions, duplications AND loss of heterozygosity
- Can flag UPD, consanguinity, triploidy

uniparental disomy

→ a certain probe binds to a certain locus on a certain region which could be homozygous for the 1st allele or the 2nd allele or heterozygous.

CMA: First-Line for ID/DD/ASD/MCA

1st line became NGS

ACMG, AAP, ACOG, and ISCA consensus recommendation

vs. 3% for routine karyotype in the same population

↳ (extra): means that if you test 100 children from the same population mentioned above, a traditional karyotype will only find the genetic cause in 3 of them.

Order CMA when…

- Unexplained developmental delay or intellectual disability (could be due to CNV (deletion/duplication more than 25-50 kb))
- Autism spectrum disorder without obvious syndromic diagnosis
- Multiple congenital anomalies not fitting a recognizable syndrome
- Dysmorphic features without a clear clinical diagnosis
- Fetus with structural anomalies on ultrasound (often replaces karyotype prenatally)
- After stillbirth or anomalous fetal demise

Interpreting a CMA Result

* the larger the size of CNV the more serious it could be especially if it encompasses gen-coding genes, or if it is inherited or not.
(if a parent has the same variant & they are healthy then it can imply that this mutation is NOT disease-causing UNLESS it is incomplete penetrance or variable expressivity)

American College of Medical Genetics.

ACMG 5-tier CNV classification (this classification also applies to SNV)

Pathogenic	Well-established deletion/duplication causing a known syndrome (e.g. 22q11.2 deletion, 7q11.23 duplication).
Likely pathogenic (→ deletion affects a haploinsufficient gene; → duplication affects a triplosensitive gene.)	Overlaps a known disease region or contains haploinsufficient gene(s); evidence suggests causation.
Uncertain significance (VUS)	Novel or rare CNV with insufficient evidence; may require parental testing or follow-up.
Likely benign	Reported in healthy controls or population databases at low frequency without phenotype.
Benign	Common copy-number variant in population (e.g. DGV, gnomAD-SV); no clinical relevance.

→ known to cause DiGeorge

one copy is NOT enough (in case of deletion)

OR triplosensitive gene → 3 copies cause a disease.

↳ NOT enough knowledge established

* there are many regions if they have deletions/duplication with magnitude of kilobases or even megabases they remain benign.

i.e: the lower 1/2 of the q arm of ch. Y is polymorphic. (any gain or loss is benign)

Reporting note: Classification uses size, gene content, inheritance pattern, and overlap with established databases (DECIPHER, ClinGen, ClinVar). De novo CNVs are more likely pathogenic than inherited ones.

platform where health care services can share what variants they identified including CNVs.

What CMA Cannot Detect

Balanced rearrangements

Reciprocal translocations, inversions — no change in DNA dosage

Single nucleotide variants

Point mutations and small indels < probe resolution

Low-level mosaicism

Generally needs > 10–20% mosaicism to be reliably detected

Triploidy (aCGH only)

69,XXX or 69,XXY shows normal ratio against reference; SNP array does detect it

Methylation defects

Imprinting disorders (Prader-Willi, Angelman) — needs methylation-specific testing

Repeat expansions

Trinucleotide repeat disorders (Fragile X, Huntington) — needs targeted methods

Microarray Comparative Genomic Hybridization (aCGH)

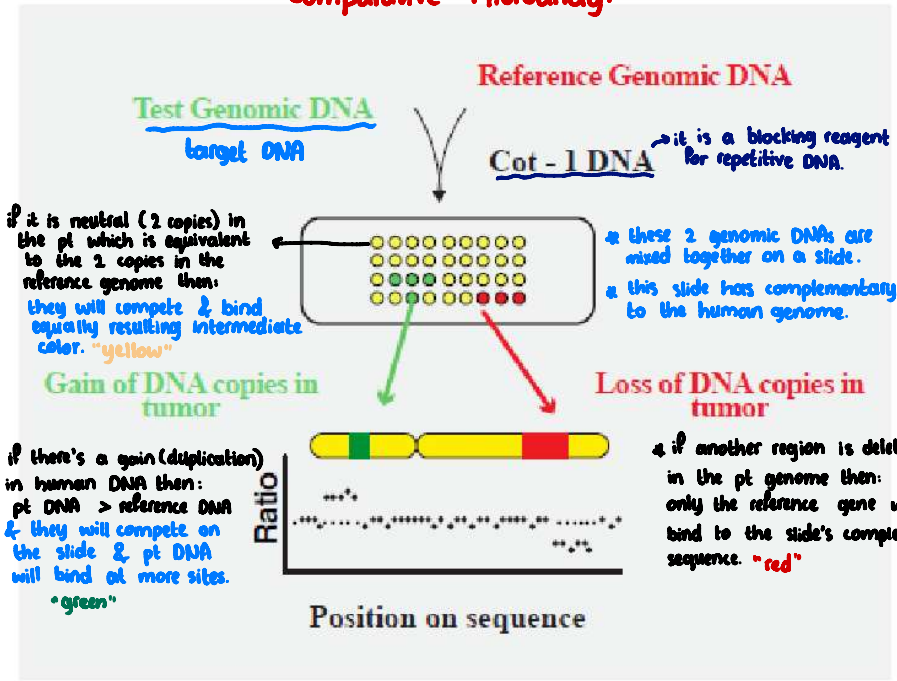
- Detects genome-wide copy number variations (deletions, duplications, gains, losses)
- Higher resolution than conventional chromosome banding
- Does not require prior knowledge of specific chromosomal abnormalities → advantage over FISH
- Uses labeled DNA from patient samples; no cell culture needed
- Hybridization occurs on glass/silicon slides printed with thousands of DNA probes

Interpretation (for this example)

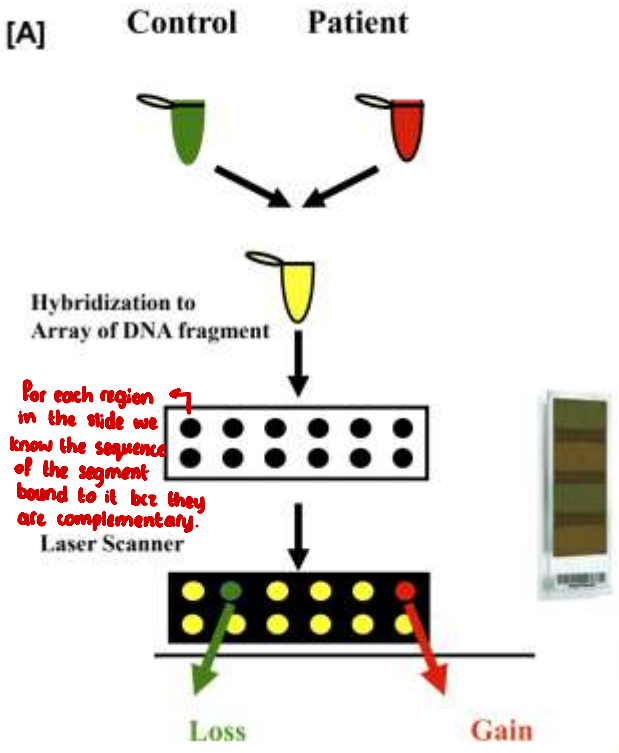
- green: pt DNA > reference DNA = Gain
- red: reference DNA > pt DNA = Loss
- yellow: pt DNA = reference DNA = Neutral

color interpretation is NOT the same always, depends on the color assigned to the pt DNA & control DNA

Comparative Microarray:

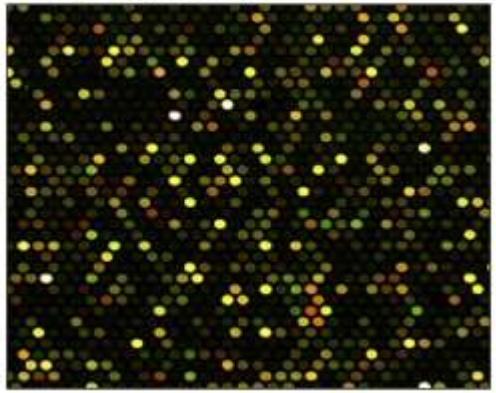


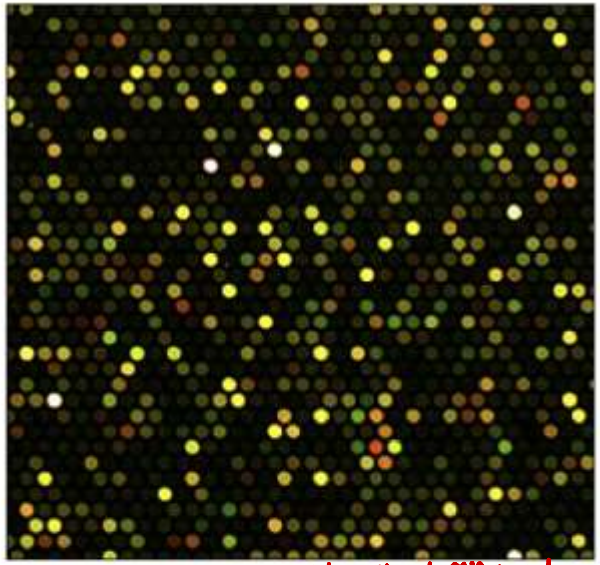
[A]

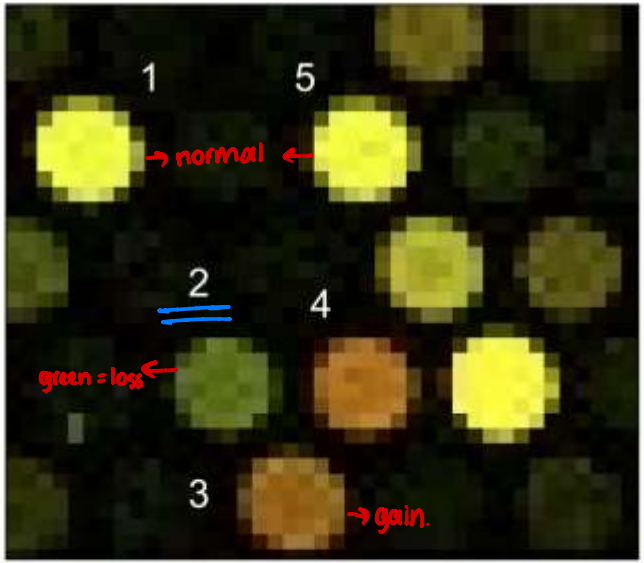


[B] Laser Scanner

[C] Actual Array

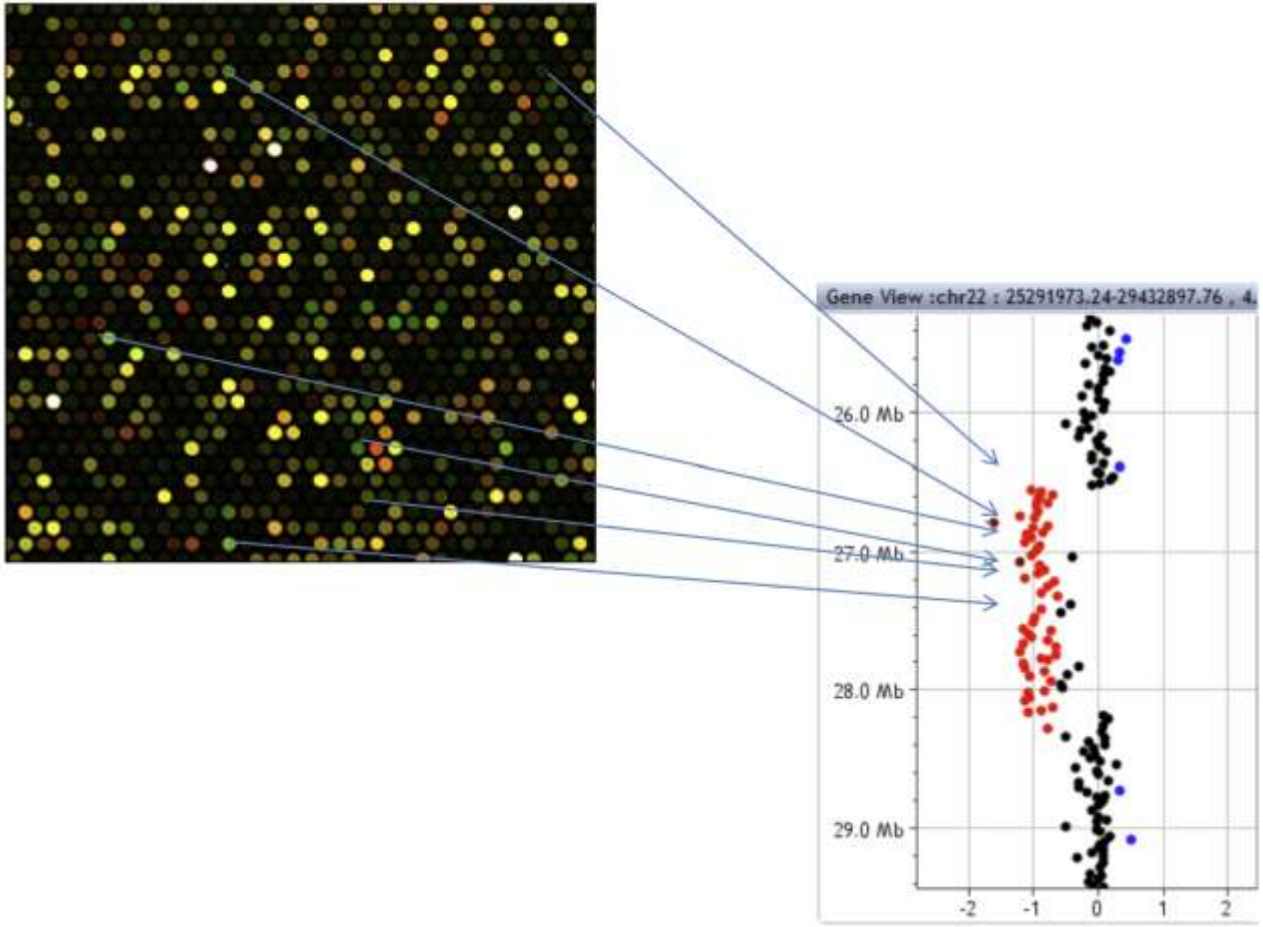






* here the pt DNA is red & control DNA is green.

Spot	position	Intensity Red patient	Intensity Green control	Ratio	Log_2	In the threshold? -0.5-0.3	Interpretation
1	1p22	656	632	1.0	0.05	yes	normal
2	Xq28	302	583	0.5	-0.95	no	loss
3	3p26	850	621	1.4	0.45	no	gain
4	4p16	826	588	1.4	0.49	no	gain
5	22q11	586	601	1.0	-0.04	yes	normal


Gene View :chr22 : 25291973.24-29432897.76 , 4.
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-2
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1
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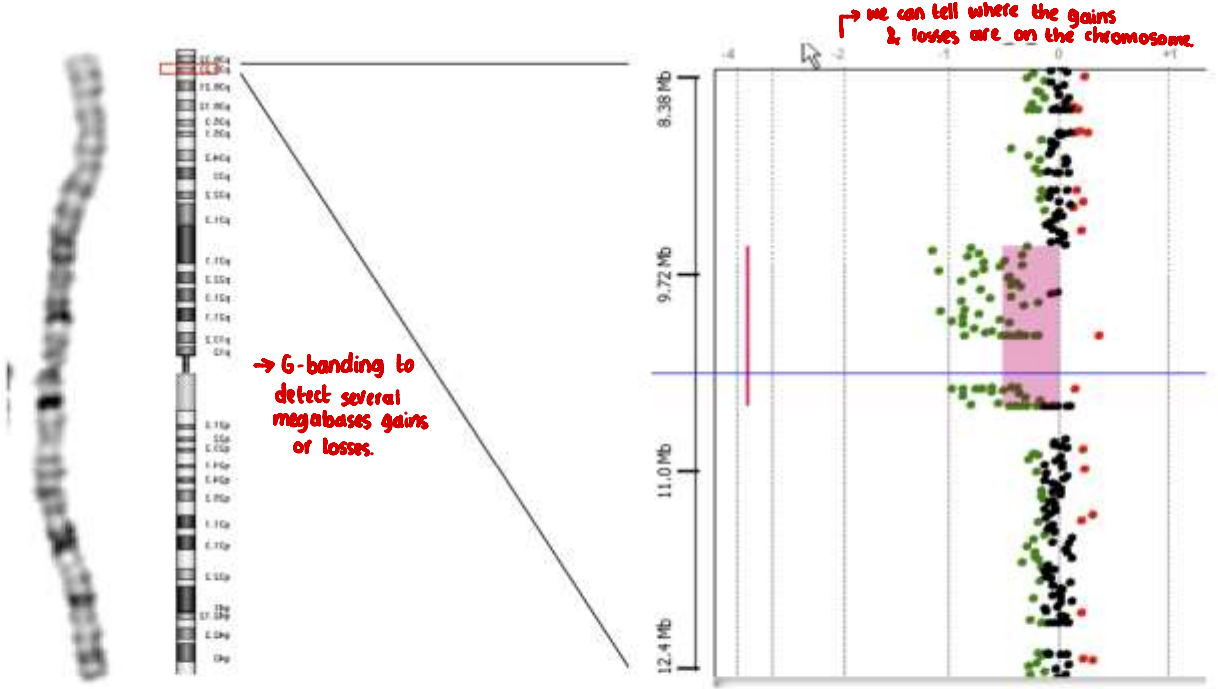
CMA can detect del/dup of ~>25-



Chromosome 1

Limits of detection for G-banded chromosome analysis is 4-5 Mb

4 Mb region of microarray data showing a 1 Mb deletion encompassing ~70 oligos

~70 probes captured this 1 Mb deletion

- Modern microarray platforms typically combine copy-number (CNV) probes with SNP probes.
- SNPs must be present in >1% of the population (most informative when present at ~50% frequency). preferably probe includes a SNP that has a frequency ≈ 50%.
- SNPs may be located in coding regions, noncoding regions, or intergenic areas between genes.
- Each SNP's possible states are assigned arbitrary allele labels (A and B).
- SNP data can identify uniparental disomy (UPD) and regions of homozygosity (ROH).
- Different microarray platforms vary in their specifications; for example, some SNP-only arrays do not require a control sample.

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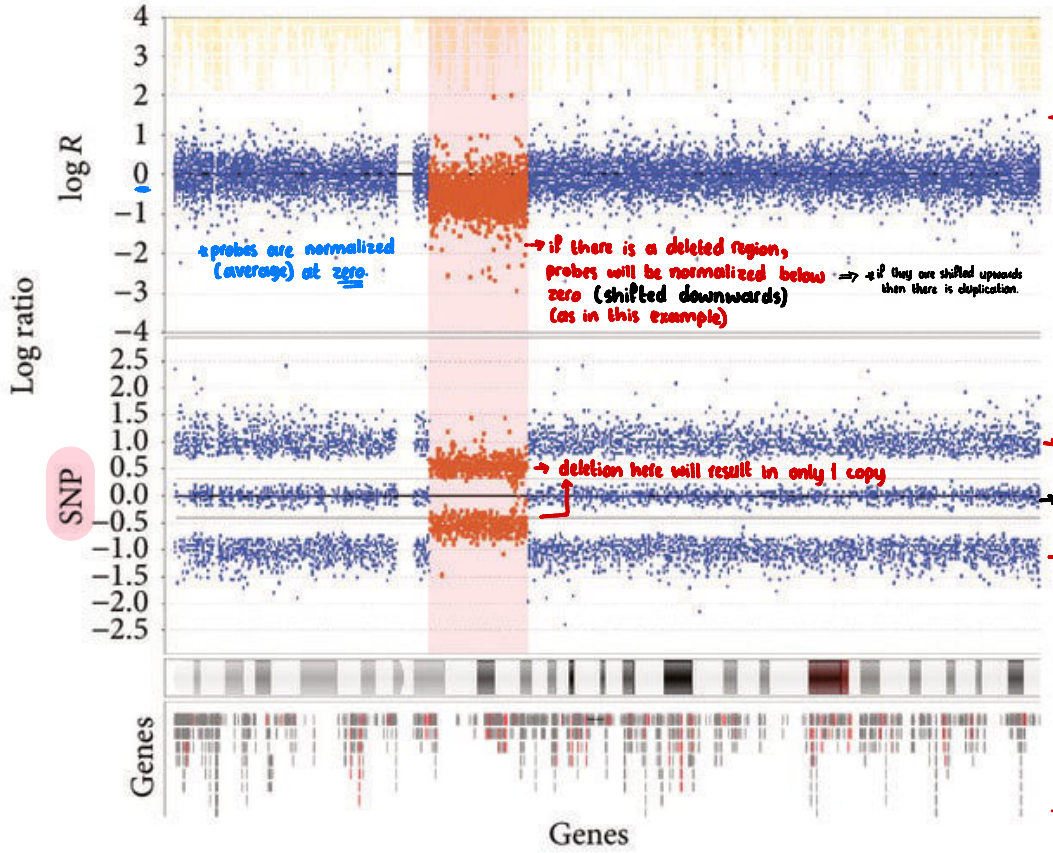
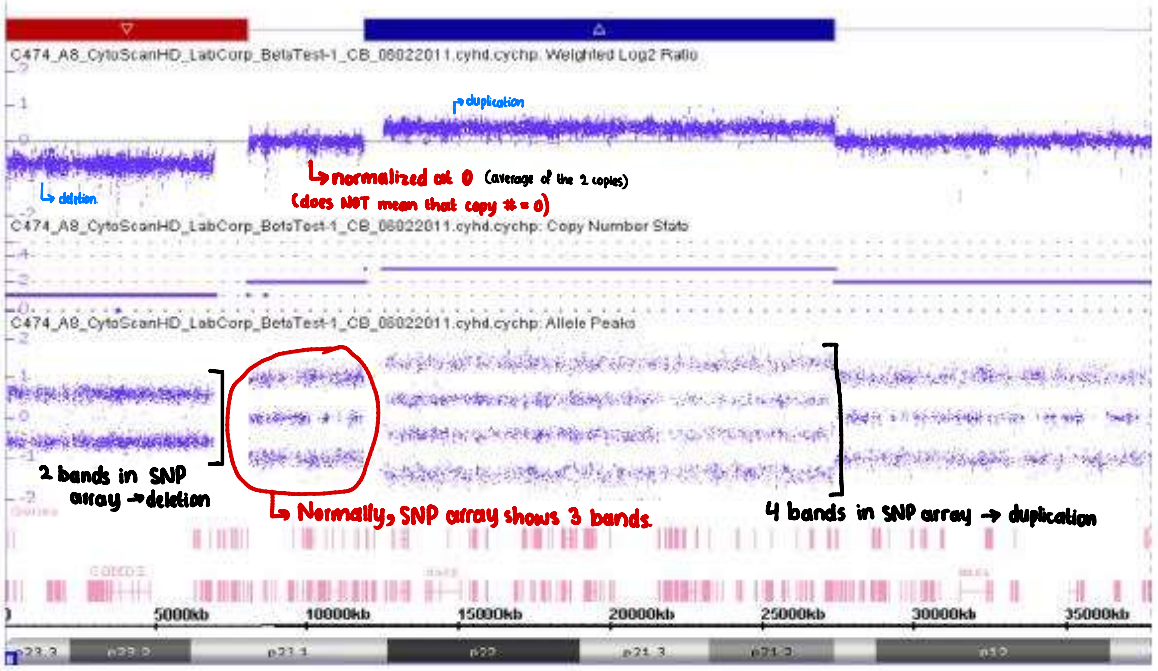


Copy number + SNP array

Copy #

Allele peaks



Interpretation of germline

Each SNP probe present in a diploid region is genotyped as:

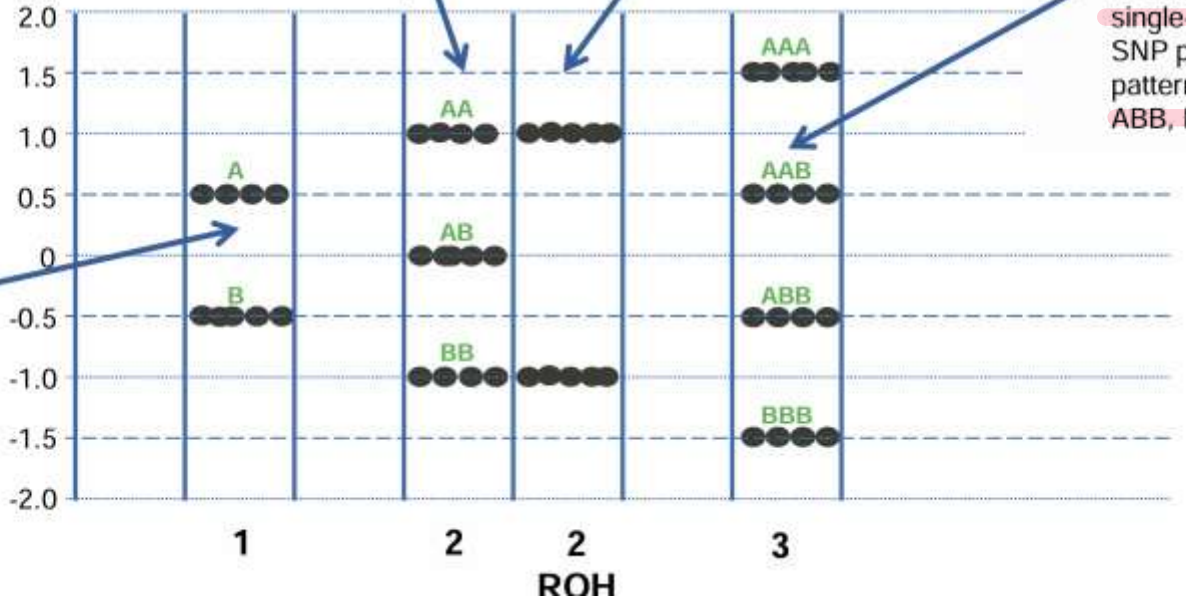
- AA (0 uncut copies), homozygous
- AB (1 uncut copy), heterozygous
- BB (2 uncut copies), homozygous

(normal copy number and SNPs = 3 lines)

Regions of Homozygosity (ROH) have no heterozygous alleles = AA or BB (2 lines)

In aberrant genomic locations harboring single-copy gains, SNP probes yield four patterns: AAA, AAB, ABB, BBB (4 lines)

In aberrant genomic locations harboring single-copy losses, SNP probes are either cut or uncut and the resulting genotype is simply A or B (2 lines)



In amplified regions (typically tumor samples), additional A or B alleles are detected by the SNP probes and the genotypes become increasingly more complex

Copy Number

PART 4

Next-Generation Sequencing

Massively parallel sequencing — panels, exomes, and whole genomes

From Sanger to Massively Parallel Sequencing

* you design primers that surround a certain region that are sequenced
(if the nucleotides/monomers are colored then you can detect signals of nucleotides then figure out the sequence)

↳ another name for NGS
↳ bcz in the same reaction (tube) you can sequence many different regions at the same time.
(we can even sequence multiple genomes for different pts simultaneously)

Sanger sequencing (1977) reads one DNA fragment at a time and remains the gold standard for confirming single variants. Next-generation sequencing reads millions of fragments simultaneously, making whole-exome and whole-genome analysis clinically feasible and affordable.

* Sanger is not practical for sequencing the entire genome (NGS instead) BUT sometimes we only want to sequence a certain region then Sanger can be used. ⇒ i.e: you sequenced the entire genome for a family member with a genetic disease & you captured a variant & knew its location then you can just Sanger sequence the family members for that variant.

Feature	Sanger sequencing	Next-generation sequencing
Reads per run	1 fragment (~800 bp)	Millions of fragments in parallel
Throughput	Low	Very high
Cost / base	High	Very low (and falling)
Best use	Variant confirmation, single-gene tests	Panels, exomes, whole genomes
Sensitivity for mosaicism	~ 15–20%	≥ 1–5% (depending on depth)
Turnaround	Hours–days	Days–weeks (clinical pipelines)

↳ DNA polymerase that is used to amplify the region between primers can reliably amplify around 800 bases before becoming inefficient. (human genome is 3 billion bases)
* it took 10-20 years to Sanger sequence the human genome across 4 different countries: Germany, UK, USA, Japan.

↳ cost is high if we want to sequence many regions BUT it is low if we want to sequence a few regions each one is < 1 kb.

↳ If the gene is small (i.e: 1 or 2 exons) then Sanger is appropriate to use BUT if the gene was large & carried many exons & each exon is big then NGS is better.

if we want to confirm a certain variant that we know where it is located & we design primers to surround/flag that variant

↳ Sanger is suitable for mosaicism that is >20%.

↳ if you have primers you can do it on the same day.

↳ not long ago, it was included in clinical practice not just research.

↳ choose a group of genes we want to sequence.

* the standard turnaround time for NGS if it is not rapid is around 4-6 weeks. (bcz samples would be grouped together & run in sequence to be more cost-effective)

↳ nowadays can take a few days from recieving the sample, extracting DNA, sequencing & analyzing the sequence data & interpreting it to announce preliminary results at least. ⇒ rapid NGS is used in NICU (Neonatal Intensive Care Unit) within 1 week at least.

The NGS Workflow

From sample to clinical report

Note #1:
"Depth of coverage" or "average coverage":
each region/gene/exon in the human genome will be sequenced more than once. Why? to ensure that each time it is sequenced, the sequence data is calling the mutation over & over again which gives confidence that there is a true variant (NOT an artifact or a certain glitch)

Note # 3:
Somatic cancer variants precisely → Cancer is a different story bcz it is not just homozygous or heterozygous in the tissue, rather it is a percentage. For a certain coordinate, a variant could be found in only 10% of tumor tissue & 90% is reference (normal sequence) & this is called "Allele Fraction"
a variant could be 100% in tumor cells (all tumor cells have the variant) or it could be heterozygous for all cancer cells then it is 50% or it could be any other percentage such as 10% (only a subpopulation develops the variant)
↳ Remember tumor clonality

1 Library preparation

Genomic DNA is fragmented; adapters with sample barcodes are ligated.

2 Target enrichment (optional)

Capture probes pull down the regions of interest — panel or exome. WGS skips this.

3 Cluster generation

Fragments amplified on a flow cell, generating clonal clusters of identical DNA.

4 Sequencing by synthesis

Fluorescent nucleotides incorporated one base at a time; each cycle imaged.

5 Alignment

Short reads mapped to a reference genome (GRCh38).

6 Variant calling

Differences from reference identified: SNVs, indels, CNVs, structural variants.

7 Annotation & filtering

Variants linked to gene/transcript, population frequency, prior reports.

8 Clinical interpretation

ACMG criteria applied; report issued; Sanger confirmation if needed.

Note #2:
↳ average coverage (how many times sequencing should be done) is different depending on type of variant.

Coverage matters: average read depth ≥ 30× for germline, ≥ 100× for somatic; uniformity is as important as the mean — gaps cause false negatives.

30x for the genome
100x for the exon.

Panels, Exome & Genome

Three flavors of clinical NGS

CF is only caused by 1 gene (CFTR) BUT it is suitable to test it using NGS. Why? bcz CFTR gene contains MANY exons. (NOT practical to run lots of Sanger)

if ddx is NOT clear & we don't have a group of genes in mind then we can do exome testing which analyzes variants in the coding regions for ALL the genes.

Targeted panel

Tens to hundreds of curated genes

Coverage
5–500 genes

Genome fraction
< 0.1%

the smaller the region you sequence the deeper you can cover it (the more you can read it over & over again therefore the higher confidence you have)

Depth
Very deep (200–1000×)

there are many panels i.e: inborn errors of metabolism, ophthalmic, etc...

Best for
Well-defined phenotype (e.g. hereditary cancer, cardiomyopathy, hearing loss)

100 genes

Nowadays, genes are selected for a panel based on exon chemistry → you sequence all of the exons for all of the genes → then you can select a group of genes (panel) to test for.

Yield
Variable; high when phenotype is specific

Cost
Lowest

Whole exome (WES)

~ 20,000 protein-coding genes

Coverage
Exonic regions (~ 1–2% of genome)

Genome fraction
1–2% → exons of the 20,000 pln-coding genes represent only up to 2% of the genome.

Depth
Moderate (80–150×)

practically, if we see a mutation in only 1 read out of 100 reads → we dismiss it (artifact)

BUT if we see a mutation in 50 reads out of 100 reads then it is a true heterozygous variant.

if a variant was seen in 100 reads out of 200 then it's a homozygous variant.

Best for
Suspected Mendelian disease without phenotypic certainty

Yield
25–40% in suspected genetic disease

Cost
Moderate

Whole genome (WGS)

Coding + non-coding sequence

Coverage
Whole genome → equivalent to >100 GB in cloud storage. (exomes ≈ 10 GB)

Genome fraction
~ 100%

Depth
Lower (30–40×) but uniform

to do 100x consumes a lot of reagents & cloud storage.

i.e: for the same run where you can sequence 2 genomes, you can sequence 15 exomes (chemically speaking)

Best for
Critically ill neonates; structural variants; non-coding regulatory variants

Yield
+5–10% incremental over WES

some variants are in non-coding regions.

Cost
Highest, but falling rapidly

Variant Types Detected by NGS

SNVs

Single-nucleotide variants — substitutions. The bread and butter of clinical NGS.

Small indels

Insertions/deletions of a few base pairs; may cause frameshift if not multiples of 3.

CNVs

↳ recently added to NGS utilities.

Copy-number variants — increasingly callable from NGS depth-of-coverage data.

Structural variants

↳ includes all structural rearrangements (i.e: inversion translocation) & CNVs.

Larger inversions, translocations, complex rearrangements; better detected with WGS or long reads.

NGS still does NOT call them perfectly (yet).

Mitochondrial variants

If mtDNA is included in the capture; heteroplasmy quantifiable at deep coverage.

Repeat expansions

↳ better to confirm on another platform.

remember NGS is sequence-based (NOT probe-based) & it depends on DNA polymerase which is weak in repeat regions.

Historically poorly detected by short-read NGS; emerging algorithms and long reads are changing this.

NGS: Strengths & Limitations

Strengths

- Detects single-base resolution variants across thousands of genes simultaneously
- Quantitative: variant allele fraction informs mosaicism, tumour purity, heteroplasmy
- Re-analysis possible as new gene–disease associations emerge
- Trio testing (proband + parents) dramatically improves yield and shortens diagnostic odyssey
- Same workflow scales from a 5-gene panel to a whole genome
- Falling cost — WGS now under US$1000 in many labs

Limitations

- Misses some structural variants and large CNVs (especially with short reads)
- Poor coverage of GC-rich regions, repeats, pseudogenes (e.g. PMS2, SMN1, CYP21A2)
- Trinucleotide repeat expansions and methylation defects need targeted assays
- Mitochondrial variants need a dedicated workflow
- VUS rate is high — particularly for under-studied populations
- Incidental / secondary findings raise complex consent and counselling issues

ACMG/AMP Variant Classification

Standardized framework for clinical sequence variants

Pathogenic (P)	Sufficient evidence the variant causes disease. Report and act on it.
Likely pathogenic (LP)	≥ 90% certainty of being disease-causing. Treated clinically as pathogenic.
Uncertain significance (VUS)	Insufficient or conflicting evidence. Do NOT use for clinical decisions; may be re-classified.
Likely benign (LB)	≥ 90% certainty of being benign.
Benign (B)	Sufficient evidence the variant is not disease-causing.

→ Same clinical management

→ perceived as non-disease causing.

Evidence categories combined to assign tiers:

→ those are algorithms that can predict.

→ studies that would study the impact by studying the RNA, the ptn, animal models, tissue culture to see what that variant would cause.

→ if there is a variant in a dominant gene that is fully penetrant in healthy individuals of the family then we don't worry about it if the pt has it.

Population data (allele frequency) · **Computational** (in-silico predictions) · **Functional** (experimental studies) · **Segregation** (in families) · **De novo** occurrence · **Allelic** data · **Other databases** (ClinVar)

→ population data: if a variant is v. common in the population & the disease is rare then it is a benign variant (otherwise the disease would've been common)

Variants of Uncertain Significance (VUS)

The most challenging result for the clinician

> *A VUS is a variant for which there is insufficient or conflicting evidence to classify as benign or pathogenic. It is NOT a positive result and should not be used to make clinical decisions.*

Common pitfalls

- Treating a VUS as if it were pathogenic — risk of overdiagnosis, unnecessary surgery, anxiety → it could also unnecessarily impact pt management or therapy regimen.
- Cascade testing in relatives — confirms nothing and adds confusion → it could lead to an answer or add more confusion.
- Modifying surveillance or therapy based on a VUS
- Failure to re-evaluate the variant over time

Best practice

- Manage the patient on phenotype and family history — not the VUS → sometimes you can clinically consider VUS variant as the cause of disease if there were many other supportive indications.
- Consider segregation testing if the family is informative
- Re-query the laboratory and ClinVar periodically (~ every 1–2 years)
- Submit variant evidence to public databases to aid reclassification
- Document clearly and counsel that the result is non-diagnostic

Secondary & Incidental Findings

IMP.

there is a certain list of genes that the pt has a choice to be aware if they have pathogenic or likely pathogenic variants in those genes. ⇒ having the right to know if you have pathogenic or likely pathogenic variants in genes that are NOT related to the disease chief complaint could help with early management specifically for cancer & cardiac diseases.

Unintended discoveries from genome-scale testing

When you sequence an exome or genome, you may find clinically important variants outside the indication for testing. ACMG maintains a list of medically actionable genes (currently ~ 80) for which findings should be reported to the patient — unless they opt out.

↳ i.e: WES or WGS is requested for specific cases such as newborns with congenital heart disease (bcz it involves a large group of genes), or dysmorphic features or neurological manifestations (i.e: seizures), etc... ⇒ You will ONLY recieve in the report variants in genes related to the chief complaint even though there could be other pathogenic variants in genes BUT those genes do NOT cause the disease the pt is being tested for. ⇒ EXCEPT if the family prefers & requests for secondary findings as well.

↳ variant that are reported for the genes relevant to the disease are pathogenic, likely pathogenic & VUS variants. (benign & likely benign variants are NOT reported)

Examples of ACMG actionable categories

« the pt has the choice to recieve info about them.

Hereditary cancer

BRCA1, BRCA2, MLH1, MSH2, MSH6, PMS2, APC, TP53, MEN1, RET

Cardiomyopathies

MYH7, MYBPC3, TNNT2, TNNI3, LMNA, DSP

Arrhythmias

KCNQ1, KCNH2, SCN5A (long QT); RYR2 (CPVT)

Aortopathies

FBN1 (Marfan), TGFBR1/2 (Loeys-Dietz), ACTA2

Familial hypercholesterolemia

LDLR, APOB, PCSK9

Malignant hyperthermia

RYR1, CACNA1S

PART 5

Putting It All Together

Choosing the right test for the clinical question

Master Comparison Table

	Karyotype	FISH	CMA	NGS
Resolution → numbers in this table are inaccurate.	5–10 Mb	~ 100 kb	10–400 kb	1 bp (single base)
Genome view	Whole	Targeted (locus)	Whole (CNV only)	Whole (sequence)
Detects balanced rearrang.	Yes	Sometimes	No	WGS partially
Detects point mutations	No	No	No	Yes
Detects CNVs	If > 5 Mb	At targeted loci 100kb	Yes (sub-Mb) ≥25 kb	Yes (variable) detects ≥ 1 kb but it has low sensitivity & high noise.
Detects mosaicism	≥ 10–20%	Possible	≥ 10–20%	≥ 1–5% with depth
Cells must divide	Yes	No	No	No
Turnaround	5–14 d → depends on the tissue you are culturing & inducing.	1–3 d	5–10 d	1–6 wk (clinical) rapid NGS is within 1 week otherwise 4-6 weeks.
Relative cost	$	$	$$	$$ – $$$$

* karyotype & FISH are more affordable than CMA.

* Note: you can do NGS or CMA to detect Down Syndrome in a pt but you can simply capture it with karyotype.

Choosing the Right Test

v. helpful

A simplified clinical algorithm

Suspected aneuploidy, balanced translocation, or recurrent miscarriage?	**Karyotype ± FISH** *Karyotype is unique in detecting balanced rearrangements; FISH adds speed for known targets.*
Unexplained ID / DD / ASD / multiple congenital anomalies?	**Chromosomal microarray FIRST** more updated (nowadays): Exome testing + CMA *ACMG first-line recommendation; 15–20% yield vs ~ 3% for karyotype.*
Recognizable single-gene syndrome (Marfan, CF, Duchenne)?	**Targeted gene test / panel** *Highest yield when phenotype clearly points to a defined gene or pathway.* → suitable to confirm an already suspected disease. (i.e: Marfan panel for MS)
Phenotype suggestive of a genetic disorder but non-diagnostic; first-tier tests negative? (→ broad ddx.)	**Whole exome (trio if possible) → Whole genome** *Trio WES yields 25–40% diagnosis; WGS adds non-coding and structural variants.*
Critically ill neonate in NICU with unexplained presentation?	**Rapid trio WGS (or WES)** *Results in days; can change management — antibiotics, surgery, withdrawal of care.*

Case 1: Pediatric Diagnostic Odyssey

Apply what you've learned

Vignette

A 4-year-old boy presents with global developmental delay, microcephaly, hypotonia, and dysmorphic features (broad forehead, low-set ears, widely spaced eyes). He has no recognizable syndrome. Hearing screen, metabolic panel, brain MRI, and Fragile X testing are all normal. Parents are non-consanguineous and healthy.

Q: What is your first-line genetic test?

A	**Karyotype**	*Low yield (~ 3%) in this setting; misses sub-microscopic CNVs that explain most of these phenotypes.*
B	**Chromosomal microarray** → used to be correct	*First-line per ACMG/AAP guidelines for unexplained ID/DD/ASD/MCA. Yield 15–20%.*
C	**Whole-genome sequencing** → correct now.	*Powerful, but reserved for after first-tier work-up (and trio testing) when CMA negative — except in critical care settings.*
D	**Targeted Fragile X repeat expansion**	*Already done in this case (normal) — and would not explain the broader phenotype.*

Case 2: Hereditary Cancer

Family history changes everything

Vignette

A 38-year-old woman is diagnosed with triple-negative breast cancer. Family history: mother with ovarian cancer at 52, maternal aunt with breast cancer at 41, maternal grandfather with pancreatic cancer at 60. Ashkenazi Jewish ancestry.

Which testing strategy is most appropriate?

Multi-gene hereditary cancer panel by NGS

Why a panel and not just BRCA1/2? Phenotype overlaps with several syndromes — BRCA1/2 (HBOC), PALB2, CHEK2, ATM, TP53 (Li-Fraumeni), Lynch syndrome (MLH1/MSH2/MSH6/PMS2). A 20–80 gene panel covers the differential in a single, cost-effective assay.

Founder mutations: Ashkenazi heritage prompts targeted analysis of BRCA1 c.68_69delAG, c.5266dupC and BRCA2 c.5946delT — three variants account for ~ 90% of HBOC in this population.

Case 3: Prenatal Diagnosis

Layering tests as information evolves

Vignette

A 28-year-old woman, G1P0, at 20 weeks gestation. Anatomy scan shows a conotruncal cardiac defect (tetralogy of Fallot) and an absent thymus. Non-invasive prenatal testing (cell-free DNA) at 12 weeks was low-risk for trisomies 13/18/21.

blood collected from the mother (fetal DNA leaks into maternal blood)

"NIPT"

→ NIPT did not provide an explanation in this case for the abnormalities detected

Tiered diagnostic approach

1. **Diagnostic amniocentesis —** Provides fetal cells; consent for genetic testing.
2. **Rapid FISH on uncultured amniocytes —** Probes for 13, 18, 21, X, Y AND a 22q11.2 LSI probe. Conotruncal defect + absent thymus is highly suggestive of 22q11.2 deletion (DiGeorge). Results in 24–48 h.
3. **Chromosomal microarray —** If FISH negative: CMA defines the full extent of any deletion/duplication and screens for other CNVs.
4. **Consider WES / WGS —** If first-tier tests negative and phenotype suggests a Mendelian disorder (RASopathies — Noonan; CHARGE — CHD7).