

Molekulární cytogenetika (FISH, array CGH, SNP array)

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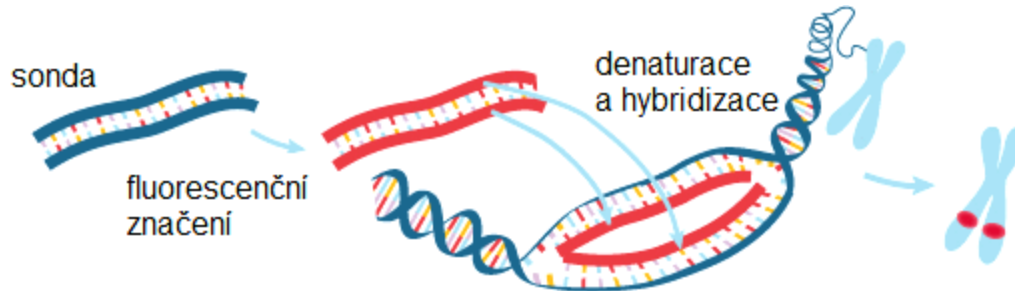
Oddělení lékařské cytogenetiky,
Ústav biologie a lékařské genetiky
2. LF UK a FN Motol

Fluorescenční hybridizace *in situ* - FISH

(„Fluorescent in situ hybridization“)

Princip metody:

- přímo či nepřímo fluorescenčně značené jednořetězcové DNA sondy hybridizují na specifické sekvenční vlákna DNA fixovaných jader nedělících se buněk (interfázní FISH) a sekvenční DNA v metafázních chromozomech (metafázní FISH)



1. denaturace sondy a cílové DNA
2. hybridizace sondy a cílové DNA – komplementarita
3. odstranění nespecifických signálů

Materiál:

- periferní a pupečnicková krev, choriové klky, plodová voda, bukalní stěr, materiál z potratu, vzorky z parafinových bločků (xylen)
- nativní vzorky - pepsin

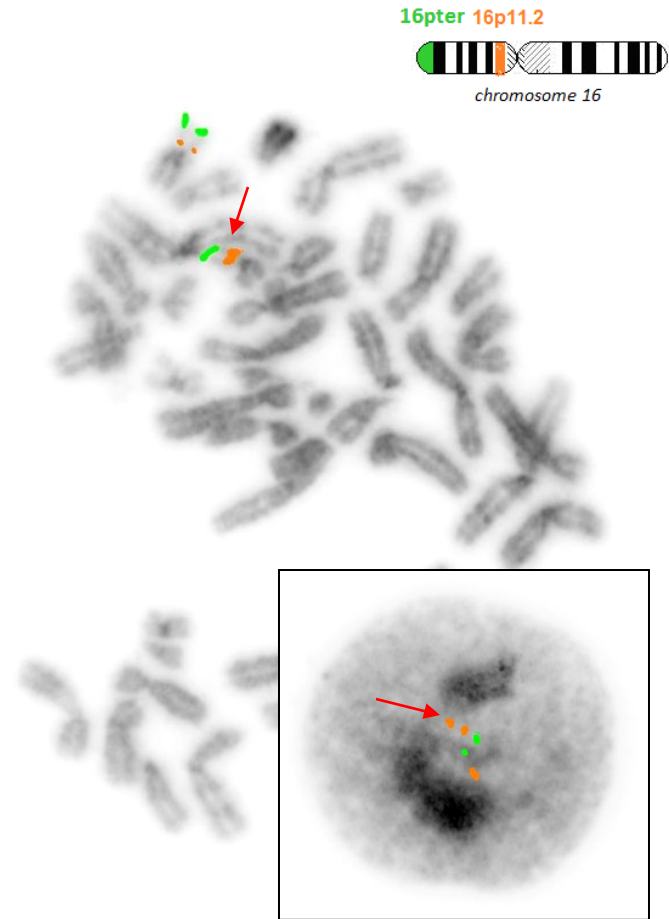
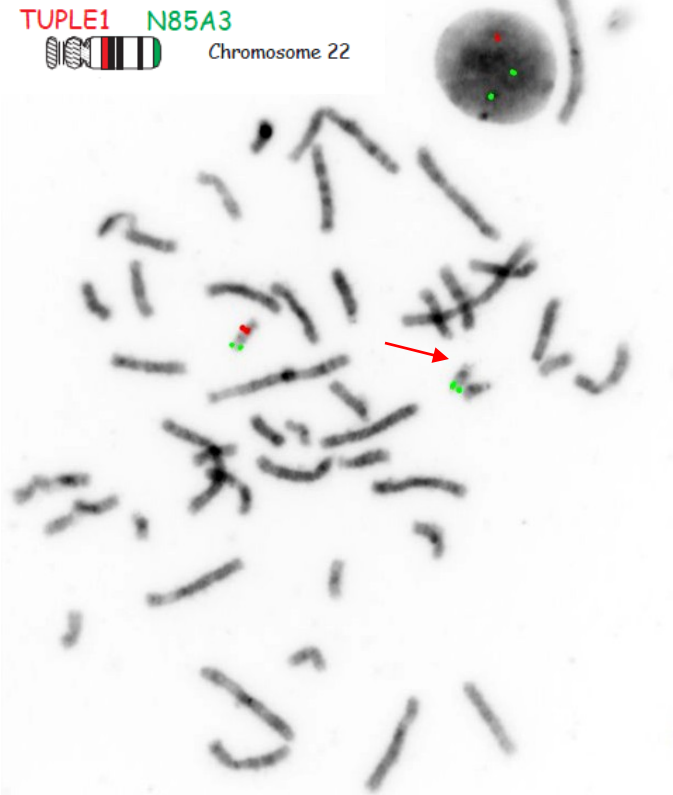
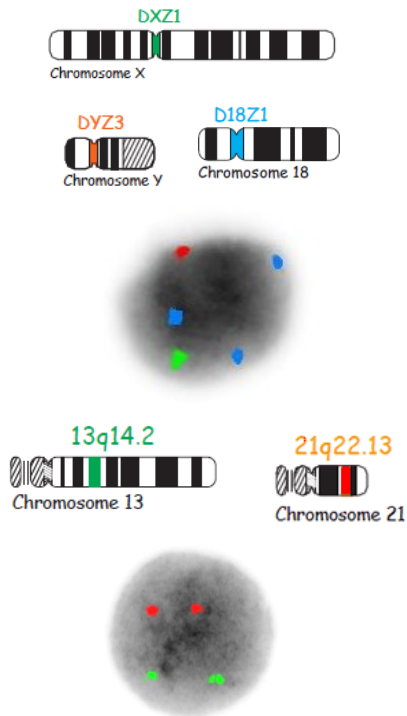
Sondy:

- nutnost znát cílovou sekvenci v genomu - vždy je třeba předem určit, jaké lokusy budou vyšetřeny - hodnotíme přítomnost, počet, lokalizaci a intenzitu signálů

- sondy o délce několika kb až 1 Mb naštěpené na fragmenty o délce 200 až 400 pb

- 1) **satelitní sondy** – centromerické (α -satelitní DNA – unikátní pericentromerické oblasti) a telomerické (repetice 6pb „TTAGGG“)
- 2) **lokus specifické sondy**
- 3) **celochromozomové („malovací“) sondy**

FAST FISH – záchyt nejčastějších aneuploidií



Výhody:

- rychlost a cena
- možnost hodnotit interfázni jádra
- přesná detekce mozaicizmu ve vzorku
- balancované aberace cíleně detekovatelné
- polyploidie detekovatelné

Nevýhody:

- nutnost znát cílovou sekvenci v genomu
- informace pouze o daných lokusech

Modifikace metody FISH:

WCP („whole chromosome painting“; obarvení celého chromozomu)

M-FISH („multicolour FISH“; mnohobarevná FISH)

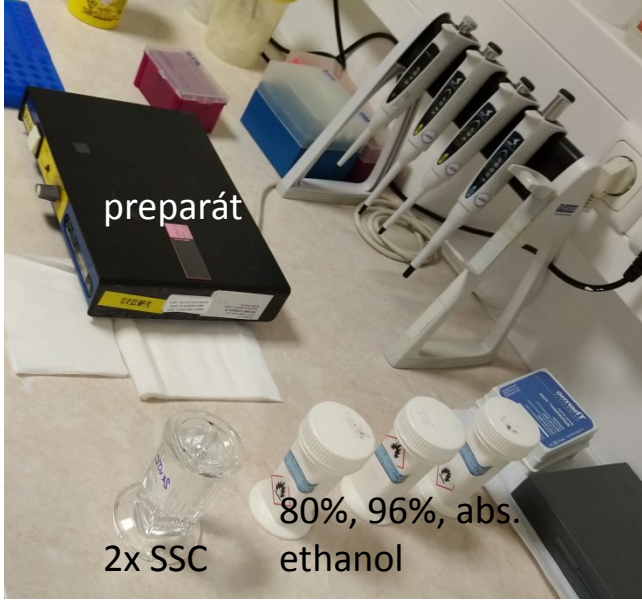
SKY („spectral karyotyping“; spektrální karyotypování)

M-BAND („multicolour banding“; mnohobarevné pruhování)

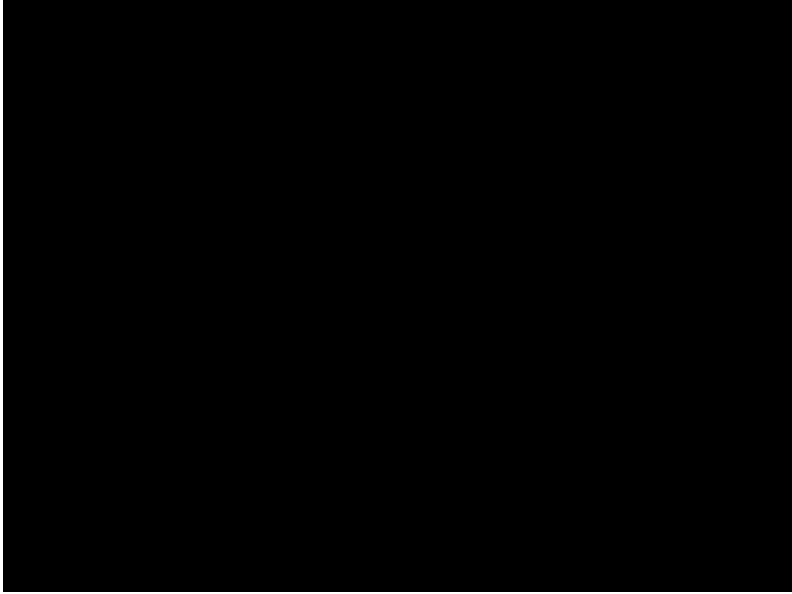
- aplikace na metafázni chromozomy odhalí a upřesní strukturní přestavby chromozomů - využití především v onkocytogenetice

Laboratorní postup – 1. den:

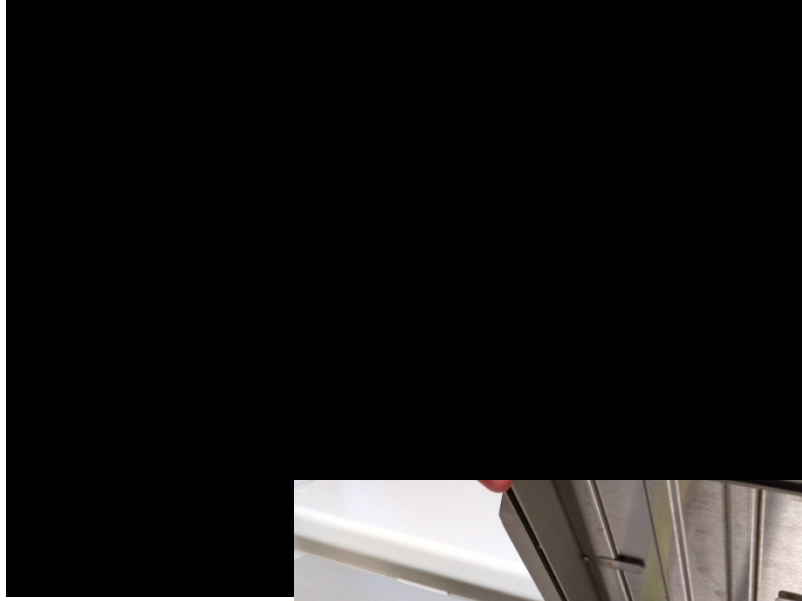
1. dehydratace preparátu ↱



3. utěsnění krycího sklíčka ↻



2. aplikace sondy ↲



4. denaturace a hybridizace:
75 °C/ 3 min. -> 37 °C/ 2-16 hod.



Laboratorní postup – 2. den:

4. aplikace DAPI (4',6-diamidin-2-fenylindol)



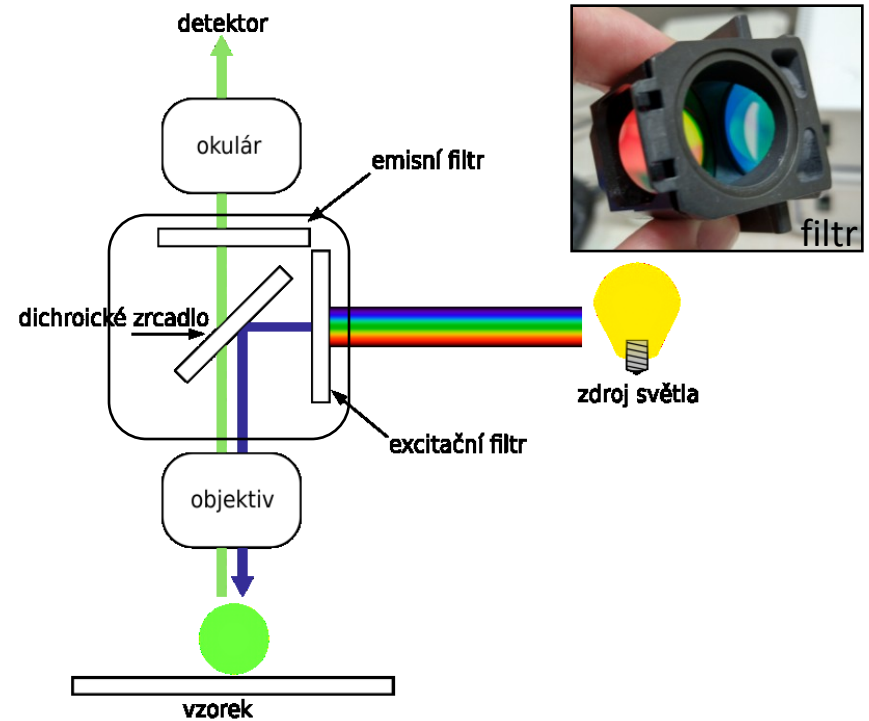
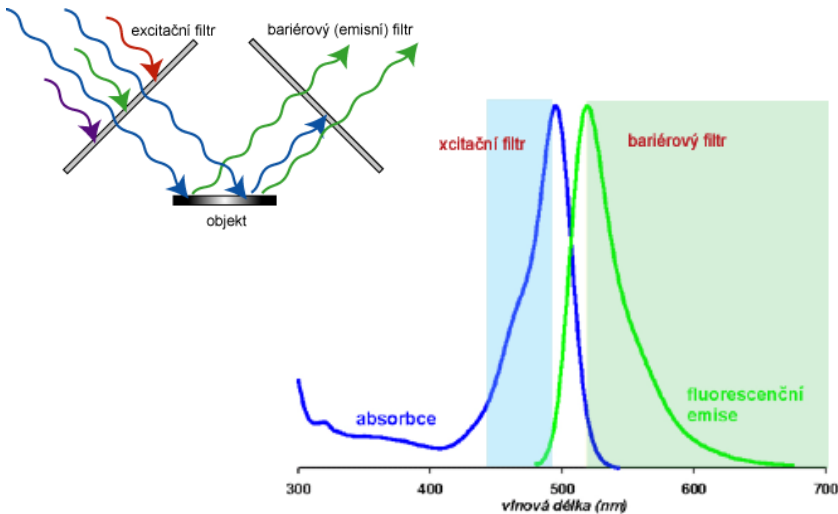
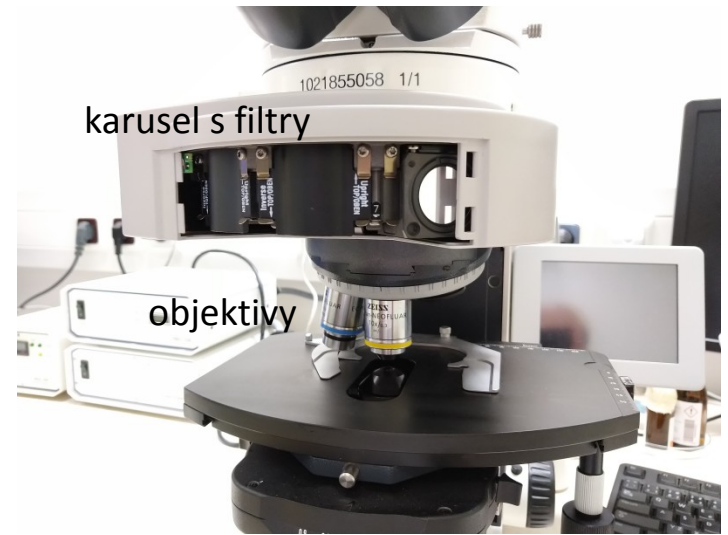
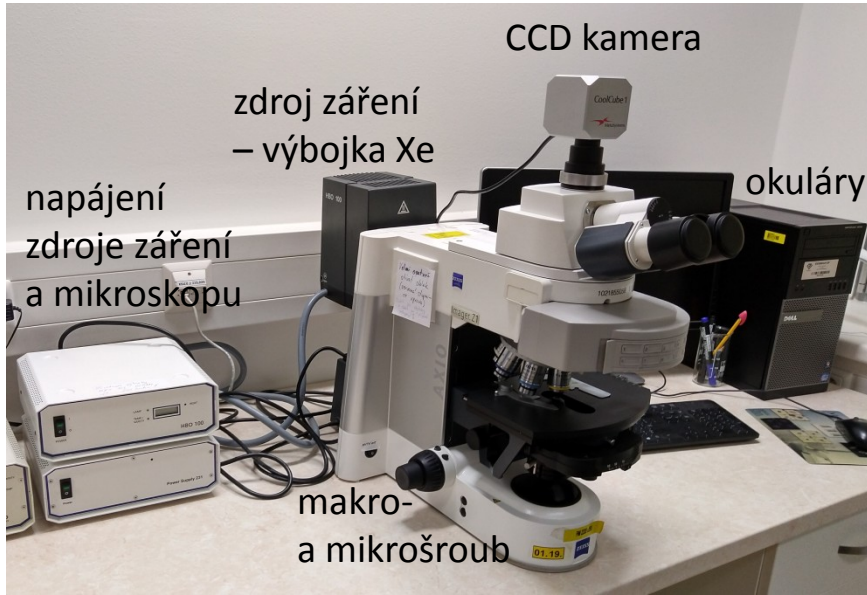
5. stržení krycího sklíčka



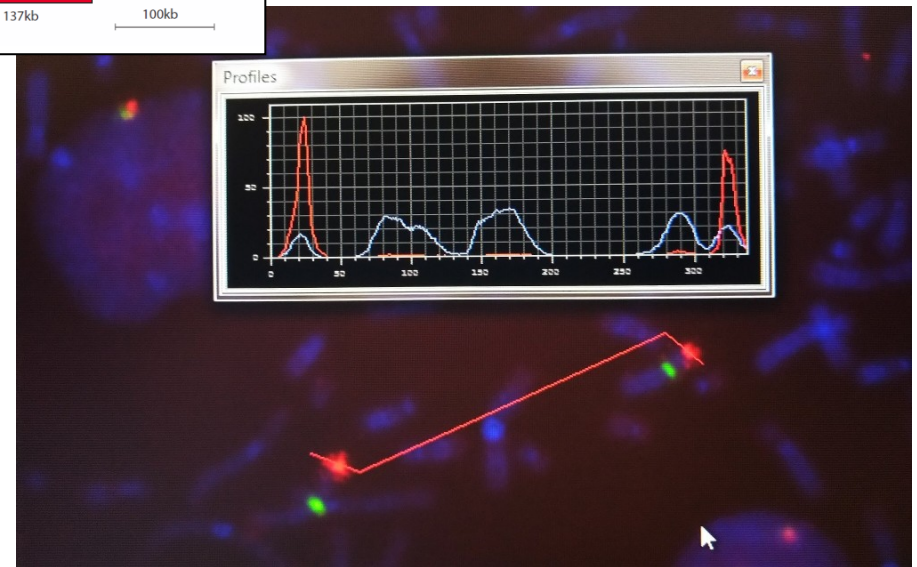
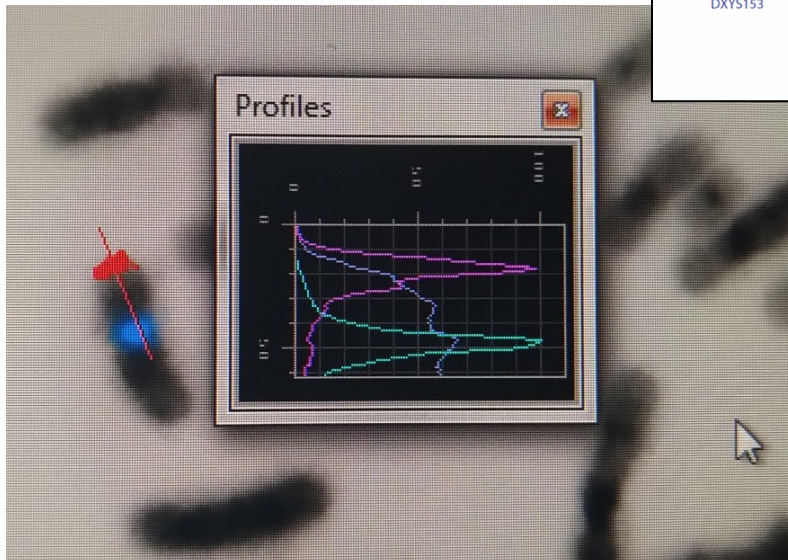
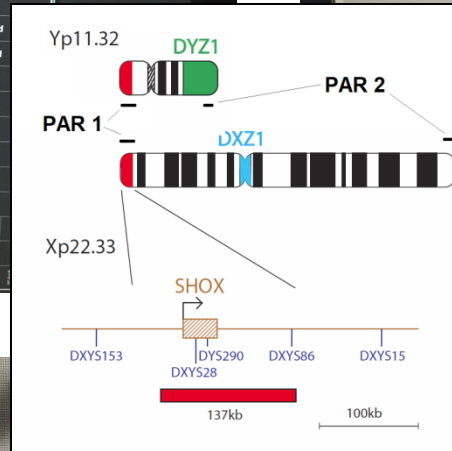
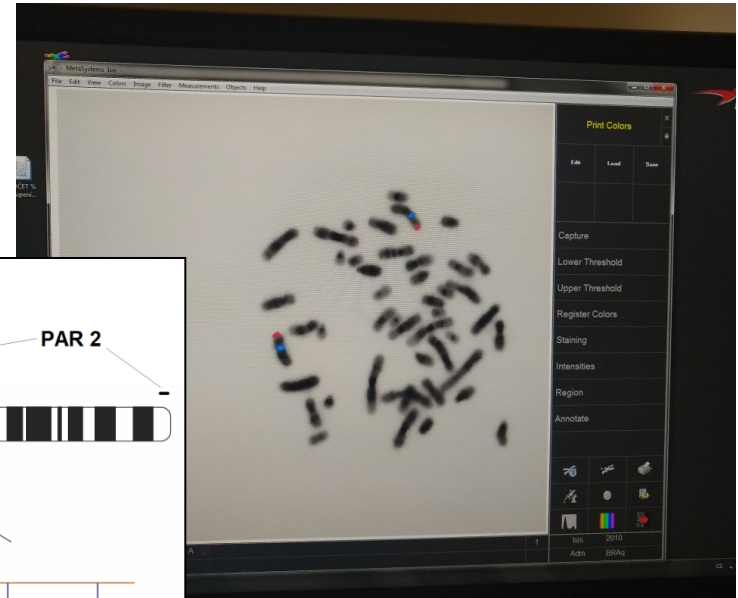
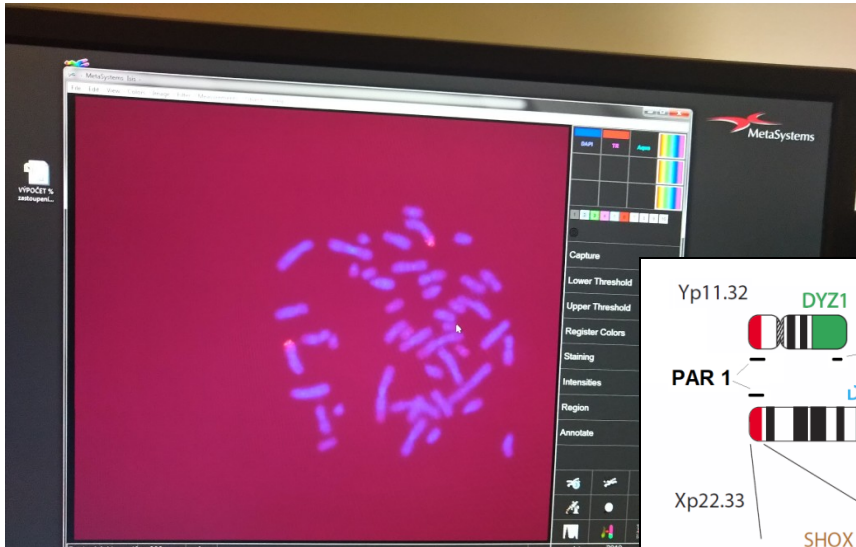
6. odmytí nespecifit: 0,4x SSC - 72 °C/ 2 min.



Fluorescenční mikroskop:



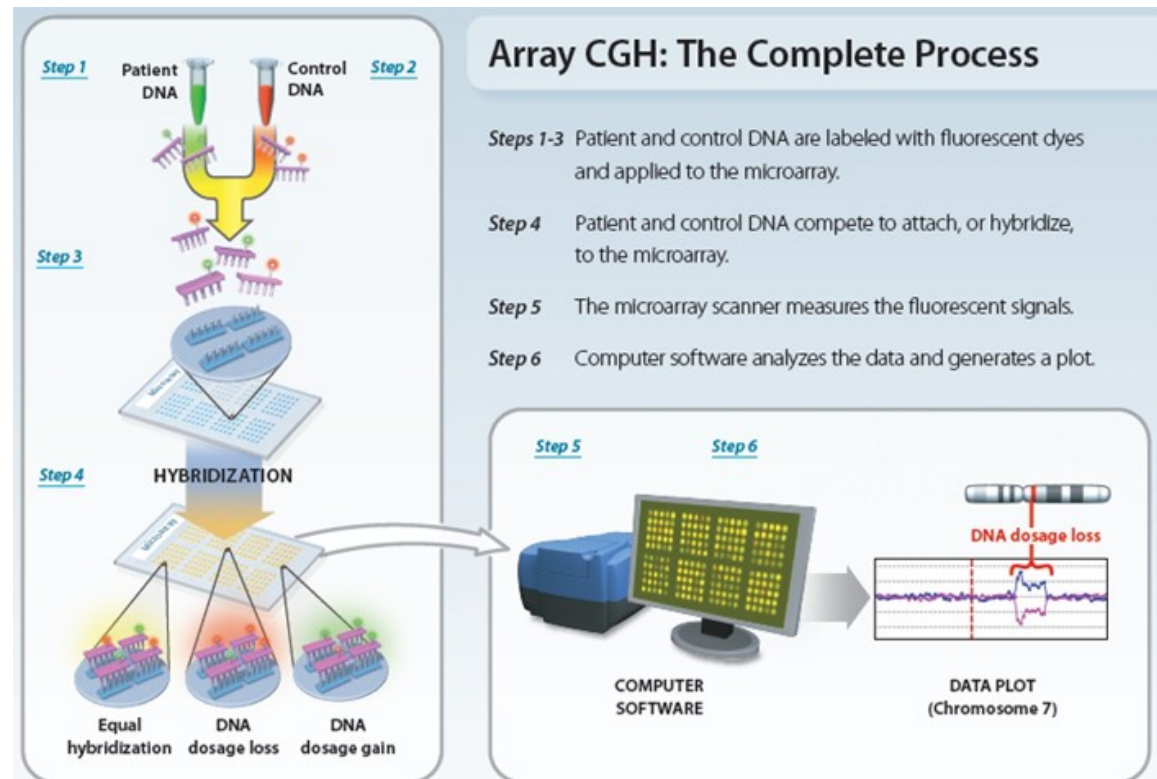
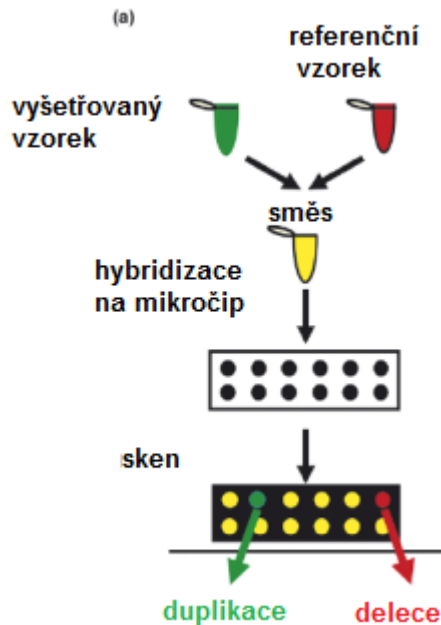
Analýza – software (Isis - MetaSystems, Lucia Cytoogenetics ad.)



Komparativní celogenomová hybridizace na čipu – *array CGH* („array Comparative Genomic Hybridization“)

Princip metody:

- odlišnými fluorochromy značené vyšetřovaná a referenční DNA kompetitivně kohybridizují se sondami na mikročipu
- vyšetření celého genomu a odhalení submikroskopických zisků či ztrát genetického materiálu, tzv. variant v počtu kopií genomových segmentů (CNV; „copy number variations“)

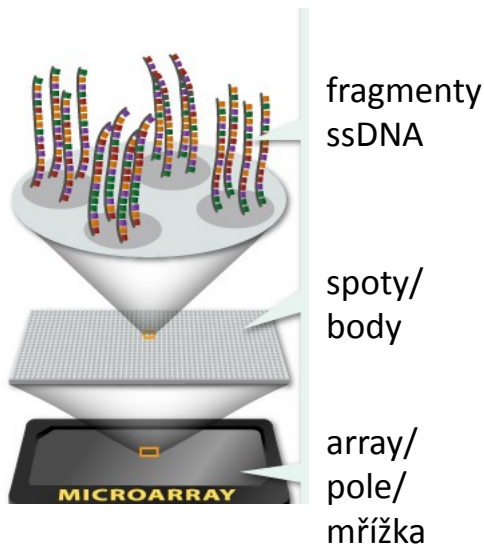


<https://www.youtube.com/watch?v=s7WWd7zsCdU>

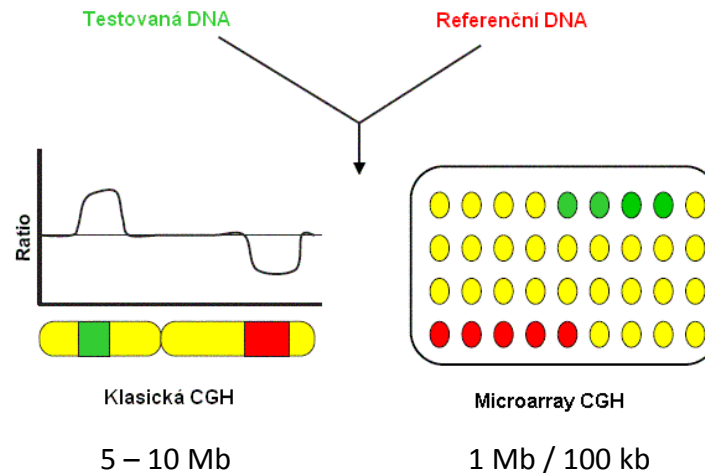
Sondy:

- mikročip je v podstatě podložní sklo s pevně navázanými sondami - jednotlivé body (spoty) mikročipu jsou tvořeny klony:

- 1) sondy BAC** - krátké úseky lidské genomové DNA o velikosti 75 až 200 kb amplifikované pomocí umělých bakteriálních chromozomů (BAC; „bacterial arificial chromosome“) – *knihovna BAC (označení RP_číslo) – nejdříve tvorba sond pro FISH*
- 2) oligonukleotidy** - uměle syntetizované oligonukleotidy o délce jen 25 až 85 bází



Historické okénko:

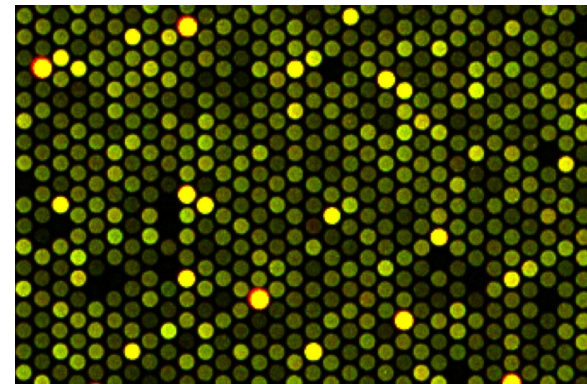
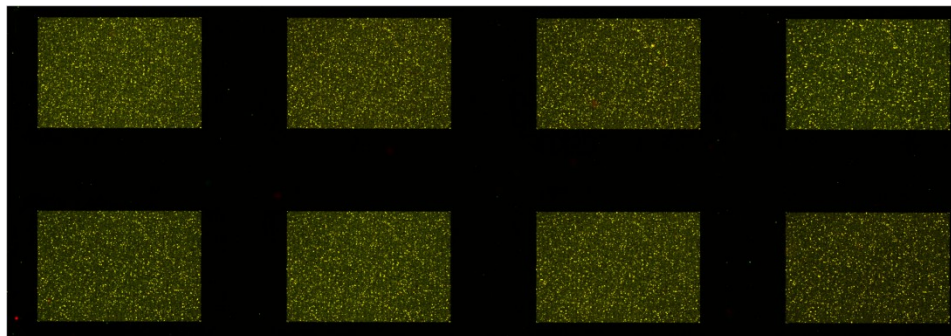
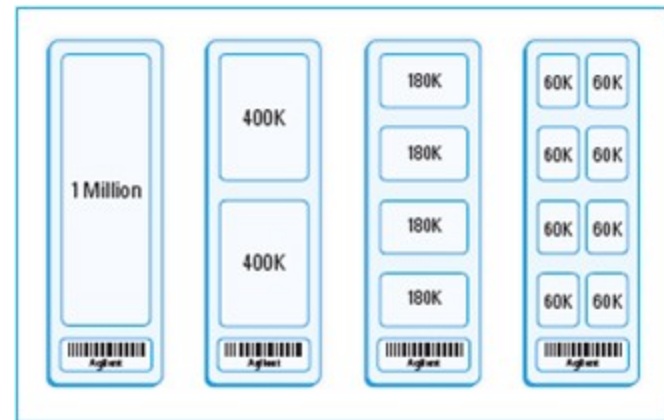
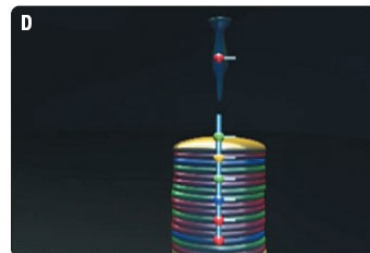
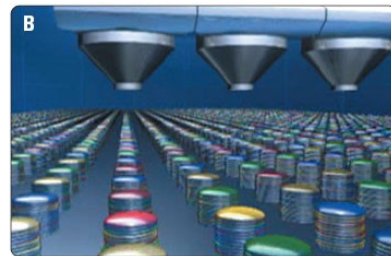
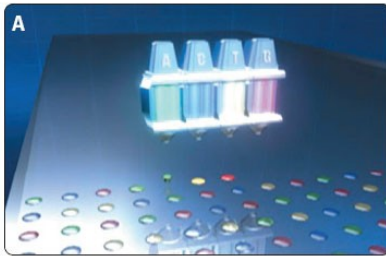
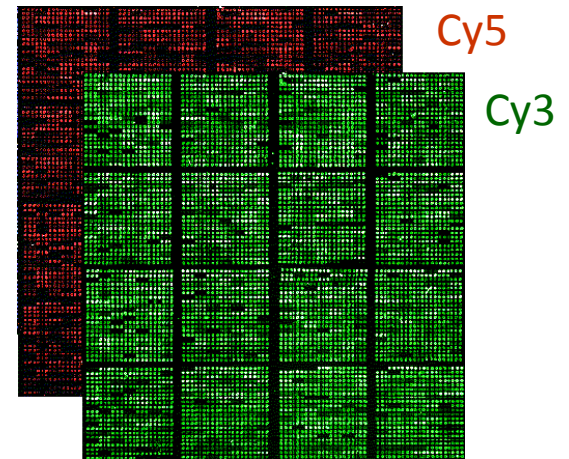


Platformy:

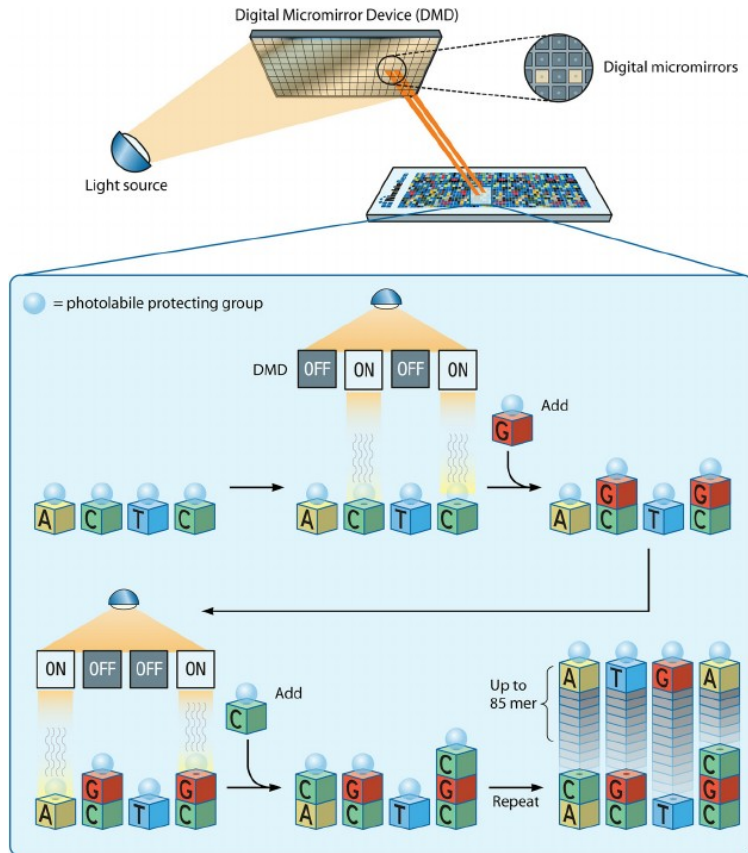
Agilent – **SurePrint** Technology (OGT, *BlueGnome*):

Odkaz na video:

<https://www.youtube.com/watch?v=eSr5CxAdiww>

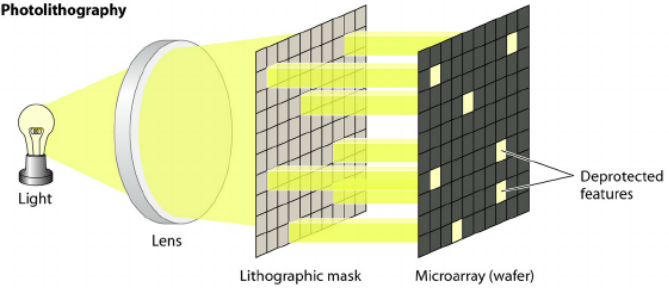


Roche – NimbleGen technology:

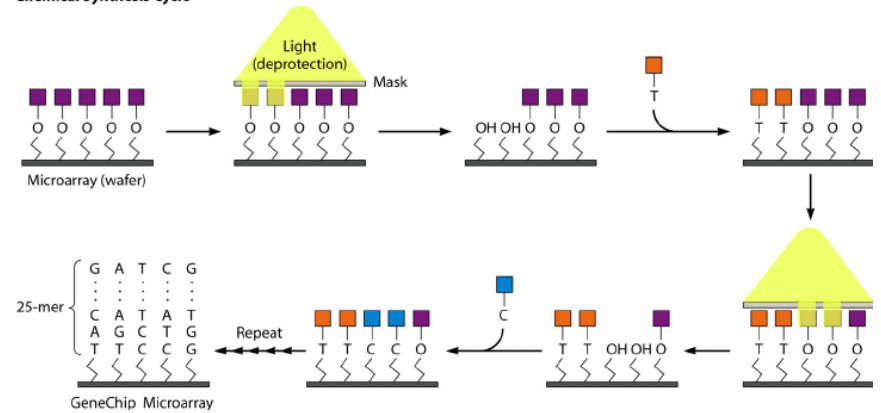


Affymetrix – GeneChip technology: (ThermoFisher)

Photolithography



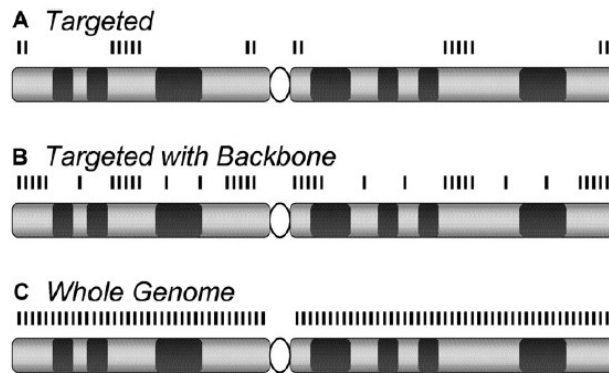
Chemical Synthesis Cycle



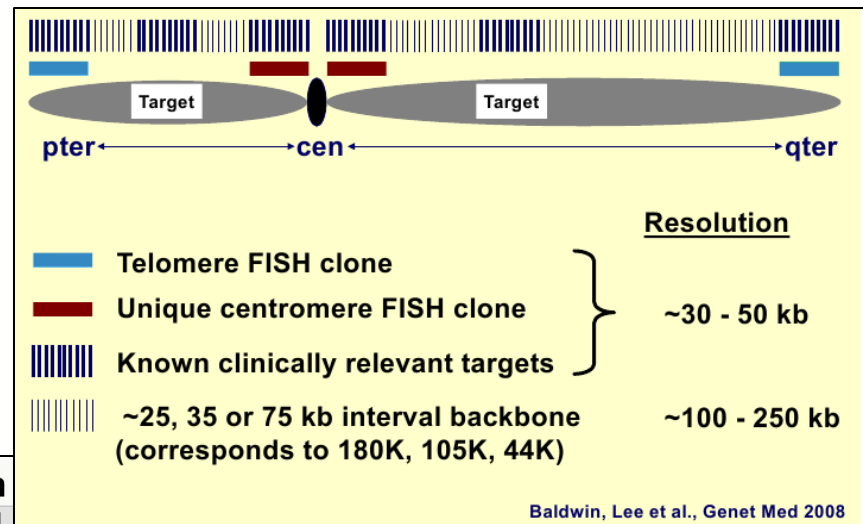
Designy:

- mikročipy navržené k vyšetřování klinicky významných genů a regionů genomu pro postnatální a prenatální genetiku (tzv. oblasti ISCA), dále pro preimplantační genetiku, onkogenetiku, kardiogenetiku, neurogenetiku ad. („*custom*“ čipy)

Nutnost sjednotit aCGH design, protokol a interpretaci výsledku vedla ke vzniku **ISCA**:
 „International Standard Cytogenomic Array Consortium and Public Database „



(zdroj: Miller et al, Am J Hum Genet, 2010)

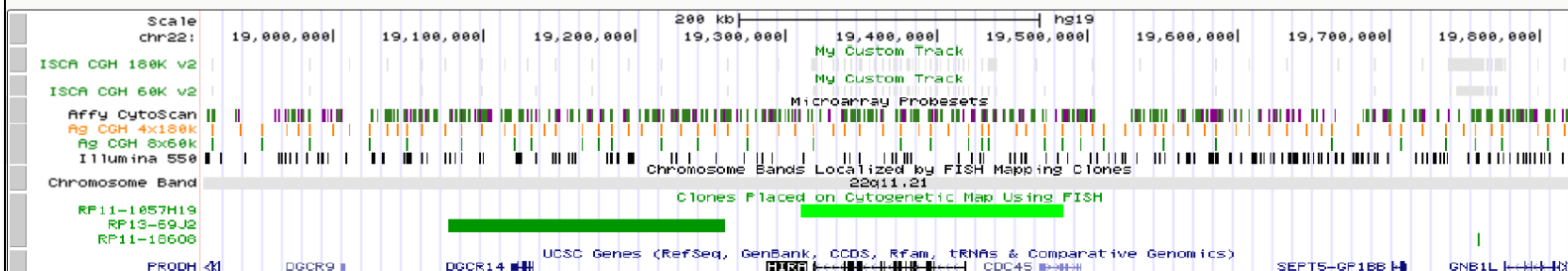


UCSC Genome Browser on Human

move <<<< << < > >> >>>> zoom in 1.

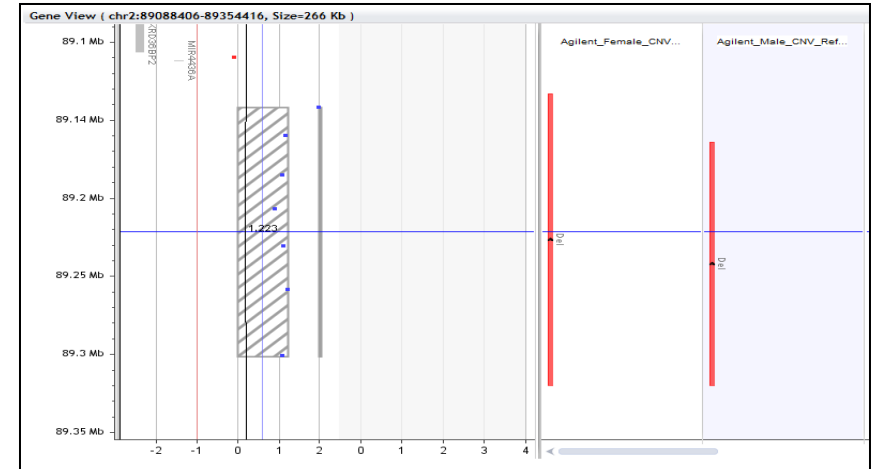
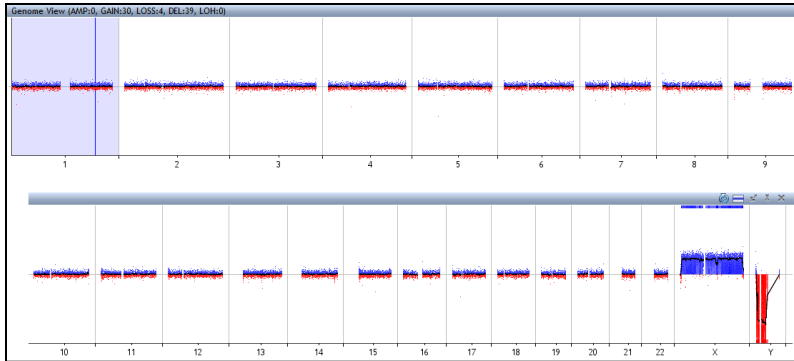
chr22:18,914,240-19,823,203 908,964 bp. HIRA (Homo sapiens histone cell cycle regulator (HIRA), mRNA.) go

chr22 (q11.21) 22p13 22p12 22p11.2 22q11.21 11.23 22q12.1 q12.2 22q12.3 22q13.1 q13.2 22q13.31



Referenční DNA:

- komerčně dodávané (Agilent, OGT, Kreatech)



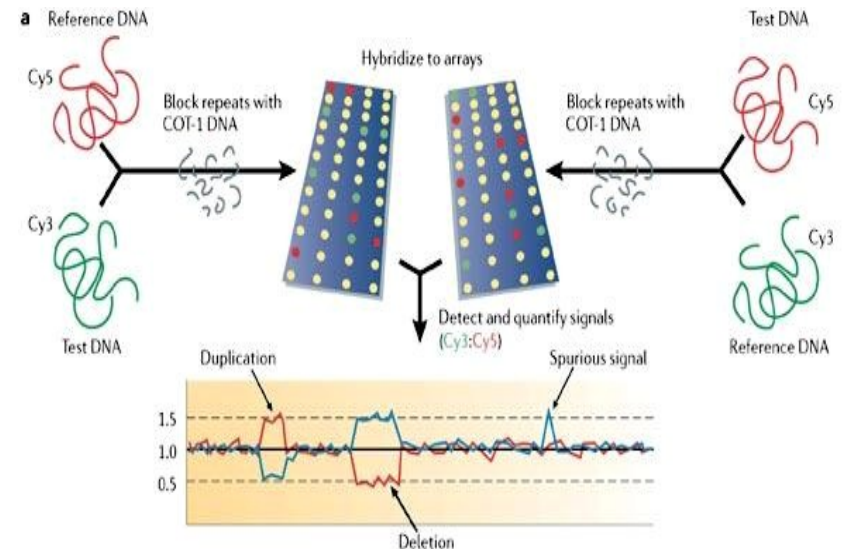
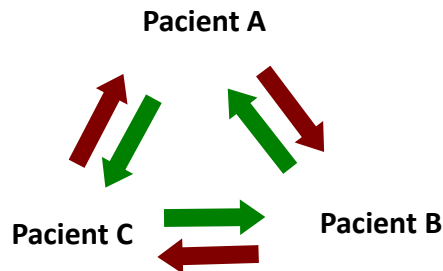
- normální jedinec

- *kdo je normální?*

- směs (pool) více jedinců

- *kolika? jakých? jaké množství?*

- jiný pacient - **trojbodové kruhové uspořádání**



Indikace k vyšetření metodou array CGH

• Postnatální:

materiál – venózní krev, bukální stěr, biopsie kůže ad. (tkáň plodu)

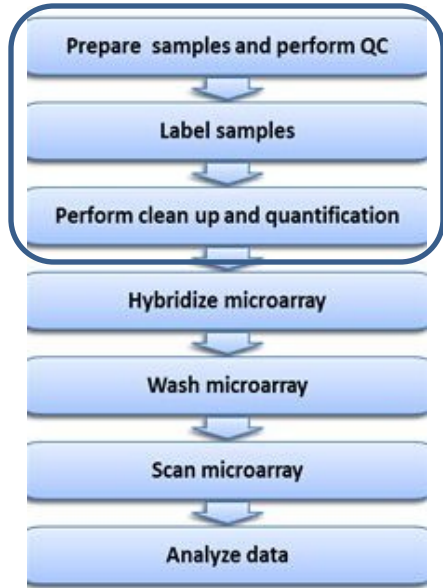
- pacienti bez kauzálního nálezu splňující indikační kritéria:
 - opoždění psychomotorického vývoje, intelektuální nedostatečnost, poruchy autistického spektra, ADHD, poruchy řeči a učení...
 - abnormality CNS (ageneze *corpus callosum*, sy Dandy-Walker), záchvaty, hypotonie, hypertonie...
 - VVV (např. srdce, gastrointestinálního/urogenitálního traktu, končetin a další)
 - makrocefalie/mikrocefalie, faciální stigmatizace, dysmorfické rysy...
 - prenatální/postnatální porucha růstu, neprospívání...
- ke zpřesnění nebalancovaných přestaveb, zejména jejich rozsahu a vyloučení případné další kryptické přestavby
- s nálezem balancované aberace, ale s abnormálním fenotypem (až 40 % je jen zdánlivě balancovaných)
- segregace CNV v rodině

• Prenatální:

materiál – choriové klky, plodová voda, pupečnicková krev

- UZ nález (> NT, oligo/polyhydramnion, hydrops, VCC, VVV CNS/GIT/končetin ad.)
=> vyšetření není podmíněno normálním karyotypem
- *de novo* marker chromozom, *de novo* translokace u plodu
- nejasný nebo neúplný karyotyp (komplexní přestavba, podezření na intersticiální aberaci ad.)

Postup metody array CGH:



koncentrace,
čistota;
elfo – fragmentace
(resp. degradace)



specifická restrikce
(*Alu I* + *Rsa I*) /
nespecifická fragmentace
vysokou teplotou



(elfo)

denaturace a značení
(náhodné primery + dNTP +
Cy3-dUTP + **Cy5-dUTP** +
Klenowův fragment)

purifikace a kvantifikace

smísení značené
DNA vzorku se
značenou
referenční DNA

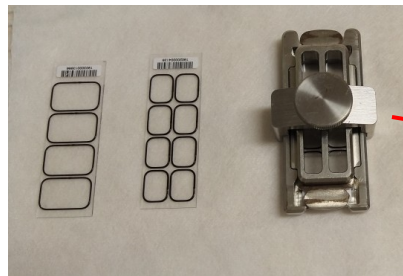


pre-hybridizace
s **COT DNA**
(blokace
repetitivních
sekvencí)





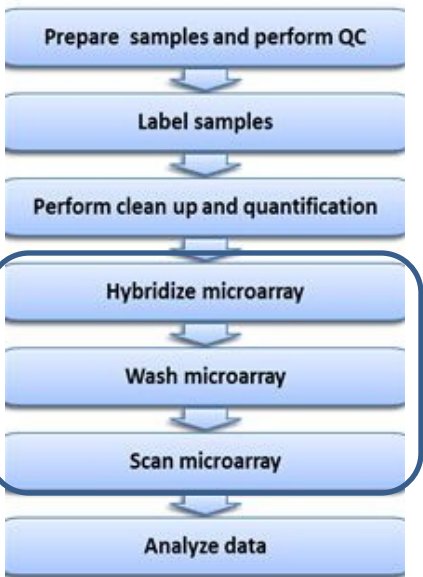
„kapání čipu“
a
hybridizace
(video čas 0:00 – 8:48)



Odkaz na video:
<https://www.youtube.com/watch?v=zVdihEVbrBY>



„odmytí čipu“ –
Wash Buffer 1 a 2
(video čas od 8:49)



analýza



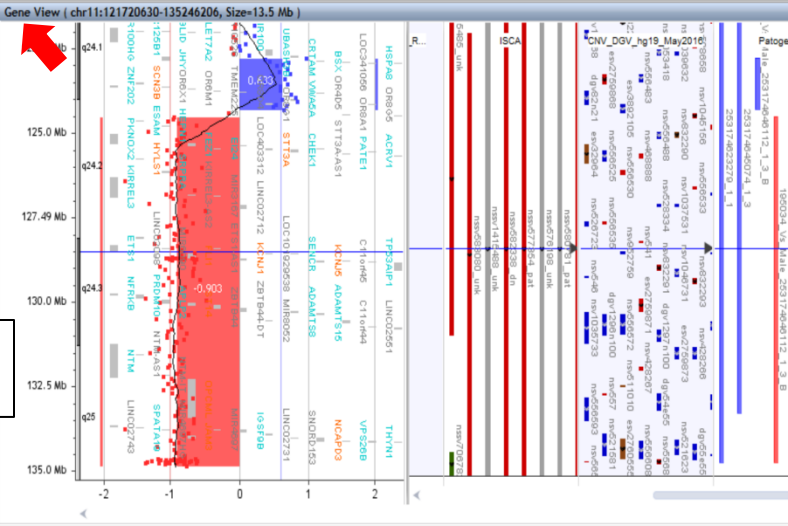
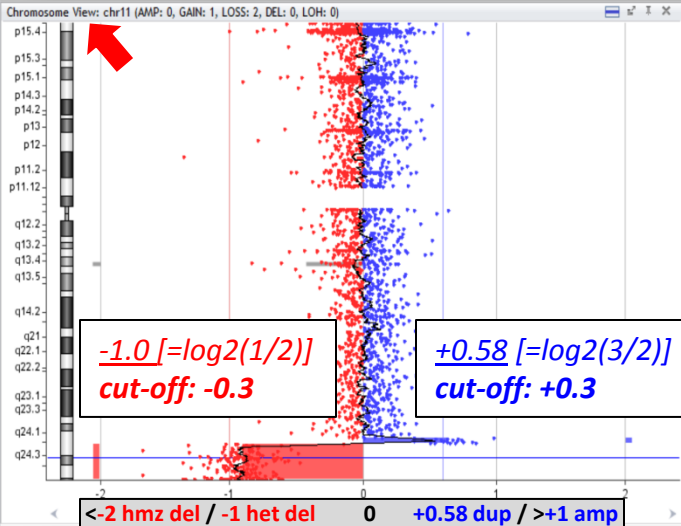
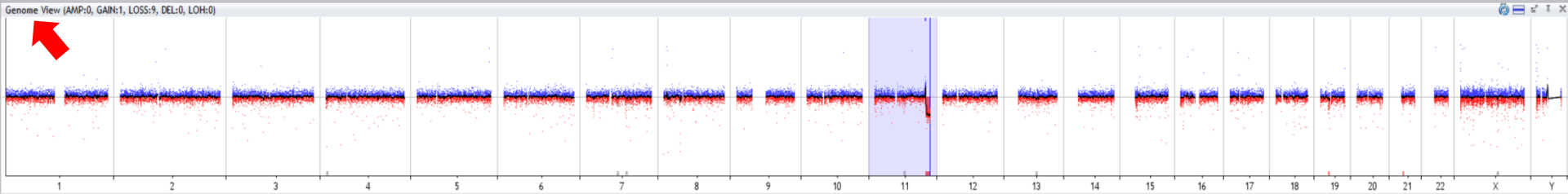
skenování



Analýza: inv dup del 11q – VCC (koarktace aorty), faciální dysmorfismus, cyanóza, abnormality končetin => dg. **Jacobsenův syndrom**

Zápis ISCN (2016): arr[GRCh37] 11q23.3q25(118210301_133382894)x3, 11q25(133477198_134868407)x1

Legacy Triage View : 195034_Vs_Male_253174646112_1_3_B [Reviewed:FNMTOL2W/zunova54357]



- genomové prohlížeče (např. UCSC Genome Browser)
- on-line databáze (DGV, OMIM, Decipher, ClinGen ad.)
- GeneReviews, PubMed, Google Scholar

Chromosome	Start	Stop	Cytoband	Gene Name	Size(kb)	Type	#Probes	Mean Log Ratio/LO...	State	Suppress	Classification	Count	pval	Action
chr4	1,986,020	1,988,866	p16.3	NELFA,MIR943	2,847	Loss	6	-0.336	Algorithm Generated	<input checked="" type="checkbox"/>		1	3.258E-5	Edit Notes
chr7	75,391,843	75,585,312	q11.23	CCL26,CCL24,RHBDD2,POR,MIR4651,SNORA14A	193.47	Loss	4	-0.479	Algorithm Generated	<input checked="" type="checkbox"/>		1	2.17E-6	Edit Notes
chr7	96,816,211	97,015,831	q21.3		199.621	Loss	4	-0.424	Algorithm Generated	<input checked="" type="checkbox"/>		1	2.282E-5	Edit Notes
chr11	71,126,259	71,147,162	q13.4	DHCR7,FLJ42102	20,904	Loss	5	-0.418	Algorithm Generated	<input checked="" type="checkbox"/>		1	3.38E-6	Edit Notes
chr11	122,791,944	124,324,762	q24.1 - q24.2	JHY,BSX,HSPA8,CLMP,SCN3B,ZNF202,VVASA,LOC1532.819	1,532.819	Gain	24	0.633	Algorithm Generated	<input type="checkbox"/>	Pathogenic	1	1.081E-47	Edit Notes
chr11	124,540,224	134,868,407	q24.2 - q25	SIAE,SPA17,NRGN,VSIG2,ESAM,ROBO3,ROBO4,HEI10,328.184	10,328.184	Loss	191	-0.903	Algorithm Generated	<input type="checkbox"/>	Pathogenic	1	4.9E-324	Edit Notes
chr13	63,928,636	64,739,368	q21.31	LINC00395,OR7E156P,LOC102723968,LOC647264,LI810.733	810.733	Loss	14	-0.276	Algorithm Generated	<input checked="" type="checkbox"/>		1	4.159E-7	Edit Notes
chr19	20,630,736	20,760,498	p12	ZNF737	129.763	Loss	3	-0.709	Algorithm Generated	<input type="checkbox"/>	Artefakt	1	1.99E-6	Edit Notes
chr21	19,886,429	20,020,474	q											

- | | | | | |
|---|---|---|--|---|
| <p>Pathogenic</p> <ul style="list-style-type: none"> • Overlapping CNV of known genomic syndrome • Overlapping CNV of patients reported in database (es. DECIPHER) | <p>Likely Pathogenic</p> <ul style="list-style-type: none"> • Reported in a single case report with similar phenotype with patient • CNV include a relevant gene | <p>VOUS</p> <ul style="list-style-type: none"> • Reported in contradictory publications • Genes unknown to be dosage sensitive | <p>Likely Benign</p> <ul style="list-style-type: none"> • Large CNV (no genes/gene poor/absence of regulatory elements) • No benign CNV | <p>Benign</p> <ul style="list-style-type: none"> • Known benign CNV |
|---|---|---|--|---|
- (zdroj: Scioni et al., 2018)

„Troubleshooting“:

What's wrong with my arrays?



	PROBLEM	IDENTIFIER	SOLUTION
DNA Sample QC 	Low A260/280 values	Protein Contamination	Re-purify samples using proteinase steps
	High A260/280 values	RNA Contamination	Ensure that your DNA extraction protocol includes RNase
	Low A260/230 values	Contamination of salts or solvents (e.g. Phenol)	Re-purified by ethanol precipitation Resuspending the DNA in TE buffer
	Inaccurate Sample Concentration	High DNA concentration (>350ng/μl)	Dilute DNA 1:2 in water or suitable buffer
Labeling reaction 	Low Dye Incorporation— (poor pmol/μl or DNA concentration values)	The wrong temperatures or times are being used	Check temperatures with a calibrated thermometer Check incubation times against protocol
		Incorrect volumes used in mastermix preparation	Check correct volumes are being added Check pipettes are calibrated correctly
		Insufficient mixing of samples, reagents and mastermixes	Gently vortex all reagent tubes (except Klenow) Flick mix Klenow tube Briefly spin to drive contents off tube walls
		Too much exposure to light or air	Use a closed thermal cycler with heated lid
		Loss of solution from evaporation	Use PCR machine with a heated lid If using tubes, make sure lids are tightly closed If using plates, use caps not a plate sealer
Hybridisation and Wash 	Black holes on array	Low volume of hybridisation solution	Ensure the correct volume of hybridisation solution has been used Check no leakage of hybridisation solution has occurred
	Non-uniform signal intensities	Split, deformity or crack in backing slide	Check the backing slide seal is intact and has not cracked Report any gasket slide failures to support@ogt.com
	Bubble Scanning/Scotching	Jig assembly untouched for too long after hybridisation Oven rotation malfunction	Check oven rotators are working Remove jigs from oven one at a time Disassemble under wash buffer rapidly
	Fluorescent smears across the slide	Wash-step contamination with fluorescent material Dried-out arrays during the hybridisation or wash steps	Ensure dishes are regularly cleaned with appropriate solvent Ensure clean gloves, forceps and dishes Carry out additional acetone/wash for 1min at room temp
	High Background Signal	Wash-step contamination with fluorescent material Wash conditions not stringent enough	Ensure dishes are regularly cleaned with appropriate solvent Ensure clean gloves and forceps Check stirrer is producing a vortex prior to adding slides in wash buffer Check temperature of oven and washes
	Poor Signal intensity	Overly stringent wash or hybridisation conditions Cy5-labelled DNA wps exposed to light	Check protocol for correct wash instructions Cover tubes with foil or use amber tubes Check temperature of oven and washes
Ozone 	Low Cy5 signal towards the edges of a feature	Wet ozone: outer edges of features dry quicker than inside, exposing edges to ozone	Ensure slides are scanned immediately after washing
	Low Cy5 signal gradient with more signal loss at one end of the slide	Dry ozone: Degradation during scanning, with exposed end degrading quicker	Enclose scanners in a box with ozone scrubbers

Important QC metrics

DIRS values

This is perhaps the most important QC metric and calculates the ratio of probe to background signal on an array. A poor DIRS value (below 0.5) will mean that it is more difficult to accurately call amplifications or deletions. The DIRS value should be >0.5. Higher values can indicate poor quality DNA.

To detect very small deletions, a DIRS value of <0.2 may be required. An excellent array would have a DIRS value of around 0.75, although for some sample types (e.g., formalin fixed paraffin embedded), this may be difficult to achieve. Check the quality of the DNA as a high percentage requires gel for degradation. If the DNA is degraded, shown by a smear on the gel, the DIRS values will be low.

Signal to Noise

This value is calculated by dividing the signal intensity by the background noise and indicates how clearly the spots can be detected above the background level. This metric is dependent on how well the sample labelling and washing steps worked. It is often easier to look at this metric first and then, if it does not pass, identify where the problem occurred by looking at the background noise and the signal intensity.

An excellent value for signal to noise would be above 100, between 100 and 50 is good but below 50 is poor. It is difficult to reliably detect deletions on arrays where the Signal-to-Noise is <50.

Background Noise

This metric is calculated as the standard deviation of multiple control probes on the array. The values are needed for the green and red channel and can be identified using Excellent, Good and Poor.

The values will depend on the array format being used. A poor background does not necessarily indicate that the array has failed. This is a secondary metric as it is incorporated within the Signal-to-Noise metric.

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 E: products@ogt.com

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 Technical support: support@ogt.com



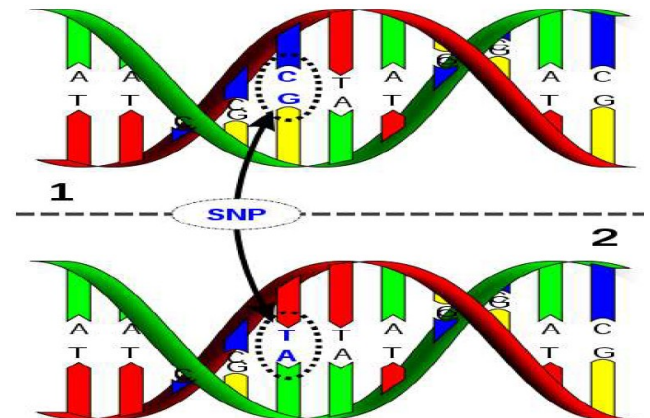
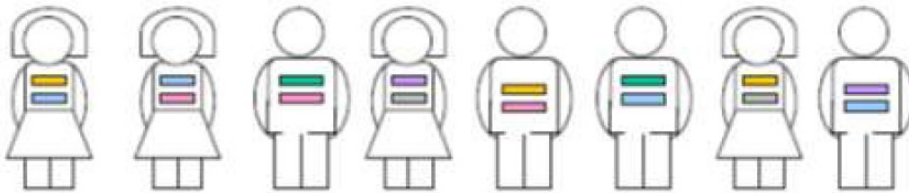
CytoSure™

SNP array / array CGH+SNP

SNP – „single nucleotide polymorphism“ – záměna jednoho nukleotidu s frekvencí v populaci > 1 %, polymorfizmus (vs. susp. mutace < 1 %)



A/G G/T A/C C/T

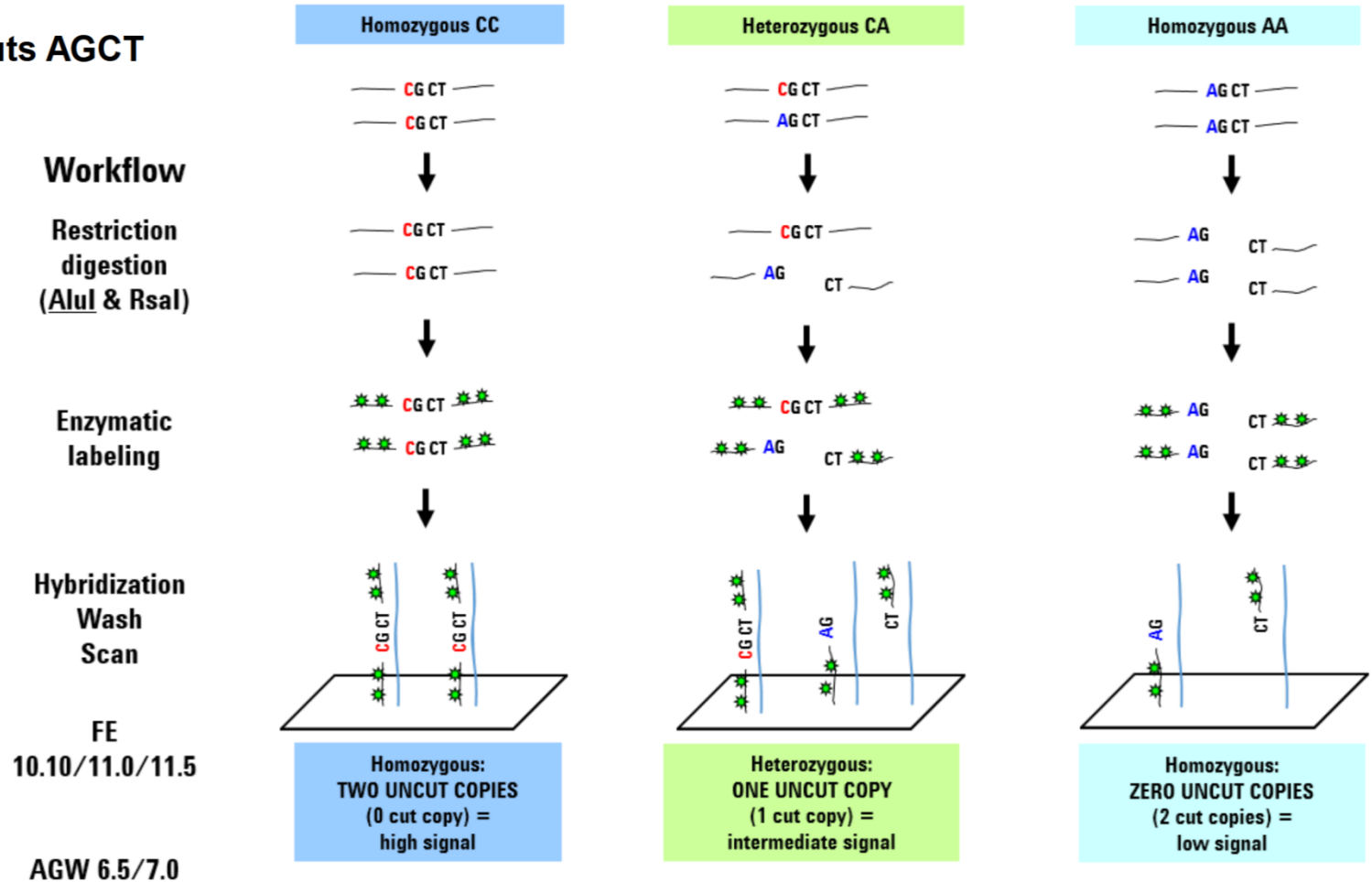


Princip metody:

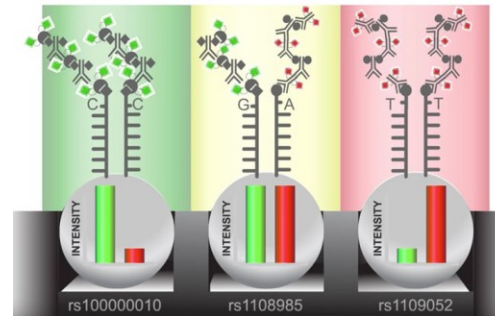
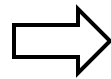
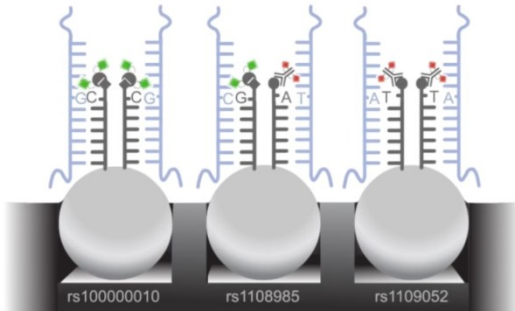
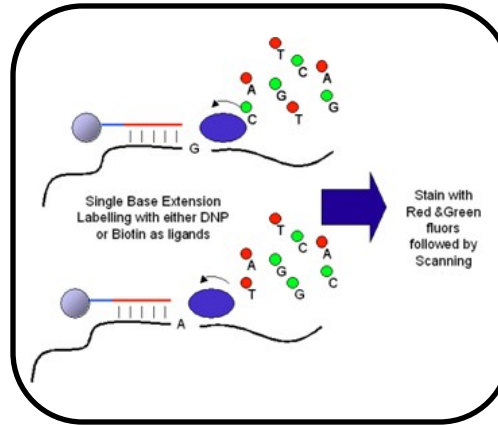
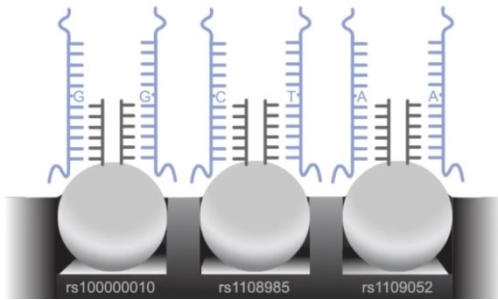


Agilent Technologies

Alu I & Rsa I cuts AGCT



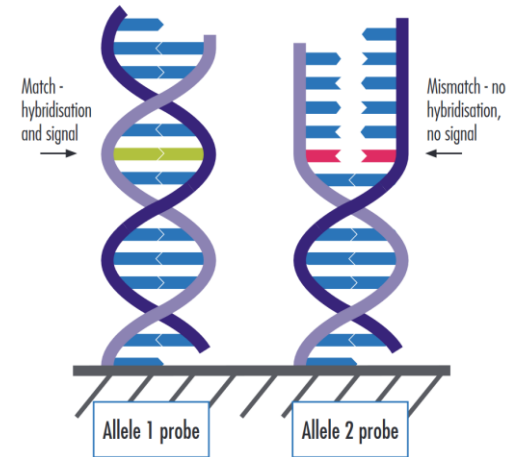
CGH+SNP - komparativní metoda - nutnost kontrolní DNA se známým genotypem



SNP array - nekomparativní metoda - bez kontrolní DNA
 - relativní detekce CNV dle intenzity signálů

Odkaz na video:

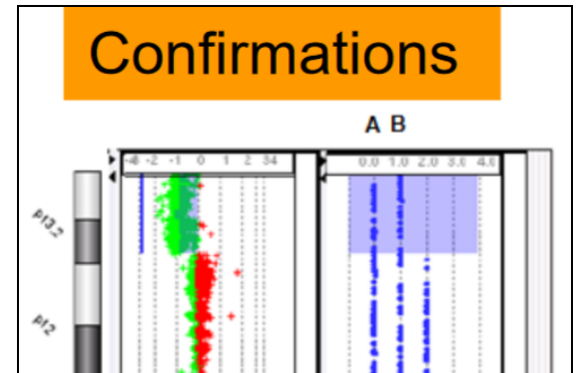
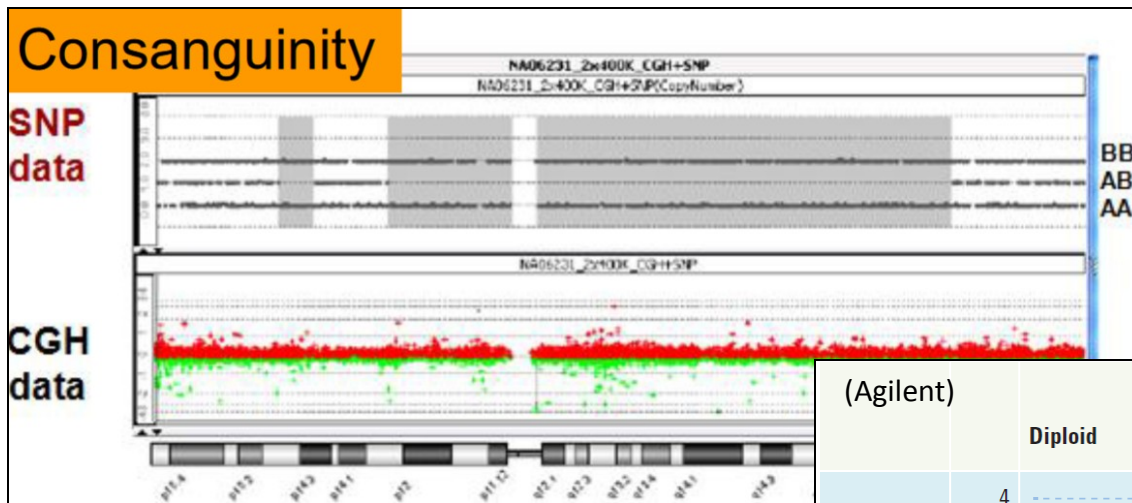
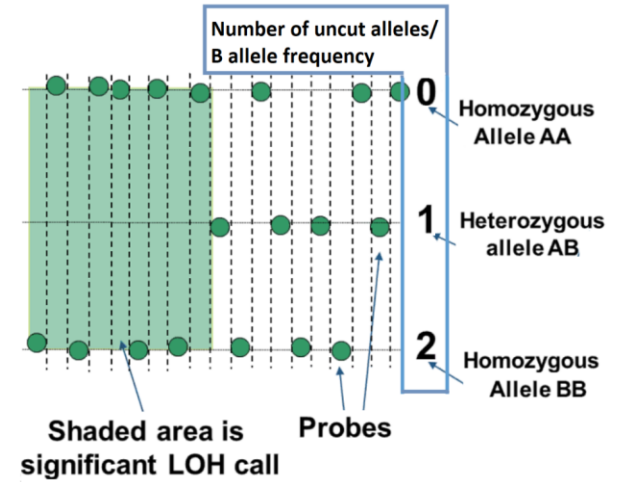
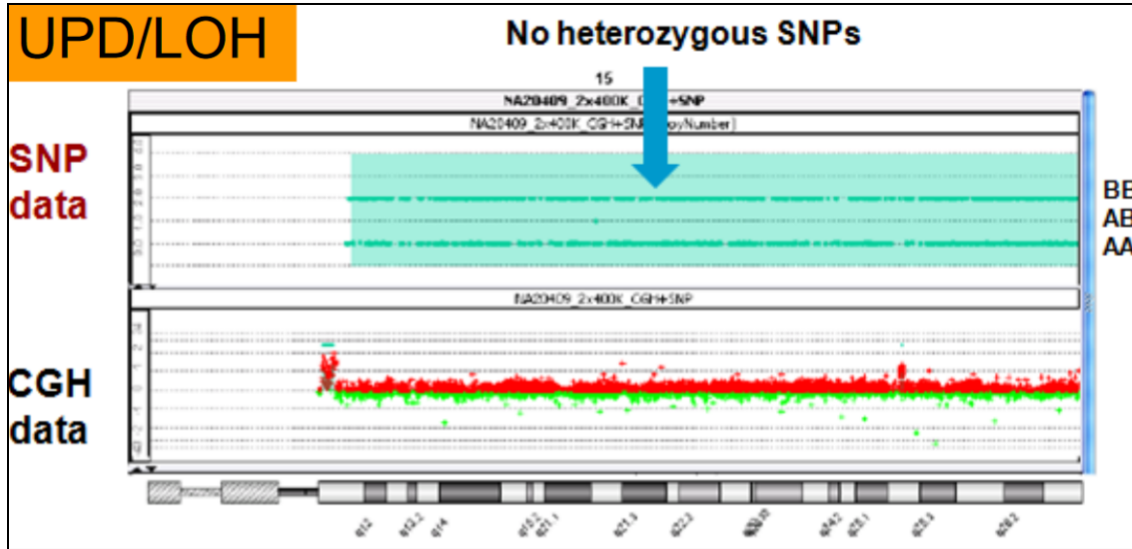
<https://www.youtube.com/watch?v=IVG04dAAyvY>



CGH+SNP

- komparativní metoda - kontrolní DNA bez známého genotypu

LOH – „loss of heterozygosity“ – ztráta heterozygotnosti:



(zdroj: agilent.com)

(Agilent)		Diploid	Hemizygous deletion	Trisomy	Tetrasomy (identical copies)	Tetrasomy (non-identical copies)
No. of uncut alleles	4	—	—	—	—	—
	3	—	—	—	—	—
	2	—	—	—	—	—
	1	—	—	—	—	—
	0	—	—	—	—	—

Frekvence alely B (BAF; „B allele frequency“):

B-allele frequency calculation
 $[B]/[A] + [B]$

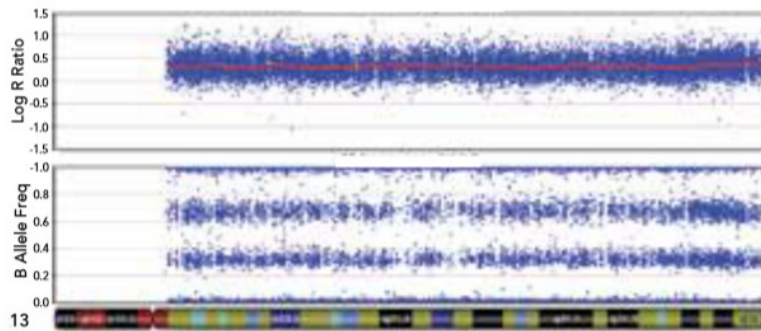
Genotype AA = $[0]/[2 + 0] = 0$

Genotype AB = $[1]/[1 + 1] = 0.5$

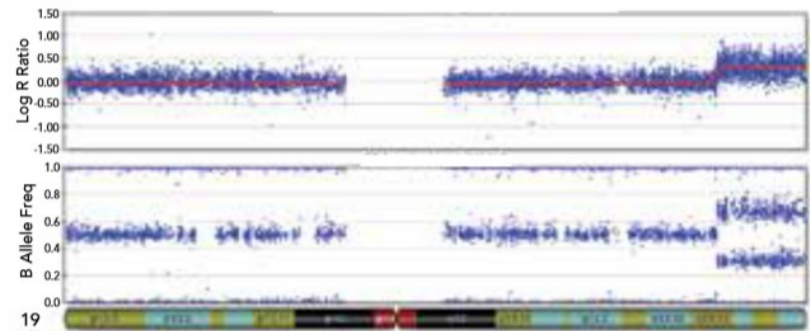
Genotype BB = $[2]/[0 + 2] = 1$

(Illumina, OGT)		Diploid	LOH/ Hemizygous deletion	Trisomy	Tetrasomy (identical copies)	Tetrasomy (non-identical copies)
BAF	1	BB	B	BBB		
	0.75					
	0.50	AB		ABB		
	0.25			AAB		
	0	AA	A	AAA		

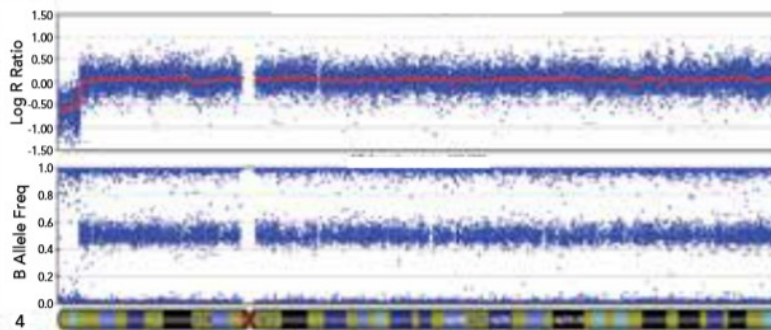
A. Trisomy 13



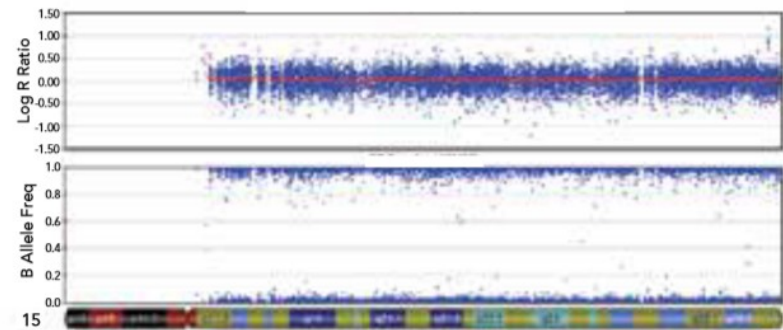
B. Partial Duplication 19q



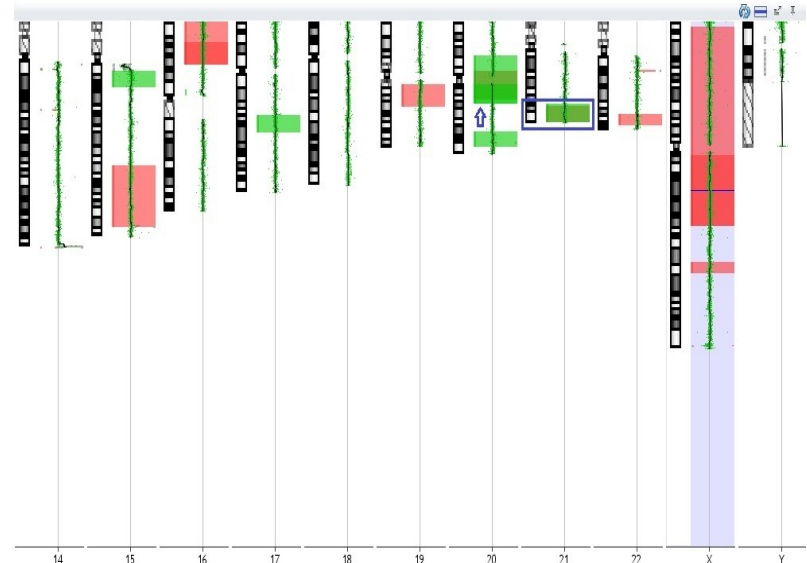
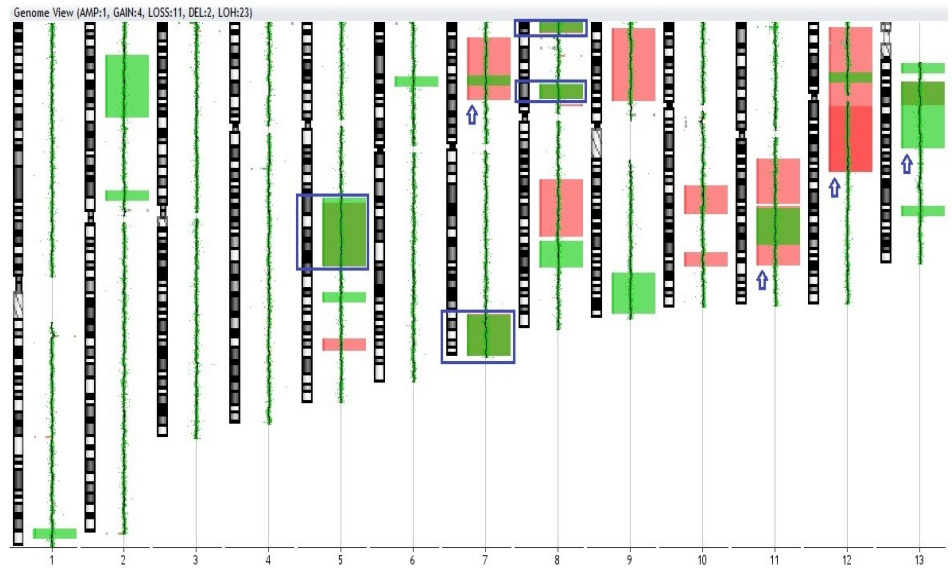
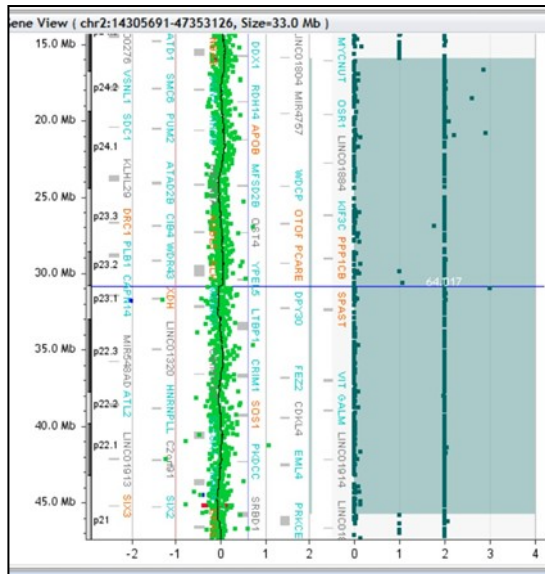
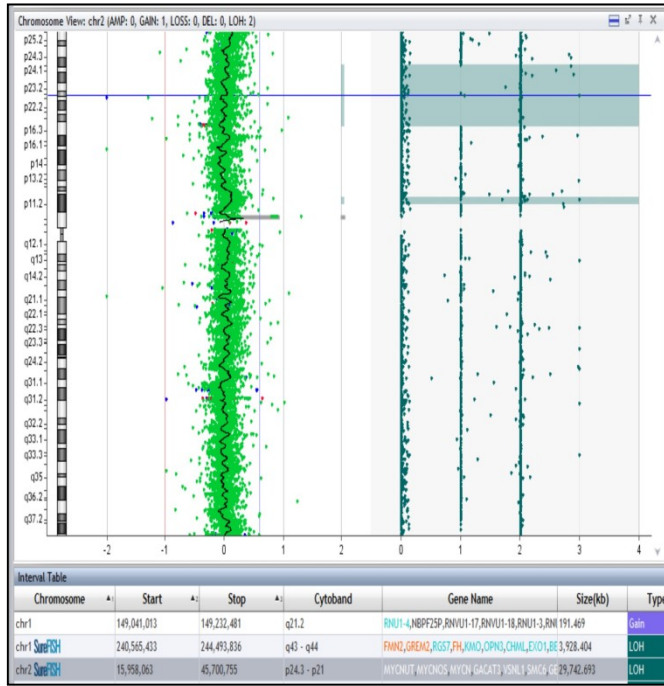
C. Partial Deletion 4p



D. UPD of Chromosome 15



Konsangvinita:



ROH sestry
 ROH bratra
 stejné ROH
 větší či menší překryv ROH

	BAC	Oligo - CN	Oligo - SNP	Oligo – CN+SNP
Practical Resolution	> 200 kb	> 10 kb	> 10 kb	> 10 kb
Genotyping	-	-	+	± / +*
UPD	-	-	+	+
Parent of ORigin	-	-	+	± / +*
Mosaicism	±	±	±	± / +**
Triploidy	-	-	-	- / +**
Input DNA	> 500 ng	> 500 ng	150 ng	> 100 ng*
DNA from various sources	+	+	+	± / +*

(zdroj: de Leeuw, prezentace: SNP array analysis and interpretation in constitutional genome diagnostics, 2016)

* Depending on the array design

** With use of B Allele Frequency improved detection rate of mosaicism AND triploidy

Výhody:

- malé množství vstupní gDNA
- vysoké rozlišení
- detekce mozaicizmu > 10 %
- rychlý výsledek (již do 2-3 dní)

Nevýhody:

- balancované aberace a polyploidie nedetekovatelné
- vysoká cena
- obtížná interpretace VOUS – **neúplná penetrance a variabilní expresivita**

Záchytnost patologií u cca 10 % vzorků (včetně VOUS cca 20 %)

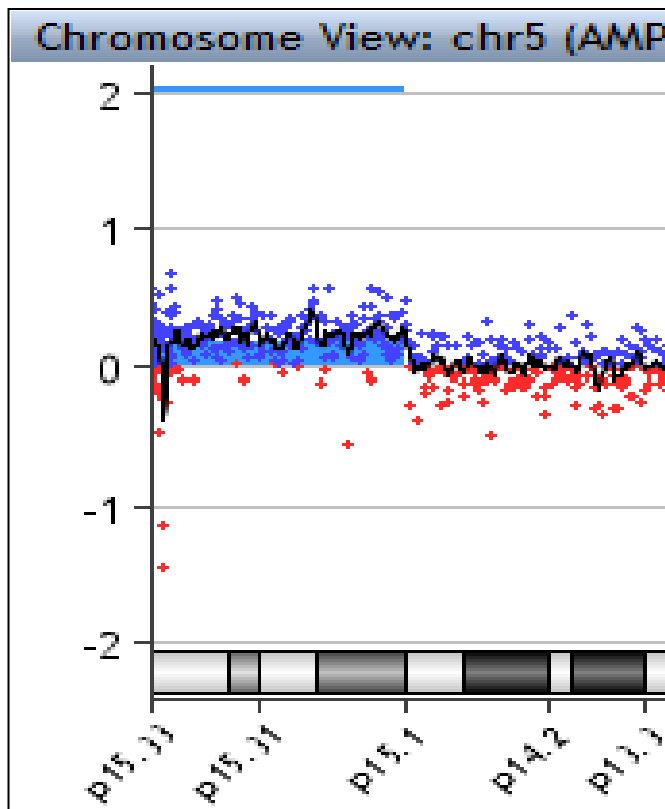
	1.	2.	3.	4.
	SurePrint G3 CGH+SNP	CytoSure ISCA UPD	250k SNP	Cytoscan HD
UniParental Disomy	+	+	+	+
Homozygous regions	±	±	+	+
Allele sharing	-	-	+	+
Parental origin of aberrant allele	-	-	+	+
To check for sample mix up or non-paternity	±	±	+	+
Low-grade mosaicism	±	±	±	+
Triploidy	-	-	-	+

(zdroj: de Leeuw, prezentace: SNP array analysis and interpretation in constitutional genome diagnostics, 2016)

1. **Agilent** – CGH sondy v ISCA regionech a „backbone“ + sondy SNP („number of uncut alleles“)
2. **OGT** – CGH sondy v ISCA regionech a „backbone“ + sondy SNP („B allele frequency“)
3. **Illumina** – SNP array („B allele frequency“)
4. **Affymetrix** – SNP array („B allele frequency“)

Kazuistika č. 1:

- Dandy–Walkerova anomálie
- opoždění psychomotorického vývoje
- faciální dysmorfie



array CGH:

- duplikace terminální oblasti 5p15.33p15.1 o velikosti 15 Mb zahrnující 69 Ensembl genů, z toho 27 OMIM genů
- dle *mean log ratio* profilu duplikace **(0,18)** se jedná o 10-20% mozaiku

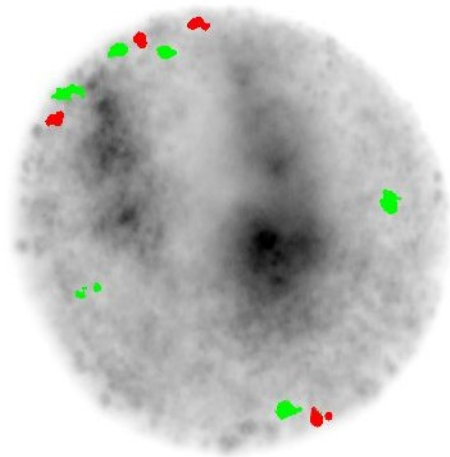
arr[GRCh37] 5p15.33p15.1(50093_15009591)x3

G-pruhování:

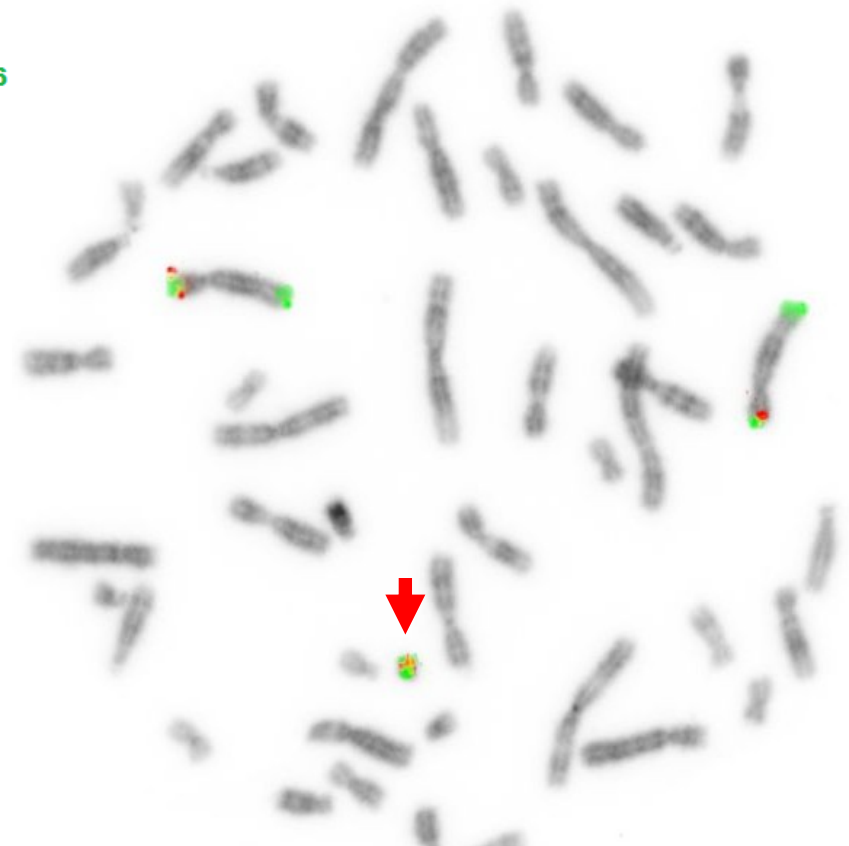
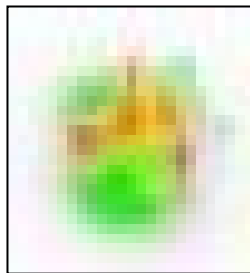
- 47,XY,+mar dn[10]/46,XY[28]

FISH:

- ish nuc 5p15.31(FLJ25076x4),5p15.2(CTNND2x4),5q35(NSD1x2)[55/414]
- ish der(5)(p15.31p15.2)(FLJ25076+,CTNND2++,FLJ25076+,NSD1-)[30/112]



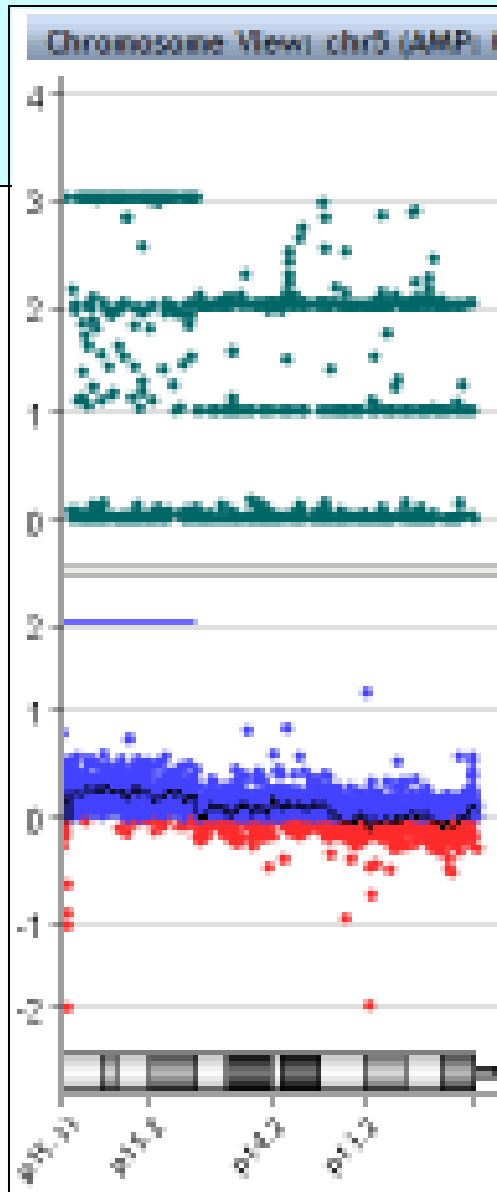
mar



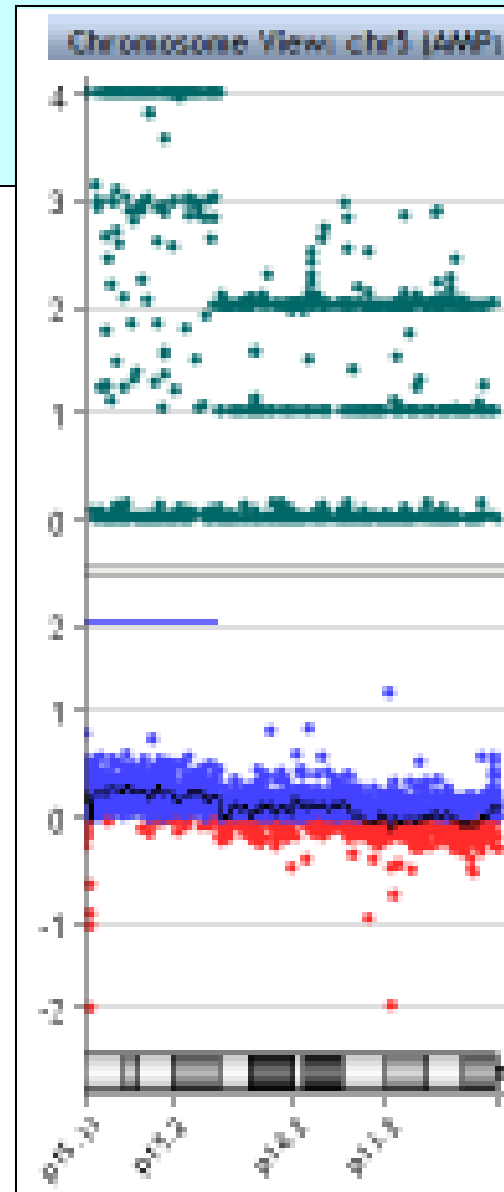
- Prokázána 15,8% mozaika aberantní buněčné linie s *de novo* nadbytečným marker chromozomem derivovaným z chromozomu 5, jenž je invertovanou duplikací oblasti 5p15.33p15.1 s neocentromerou.

array CGH+SNP:

3 kopie:



4 kopie:

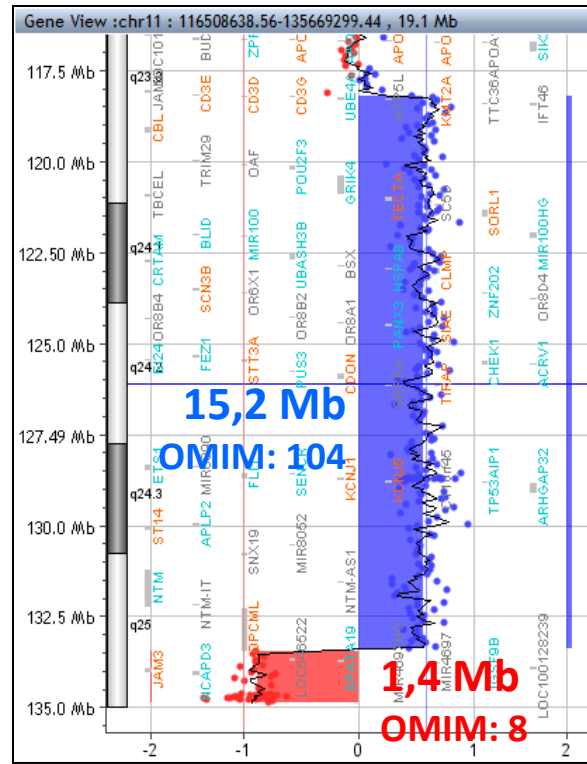
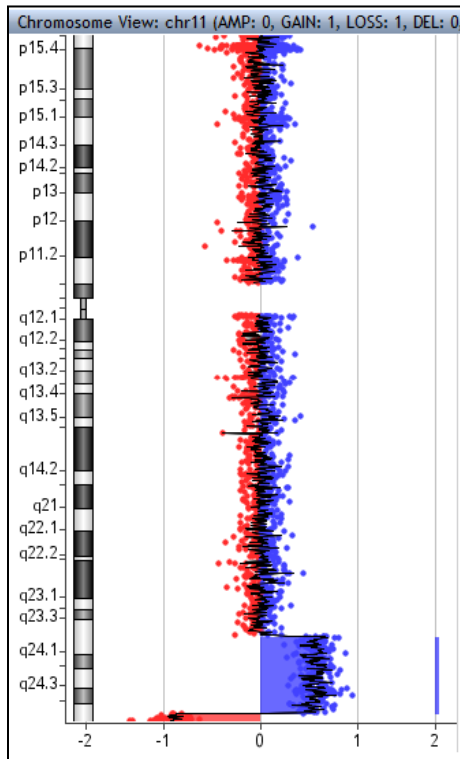


Kazuistika č. 2:

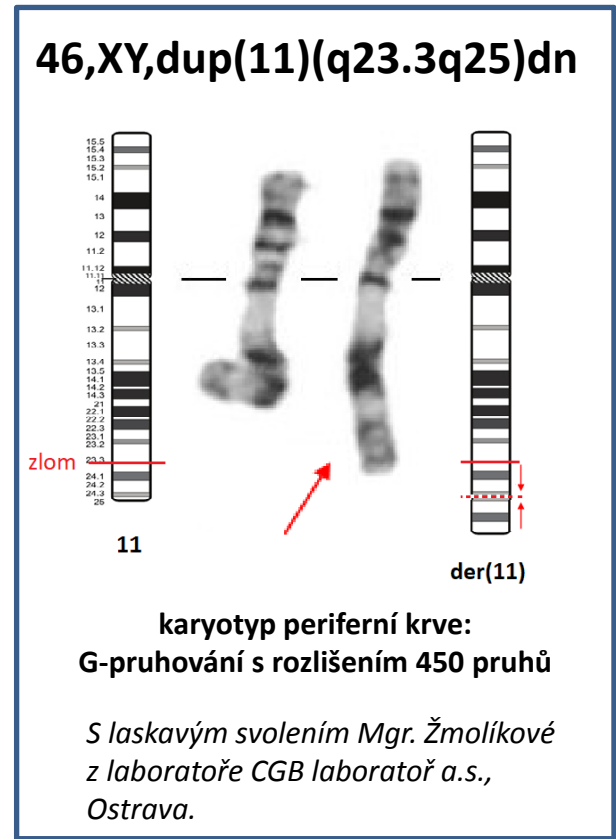
postnat.: VCC – ASD, centrální hypotonický syndrom, centrální koordinační porucha těžšího stupně, pes calcaneovalgus

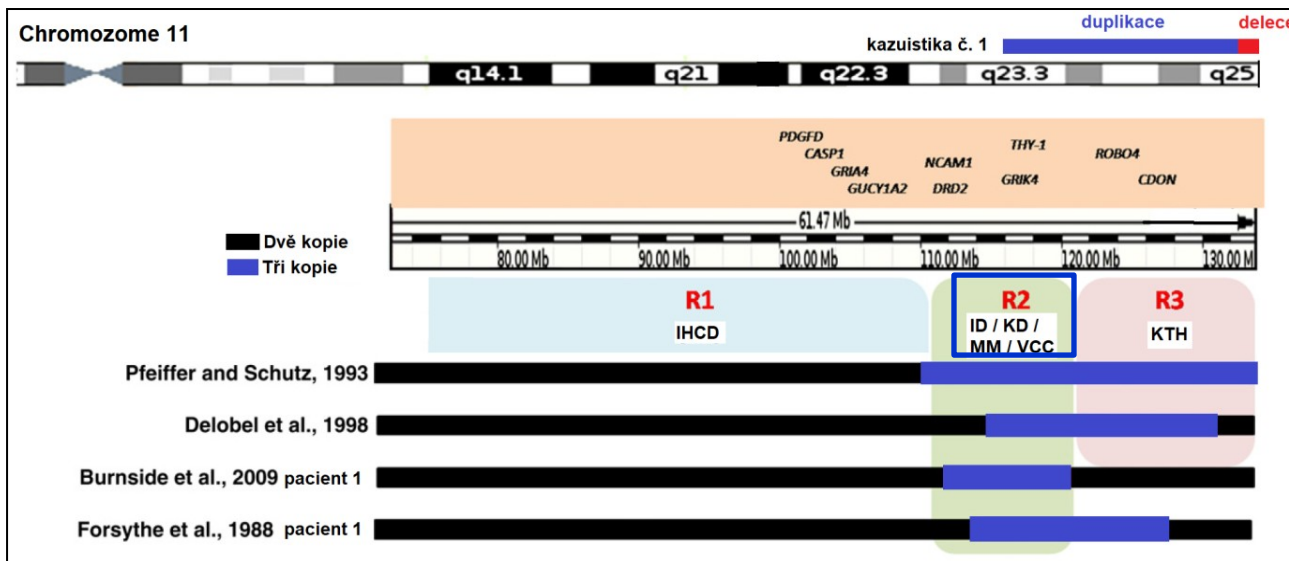
14 měsíců: kraniofaciální dysmorfie, hypotonie, statná pastózní postava, hypoplastický genitál, nesestouplá varlata, celkově opožděný psychomotorický vývoj

arr[GRCh37] **11q23.3q25(118210301_133382894)x3,11q25(133477198_134868407)x1**



array CGH periferní krve: SurePrint G3 ISCA v2 8x60K, Agilent





R2:

ID – intelektuální
nedostatečnost

KD – kraniofaciální dysmorfie

MM – malformace mozku

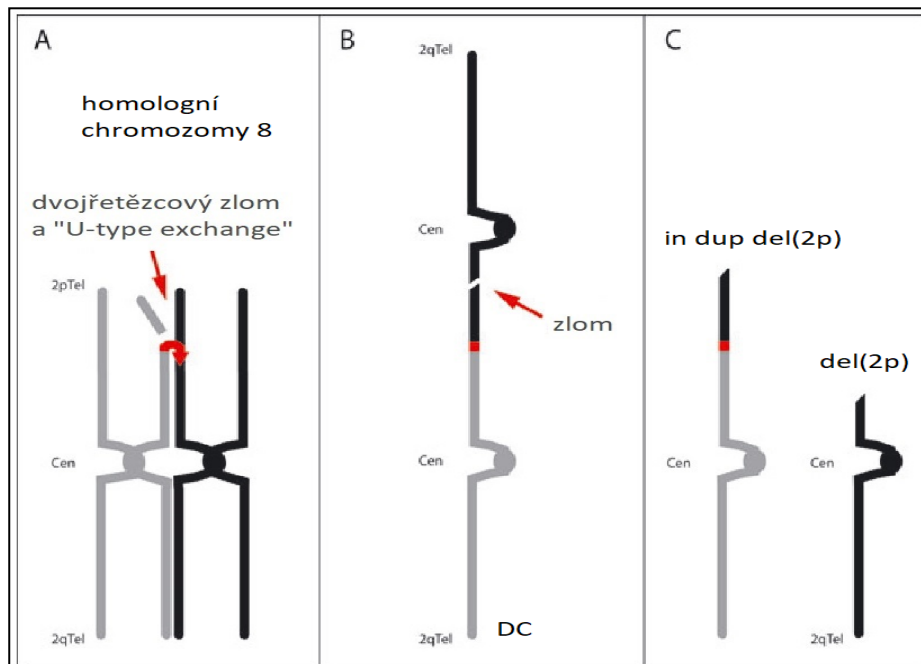
VCC – vrozená srdeční vada

R3:

KTH – kongenitální tříselná
hernie

(Ben-Abdallah-Bouhjar *et al.*, 2003; upraveno)

⇒ **patologický nález vysvětlující
fenotyp**



nerekurentní inv dup del:
dvojřetězcový zlom – fúze chromatid

(Zuffardi *et al.*, 2009; upraveno)