In situ hybridization techniques

Fluorescence in situ hybridization: FISH
Comparative genomic hybridization: CGH

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***In situ* hybridization (ISH)**

- **type of hybridization methods that uses a labeled complementary DNA or RNA probe to localize a specific DNA or RNA sequence in situ**
- **allows for precise localization of a specific segment of nucleic acid**
- **visualization of the reporter molecule allows to localize DNA or RNA sequences in a heterogeneous cell populations including tissue samples and environmental samples.**

target: cells from cell culture
mononuclear cells from peripheral blood
tissue section
array of tissue sections
bone marrow cells
blood smears etc.

In situ hybridization is distinct from immunohistochemistry, which localizes **proteins** in tissue sections.

Using DNA sequence specific probes: one type of cytogenetic techniques

Using RNA specific probes: detection of gene expression (mRNA)

***In situ* hybridization probes**

- double-stranded DNA (dsDNA) probes
- single-stranded DNA (ssDNA) probes
- RNA probes (riboprobes)
- synthetic oligonucleotides (PNA, LNA)

Labeling techniques

radioactive isotopes

^{32}P
^{35}S
^{3}H

non-radioactive labels

biotin
digoxigenin
fluorescent dye (FISH)

Cytogenetic techniques

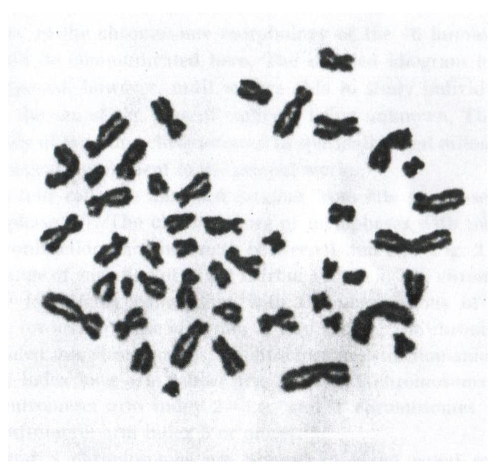
GOAL: to define abnormal chromosome number and structural alterations related to certain diseases

WHAT SAMPLES CAN BE STUDIED?

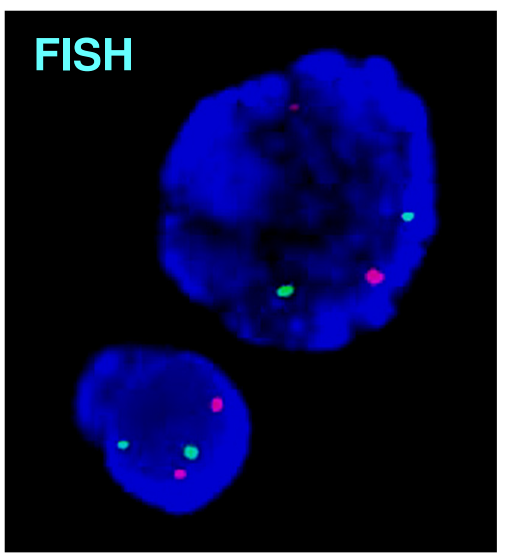
- ✓ **Cells from tissues that can be stimulated to undergo cell division in vitro**
- ✓ **It is only during mitosis of cell cycle that distinct chromosomes can be visualized under the light microscope**
- ✓ **Examples of tissues: chronic lymphoid leukemia, amniotic fluid, bone marrow, different solid tumors (very low mitotic capacity, few metaphases)**

Microscopic techniques

Classical cytogenetics



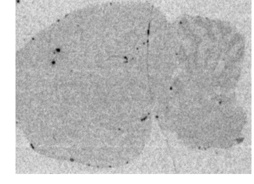
Only chromosome spreads can be analyzed
Based on: Giemsa staining

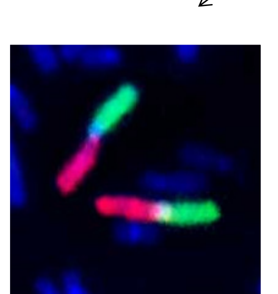


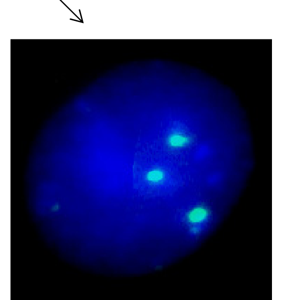
DNA fiber:	**fiber FISH**
Interphase cell nuclei:	**interphase cytogenetics**
Chromosome prep.:	**any quality of chromosomes can be analyzed**
Tissue sections:	**tissue micro-array**

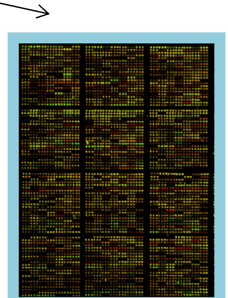
Short history of FISH

- Started with radioactive isotope labeled probes (in 1960 s)
- **Enzyme labeled DNA probes: the labeling efficiency was low**
- **Fluorescently labeled probes (the end of 1980s)**









Timeline from the discovery of DNA structure to the array technologies

Human genetics ↘ The end of the 1950s

Medical sciences ↗ *medical genetics*

Genetics is the most dynamic field of the natural sciences, and the most influential on the social area.

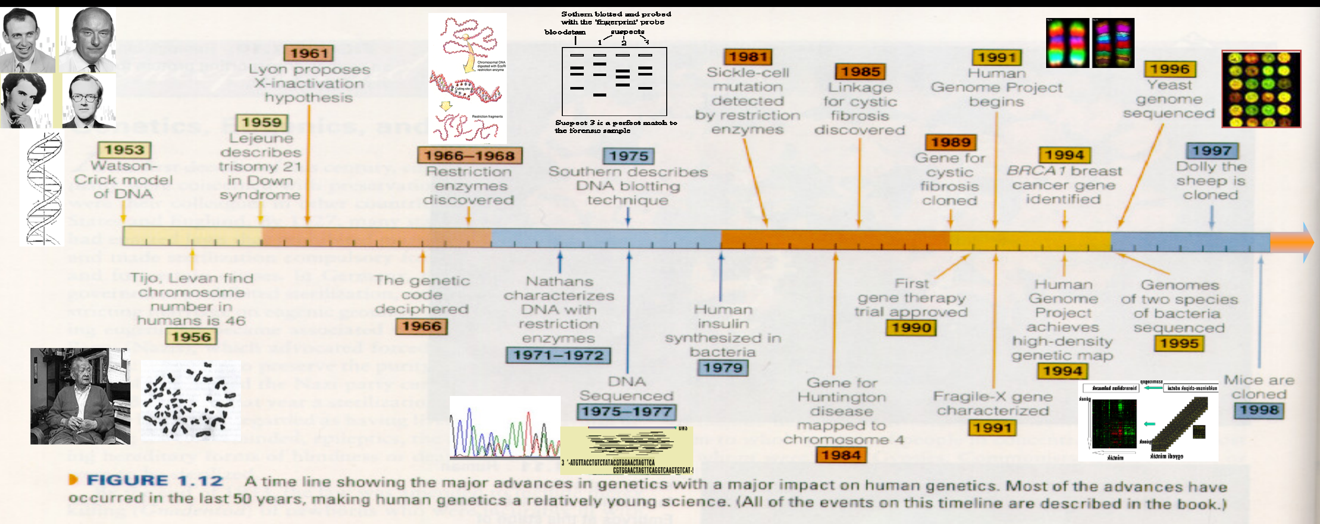
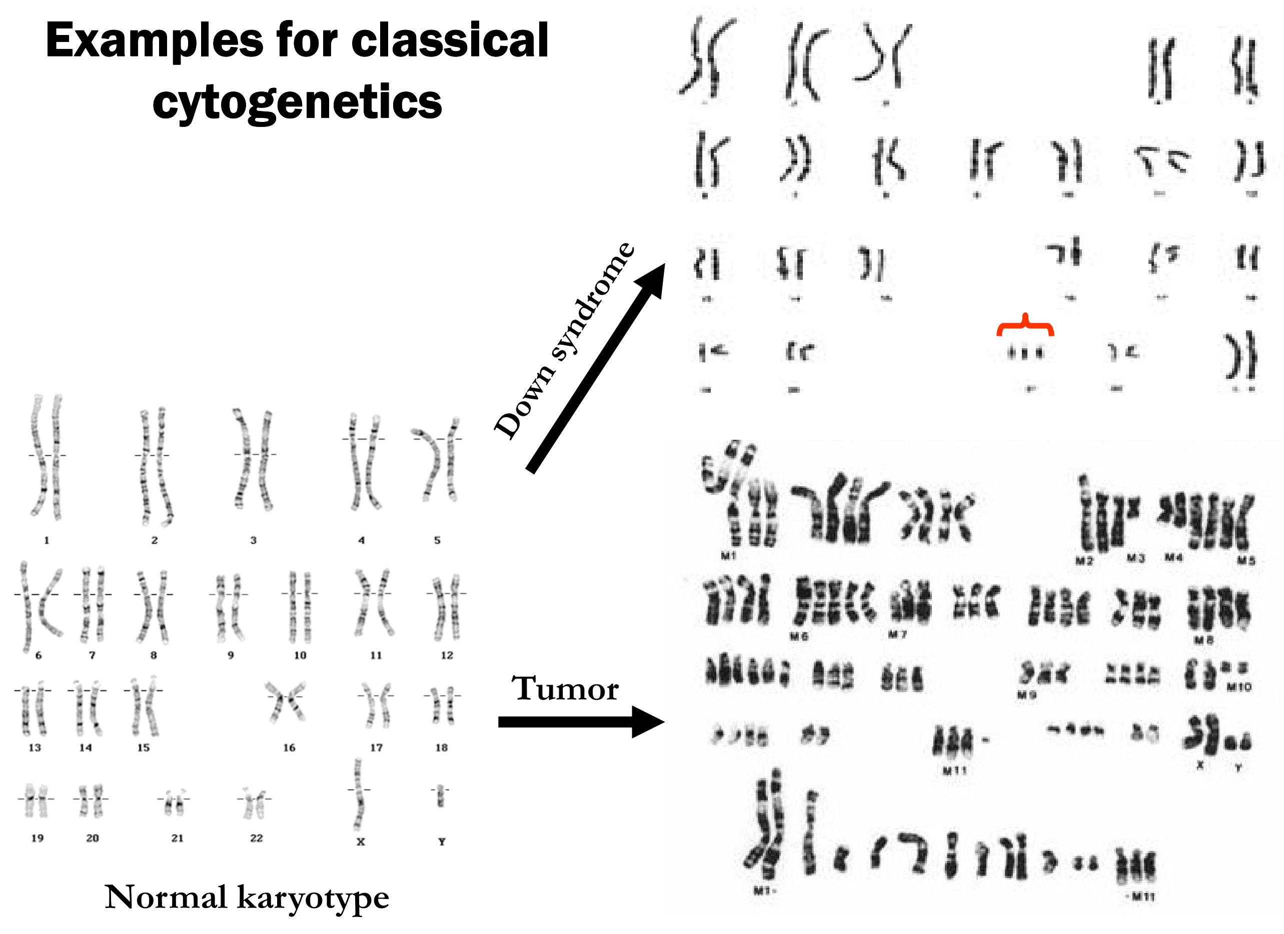


FIGURE 1.12 A time line showing the major advances in genetics with a major impact on human genetics. Most of the advances have occurred in the last 50 years, making human genetics a relatively young science. (All of the events on this timeline are described in the book.)

Widen diagnostic opportunities
More efficient screening strategies

Examples for classical cytogenetics



Problems with classical cytogenetics

- selective growing of cells isolated from tissues
- the in vitro cell line is not representative for the original tumor
- the efficiency is very low, missing genetic information for many solid tumor tissue types

INTERPHASE FISH

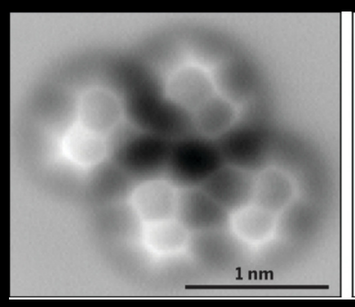
In situ hybridization

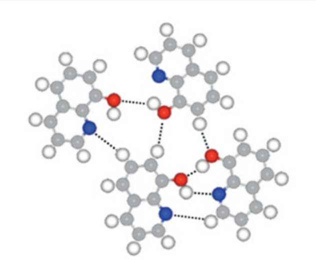
Genetic sequences

1-es váz
2-es váz
adenin
timin
guanin
citozin
hidrogénkötés

morphological localization

specific DNA sequences



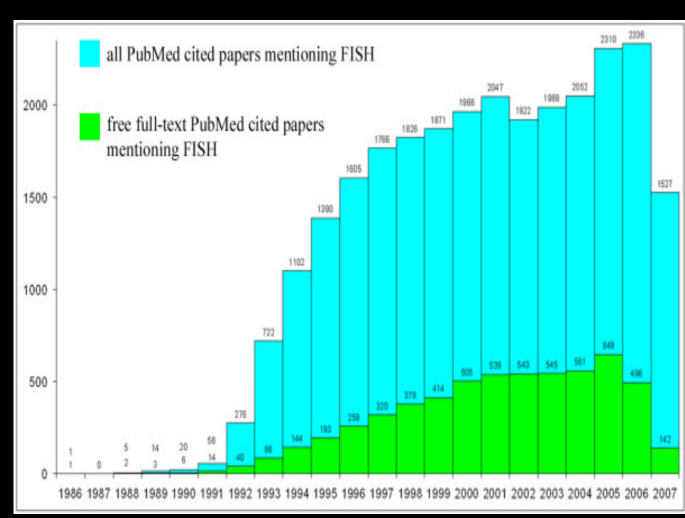


The hydrogen bonding **Intermolecular Bonding with Atomic Force Microscopy**
Science , Published Online September 26 2013

Complementary sequences of the target nucleic acids and labeled probes

- 1986 Dan Pinkel, Joe Gray

Specific aim: study of chromosome alterations in clinical samples
Personalized therapy: DNA based alterations are target of new drugs



Breakthrough in cytogenetics

Fluorescence in situ hybridization (FISH)

DNA probe double stranded with fluorochrome

Double stranded DNA target (interphase cell, chromosome, tissue section)

DNA probe

Labeling of DNA probes

2. renaturation

1. denaturation

FISH in normal cell

13-chr

21-chr

Different probes, different colors

Fluorescence in situ hybridization (FISH)

DNA probe double stranded with fluorochrome

Double stranded DNA target (interphase cell, chromosome, tissue section)

DNA probe

2. renaturation

1. denaturation

3 signals of chromosome 21

Down syndrome

FISH probes

Painting probes

Centromere specific

Telomere specific

Region, band, gene specific

a Centromere Nucleus

b

c

d

e

Chromosomal CGHCGH

FIBER FISH

ARRAY-CGH

f

g

h

EXAMPLES FOR DNA SPECIFIC PROBES ON CHROMOSOMES

a)

© Chrombios

Centromere probe

Telomere probe

NOR probe

Alu probe

b)

Whole chromosome painting probe

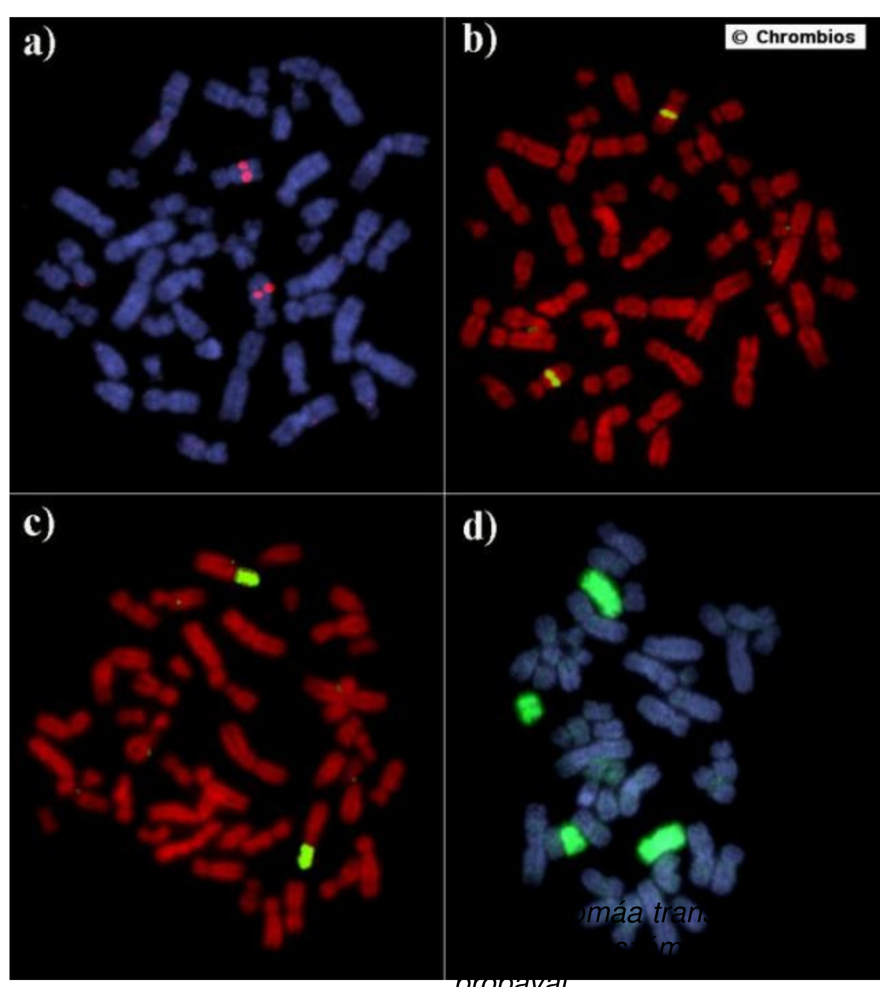
Arm specific probe

Band specific probe

Cloned DNA probe

Localization of gene specific probes on normal chromosome preparation

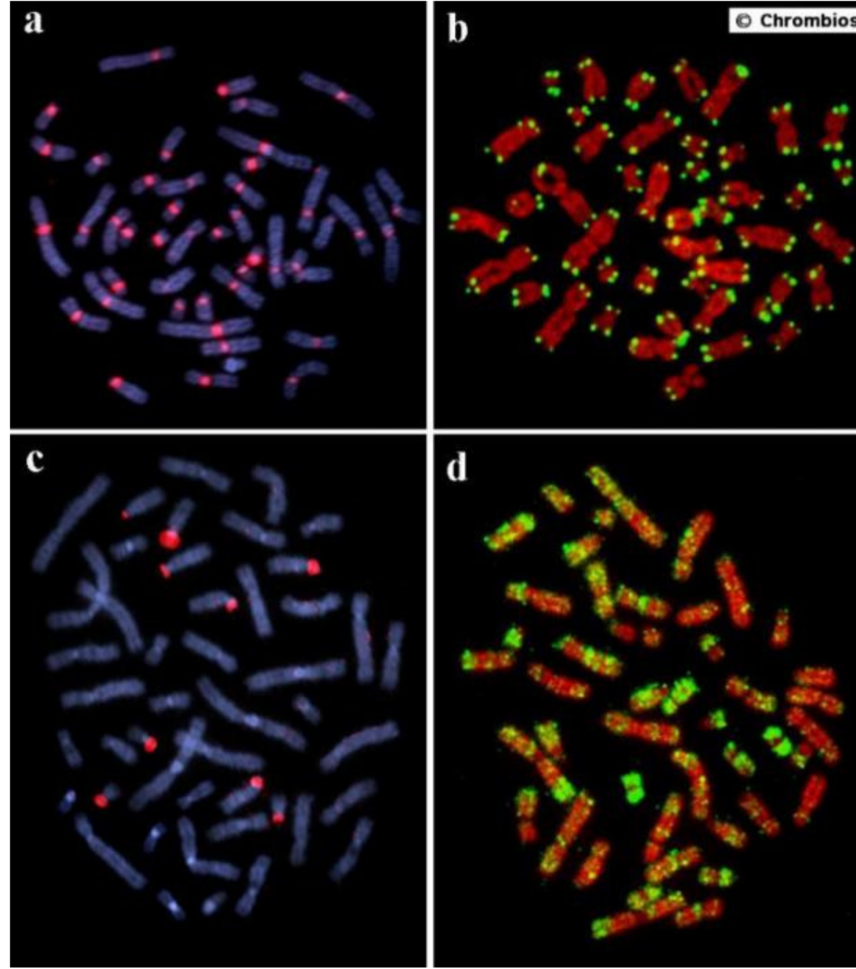
Two pairs of signals are present



Chromosome painting probes

Highly specific staining

probával



Source: chrombios

Slide based technique:

Basic science
gene mapping

Pathology, oncology

Prenatal diagnosis

Environmental toxicology

Gene mapping

Breast cancer HER-2 amplification

Down syndrome

Alterations in sperms

In solution: rare
Basic science

Chromosomal organization

How is it possible to detect the fluorescently labeled DNA probes?

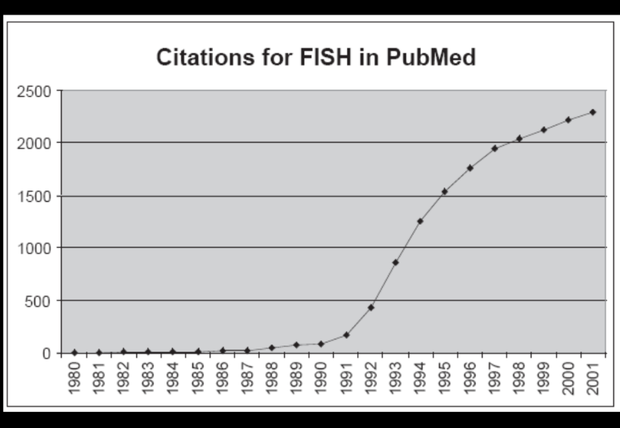
- Cytochemical techniques with several approaches
- Natural and synthetic dyes: non-specific

- Nucleotide labeling with marker molecules
  - Insertion of digoxigenin or biotin labeled nucleotides

- Indirect detection methods:
  - anti-digoxigenin
  - biotin-avidin complexes

- Direct labeled fluorescent probes
- Probe size
- Suppressive hybridization
- New labeling techniques
- Increasing number of probe combinations

  - Increasing specificity



Preparation of DNA probes

Recombinant DNA technology (cloning)

different insert sizes

plasmids (centromere specific probes, some kb)

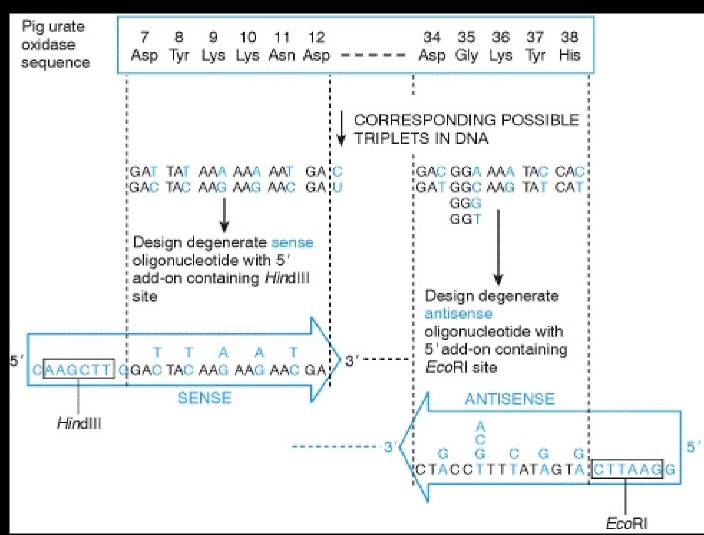
cosmids (gene specific probes, 5-40 kb)

BAC, PAC, YAC (gene specific probes, 100 kb-1 Mb)

in situ PCR

chromosome microdissection with PCR

- AMPLIFICATION OF THE PREPARED CHROMOSOMES WITH DOP-PCR (*degenerate oligonucleotide primer PCR*)
    - Degenerated primer



Preparation of DNA probes: recombinant DNA technology

bacterium
plasmid
bacterial chromosome

After insertion of DNA probes into plasmids, E.coli is transfected with the **recombinant plasmids**

DNA probe specific libraries

10^{-2}

1. **bacteria culturing**

2. plasmid isolation from the bacterial cells

3. labeling of the cleaned DNA probes using nick translation

DNA polymérase I
Escherichia coli
Polymérase
Exonucléase 5'→3'

4. probe testing, and control hybridization to normal, peripheral lymphocytes

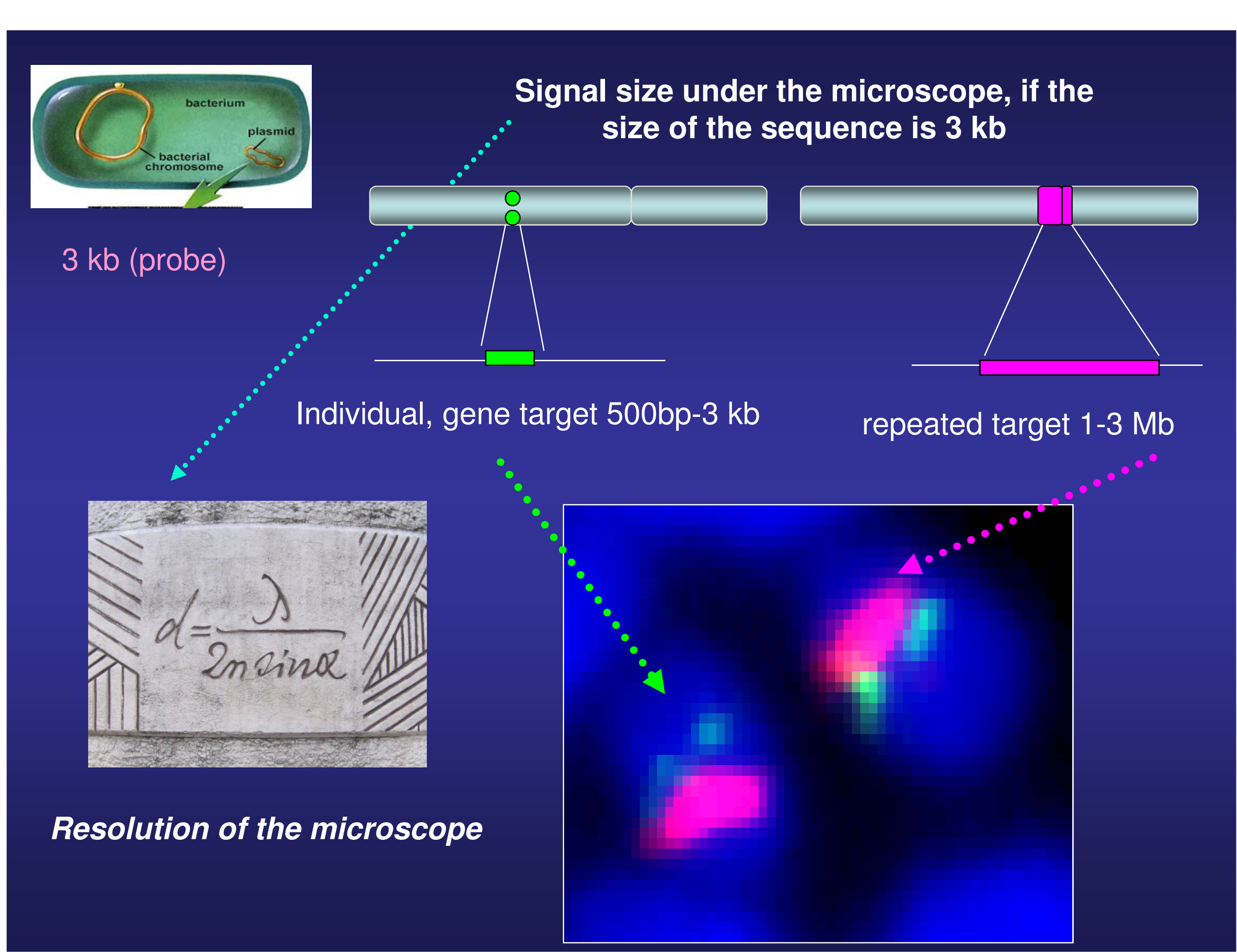
Preparation of DNA probes Flow cytometry

Hoechst 33258 Fluorescence (dot-plot)
Number of particles (continuous trace)
Chromomycin A_3 Fluorescence (dot-plot)
Fluorescence Level (continuous trace)

Flow Sorted Human Chromosome 3

Flow Sorted Human Chromosome 8

1979 Laurence Livermore National Laboratory

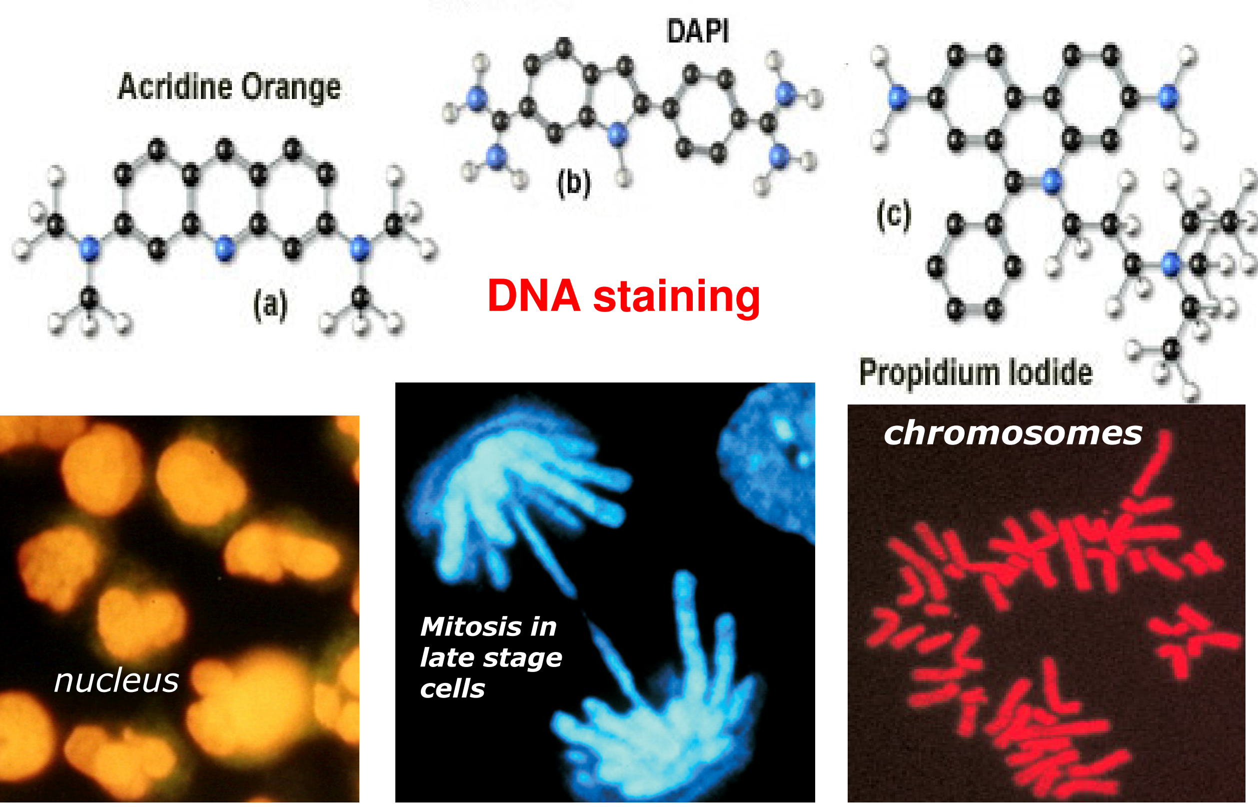


Increasing specificity: multi color application

Table 1. Selected milestones in the development of multi-target FISH

	DNA/gene	mRNA/expression
First in situ detection	Bauman et al., 1980	Singer and Ward, 1982
Two-color detection	Hopman et al., 1986	Dirks et al., 1990
Three-color detection	Nederlof et al., 1989	Dirks et al., 1991
Combinatorial color-coding (M-FISH)	Nederlof et al., 1990	Levsky et al., 2002
Ratio color-coding	Nederlof et al., 1992b	–
Combinations and ratios (COBRA)	Tanke et al., 1999	–

Labeling the nucleus with different colors



2. táblázat. A FISH módszerhez alkalmazott fluorofórok és DNS festékek gerjesztési és emissziós maximumai

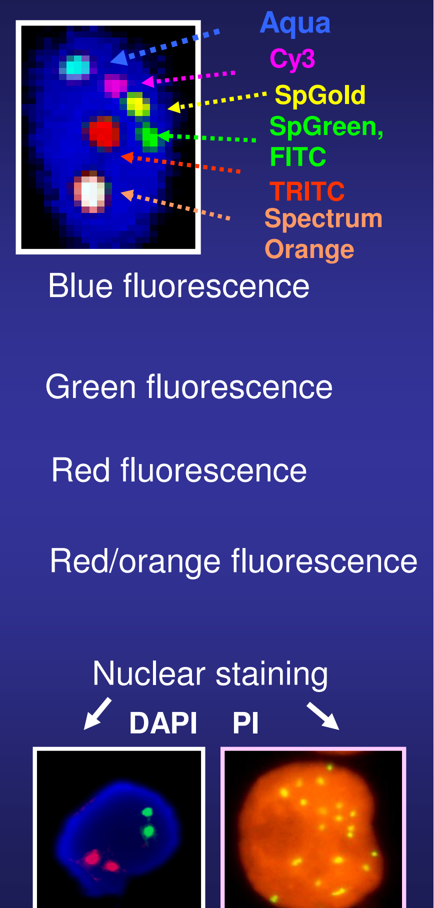
Fluoreszcens festék	Gerjesztési maximum (nm)	Emissziós maximum (nm)
Fluorofórok		
AMCA	350	450
Cascade Blue	377, 398	422
Spectrum Aqua	433	480
CY2	489	506
FITC, FluorX	495	519
Spectrum Green	509	538
TRITC	544	572
CY3	552	565
Spectrum Orange	559	588
Spectrum Red	587	612
Texas Red	589	615
CY5	648	665
DNS festékek		
DAPI	359	461
Hoechst 33258	346	460
Chromomycin A3	430	570
Propidium iodide	340, 536	617

Blue fluorescence

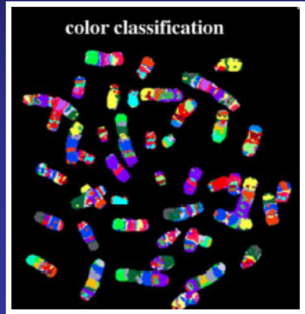
Green fluorescence

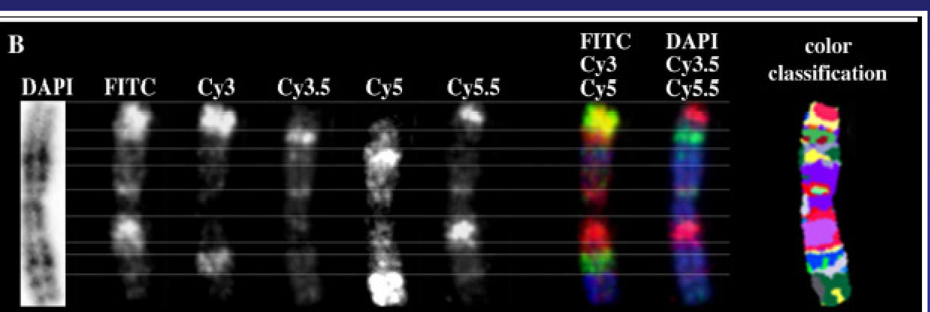
Red fluorescence

Red/orange fluorescence



Chromosome band specific probes

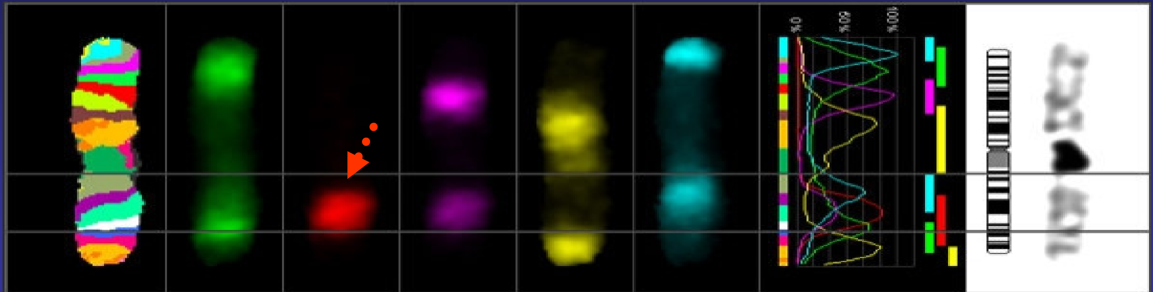




Labeling of band specific probes: a mixture of 5 fluorescent colors
labeled probes are overlapped
detection of the appropriate localization is based on color coding

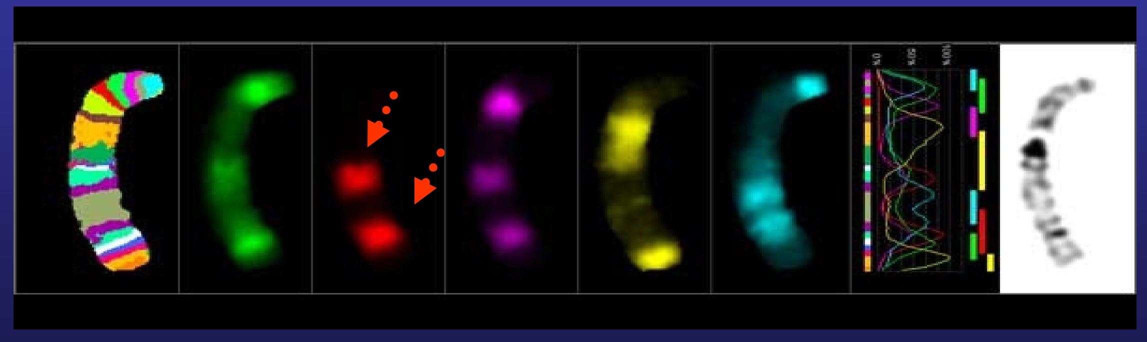
Chromosome band specific probes

Chromosome 1 *Banding probe labeling kit*



24 bands on the normal chromosomes

Chromosome 1 derived from a patient *alteration:* 1q21.3q41 duplication



Chromosome painting probes can be used only on chromosome spreads

FISH

1 2 3 4 5 6
7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 X Y

Applicable on chromosome spreads unsuitable for banding

A B C D E F G H I

BAC #131 SP6 vector T7 F18B21 BAC #97 BAC #131

2 signals vector-specific probe

vector PCR probe PAC BAC

BAC 97A23 PCR probe 17kb 80kb PAC 11C11 vector T7 SP6

P1 #1143 P1 #1107 pYAC3 pYAC3 YAC 141G6

Fiber FISH

DNA-bound probes to determine their position relative to each other

ERBB2 amplification in breast cancer cells

Centromere 17
ERBB2 17q13

Tandem repeated amplicons

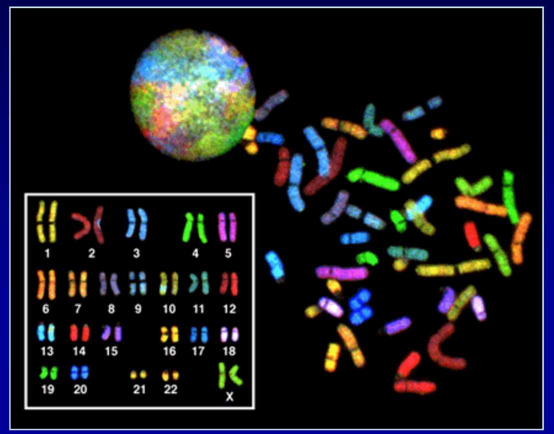
Localization of ERBB3 on the normal chromosome

ERBB2 oncogene amplification in interphase breast cancer cells

centromere 17
5 signals, aneuploidy

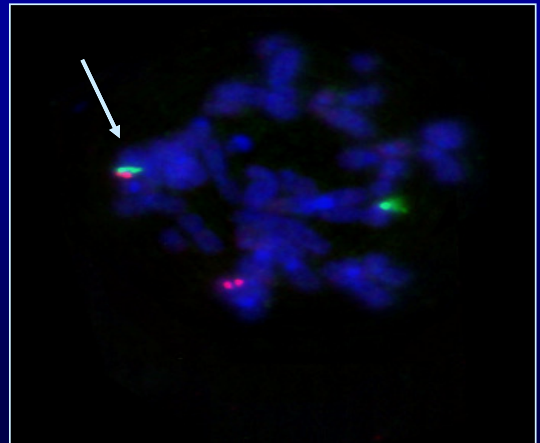
ERBB2 signal
More than 50 gene copies

Translocation detection



Chromosome painting probes:

ONLY ON CHROMOSOME SPREADS



Breakpoint specific probes:

BOTH ON CHROMOSOME SPREADS AND INTERPHASE CELLS

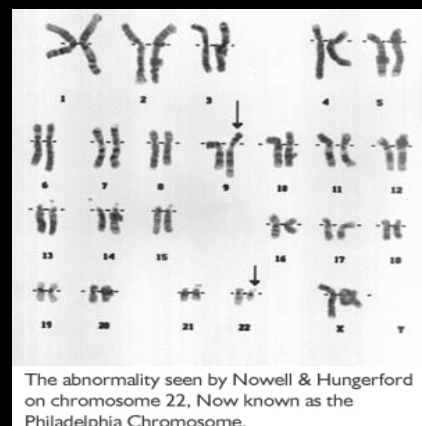
Peter Nowell David Hungerford (1960)

patients with leukemia (*chronic myelogeneous leukemia*)

cancer cells an abnormally small chromosome

Philadelphia CHROMOSOME

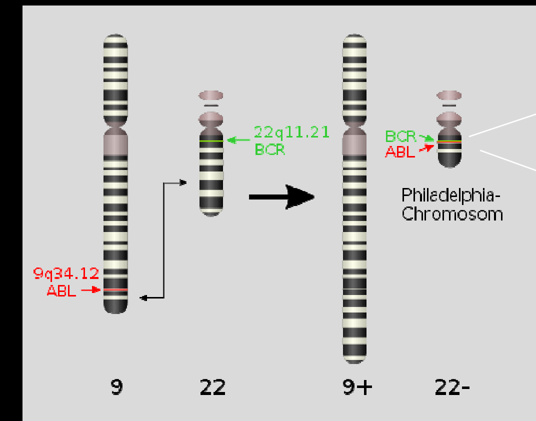
2001: drug Gleevec/Imatinib: can block the effects of the gene and stop progression of CML in 95% of patients.



The abnormality seen by Nowell & Hungerford on chromosome 22, Now known as the Philadelphia Chromosome.

Every time with patient with CML bone marrow

Janet Rowley 1972
RECIPROCAL TRANSLOCATION



BCR :("breakpoint cluster region") gene

Abl : "Abelson", leukemia virus

Ph1

Gleevec

FISH image of bcr/abl positive rearranged metaphase

http://www.uphs.upenn.edu/news/features/philadelphia-chromosome/history/photo.html
http://news.lib.uchicago.edu/blog/2009/05/11/dr-janet-rowley-to-speak-at-crerar-library/
http://upload.wikimedia.org/wikipedia/commons/4/4d/Philadelphia_Chromosom.svg

95% of patients with CML have the Philadelphia chromosome

Reciprocal translocation

Changed chromosome 9

Normal chromosome 9

Chromosomes break

Normal chromosome 22

BCR

ABL

Changed chromosome 22 (Philadelphia chromosome)

BCR-ABL

Bone marrow karyotype

9 22

Philadelphia Chromosome Produces a Hybrid Gene that Causes Cancer

BCR (chromosome 22)

ABL (chromosome 9)

Ph chromosome

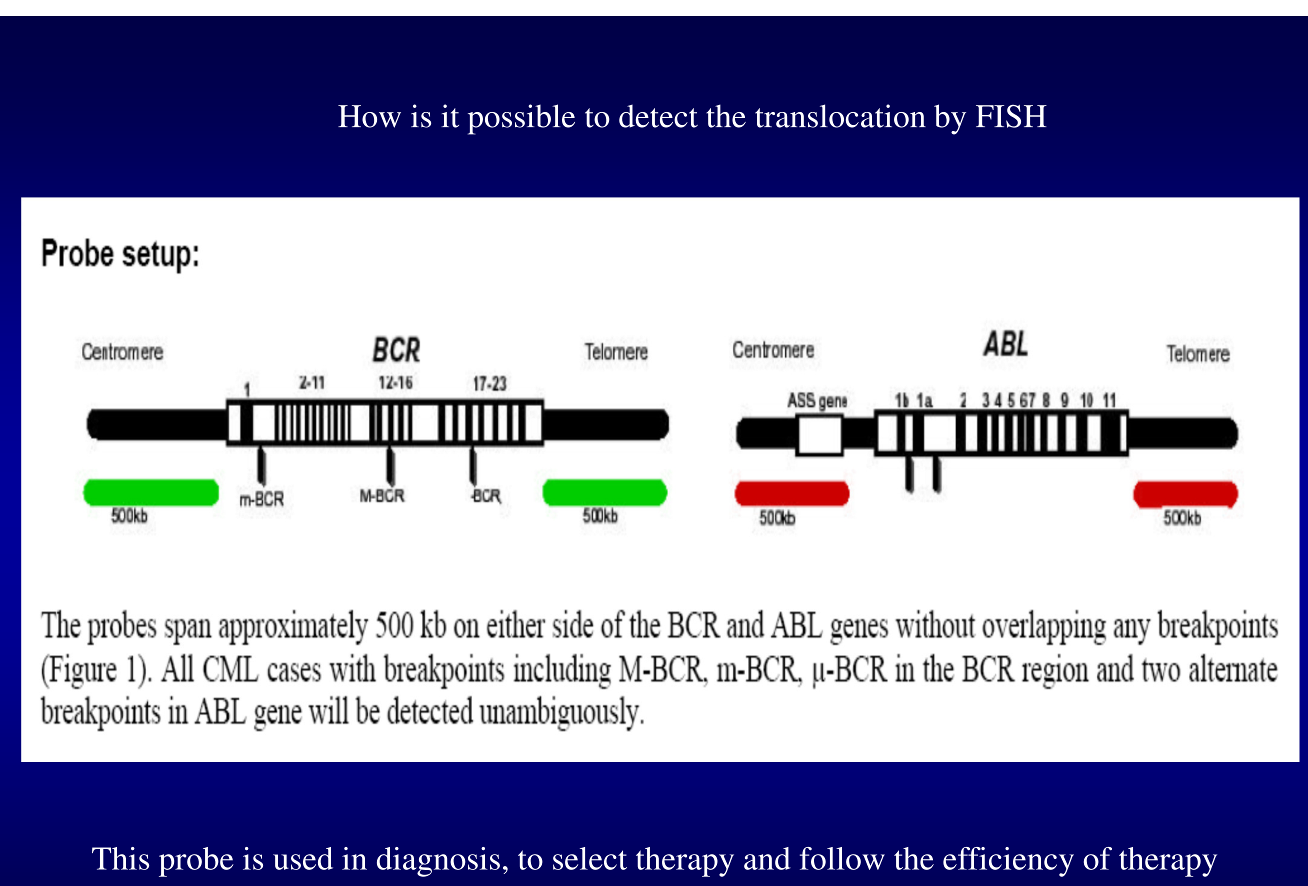
Hybrid fusion gene

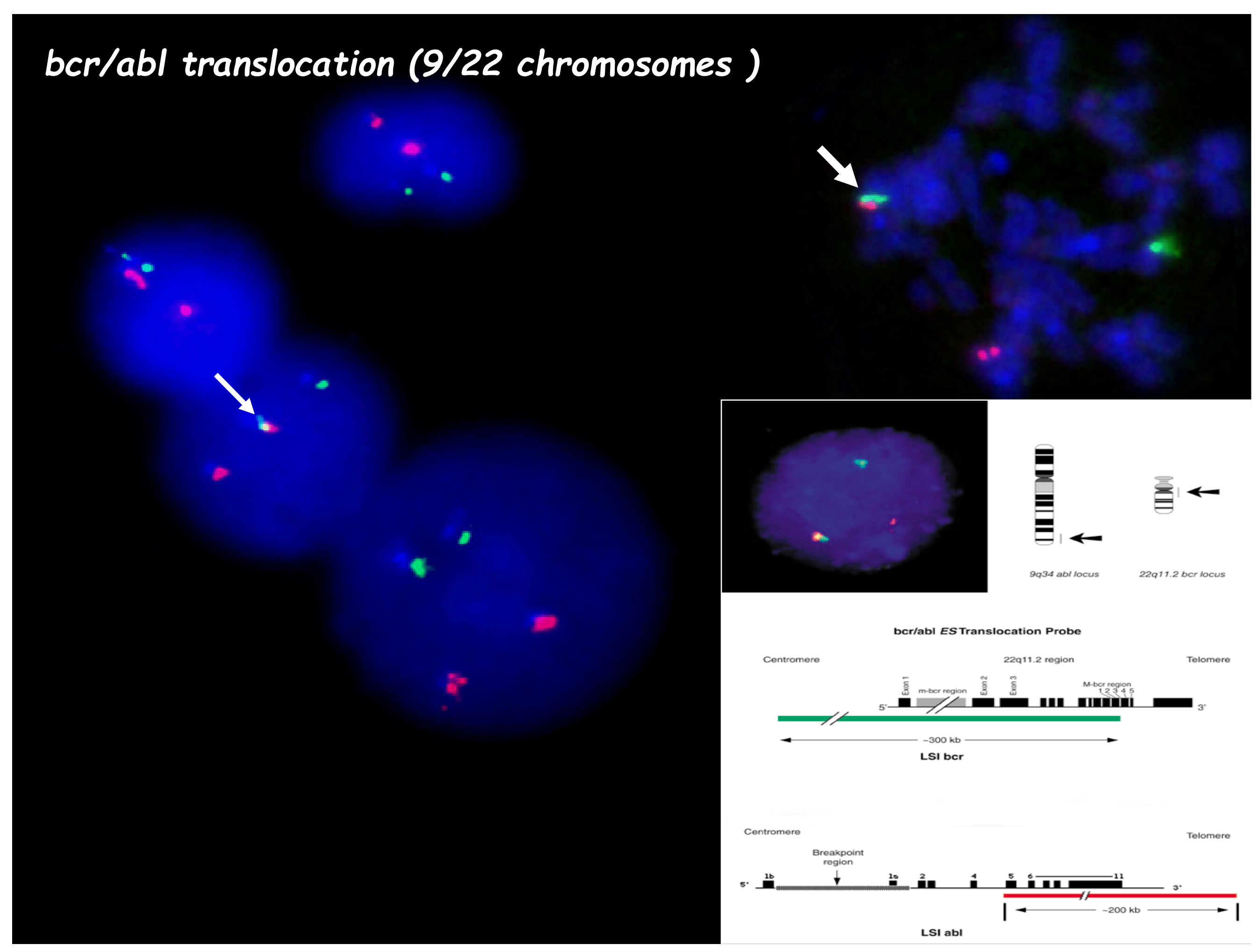
BCR-ABL protein

Fusion protein

Animal experiment

http://media.hhmi.org/hl/13Lect2.html





PARAMETERS AFFECTED THE DNA HYBRIDIZATION

- temperature, pH
- monovalent cation concentration
- denaturing agent (formamide) concentration
- DNA fragment (DNA probe) length
- DNA concentration (DNA probe)
- dextran sulphate concentration, which increases the local concentration of the probe
- posthybridization washes
- ionic strength, pH, organic and inorganic solvents ratio, temperature

DNA T_m ⇒ 0.1-0.2 M Na^+ ⇒ 90-100 °C

lost morphology

solution: an organic solvent, being able to reduce the heat stability of the double stranded DNA, therefore facilitates denaturation

formamide:

linearly reduces the DNA heat stability at a rate of 0.72 °C per % solvent in the mixture, therefore in situ hybridization can be performed at 30-45°C using 50% formamide; in practice, the hybridization temperature is between 65- 75 °C

in situ hybridization 65-75 °C ⇒ 16 - 48 hours

formamide degradation: deionized formamide

Master mix: - salts (standard saline citrate: SSC)

1xSSC: 0.15 mol/l NaCl, 0,015 mol/l Na citrate
pH: 7.00

- formamide

$$Tm = 81.5 + 16.6 \lg [Na^+] + 0.41 [\%GC] - 0.63 [\%formamide] - [Na^+] / N$$

N= hybrid length (base)

Human DNA has an avg. 40% GC content, but it can be significantly altered depending on the genomic localization

Cation concentration:

The Na^+ ion influences the Tm point.

bivalent cations: Mg^{2+} and Ca^{2+} can stabilize the double stranded DNA,

reduced hybridization efficacy in the presence of their free form

chelate formation (EDTA) !!!!

Temperature

denaturation and renaturation

Renaturation is spontaneous below the Tm point

Tm depends on the [GC] / [AT] ratio

ratio of the base pairs (%),

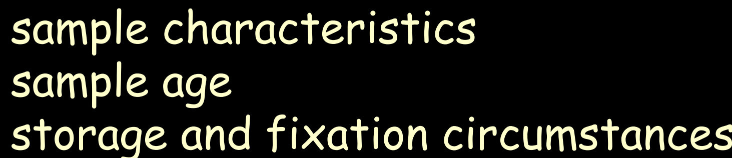
forces between the base pairs,

hidrogene bonds

Denaturation:

70% formamide,
2 x SSC, 65-75 °C
few minutes

The optimal conditions are always depend on the sample.



sample characteristics
sample age
storage and fixation circumstances

Denaturation of the sample and probe can occur:

- separately, in solution
- parallel, in a hybridization chamber

Components of the hybridization mixture: (master mix)

- 50/55% formamide
- 2xSSC (standard saline citrate; 1xSSC: 0.15 mol/l NaCl, 0,05 mol/l Na citrate)
- 10 % dextran sulphate: increases the local concentration of the DNA probes

pH: 7.00 (its preparation takes ~2-3 hours, because of the slow dissolution of dextran sulphate

+ optimal probe concentrations

Centromere specific probes: 1-4 ng

Small locus specific probes : 10-20 ng

pH:

Denaturation: FISH, CGH: pH: 7.00

Renaturation independent:
pH: 5.00-9.00

Native human cells, tissues

- Chromosome preparation from peripheric cells
- Bone-marrow derived cells
- Urine derived epithelial cells from
- Hair follicular cells
- Non-cultured cells derived from the amniotic fluid
- Sperms

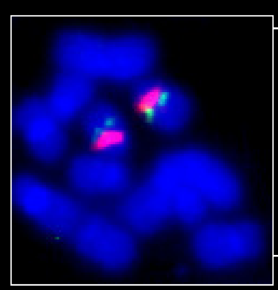
Archived samples

- Archived cytogenetic plates
- Formalin-fixed paraffin-embedded sample sections, extracted nuclei
- Combined in situ Nick Trans. and mRNA FISH
- Frozen sample sections, extracted cells

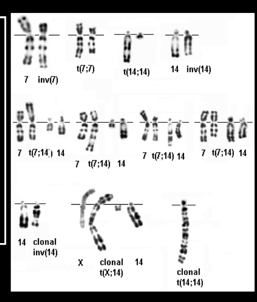
Zoology, Virology

- Zoo-FISH

Comparative genomic hybridization



Advantages of FISH against the classic cytogenetics



- ❖ Chromosomal alterations can be analyze on interphase cells
- ❖ Duration of the examination is considerably reduced (a few hours-12 hours after sampling)
- ❖ Only a few hundred cells are enough
- ❖ Complex karyotype of samples unsuitable for standard cytogenetic analysis using multicolor–FISH
- ❖ Tissue and bone-marrow smears, cytological samples
- ❖ Tissue microarray analysis enables the parallel examination of tumor progression related alterations on hundreds of samples

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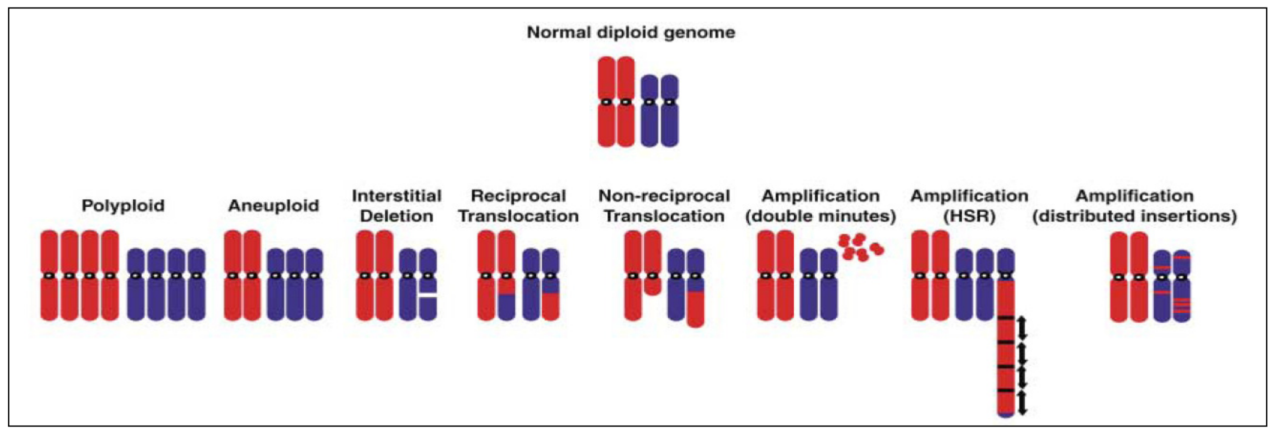
- FISH is unsuitable for the detection of **unknown** chromosomal aberrations
- DNA specific probes are worth to produce if the sequence has a known localization
- **How can we examine the disease specific genetic alterations if there is no DNA probe for them?**

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D.
Science, 1992 Oct 30;258(5083):818-21.

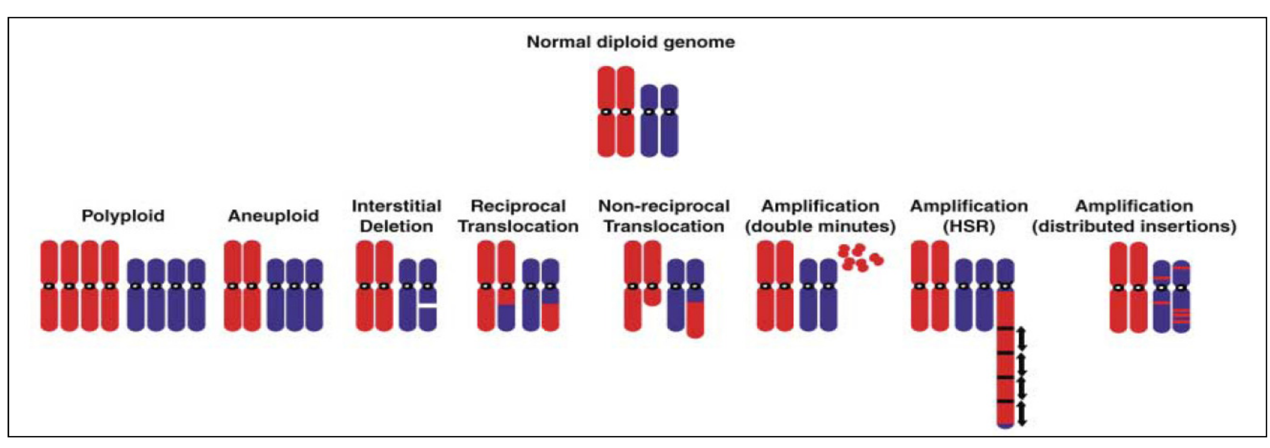
Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors *(cited: 2 282)*

Genetic alterations are a key feature of cancer cells and typically target biological processes and pathways that contribute to cancer pathogenesis



Identification of regions with Copy Number Alterations (genes) involved

- **offers a basis for better understanding of cancer development and progression**
- **provide improved tools for clinical management of cancer, such as new diagnostics and therapeutic targets**

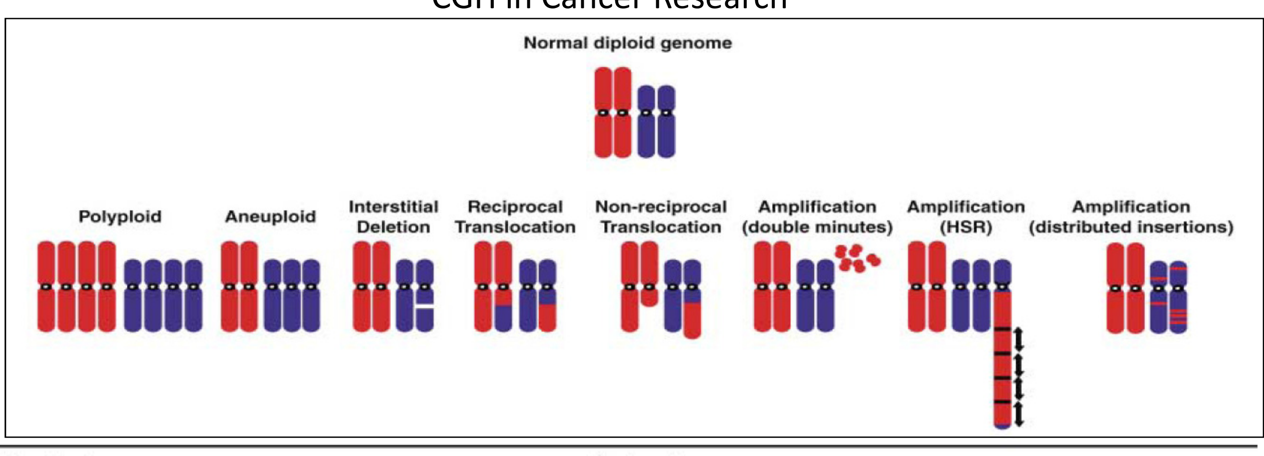


Technique	Detection							
Banding	+	+	+/-	+	+	+	+	-
SKY/FISH	+	+	-	+	+	+	+	-

advantage/ disadvantage

Discovery of new genetic alterations: difficult
Karyotyping: low number of high quality chromosomes
Molecular genetic techniques: highly focused , target one specific gene

CGH in Cancer Research

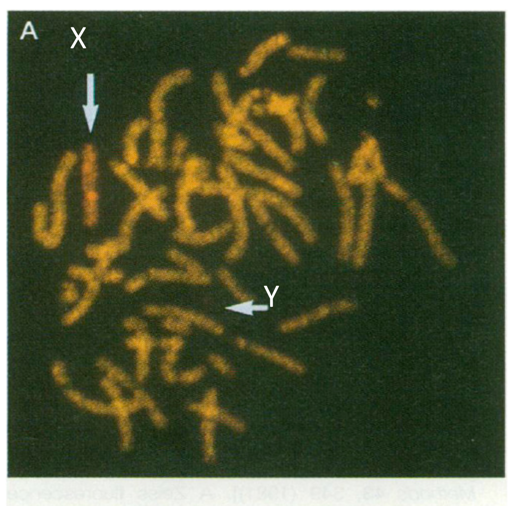


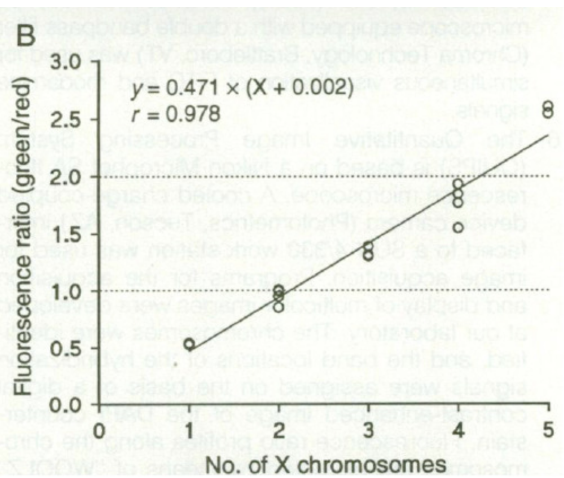
Technique	Detection							
Banding	+	+	+/-	+	+	+	+	-
SKY	+	+	-	+	+	+	+	-
CGH	-	+	+	-	+	+	+	+
LOH	-	+	+	-	+	+	+	+

Distinct advantage of CGH

- only tumor DNA is required
- archived, formalin fixed and paraffin embedded tissue can be used
- applicable for the low mitotic index of malignant cells and poor chromosome morphology and resolution
- new experimental approaches (cancer progression, disseminated tumor cells, molecular classification)

CGH in Cancer Research



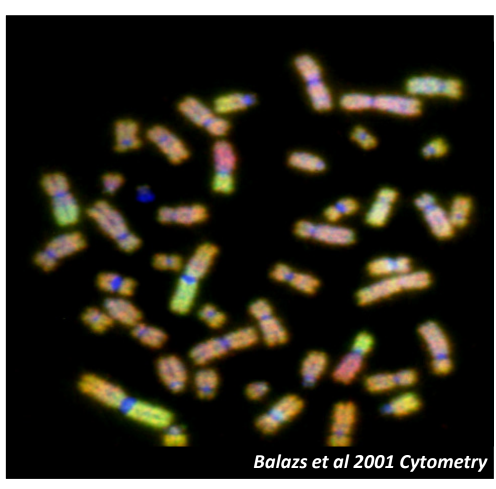


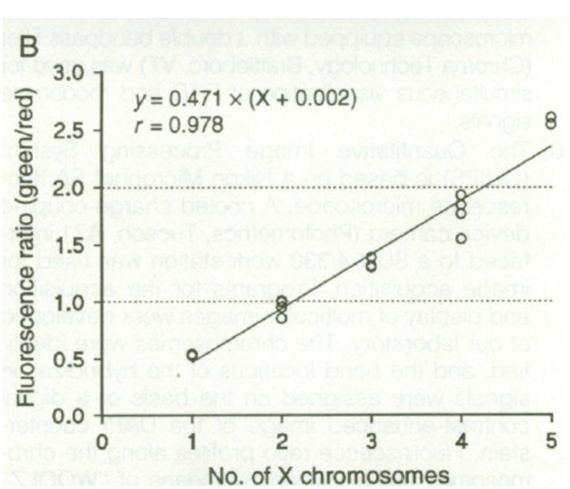
Hybridization of DNA from the 45,X0 cell line (green) and normal female reference DNA (red) to a normal male metaphase spread

Correlation of the number of X chromosomes in five fibroblast cell lines and the average green-to-red ratio of the X chromosome relative to the same ratio for the autosomes.

***Science*. 1992 Oct 30;258(5083):818-21.**

Quantitative detection of copy number alterations



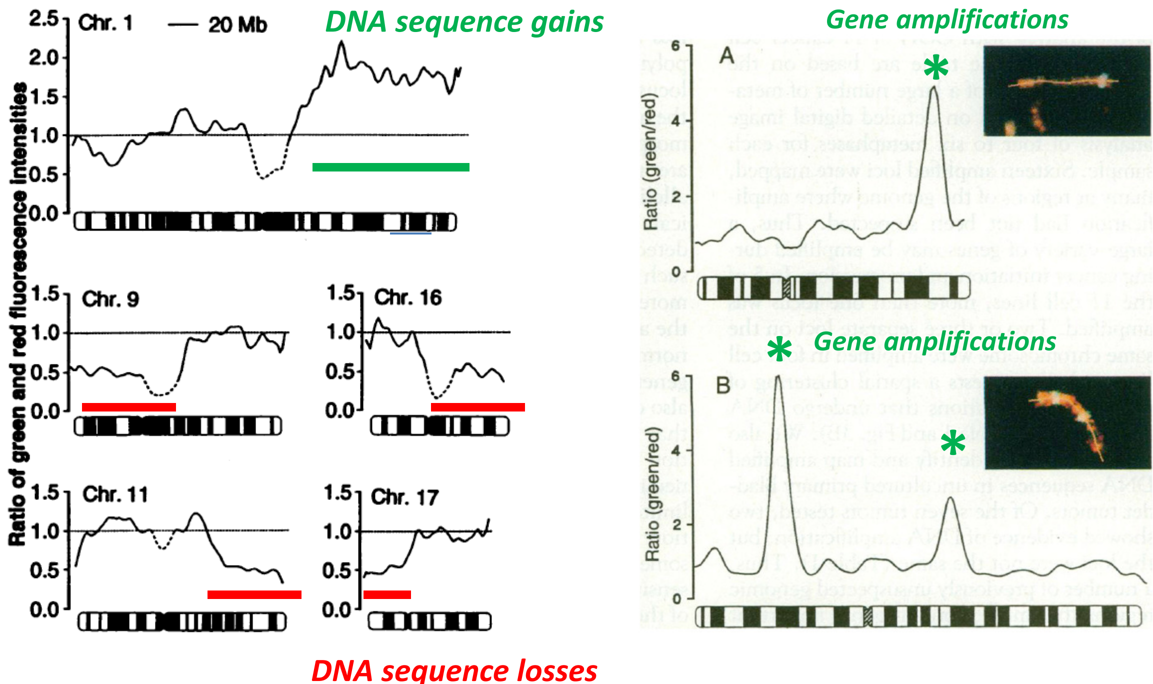


Hybridization of DNA from primary malignant melanoma (green) and normal female reference DNA (red) to a normal male metaphase spread.

Correlation of the number of X chromosomes in five fibroblast cell lines and the average green-to-red ratio of the X chromosome relative to the same ratio for the autosomes.

***Science*. 1992 Oct 30;258(5083):818-21.**

Chromosomal CGH profiles of different cancer cell lines



Resolution of chromosomal CGH: 10-20Mb

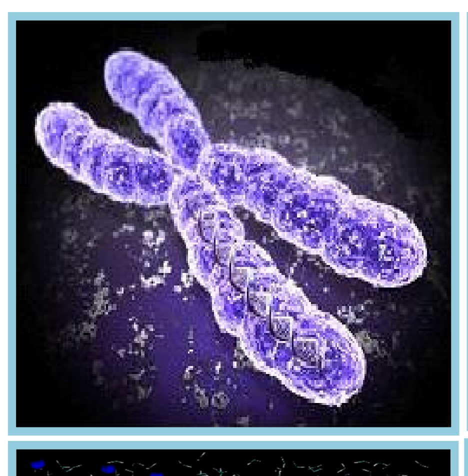
Science. 1992 Oct 30;258(5083):818-21.

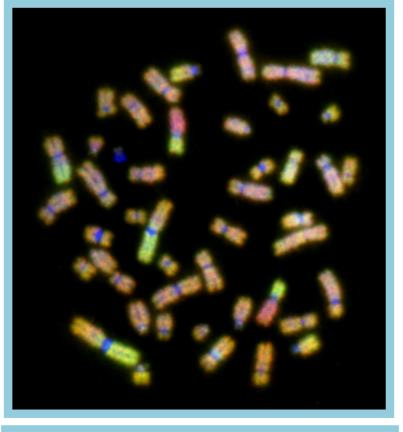
Table 1. Mapping of amplified sequences in established cancer cell lines and primary tumors by CGH. Cytogenetic information is based on the American Type Culture Collection catalog of cell lines and hybridomas (1992). DM, double minute chromosomes; HSR, homogeneously staining regions.

Specimen	Origin	Amplification by CGH*	Cytogenetic evidence of gene amplification
		Cell lines	
5637	Bladder	3p25, 6p22	DM
SK-BR-3	Breast	8q24 (*myc*), 8q21, 17q12 (*erb*B2), 20q13	—
COLO 205	Colorectal	6p21, 6q24	—
NCI-H508	Colorectal	14q12-q13	DM
SW480	Colorectal	8q24 (*myc*)	DM
SW620	Colorectal	16q21-q23	HSR
WiDr	Colorectal	8q23-q24 (*myc*)	—
SK-N-MC	Neuroblastoma	8q24 (*myc*)	DM
Calu-3	Small cell lung	8p12-p21, 8qtel, 17q12 (*erb*B2)	HSR
Calu-6	Small cell lung	13q32-q34	—
NCI-H69	Small cell lung	2p24 (N-*myc*), 2p21, 2q21	—
		Primary tumors	
UR140	Bladder carcinoma	16q21-q22	—
UR145	Bladder carcinoma	6p22	—

*The oncogene most likely involved in this amplification is shown in parentheses.

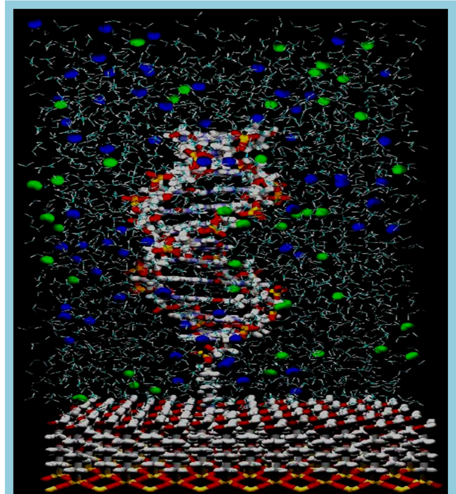
Science. 1992 Oct 30;258(5083):818-21.

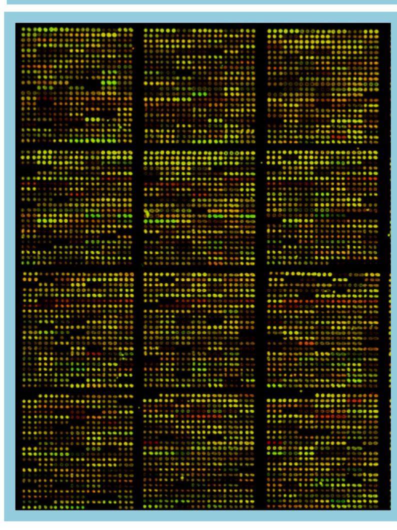




Technical difficulties and problems

cytogenetic chromosome preparation





arrays of genomic sequences replaced the metaphase chromosomes as hybridization targets

COMPARISION OF CHROMOSOMAL AND ARRAY CGH

SAME MELAMOMA DNA

Chromosome 1

primary

Log 2 Ave Raw Ratio

0 75000 150000 225000 300000

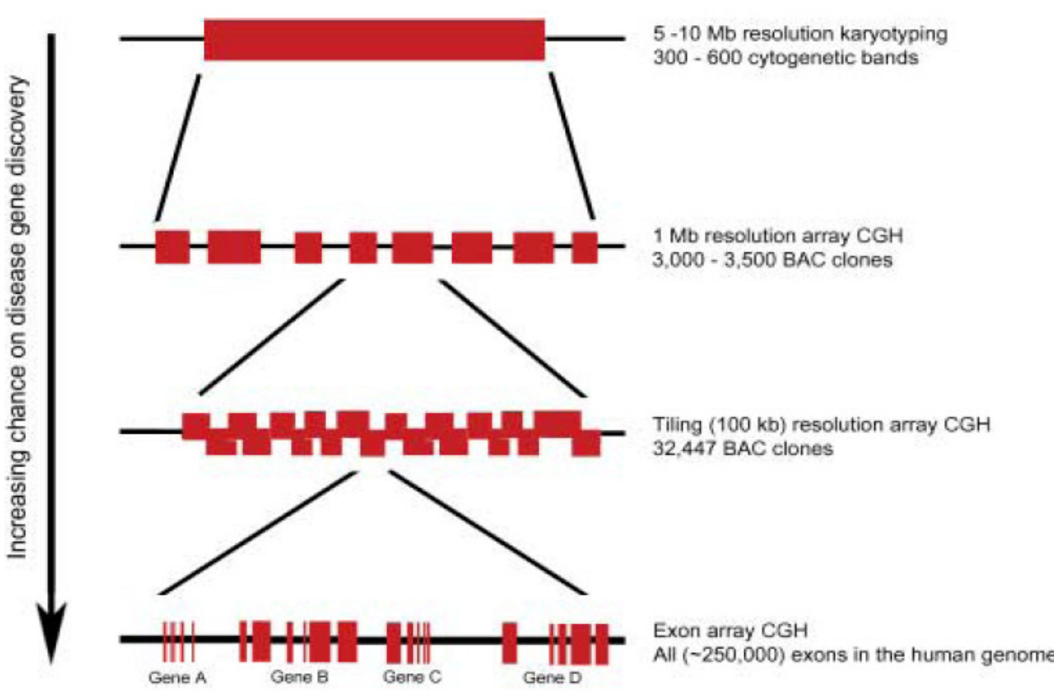
chromosomal CGH

10-20 Mb

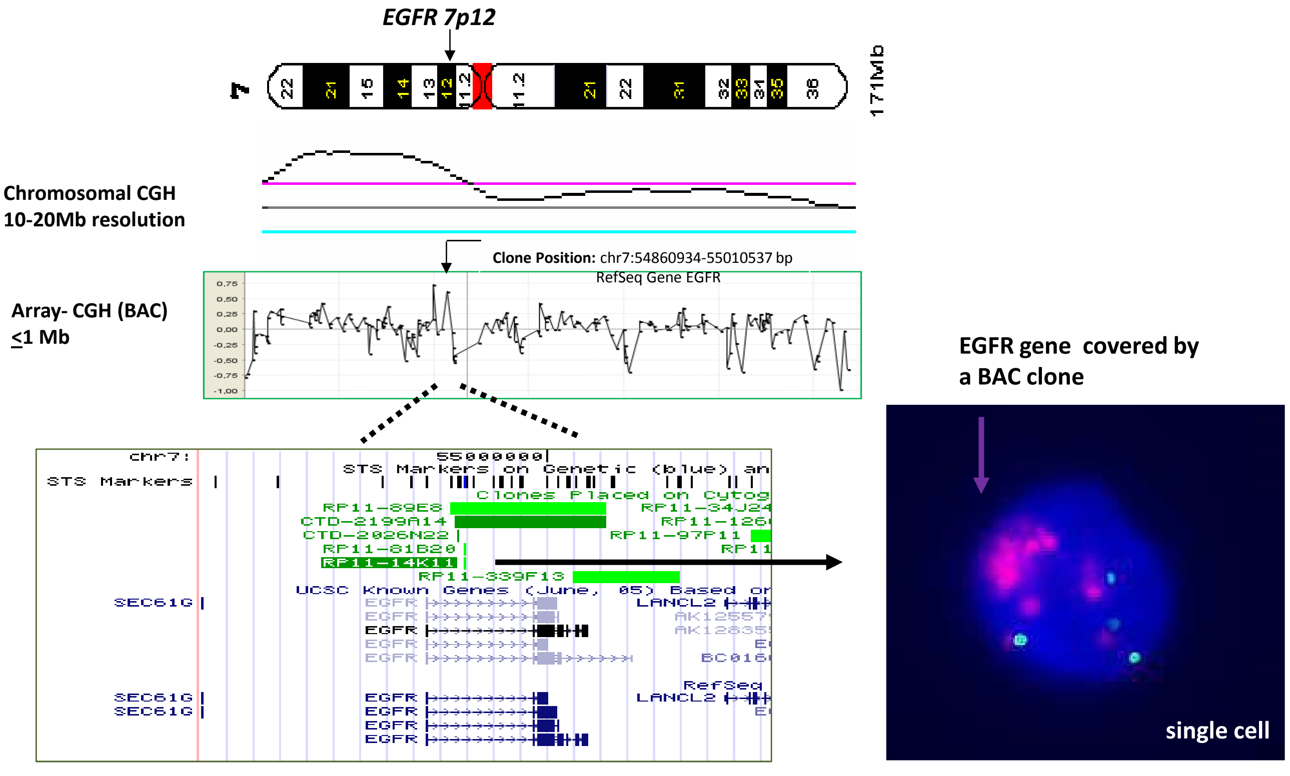
array CGH

resolution is restricted only by clone size it can be 100kb

Main advantage of array CGH



Amplification of the EGFR gene in malignant melanoma: genome and single cell level



Rakosy et al., Int J Cancer 2007.

Table 1
Comparison of array CGH technologies

Platform	Technology	Functional resolution			Sample labeling	Sample requirements	Notes
		Theoretical sensitivity	Single-copy sensitivity	Breakpoint precision			
Nimblegen 385K	Oligonucleotide (45–85 nt)	15 kb	54 kb*	24 kb	Whole genome	1–3 µg	*Single-copy sensitivity is estimated based on analysis parameters described in Selzer et al.
Agilent 244A	Oligonucleotide (60 nt)	36 kb	36 kb	56 kb	Whole genome	0.5 µg (1 µg with dye flip)	DNA amplification reduces DNA requirements to 0.1 µg of DNA per slide (0.2 µg with dye flip) (not tested in this study)
Affymetrix GeneChip human mapping 500K set	Oligonucleotide (25 nt)	41 kb	75 kb	74 kb	PCR reduction	0.5 µg	Platform is also used for LOH analysis
Submegabase Resolution Tiling (SMRT) set	Large insert clone (BAC)	50 kb	50 kb	152 kb	Whole genome	0.1 µg	High-level amplifications below 50 kb may be detectable; this is not indicated.
Affymetrix GeneChip human mapping 100K set	Oligonucleotide (25 nt)	271 kb	476 kb	528 kb	PCR reduction	0.5 µg	Platform is also used for LOH analysis
VUMC MACF human 30K	Oligonucleotide (60 nt)	1.05 Mb	1.32 Mb	1.94 Mb	Whole genome	0.3 µg	Invitrogen has recently released a 50K oligonucleotide library suitable for array CGH including intragenic oligonucleotides
Illumina Linkage IV	Oligonucleotide (40 nt)	1.35 Mb	2.66 Mb	2.06 Mb	PCR reduction	1 µg	Illumina has recently released a 100K (Infinium) assay. Both platforms are also used for LOH analysis.
UPenn	Large insert clone (BAC)	1.99 Mb	1.99 Mb	3.15 Mb	Whole genome	1 µg	Sample requirement is likely 100 ng due to use of BAC clones
Spectral Chip 2600	Large insert clones (BAC)	2.65 Mb	2.65 Mb	4.55 Mb	Whole genome	1 µg (2 µg with dye flip)	Sample requirement is likely 100 ng due to use of BAC clones
HumArray 3.2	Large insert clone (BAC)	5.07 Mb	5.07 Mb	8.75 Mb	Whole genome	0.6 µg	Sample requirement is likely 100 ng due to use of BAC clones

Genomics 89 (2007) 647–653

Global analysis of copy number aberrations and identification of putative target genes

Common tumor types: breast and colorectal cancers

Rare tumor entities: gastrointestinal stromal tumors insulinomas and ependymomas

New information on the patterns of CNA: **cancer development and progression**

Breast cancer complex pattern of amplicons: → 8 distinct amplicons 8q21-8q24

Meningiomas chromosome 1 specific tiling path array disclosed four separate commonly deleted candidate loci

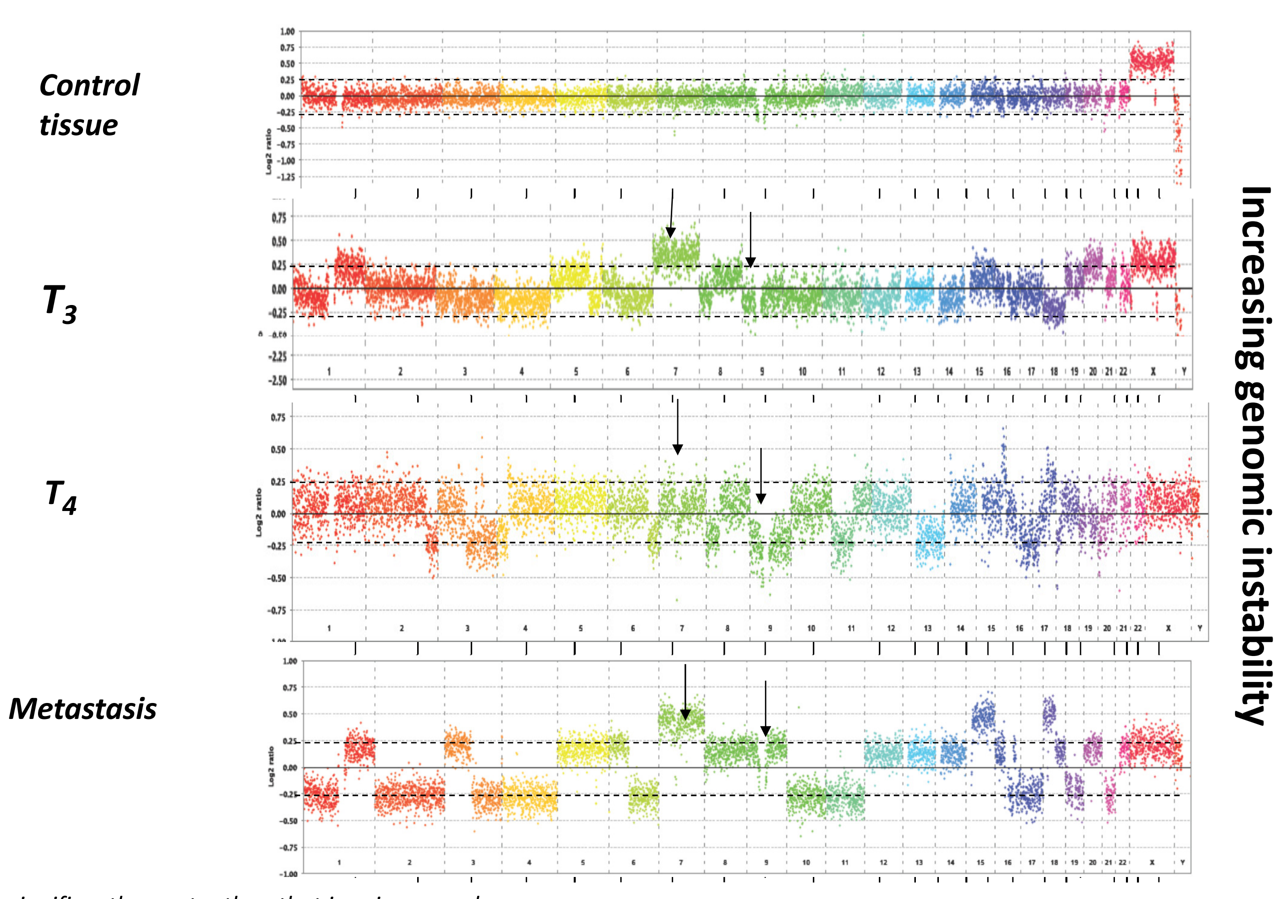
Mantle cell lymphomas homozygous deletions of 2q13targeting proapoptotic *BIM gene*
Ovarian cancers total of 27 homozygous deletions (e.g. RB1 tumor suppressor gene)
Oral cancers FAT gene, a member of the human cadherin superfamily

An array CGH based genomic instability index is predictive of clinical outcome in breast cancer and reveals a subset of tumors without lymph node involvement but with poor prognosis

- Despite entering complete substantial proportion of patients with early stage breast cancer will develop metastases
- To explore the prognostic value of genomic alterations present in primary tumors array CGH study on BAC arrays with a panel of breast carcinomas (n=135)
  - metastatic relapse
  - axillary node involvement without any recurrence
  - at least 11 years of follow-up
  - array-CGH data was used to establish a genomic instability index representative of the global level of aneusomy by chromosomal arm and of the number of breakpoints throughout the genome

Bonnet et al. BMC Medical Genomics 2012, 5:54

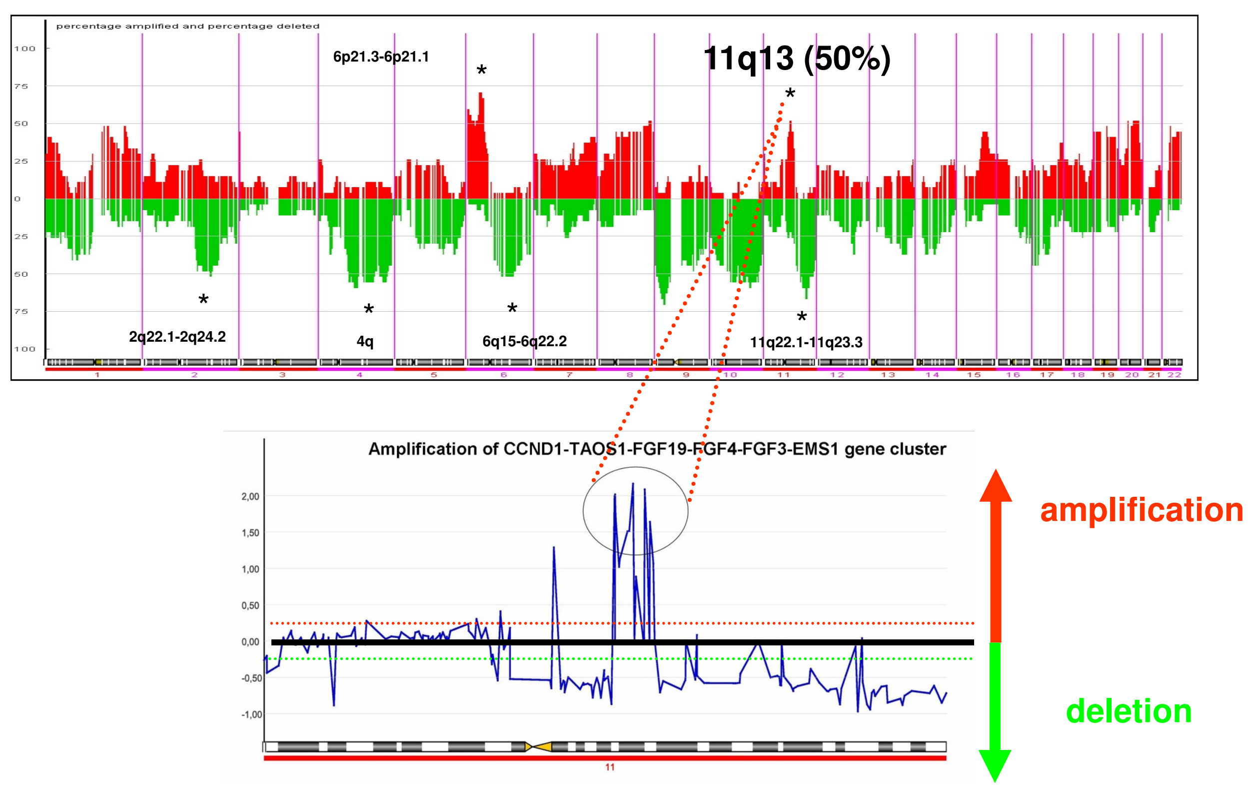
Genomic alterations and tumor progression in malignant melanomas



significantly greater than that in primary melanomas

Lázár et al. et al. 2012. Melanoma research

Genomic alterations in metastatic melanomas



Lázár et al. Modern Pathology 2009

CCND1 gene amplification

***CCND1* amplification:**
Primary tumors **32%**

c11

CCND1

Amplification of CCND1-TAOS1-FGF19-FGF4-FGF3-EMS1 gene cluster

11q13 amplicon cluster valaidation by PCR

Coamplification of genes within the 11q13 cluster resulted in shorter survival

Lázár V. ..Balázs M. 2009 Modern Pathology

Advantage of array CGH in cancer research

- Comparative genomic hybridization (cancer) publications
  - 5673 CGH
  - 1369 array CGH
- highlight
  - the overall patterns of copy number aberrations in various tumor types
- identify
  - in high-resolution-specific genetic alterations associated with
  - certain tumor entities, disease progression, therapy response, or patient outcome

Advantage of array CGH in cancer research

- aCGHdata provide **an excellent starting point** for the identification of genes involved in these aberrations
- aCGH merely points to the region of interest, and **functional analyses are always necessary to establish the actual contribution of putative target genes** to disease pathogenesis
- The data derived from aCGH studies present an essential contribution to our knowledge on cancer associated genetic aberrations and illustrate that aCGH technology still continues to function as an important tool in cancer research
- Clearly, measuring the largest possible number of tumors on the highest-resolution arrays will provide the greatest information