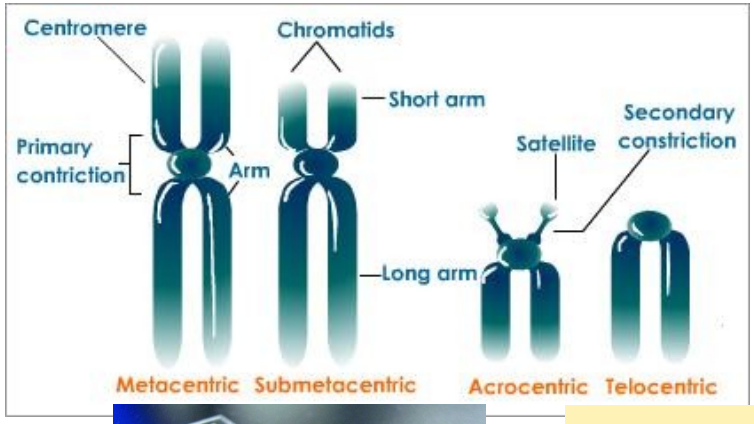
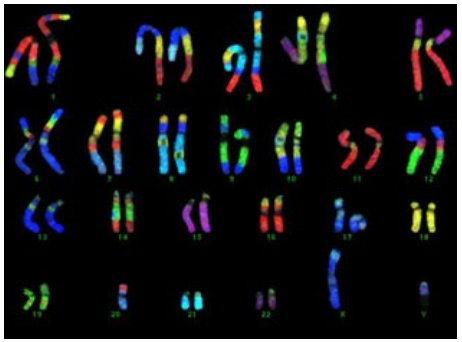
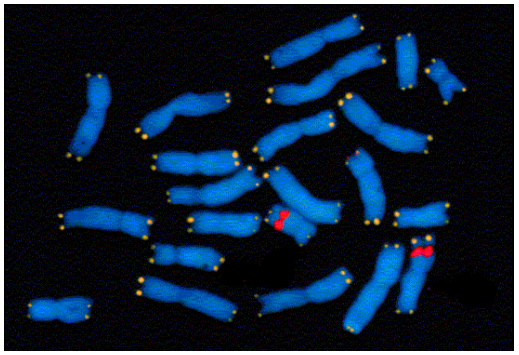
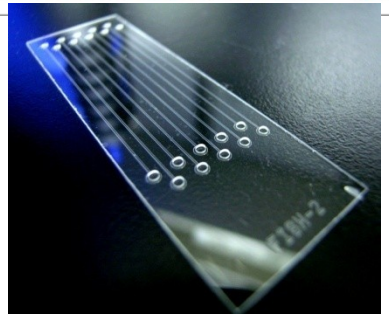
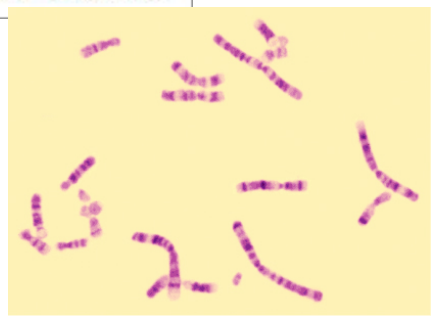
CYTOGENETIC METHODS

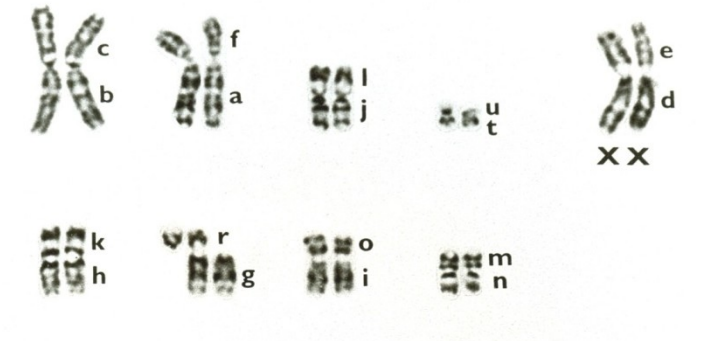












esf evropský sociální fond v ČR
EVROPSKÁ UNIE
MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY
OP Vzdělávání pro konkurenceschopnost
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

analysis of chromosome microscopic structure

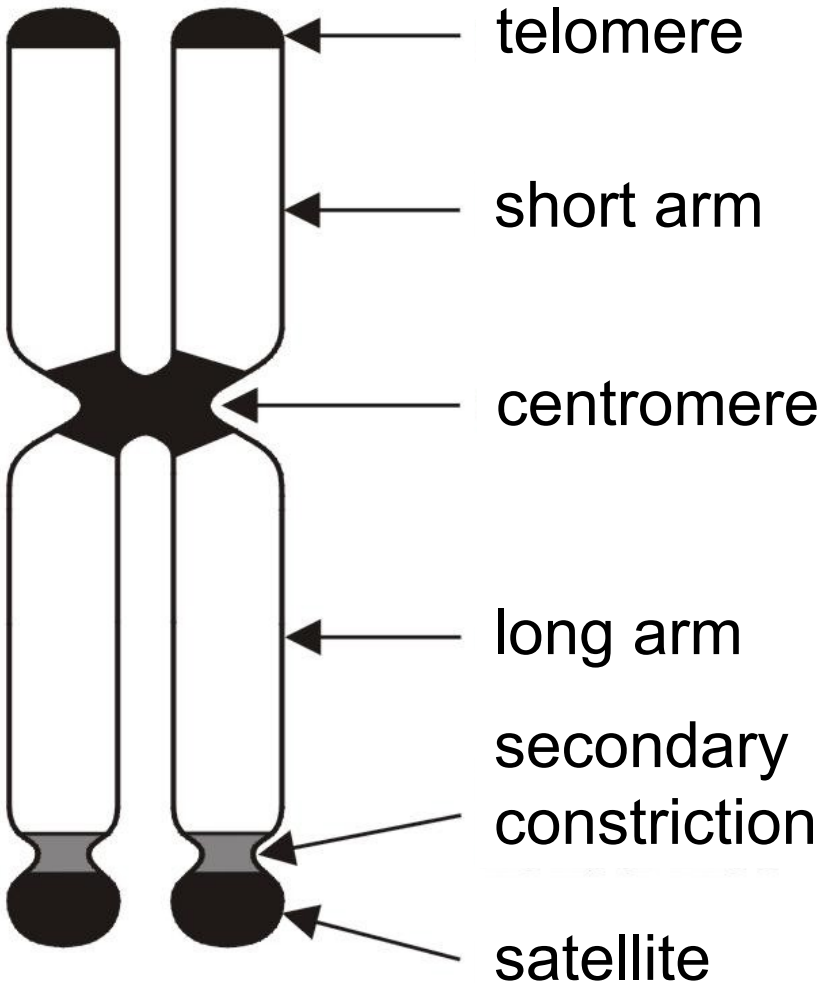
the term „chromosome“ – 1888 Wilhelm Waldeyer

chromosomal theory of heredity: 1st half of 20th century – Theodore Boveri, Walter S. Sutton, Thomas H. Morgan

study of chromosomes: karyology, cytogenetics

karyotype = arranged set of chromosomes in a cell

Structure of metaphase chromosome



Classification of chromosomes according to position of centromere:

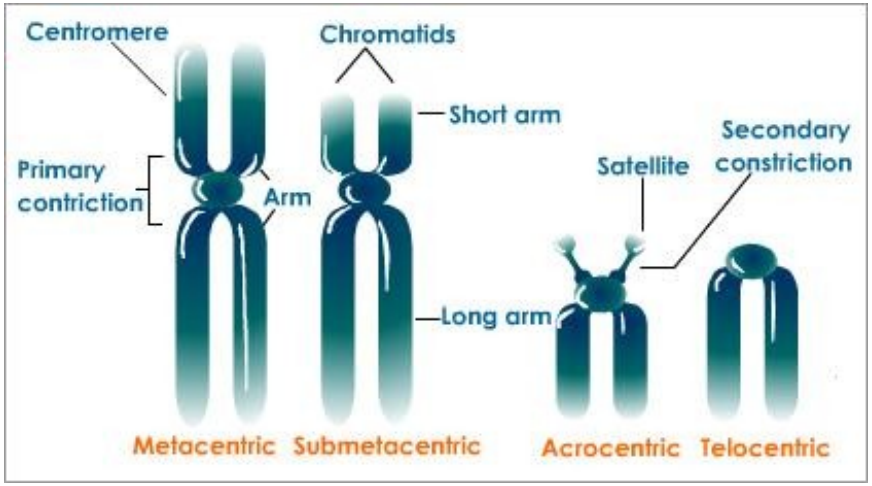
metacentric

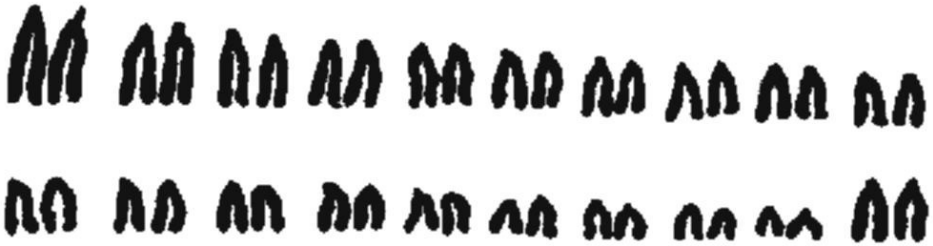
submetacentric

(subtelocentric)

acrocentric

telocentric





History of cytogenetics

Role of key technological innovations – 4-5 breakthroughs in the modern era:

1. Discovery of hypotonic treatment → spread of metaphase chromosomes
2. Cultivation of peripheral blood (leucocytes) and fibroblasts
3. Chromozome banding techniques
4. *In situ* hybridization (ISH)
5. Immunochemical methods used along with ISH → non-radioisotopic detection of hybridized probes (NISH) using different fluorochromes („chromosome painting“)

Mitotic preparations

1. Choice of tissue with high mitotic activity

root cap, embryos, larvae, regenerating tissues

adult vertebrates: bone marrow, kidney, spleen, gonads, interstitial epithelium, corneal epithelium

sometimes subcutaneous stimulation, or intraperitoneal injection of phytohemagglutinin, pokeweed (*Phytolacca americana*), or active yeast suspension

Mitotic preparations

2. Stopping of mitotic divisions *in vivo* or *in vitro*

cytostatic: colchicine, colcemid, vinblastine

in vivo: advantage: cheaper, simpler
disadvantage: necessary to sacrifice

in vitro: peripheral blood cultivation (short-term) and fibroblasts (long-term)
advantage: possibility to synchronize cell divisions → reduction of variation in chromosome condensation, increased quality, reduced consumption of cytostatic
disadvantage: more laborious, expensive, time-consuming, fewer chromosomes

Mitotic preparations

3. Hypotonization of cells

0,075 M KCl solution, distilled water also possible

4. Fixation

Carnoy mixture = methanol : acetic acid (glacial) 3:1

multiple changes

(in squash preparations: ethanol instead of methanol)

Mitotic preparations

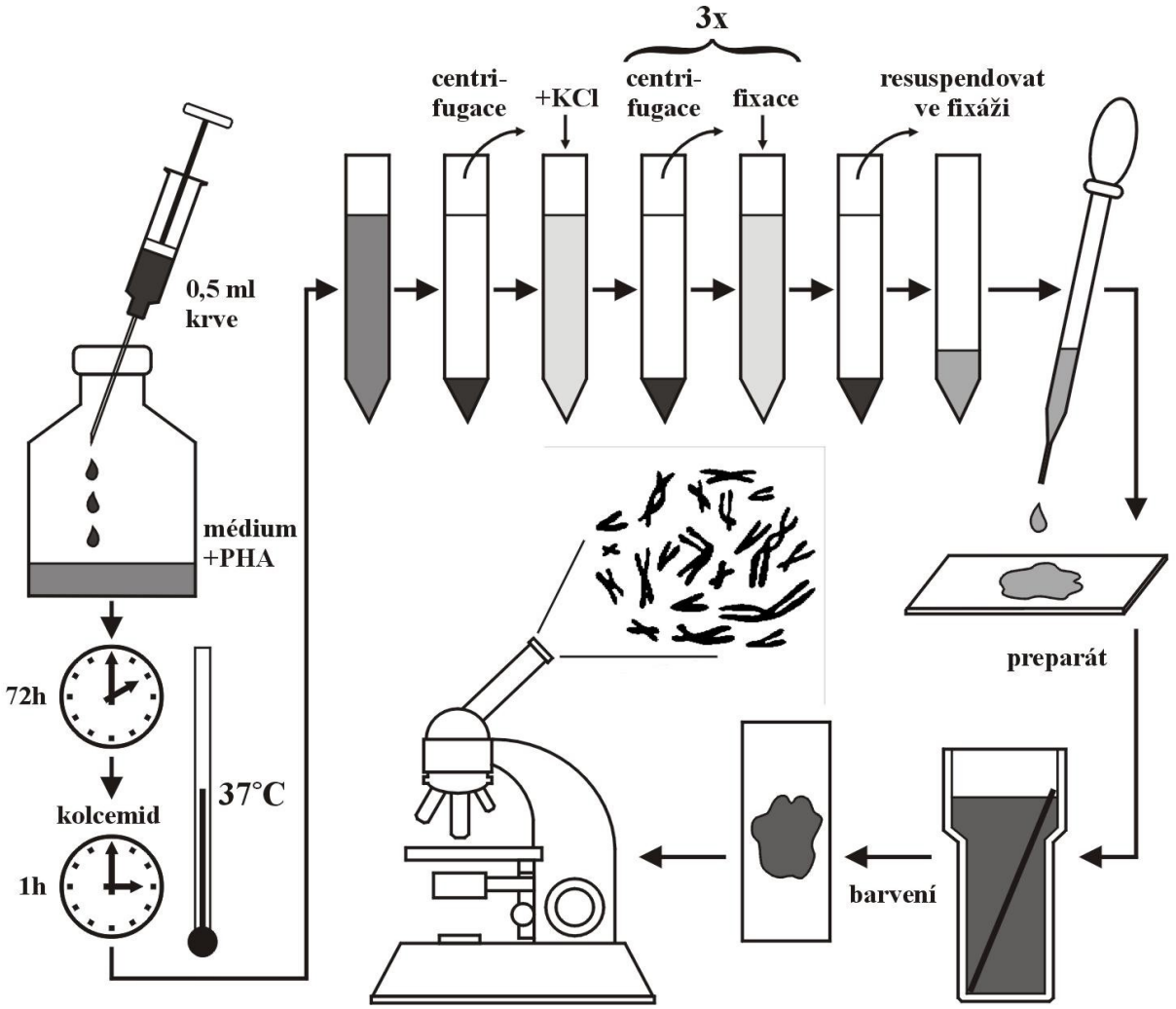
5. Slide preparation

2 basic techniques:

„squash“ (rozmačkání): maceration or gentle grinding of tissue pieces on a slide and squashing with silicone cover slip

„splash“ (nakapání): cell suspension is poured in drops onto a cover slip using Pasteur pipette → chromosome spread due to surface tension; after dripping either „air-drying“, or „flame-drying“

Blood cultivation



Meiotic preparations

testes, pollen mother cells

hypotonization with sodium citrate, procedure similar to mitotic preparation

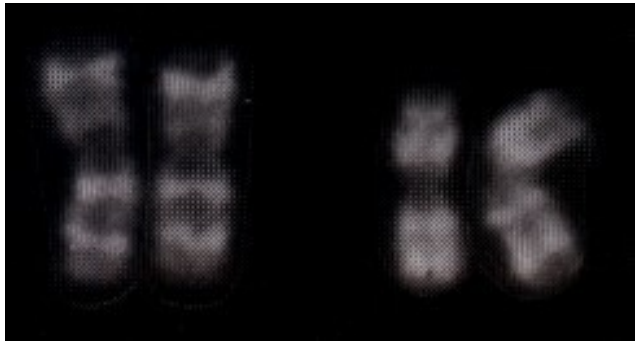
meiotic progress and importance of particular stages; synaptonemal complexes (SC), lampbrush chromosomes

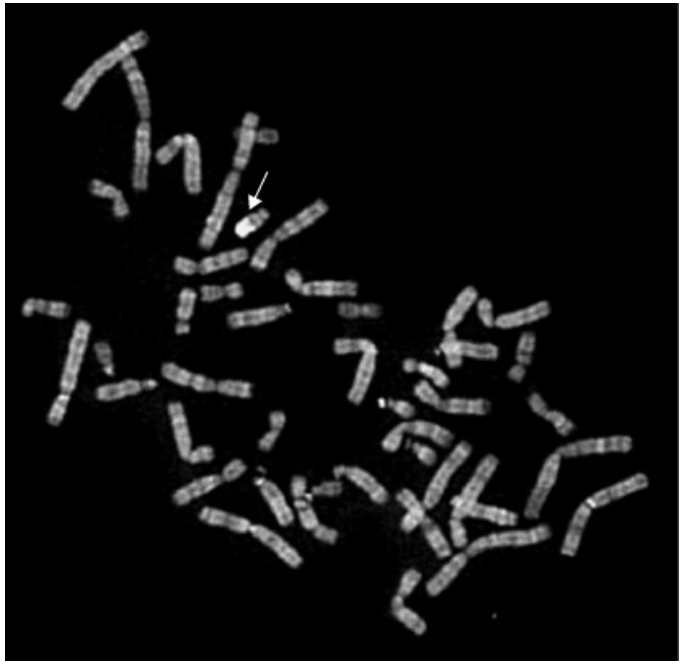
Differential staining - chromosome banding

Q-banding (quinacrine):

differential fluorochrome excitation and extinction depending on presence of AT bases

quinacrine staining, UV $\Rightarrow$ short period of visibility





Differential staining - chromosome banding

G-banding (Giemsa, GTG-banding):

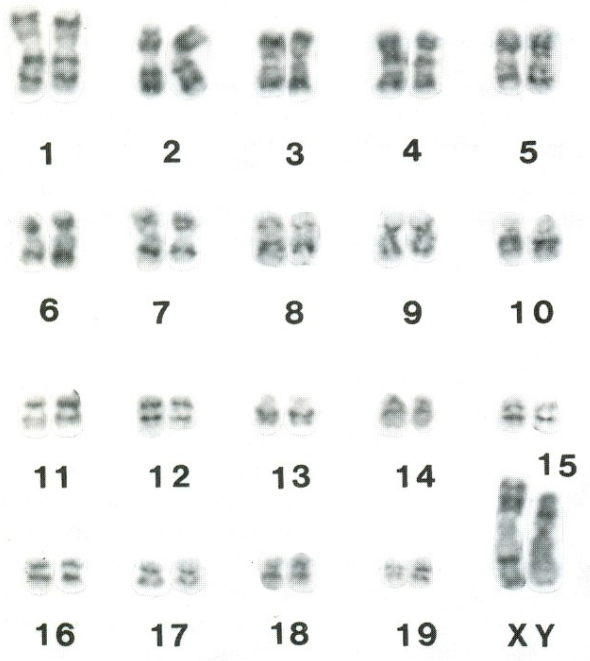
effect of denaturation agents on stability of protein and nucleic chromatin constituents

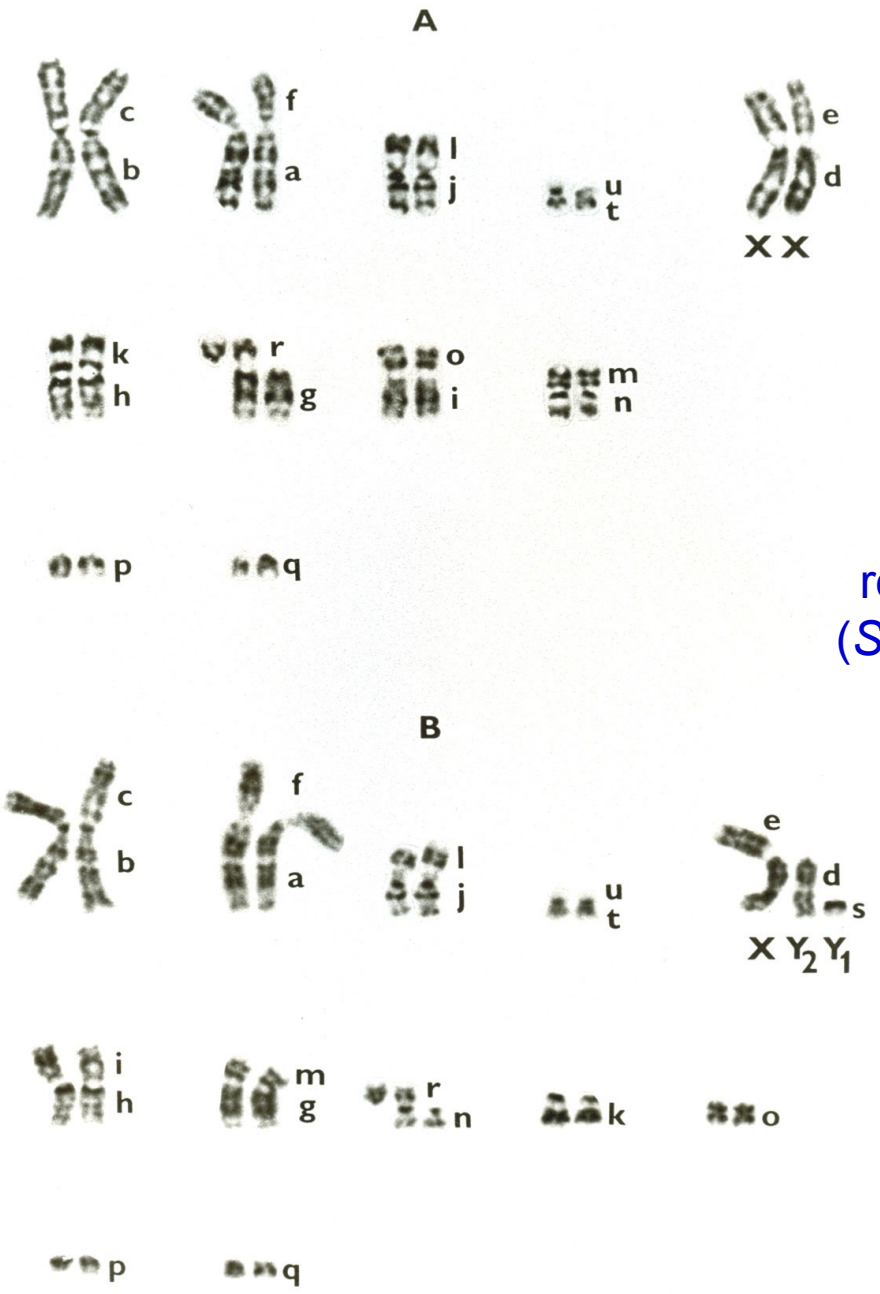
positive (dark) bands ≈ areas rich of AT bases (isochores)

effect of trypsin (chymotrypsin, NaOH)

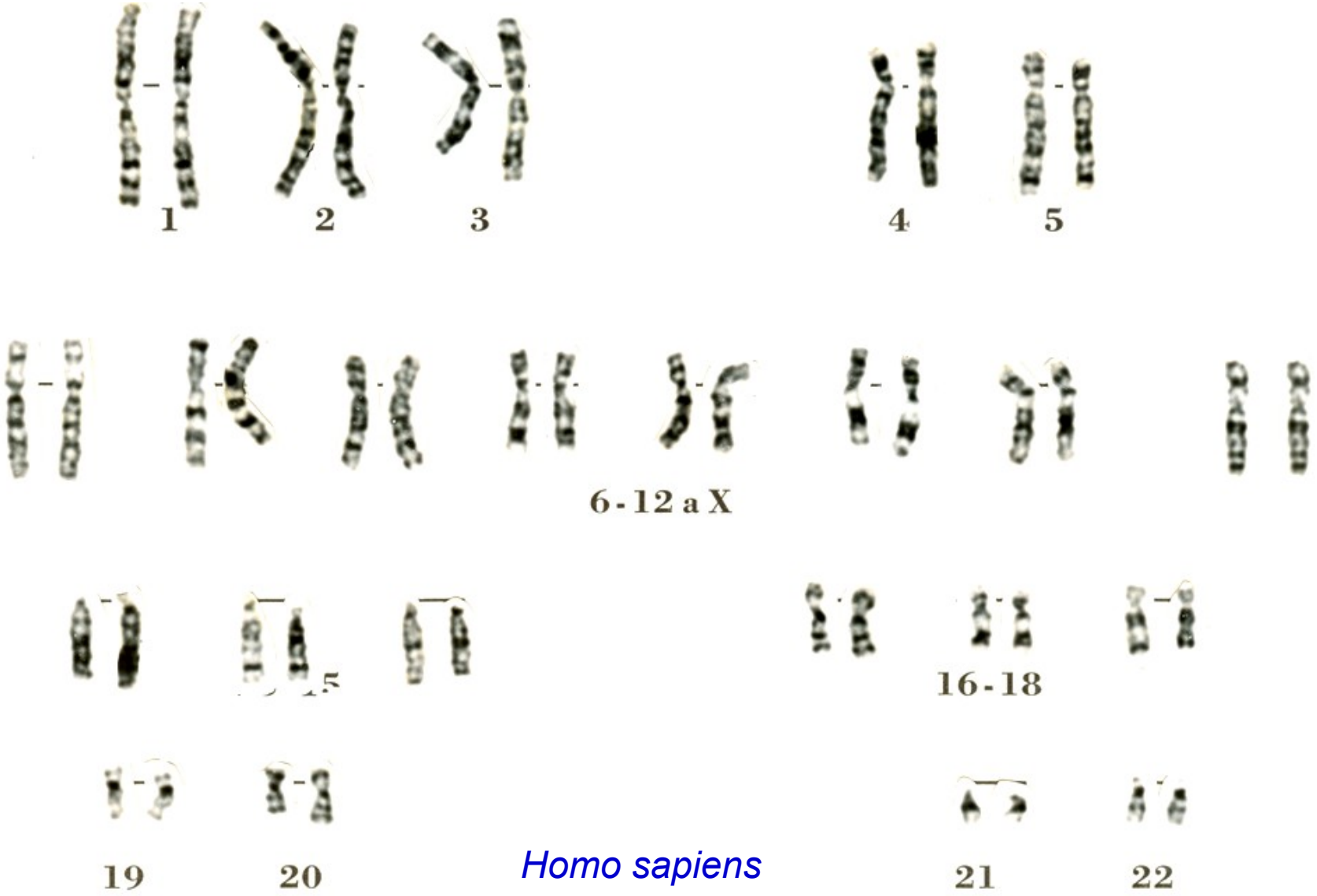
Giemsa staining

rypoš obří
(*Fukomys mechowi*)





rejsek obecný
(*Sorex araneus*)



Homo sapiens

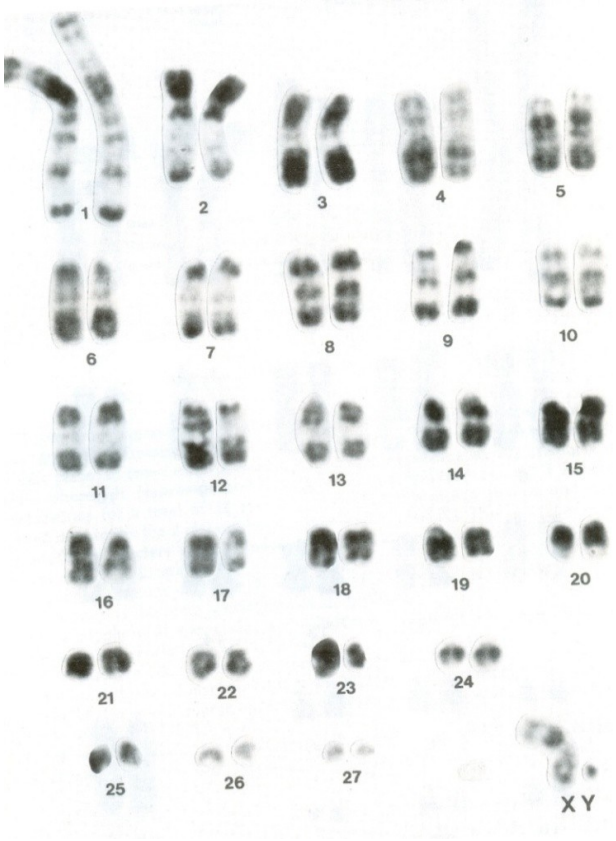
Differential staining - chromosome banding

R-banding (reverse banding):

denaturation by alcaline treatment at high temperature (80-90°C) followed by DNA renaturation

dark bands ≈ isochores rich of GC bases

Giemsa or acridine orange staining



Lemur catta

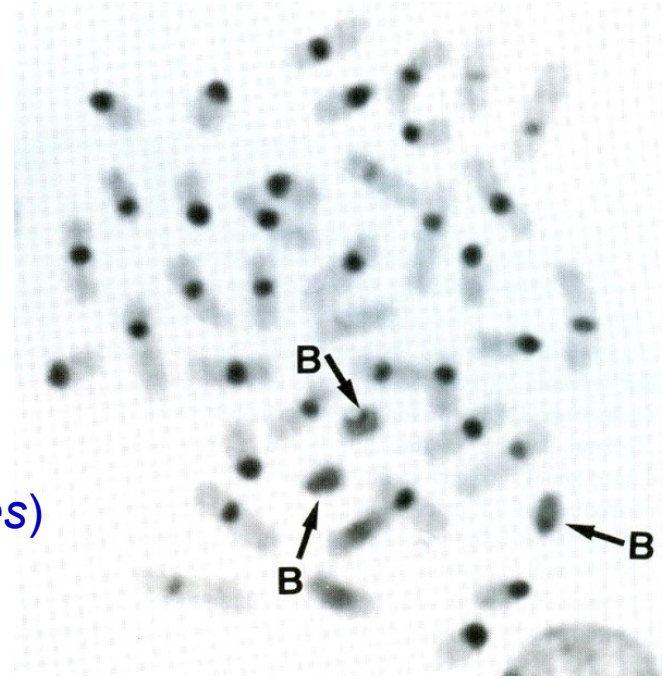
Differential staining - chromosome banding

C-banding (constitutive heterochromatin):

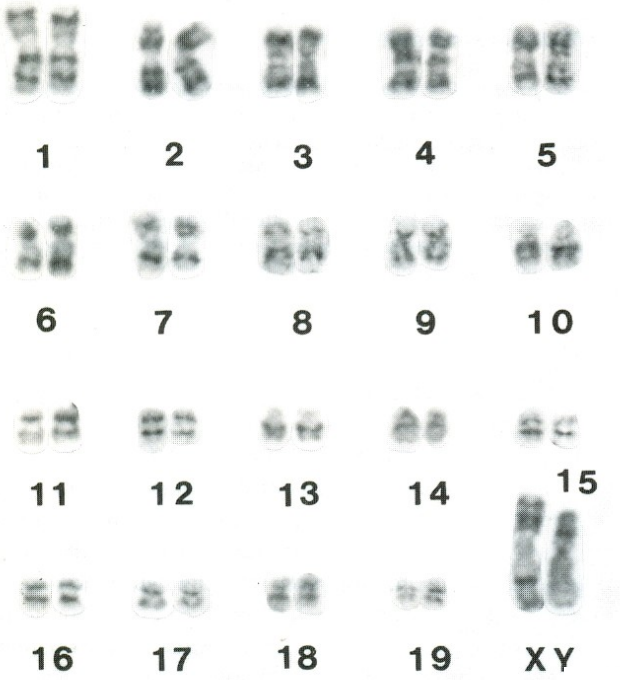
treatment first with strong acid (1M HCl), followed by alcaline ($Ba(OH)_2$) and heterochromatin renaturation in saline buffer (2×SSC) at high temperature (60°C)

euchromatin dissolving

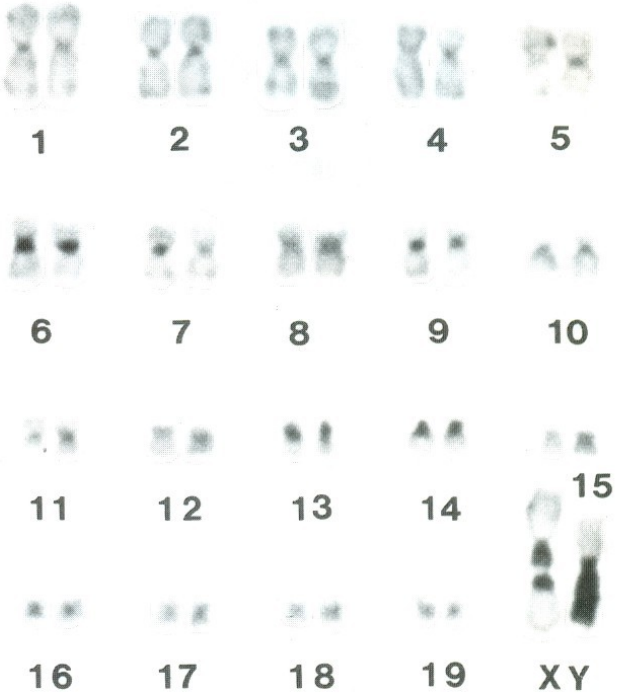
Giemsa staining
(visualization of satellite DNA)



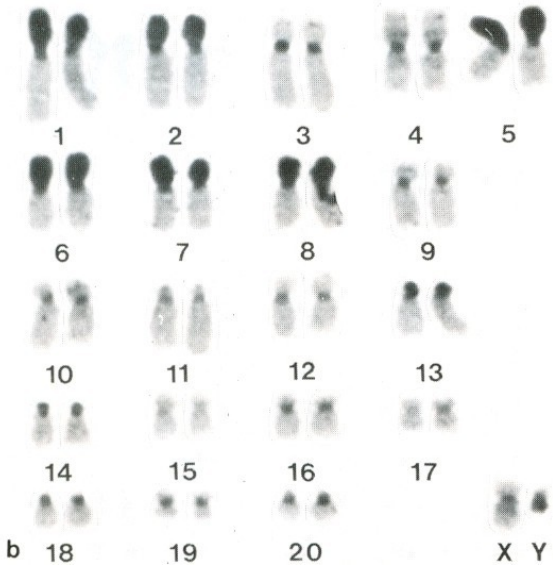
psík mývalovitý
(*Nyctereutes procyonides*)

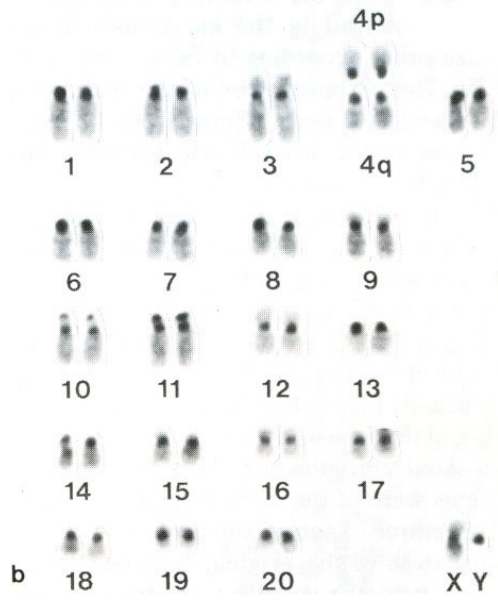


rypoš obří
(*Fukomys mechowi*)



kolčava a hranostaj

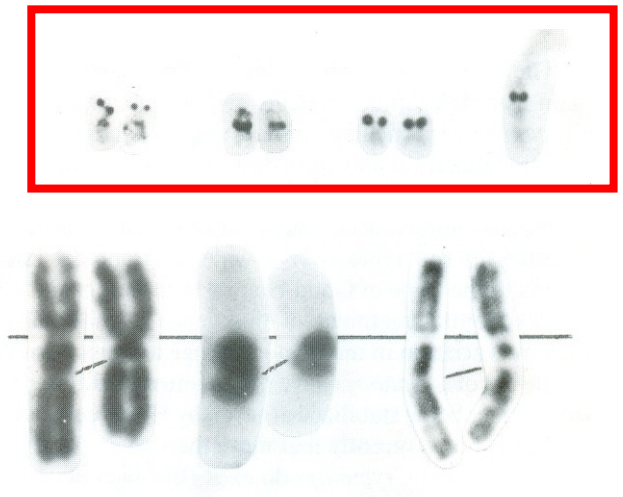




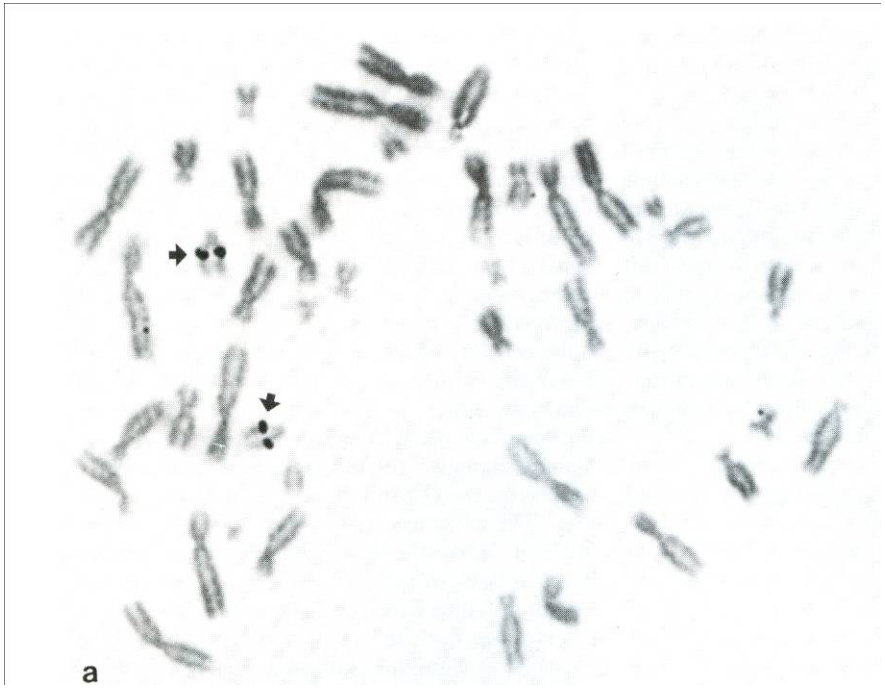
Differential staining - chromosome banding

Ag-NOR:

gelatine + formic acid, $AgNO_3$ staining
nucleolus organizer visualization (only active NORs)



rypoš obří
(*Fukomys mechowi*)

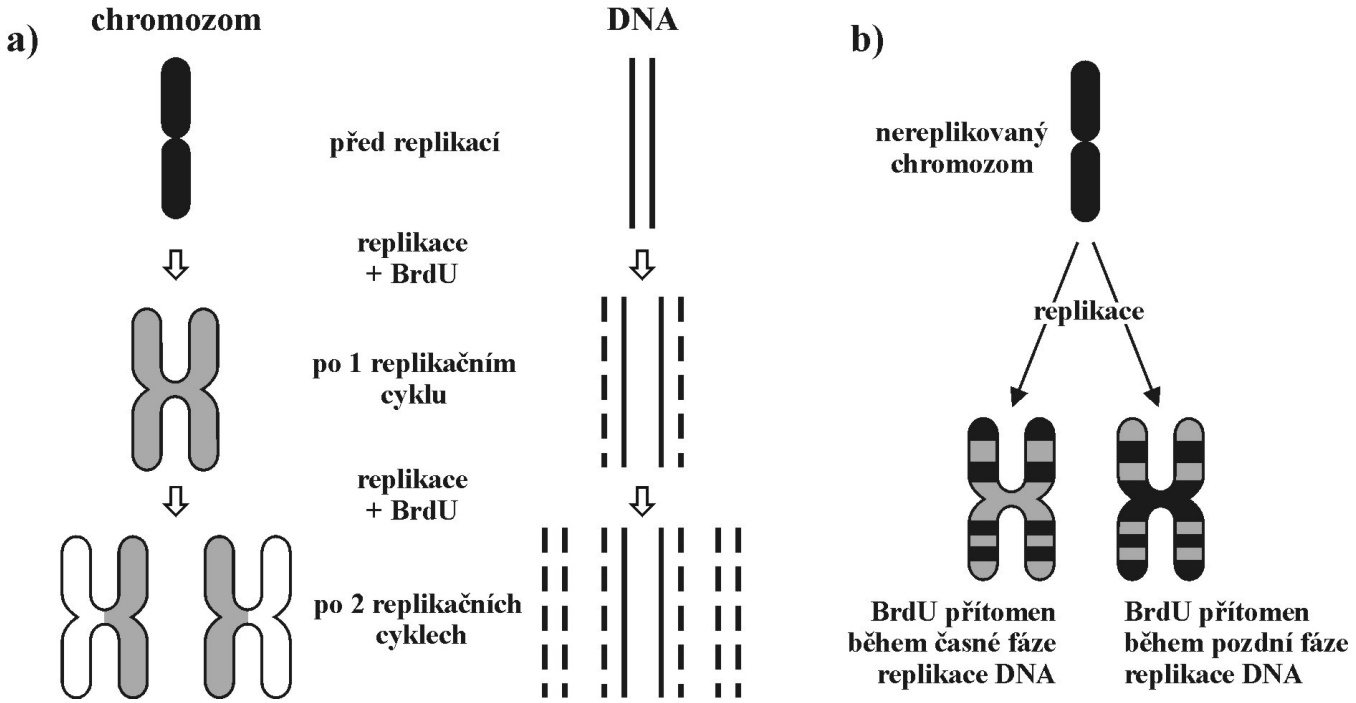


kolčava

Differential staining - chromosome banding

BrdU:

replication with artificial precursor (5-bromo-2´-deoxyuridine)
→ visualization of sister chromatid interchanges



Fluorescent *in situ* hybridization (FISH)

in situ hybridization of chromosomes with flurescently labelled probe

possibility of simultaneous application of several probes

visualization: antibodies specific for biotin (avidin, streptavidin) are conjugated either with fluorochrome (e.g. fluoresceine isothiocyanate, FITC), or enzymes (e.g. alcaline phosphatase, peroxidase), reaction with specific substrate

Fluorescent *in situ* hybridization (FISH)

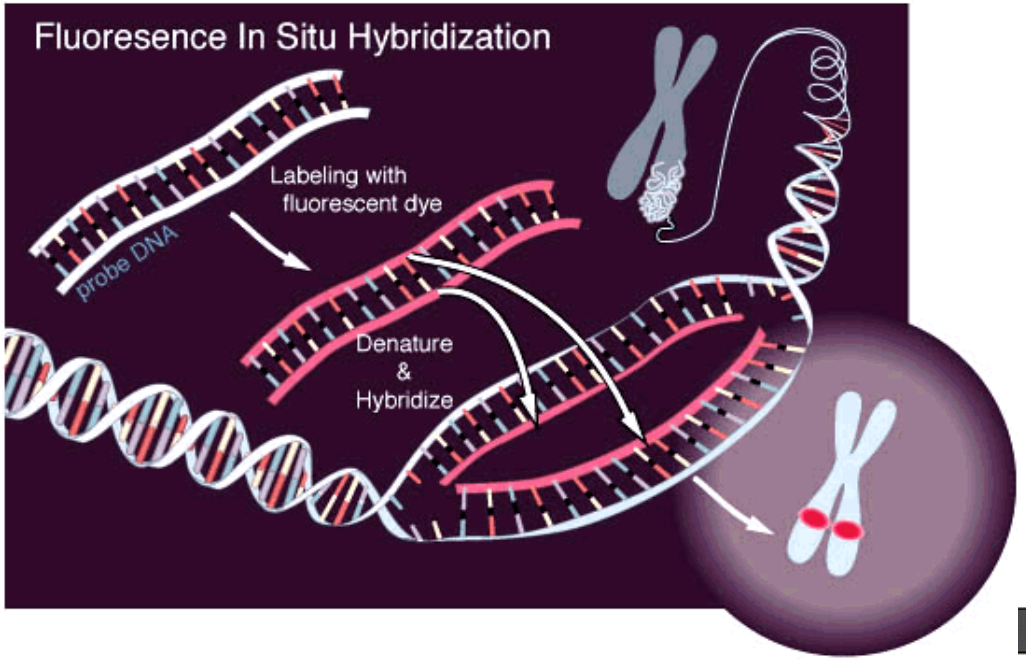
CISS, chromosome in situ supression hybridization

PRINS, primed *in situ* labelling

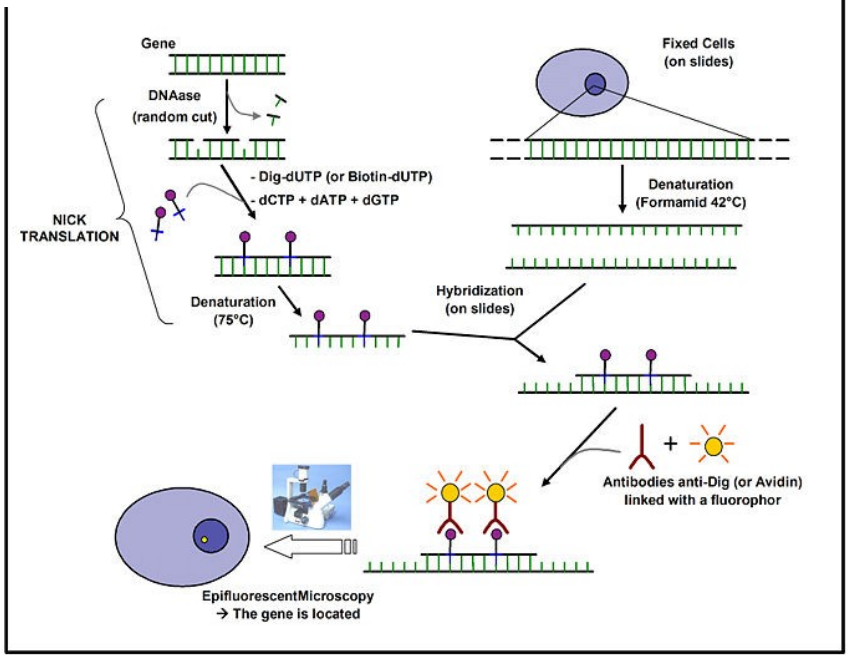
GISH, whole genome in situ hybridization

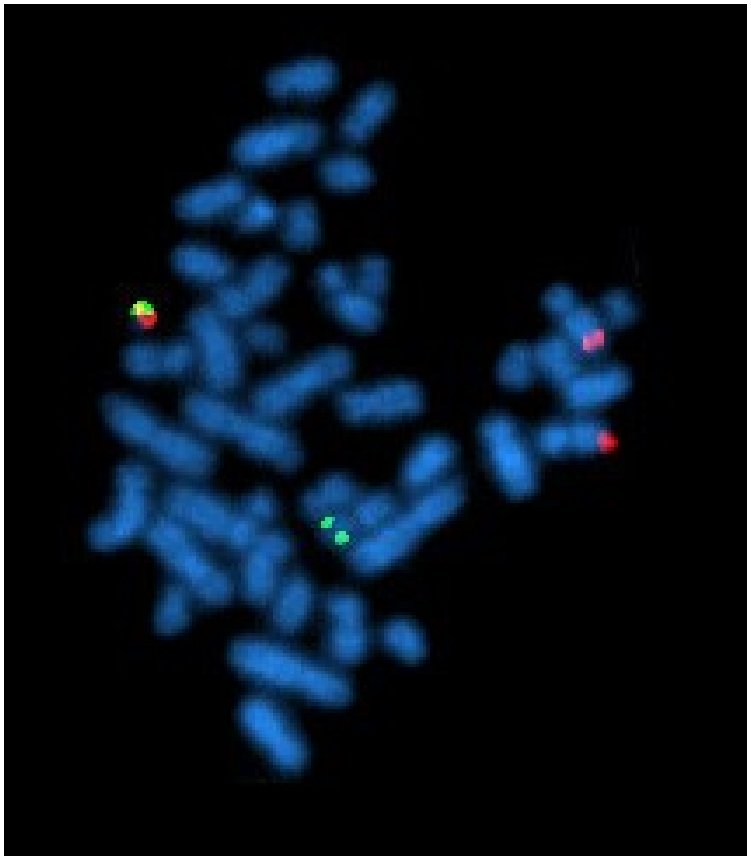
FACS, fluorescence activated cell sorting

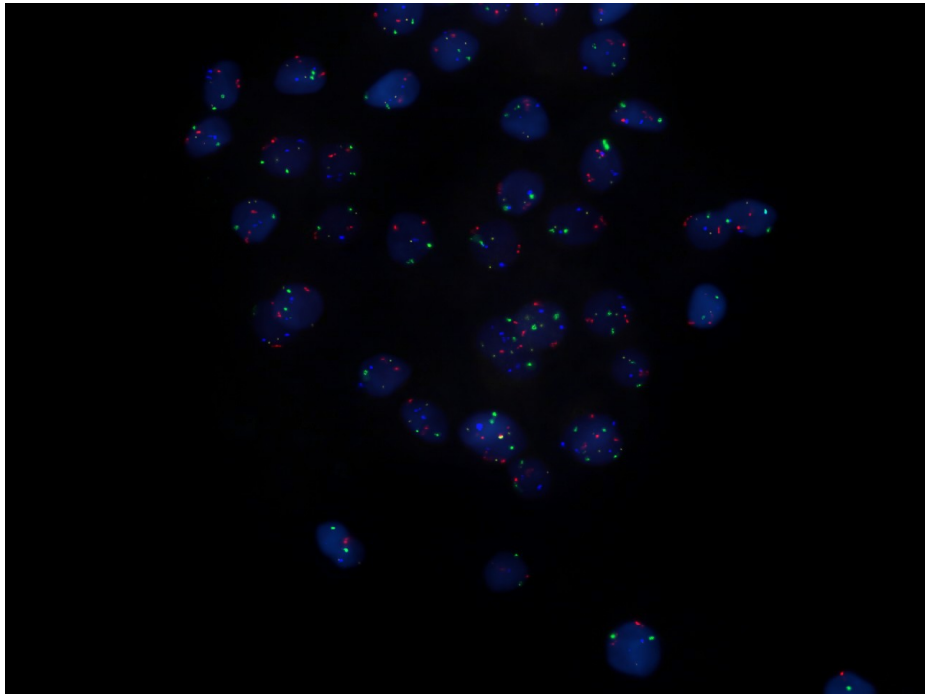
„chromosome painting“

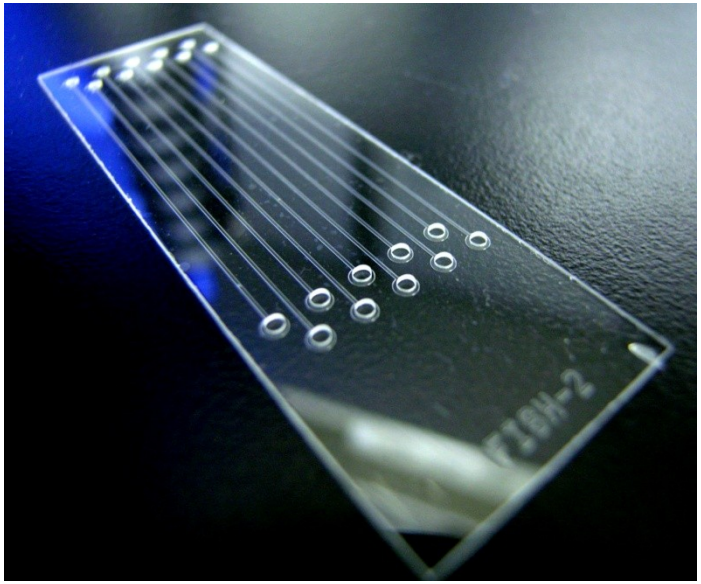


FISH (Fluorescent In Situ Hybridization)

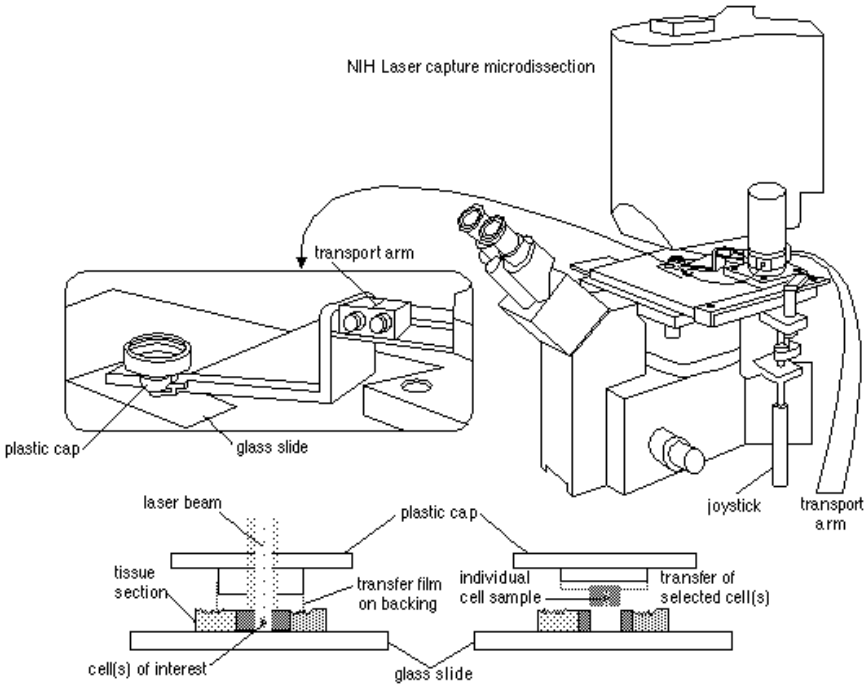


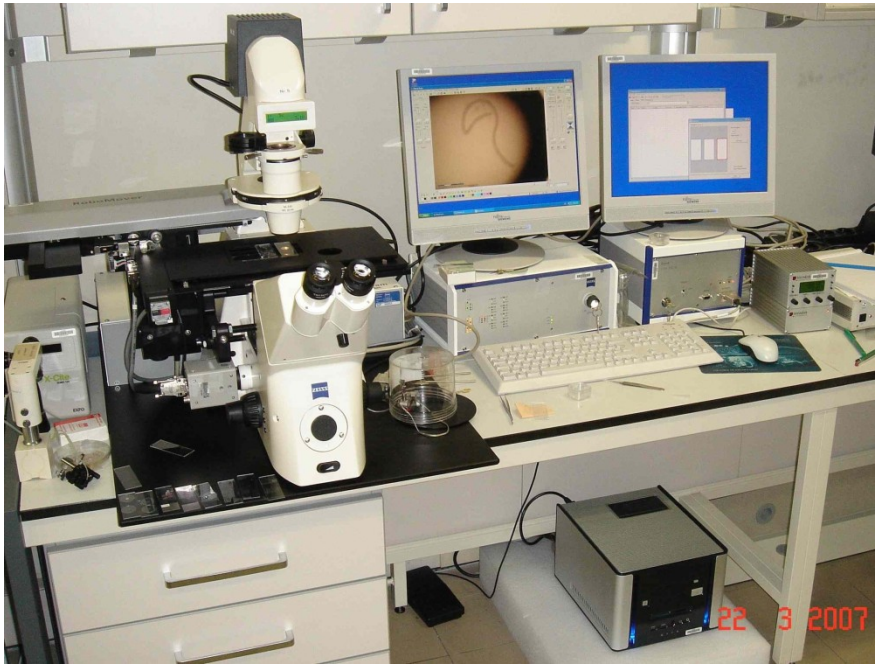


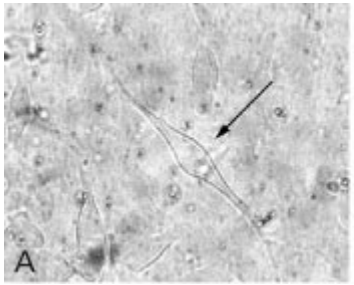


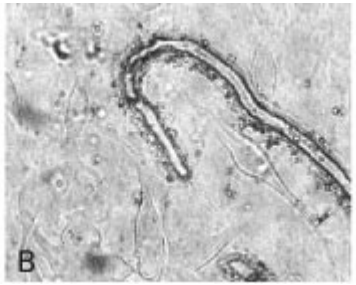

F18H-2

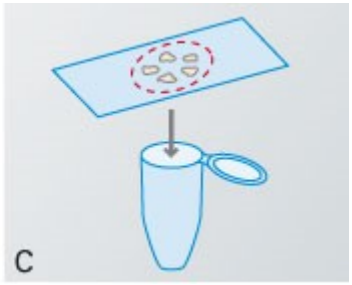
Microdissection

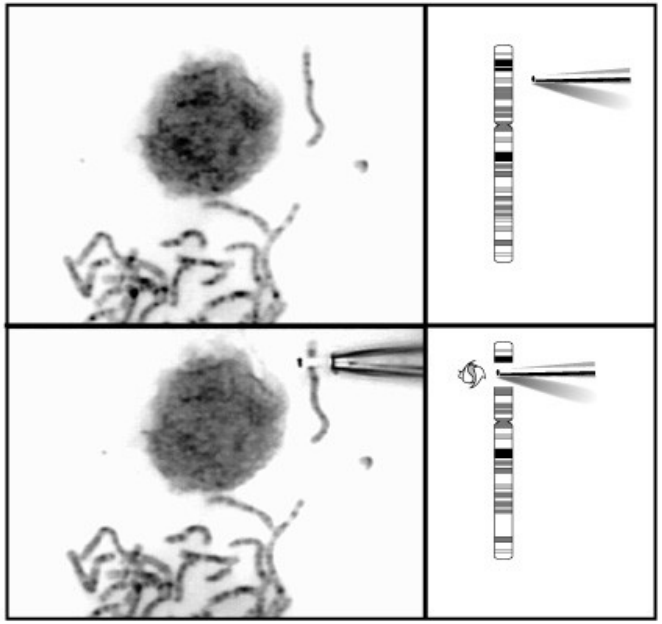






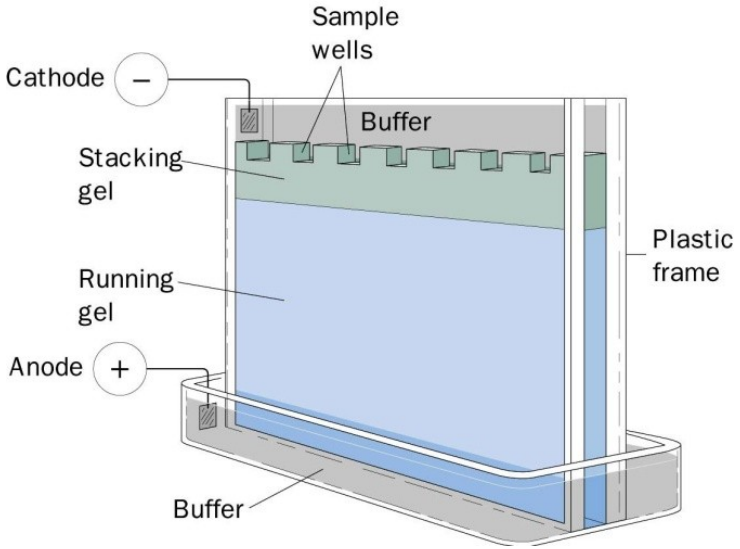


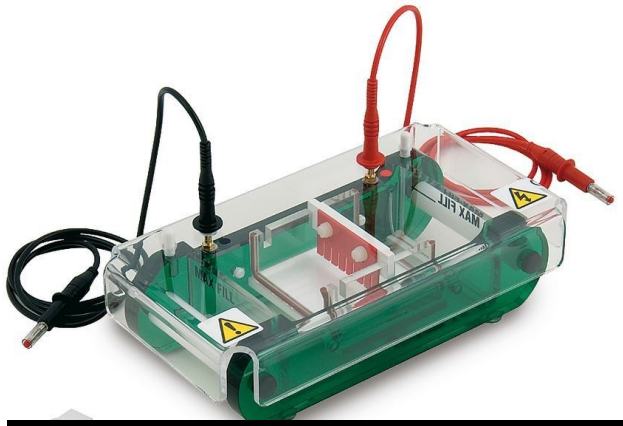


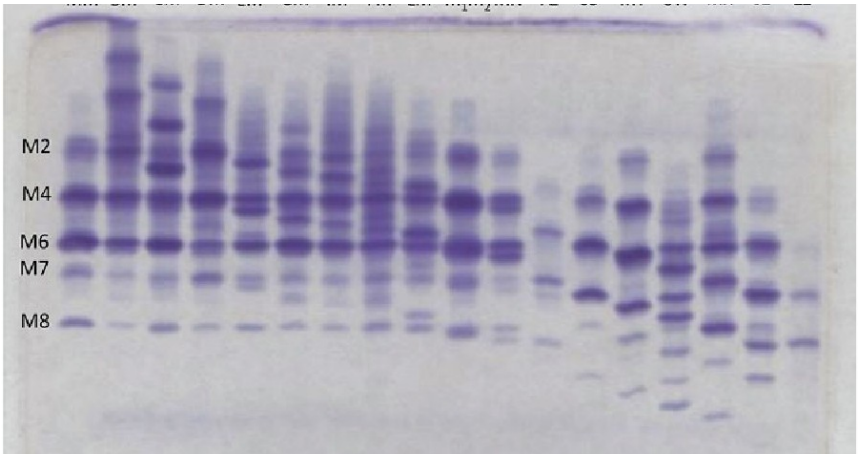


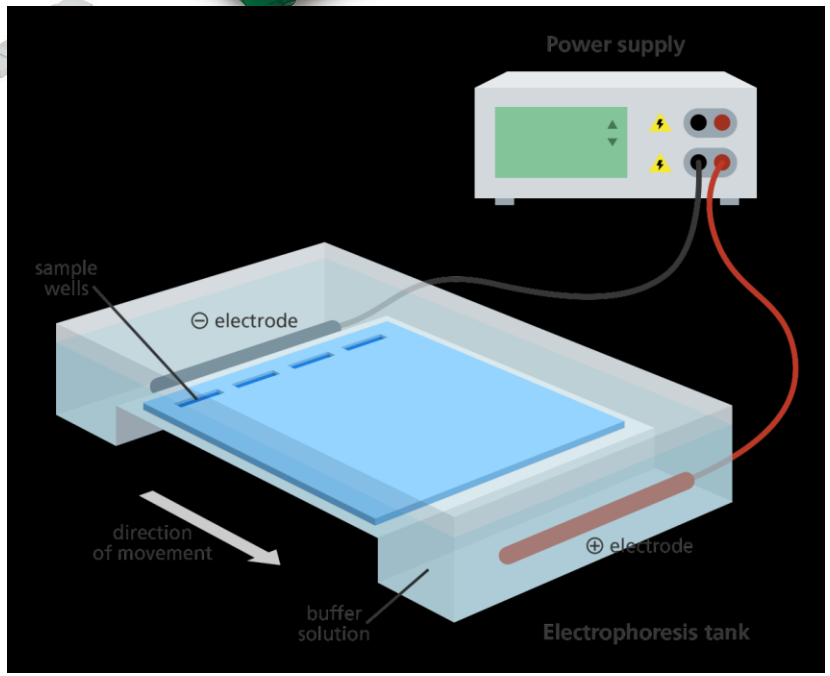
ELECTROPHORESIS

of enzymes and other proteins









electrophoresis: from Greek, "to bear electrons" = motion of particles under influence of electric field

until end of 1950´s, studies of genetic variation in natural populations only based on Mendelian morphological traits or polytene chromosomes → To what extent these traits represent real genetic variability in nature?

amino acid substitutions can be detected by sequencing – if this is impossible, we can use protein electrophoresis

of 20 AA, 3 bear positive charge (Arg, Lys, His), 2 negative charge (Asp, Glu)

besides charge, also macromolecule size and conformation (-S-S- bridges, van der Waals forces, hydrogen bonds, electrostatic forces); buffer pH

electric charge stabilization → specific buffer of high ionic strength and pH as different from given protein´s pI*) as possible: pH 3-10, most often pH 6,5-9,5

charge of most proteins at pH 8-9 negative → migration to anode

*) isoelectric point

Principle of electrohoresis known since end of 19th century

1937 - Thisselius: „moving boundary“method

1949 - Linus Pauling: filter paper - abnormal Hb (sickle cell anemia)

1955 - O. Smithies: starch

1957 - Hunter & Moeller: employment of catalytical abilities of enzymes (histochemical staining)

1966 – application on natural populations: Harry Harris (humans), Richard Lewontin & John Hubby (fruit fly)

Media (gels):

starch (SGE): molecule size + charge

cellulose acetate (CAGE): charge

agar, agarose (AGE): charge

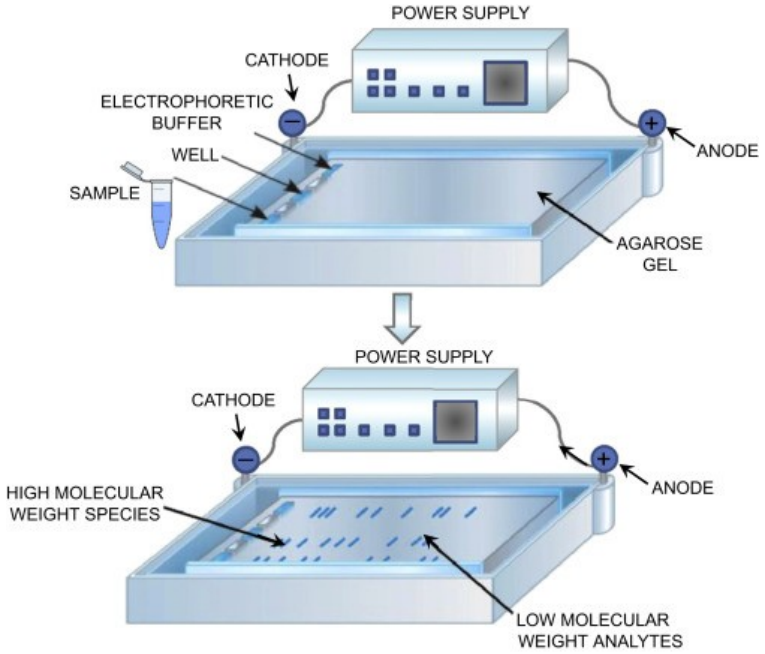
polyacrylamide (PAGE): molecule size + charge

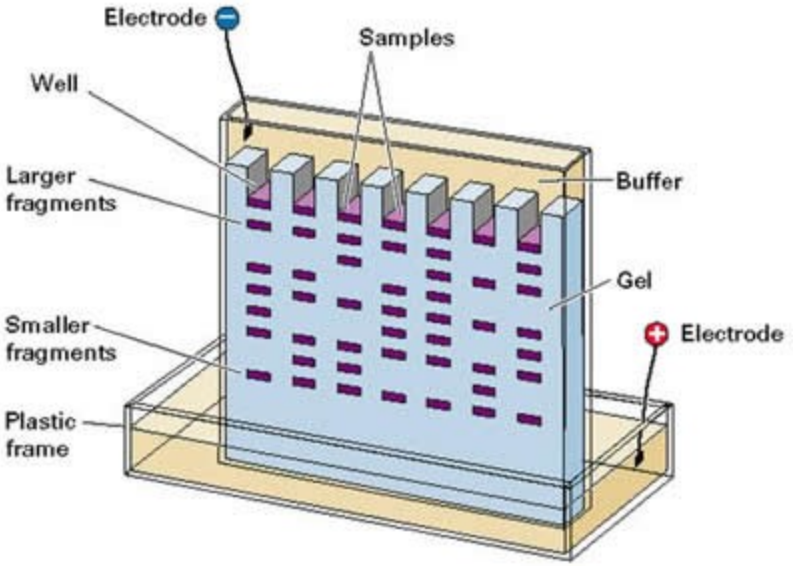
Electrophoretic methods

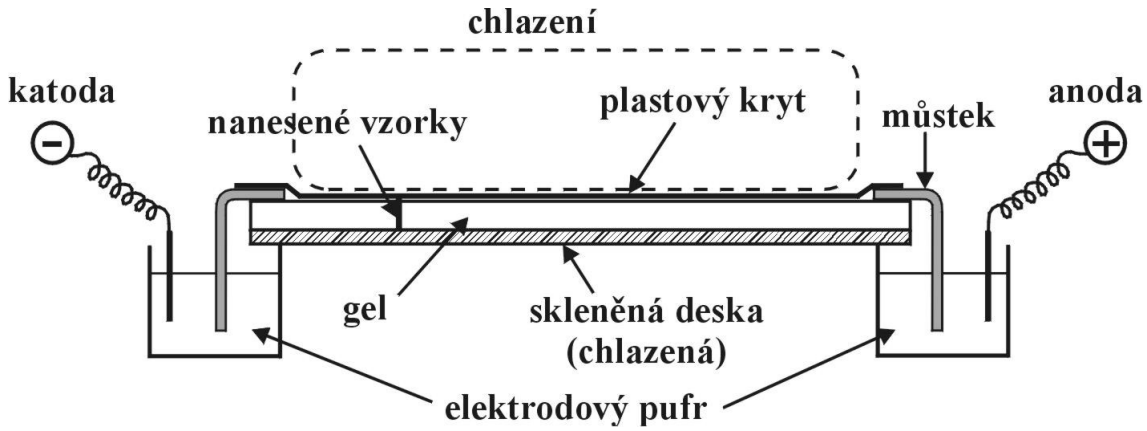
horizontal

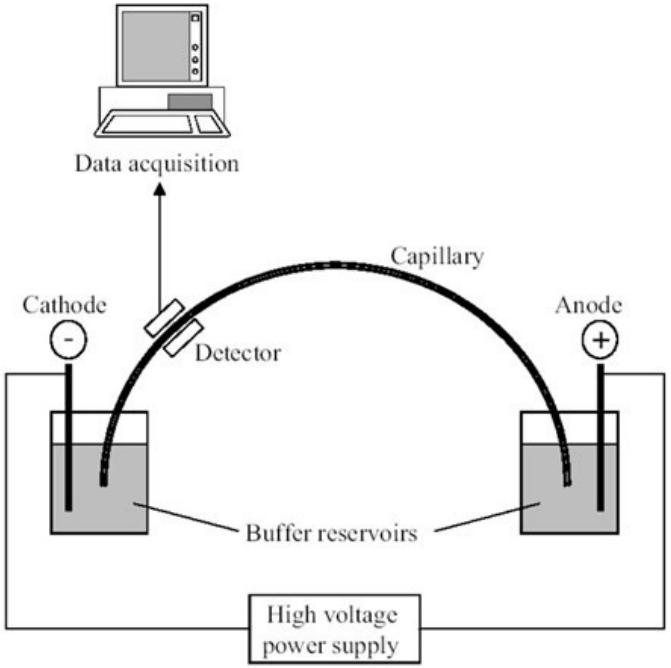
vertical

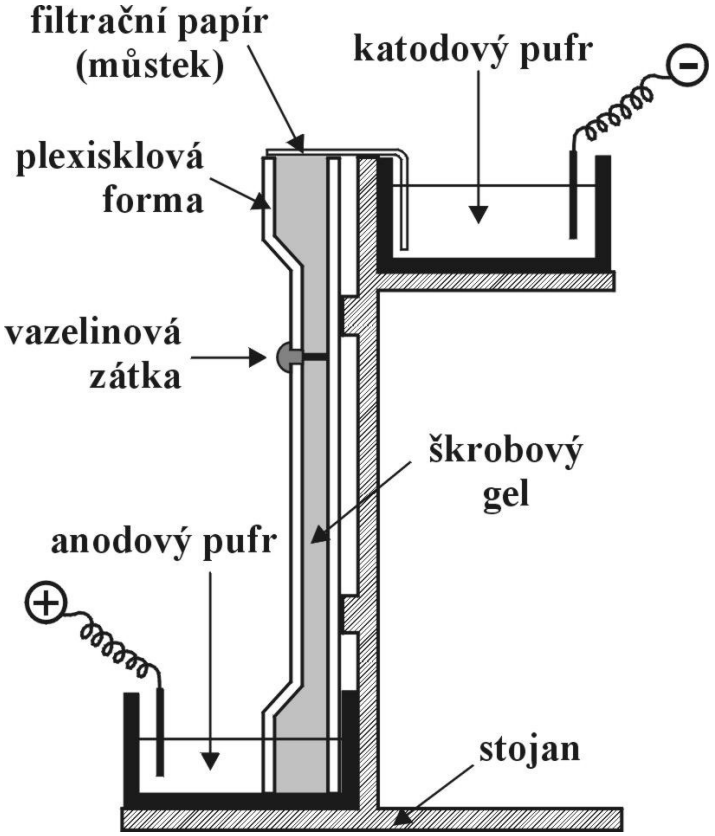
capillary






chlazení
katoda
nanesené vzorky
plastový kryt
můstek
anoda
gel
skleněná deska
(chlazená)
elektrodový pufr


Data acquisition
Capillary
Cathode
Anode
Detector
Buffer reservoirs
High voltage
power supply

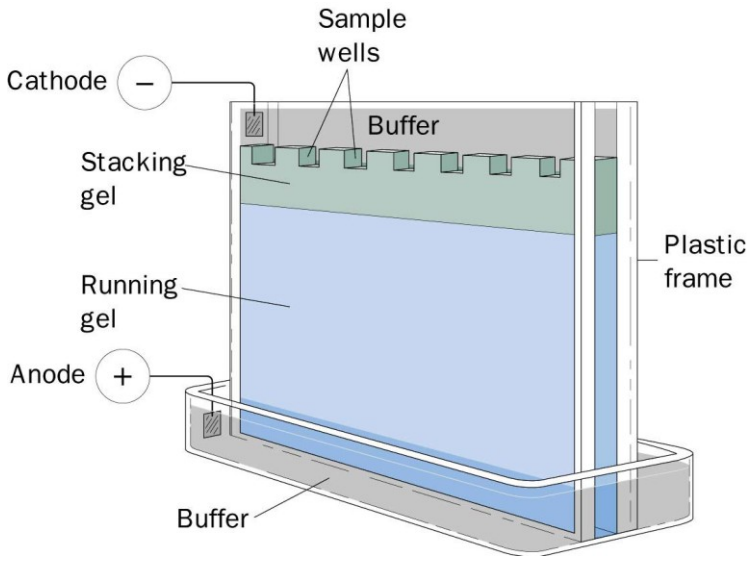

filtrační papír
(můstek)
katodový pufr
plexisklová
forma
vazelinová
zátka
škrobový
gel
anodový pufr
stojan

Electrophoretic methods

1. ELFO in continuous buffer

2. ELFO in discontinuous buffer (multiphasic ELFO):

2 gels of different concentrations - concentrating and separating gels
protein „sandwiching“ on boundary between „leading“ a „dragged“ ions;
on its own = isotachophoresis

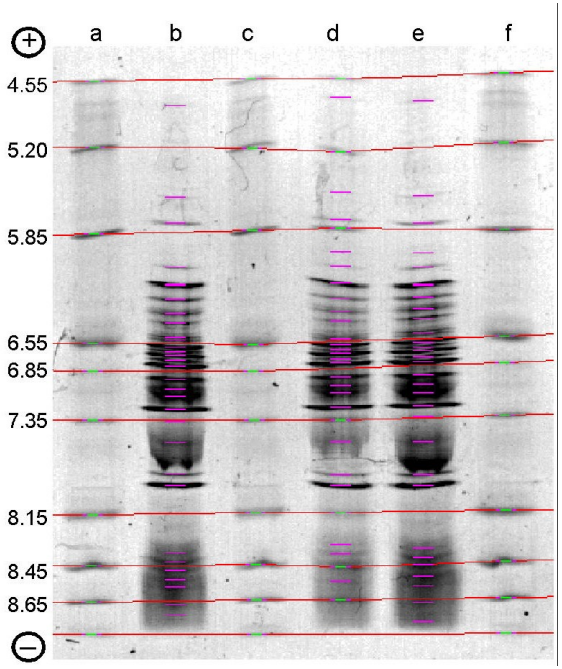


3. Isoelectric focusing, IEF:

= separation of molecules by differences in their isoelectric points

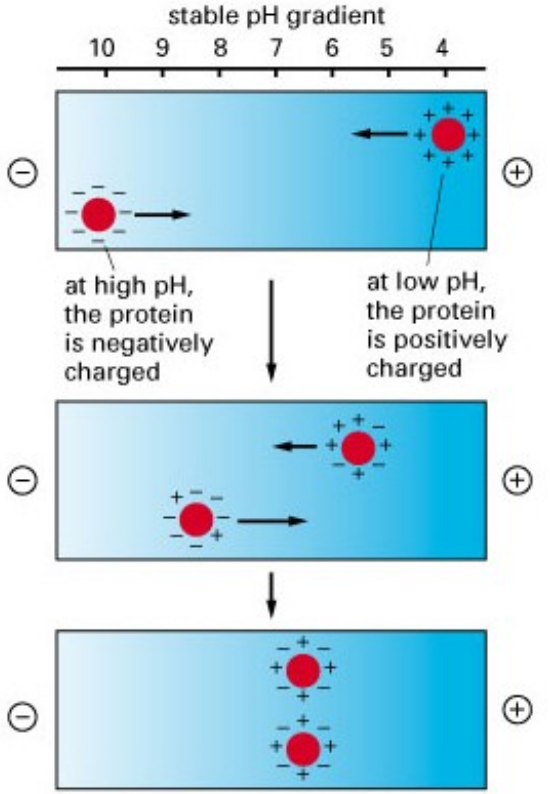
solution of ampholytes (syntetic polyamino polycarbonates) with a range of pI put in gel; in electric field → stable pH gradient; ampholytes kept in gel by strong acid at anode and strong alcali at cathode

molecules stop where zero charge (pI point)



ISOELECTRIC FOCUSING

For any protein there is a characteristic pH, called the isoelectric point, at which the protein has no net charge and therefore will not move in an electric field. In isoelectric focusing, proteins are electrophoresed in a narrow tube of polyacrylamide gel in which a pH gradient is established by a mixture of special buffers. Each protein moves to a point in the gradient that corresponds to its isoelectric point and stays there.



The protein shown here has an isoelectric pH of 6.5.

4. urea and SDS ELFO:

SDS = sodium dodecyl sulphate (= anion detergent):

can dissolve some proteins and cleave some polymers

SDS brings about strong charge of proteins, migration only based on molecular weight

urea: similar to SDS, but protein charge normal – migration based on total charge

(likewise, proteins can be denatured by increased teperature → ELFO)

5. Two-dimensional (2-D) ELFO:

electric field applied first in one direction and then perpendicularly

e.g. 1. stage = IEF, 2. stage = SDS ELFO – combination of pI and molecular weight

Electrophoretic methods

Ability to separate blood plasma proteins:

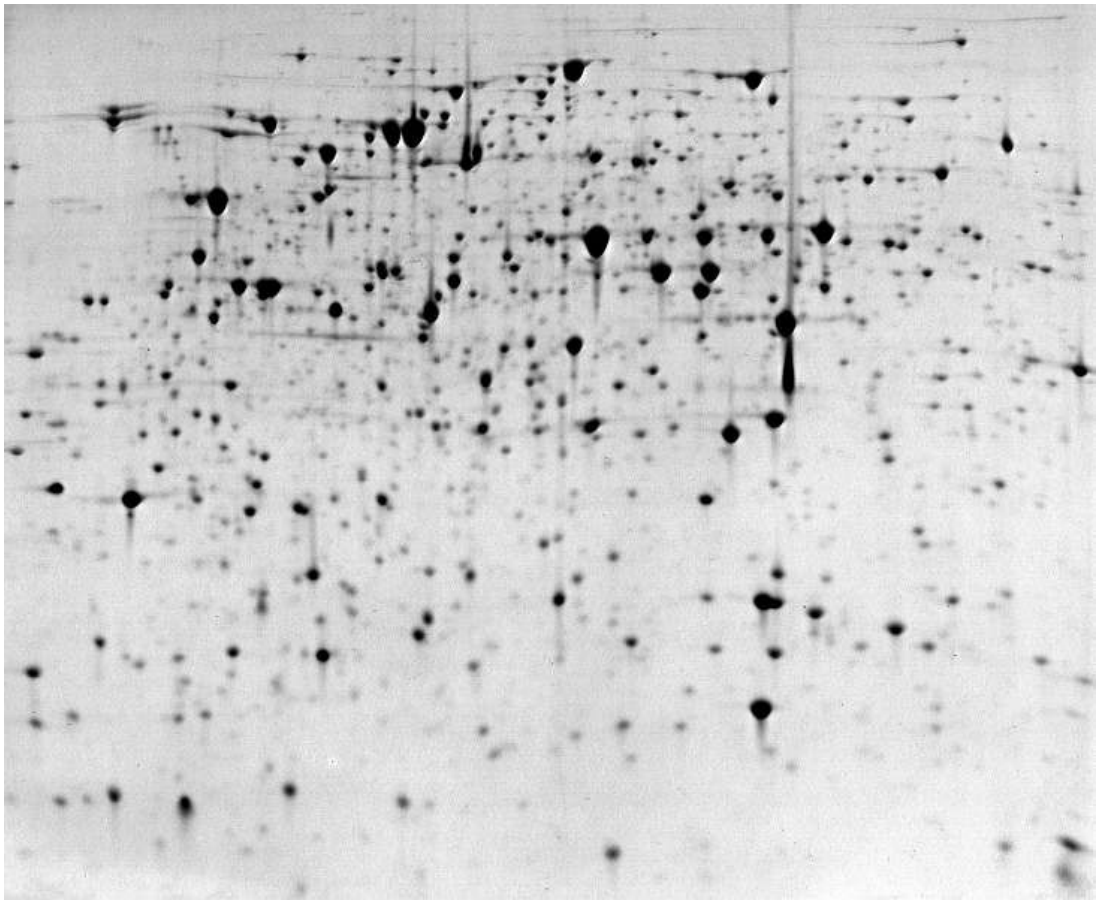
CAGE: 5 bands

SGE: 15 bands

PAGE: 19 bands

IEF > 30 bands

2-D ELFO ~300 spots
≈75-100 polypeptides



Protein detection

non-specific:

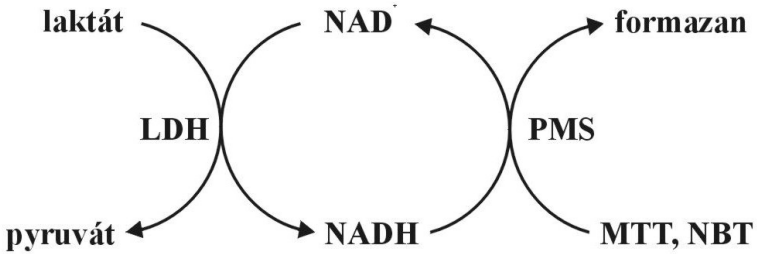
amido black, Coomassie Brilliant Blue R

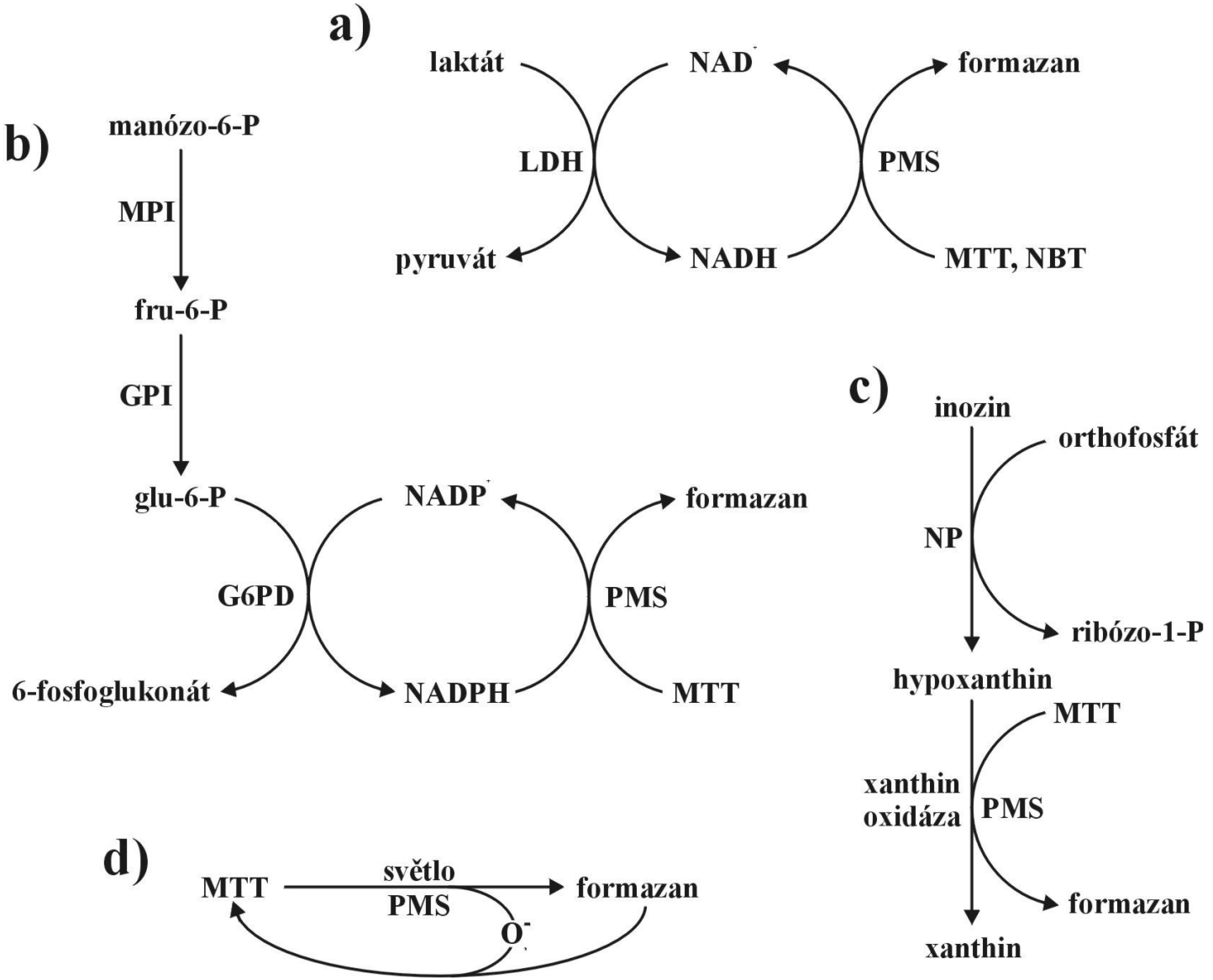
specific:

dyes for glycoproteins, lipoproteins

histochemical staining of enzymes: catalysis of specific substrate processing connected with staining reaction

- nitro tetrazolic salts (MTT, NBT) + PMS (phenazine methosulphate); Fast Blue RR; Fast Garnett GBC, Fast Black K
- reduction of NAD^+, $NADP^+$
- sometimes necessary to add other enzymes




a)
laktát
NAD
formazan
LDH
PMS
pyruvát
NADH
MTT, NBT
b)
manózo-6-P
MPI
fru-6-P
GPI
glu-6-P
NADP
formazan
G6PD
PMS
6-fosfoglukonát
NADPH
MTT
c)
inozin
orthofosfát
NP
ribózo-1-P
hypoxanthin
MTT
xanthin oxidáza
PMS
formazan
xanthin
d)
světlo
MTT
formazan
PMS
O-

stained gel = in general electrophoretogram,
if enzymes specifically stained = zymogram (enzymogram)

bands = „electromorphs“, „alelles“, „alellomorphs“

isozymes, allozymes