SVĚTELNÁ A FLUORESCENČNÍ MIKROSKOPIE

Mgr. Michal Franek
Oddělení molekulární cytologie a cytometrie

OSNOVA PŘEDNÁŠKY

1. Historie
2. Světelná mikroskopie a její modifikace
3. Fenomén fluorescence
4. GFP a Spinach
5. Konfokální fluorescenční mikroskopie
6. Imunocytochemické techniky, FISH, FRAP, FRET, live cell imaging
7. Superrezoluční fluorescenční mikroskopie

Kdy vznikl první mikroskop?

Antonie van Leeuwenhoek, Single-lens microscope, 200x magnification

Robert Hooke, Micro-graphia, 1667,
“Microscopium compositum”

Sabas Hoff
31st January 1864

MICROGRAPHIA:

OR SOME

Physiological Descriptions

OF

MINUTE BODIES

MADE BY

MAGNIFYING GLASSES.

WITH

OBSERVATIONS and INQUIRIES thereupon.

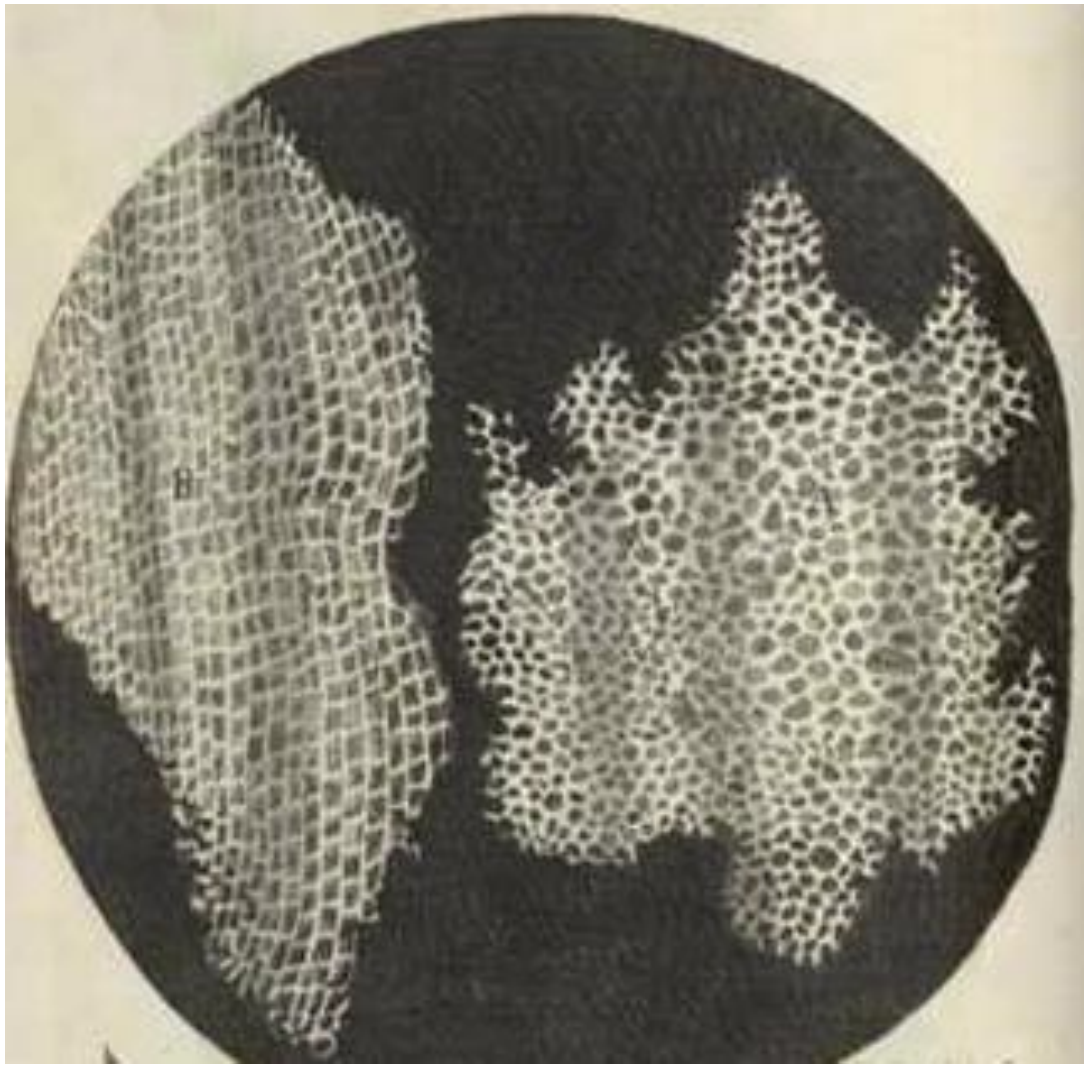
By *R. HOOKE*, Fellow of the ROYAL SOCIETY.

Non possis oculo quantum contendere Lynceus,
Non tamen idcirco contemnas Lippus inungi. Horat. Ep. lib. 1.

LONDON, Printed by *Jo. Martyn*, and *Ja. Allestry*, Printers to the ROYAL SOCIETY, and are to be sold at their Shop at the sign of S. *Paul's* Church-yard. MDC LXV.

Notes

Pozorování buněk v microscopum compositum, R. Hooke



This is Hooke's drawing of the microscopic structure of cork, showing in great detail the cell walls neatly separating the tiny sub-divisions of the plant. Until Hooke's observations, the cellular structure of living matter was unknown. It was in fact Hooke who first used the word "cell," likening what he saw through his microscope to a monk's cell in a monastery.

http://archive.nlm.nih.gov/proj/ttp/flash/hooke/hooke.html

SVĚTELNÁ MIKROSKOPIE

1. Pozorování ve světelném poli
2. Optické kontrastní metody
    1. Fázový kontrast
    2. Diferenciální interferenční kontrast
3. Pozorování v tmavém poli

CHARAKTERISTIKA OBJEKTIVŮ – NUMERICKÁ APERTURA

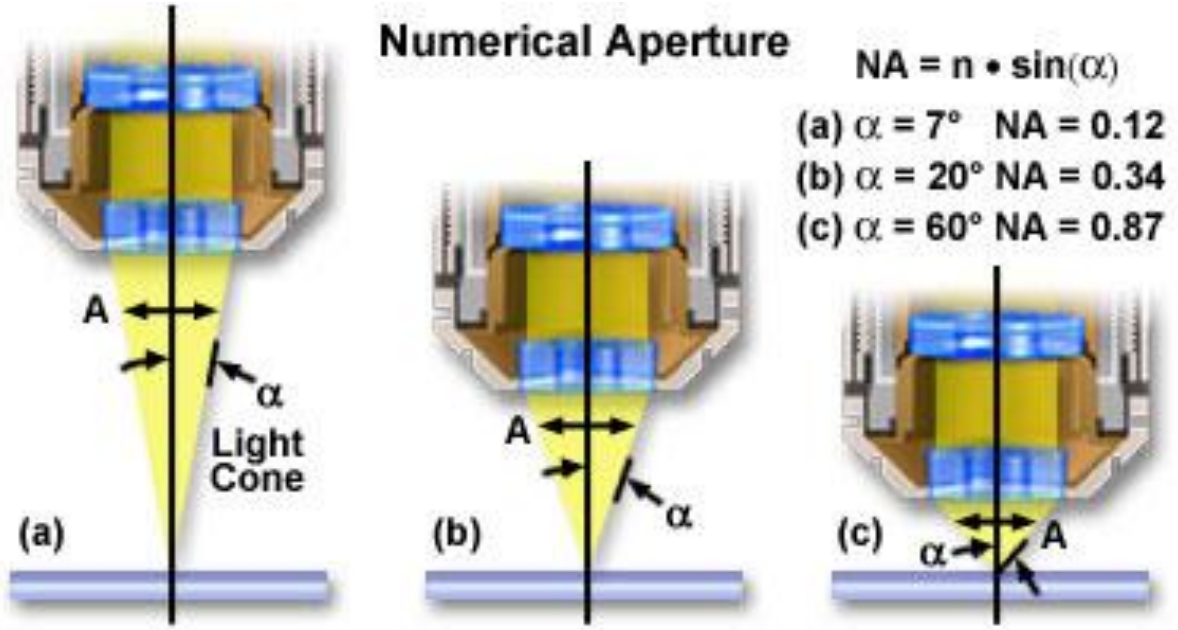


Figure 1

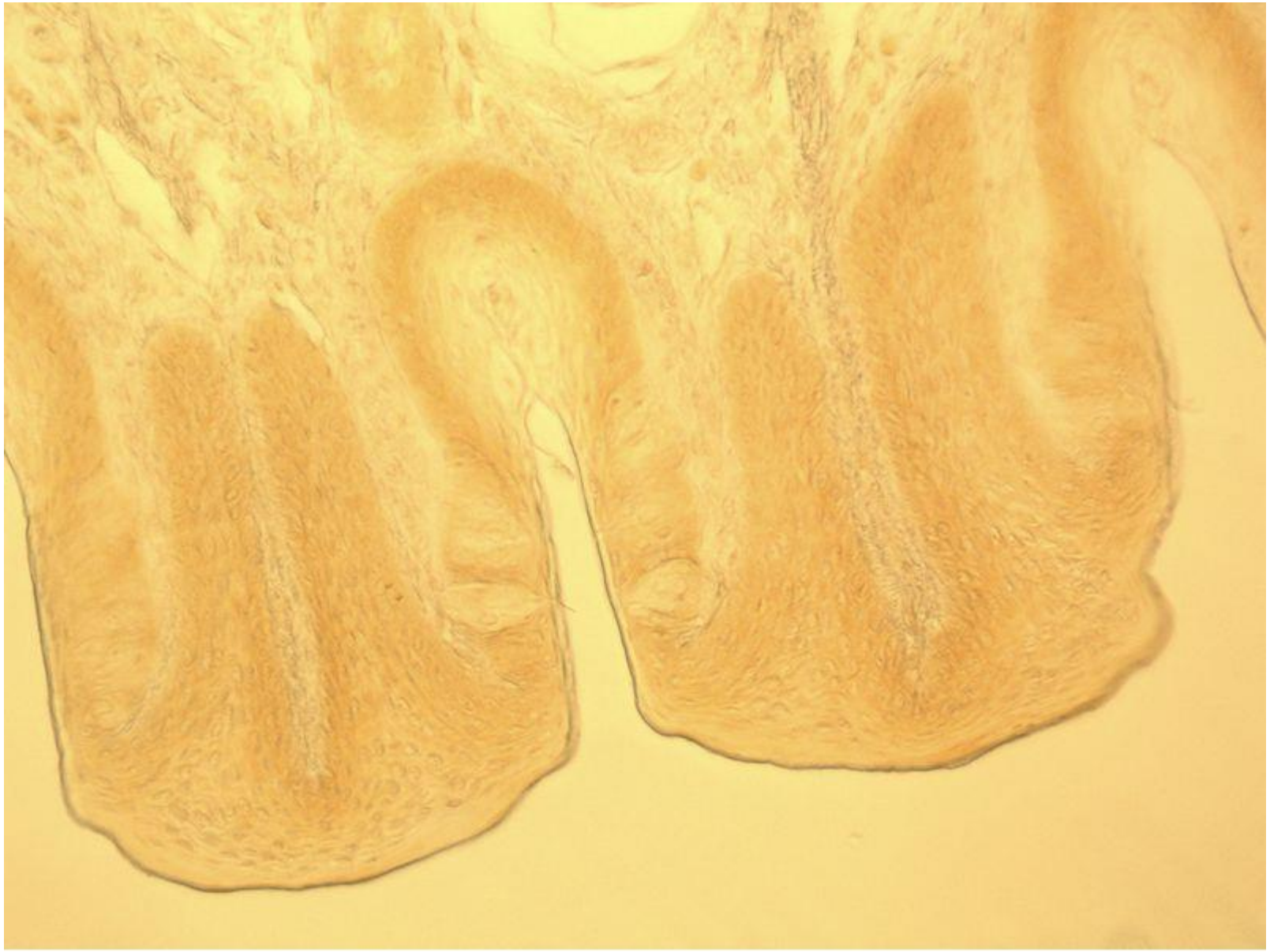
Rozlišení, R, objektivu je definováno jako

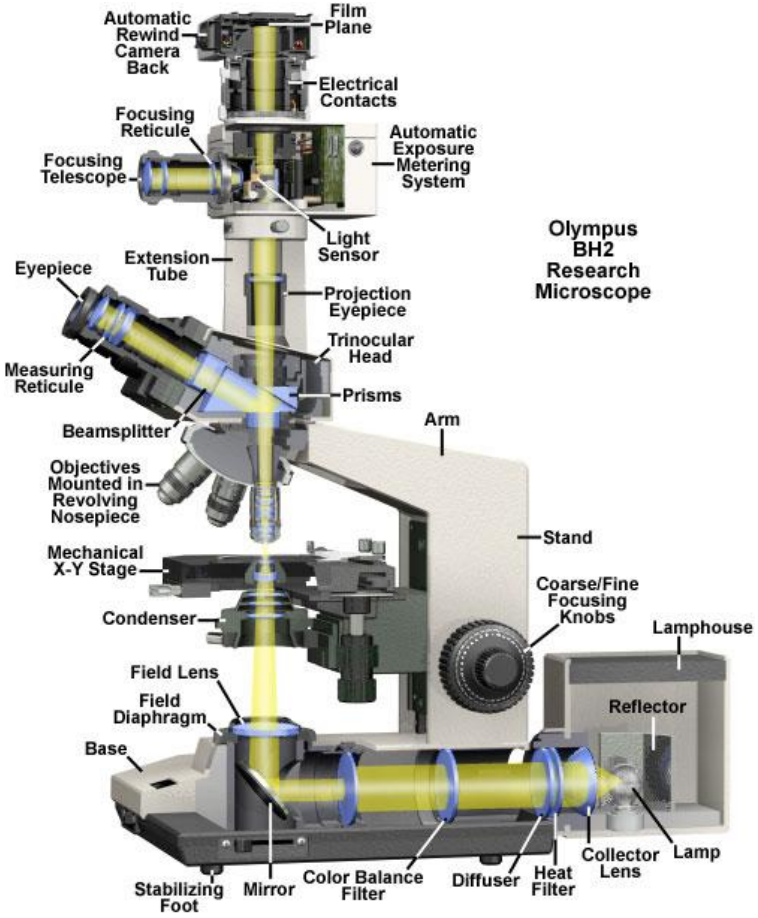
$$R = \lambda / 2NA$$

n = index lomu média

Imerzní objektivy (zvětšení 60x – 100x); NA = 1,4

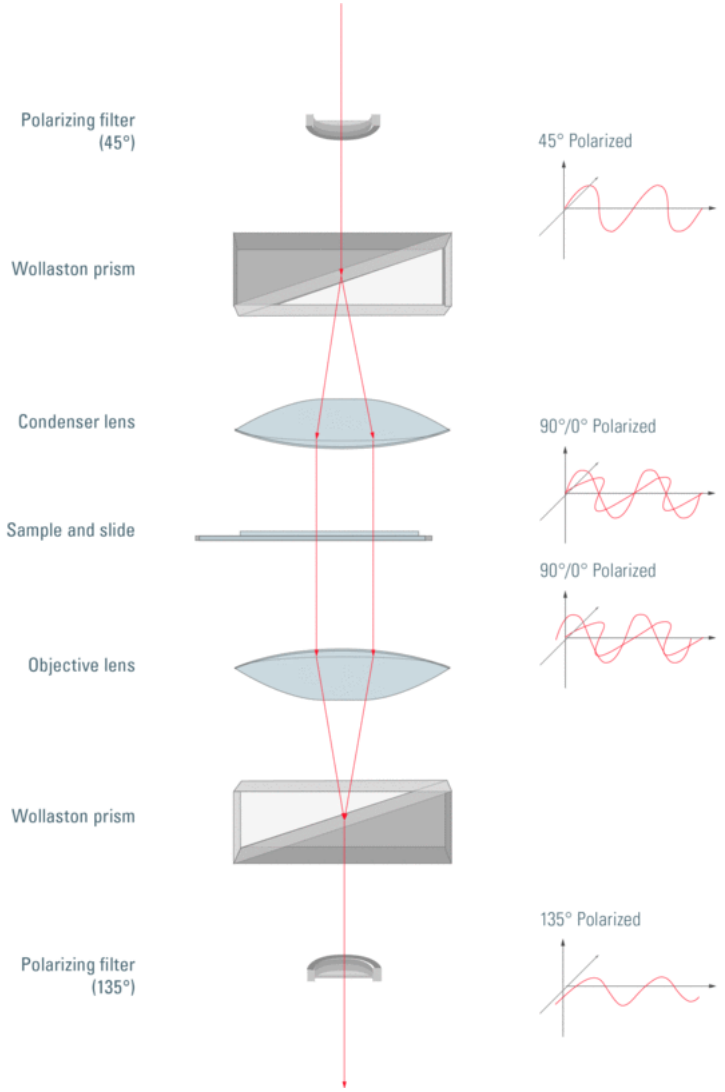
Bright-field microscopy





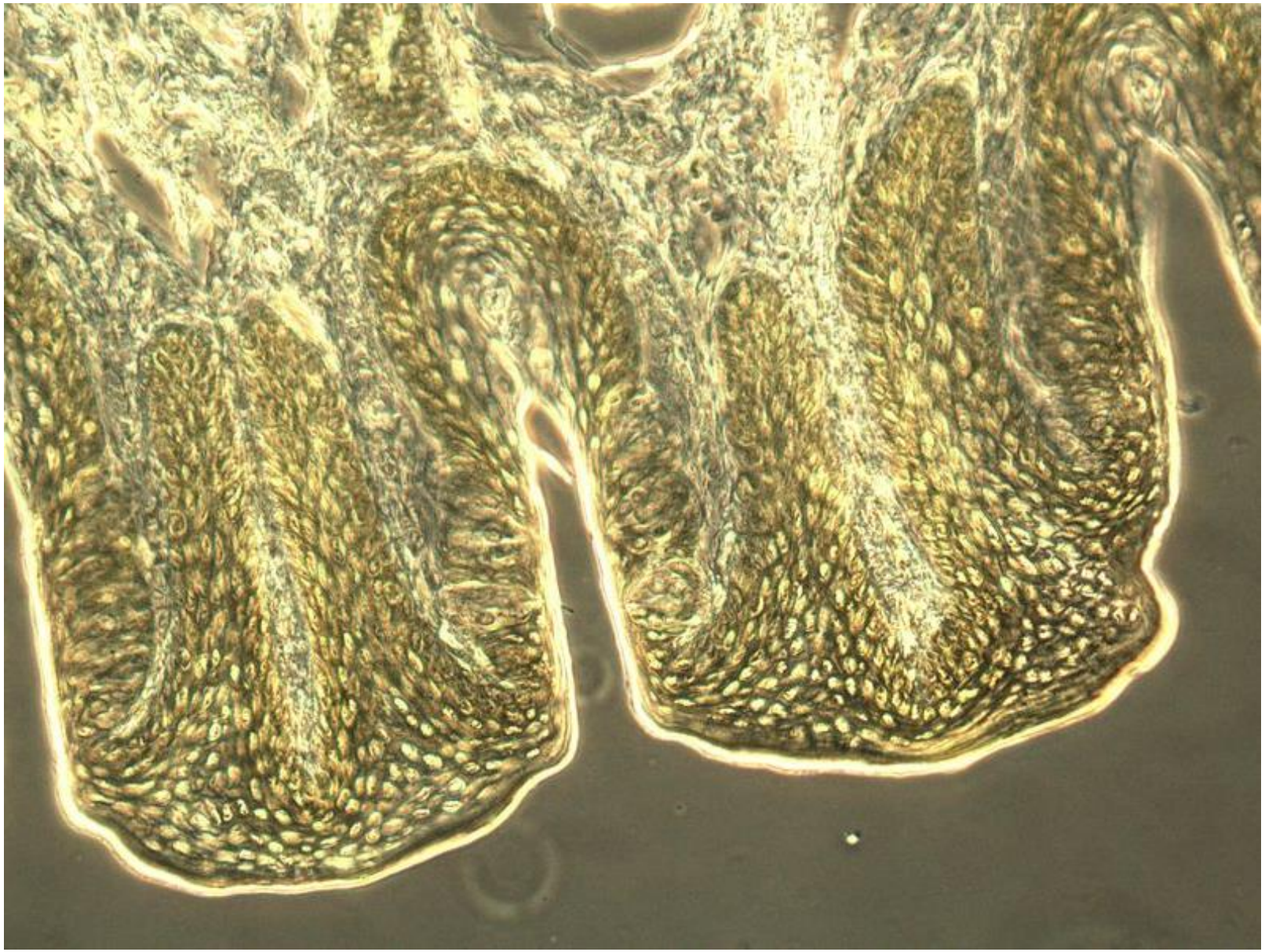
http://www.olympusmicro.com/primer/anatomy/bh2cutaway.html

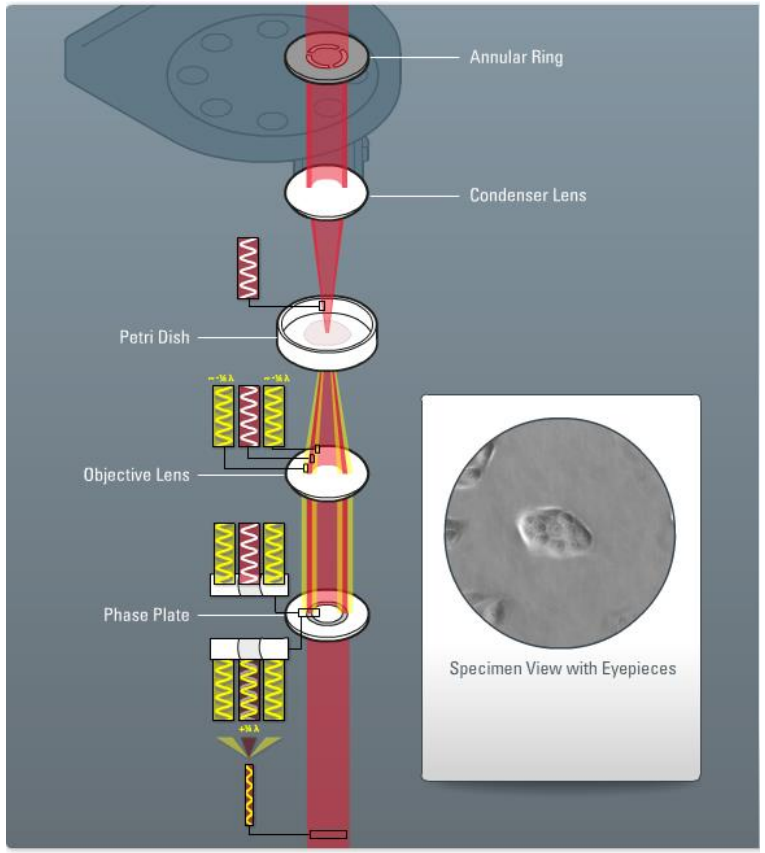
Differential interference contrast microscopy



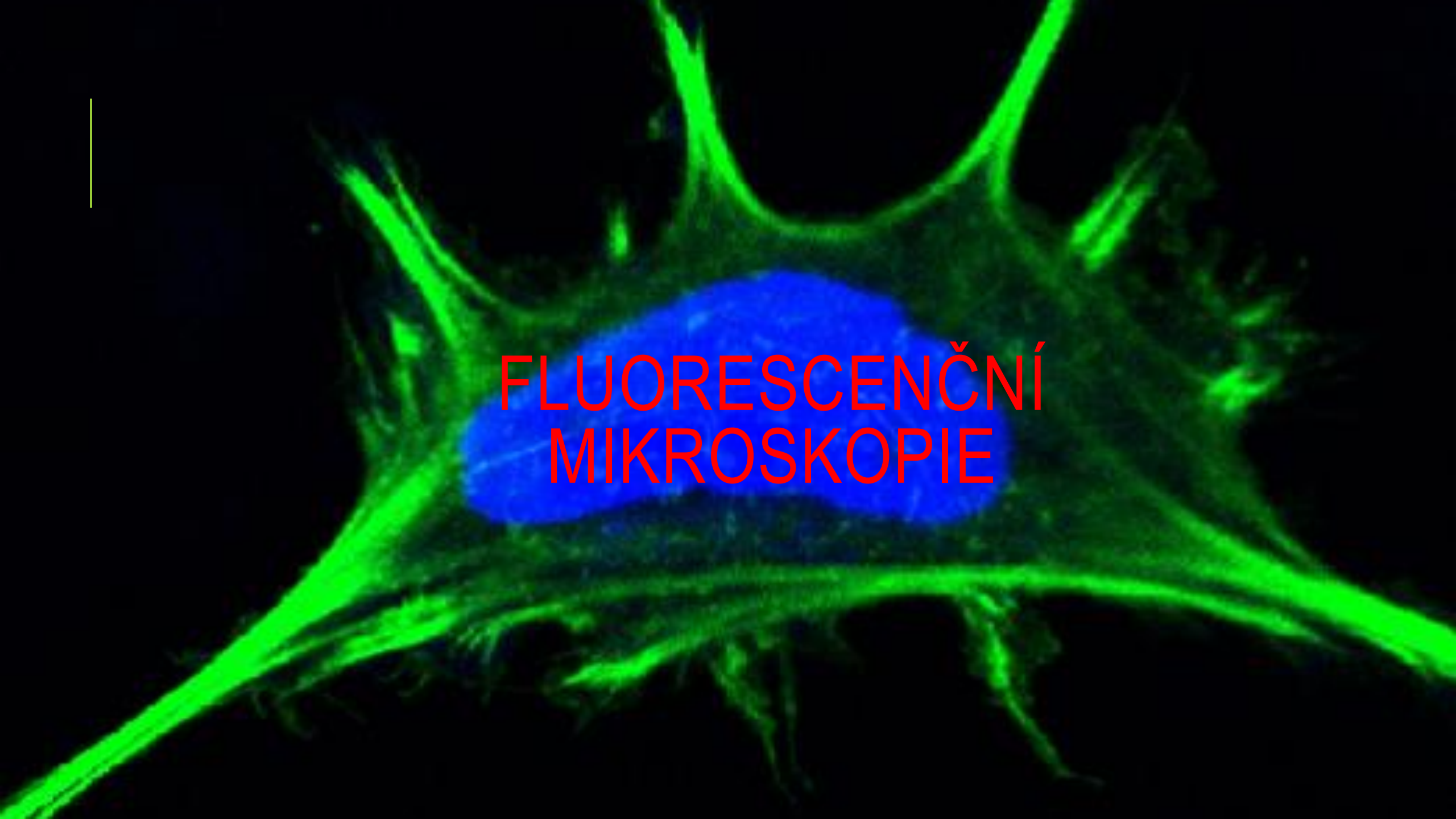
http://www.leica-microsystems.com/science-lab/differential-interference-contrast-dic/

Phase contrast microscopy





http://www.leica-microsystems.com/science-lab/the-principles-of-phase-contrast/


FLUORESCENČNÍ
MIKROSKOPIE

FYZIKÁLNÍ PRINCIPY FLUORESCENCE

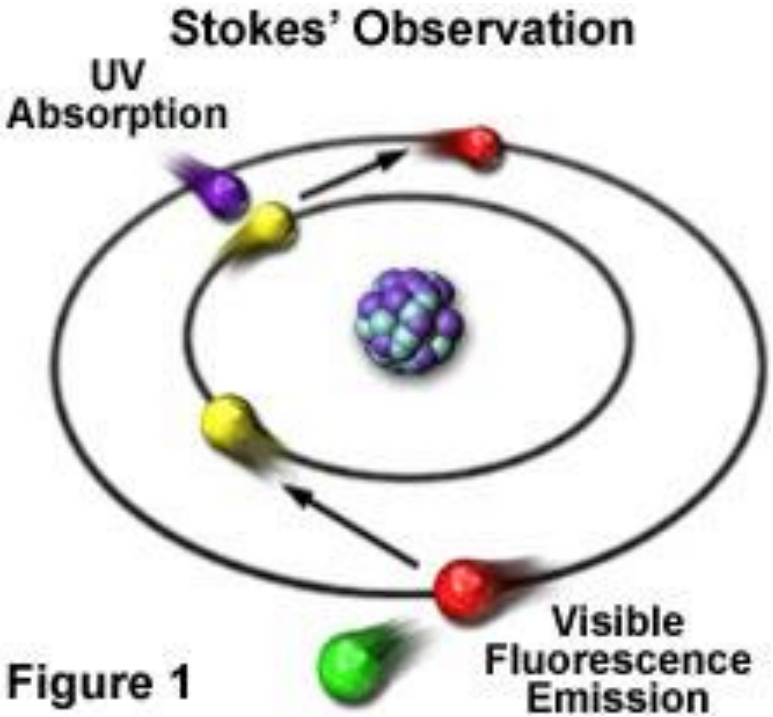


Figure 1

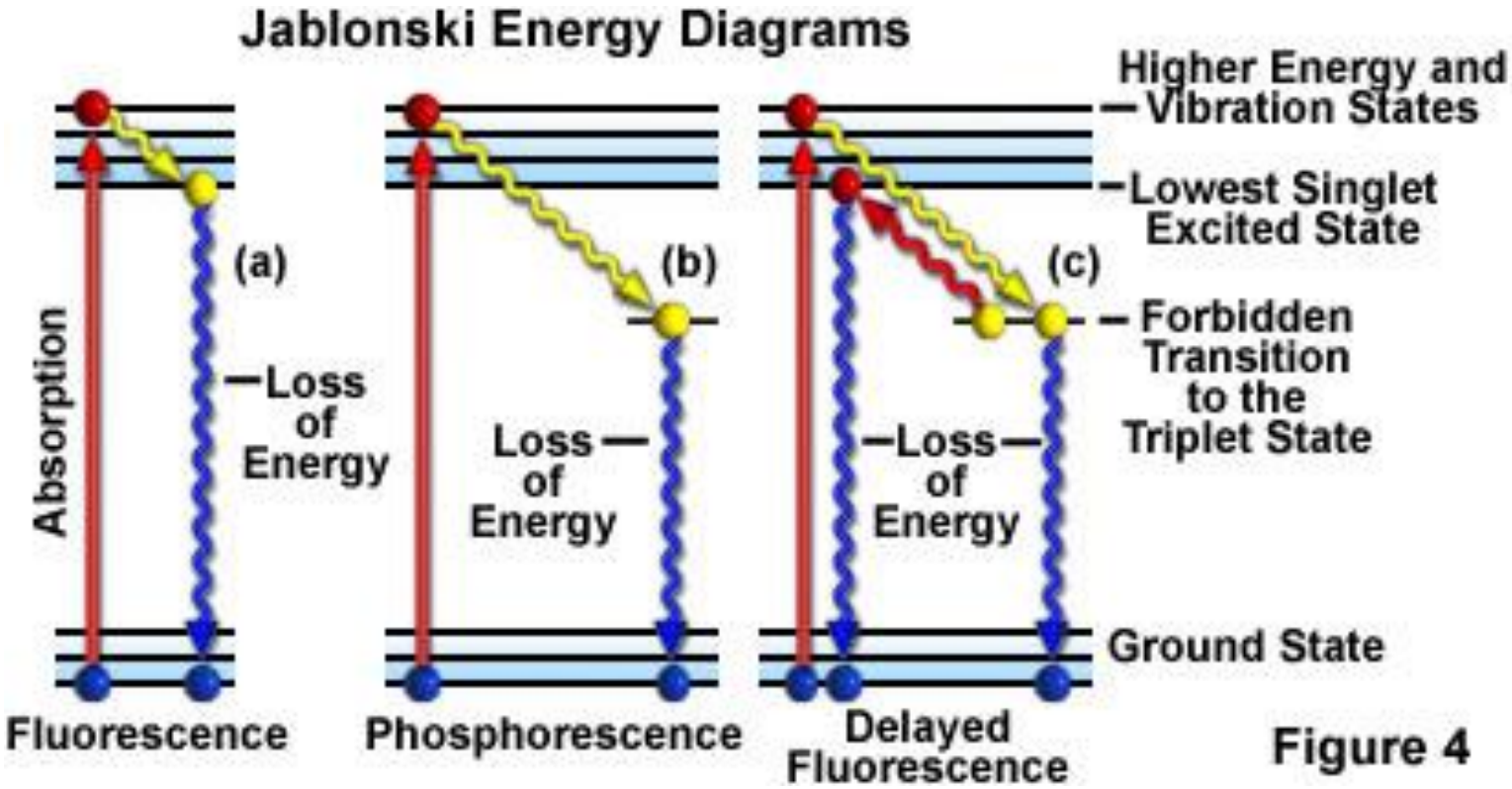
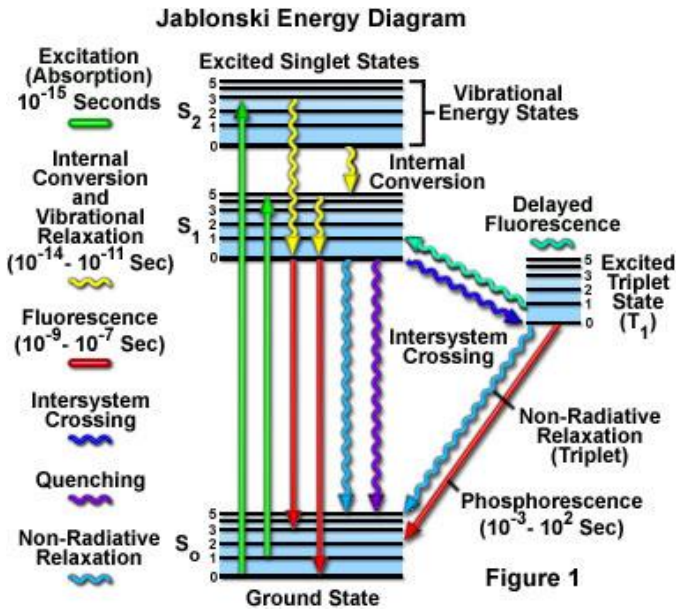


Figure 4

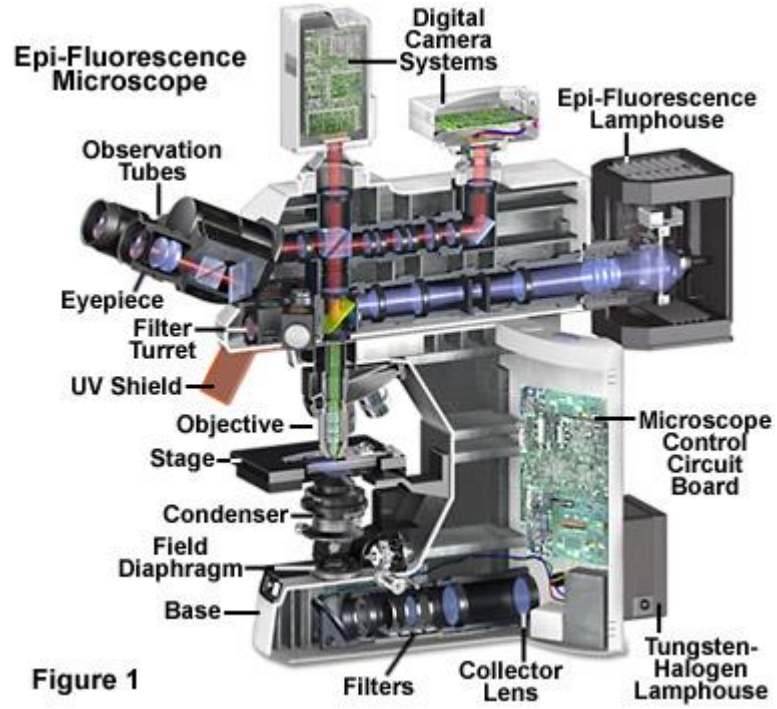
STOKESŮV ZÁKON

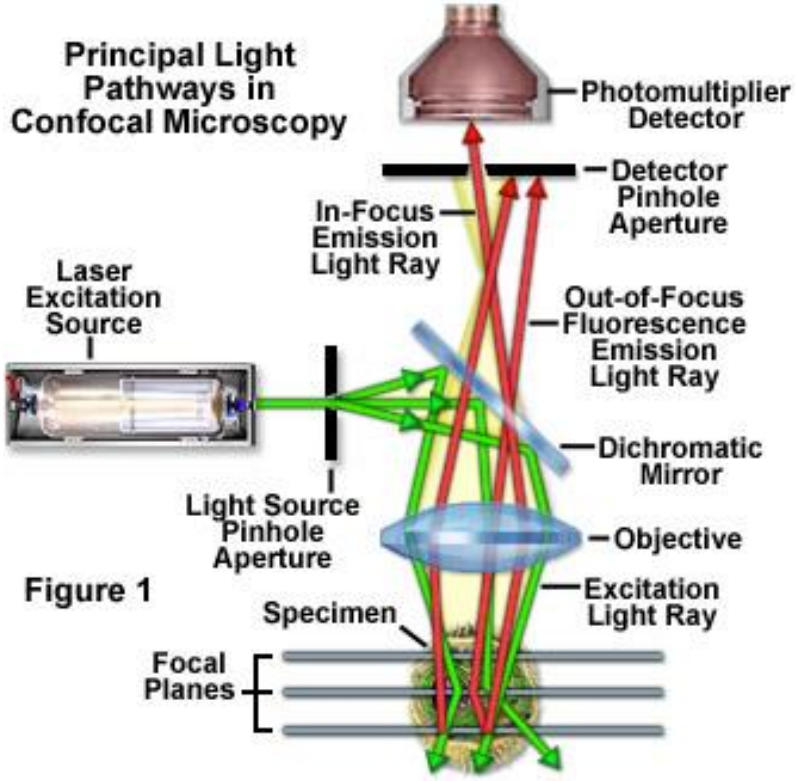
Vlnová délka emitovaného světla je vždy vyšší než vlnová délka excitačního světla (v důsledku ztráty energie elektronů při přechodu z vyšších excitovaných energetických hladin do nejnižšího stavu)

$$\lambda_{em} > \lambda_{ex}$$

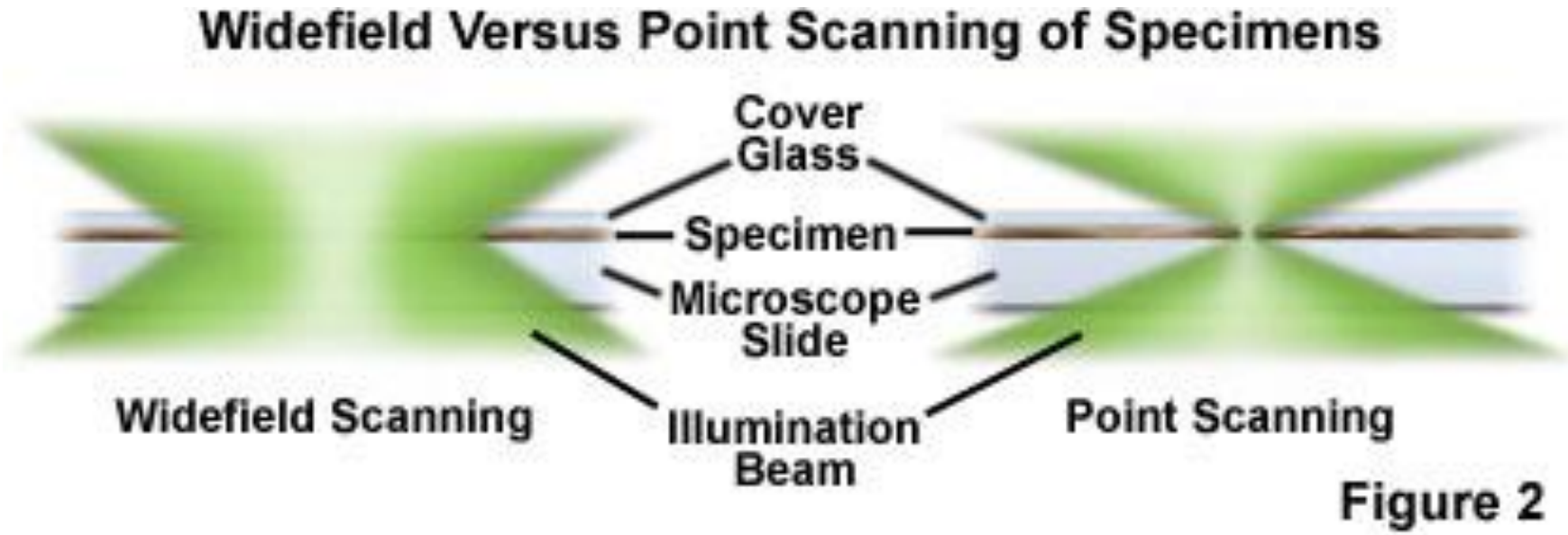


FLUORESCENČNÍ MIKROSKOP A KONFOKÁLNÍ FLUORESCENČNÍ MIKROSKOP





Rozdíl mezi widefield a konfokální mikroskopií

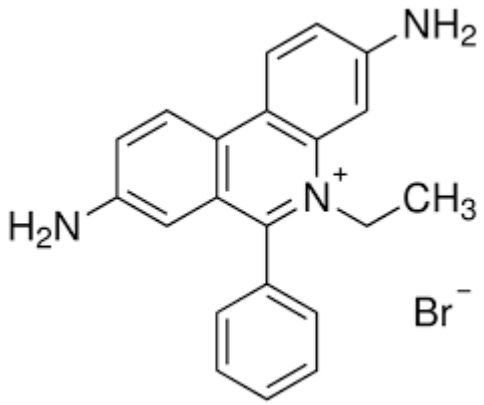


TYPY FLUOROFORŮ

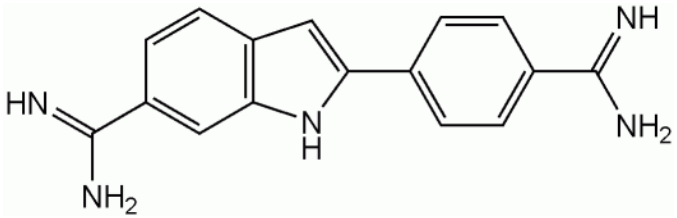
-> Aromatické organické sloučeniny (heterocykly, polyaromatické uhlovodíky)

-> Proteiny (BFP, CFP, GFP, YFP, RFP, DsRed)

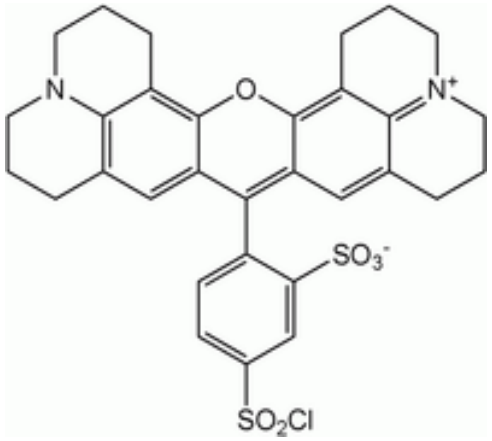
-> RNA (Spinach)



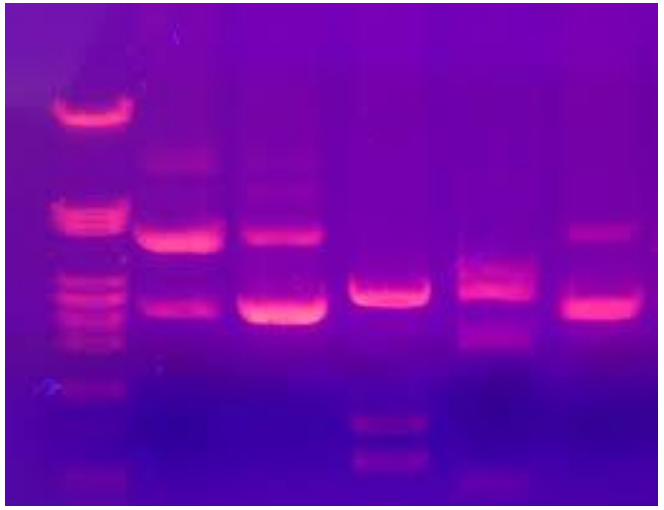
Ethidium bromid

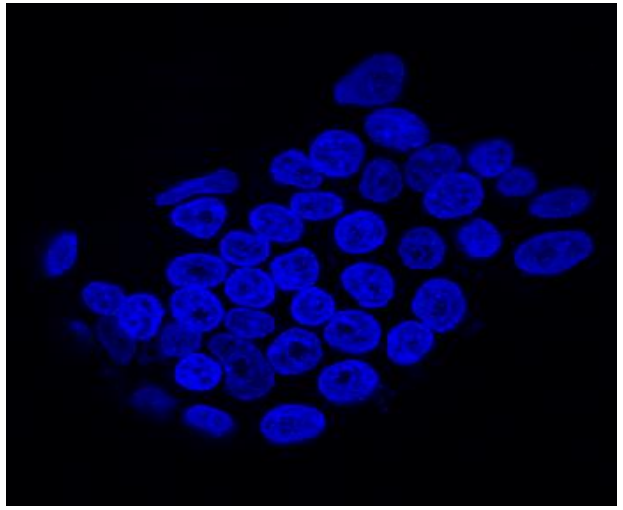


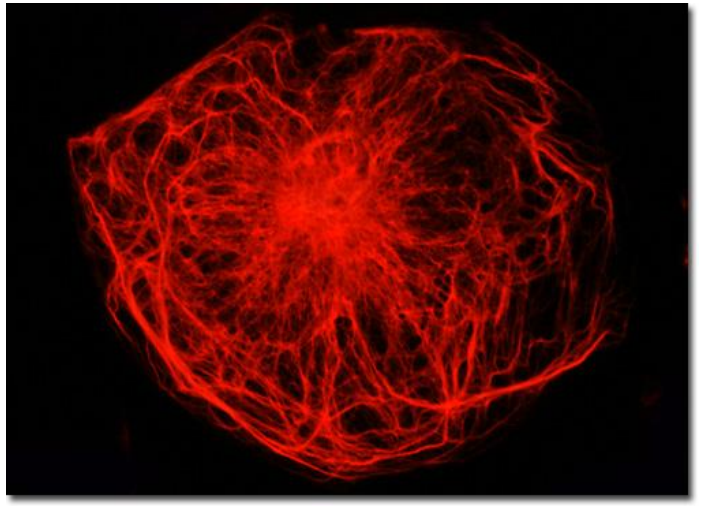
DAPI



Texas Red







Chromophore Structural Motifs of Green Fluorescent Protein Variants

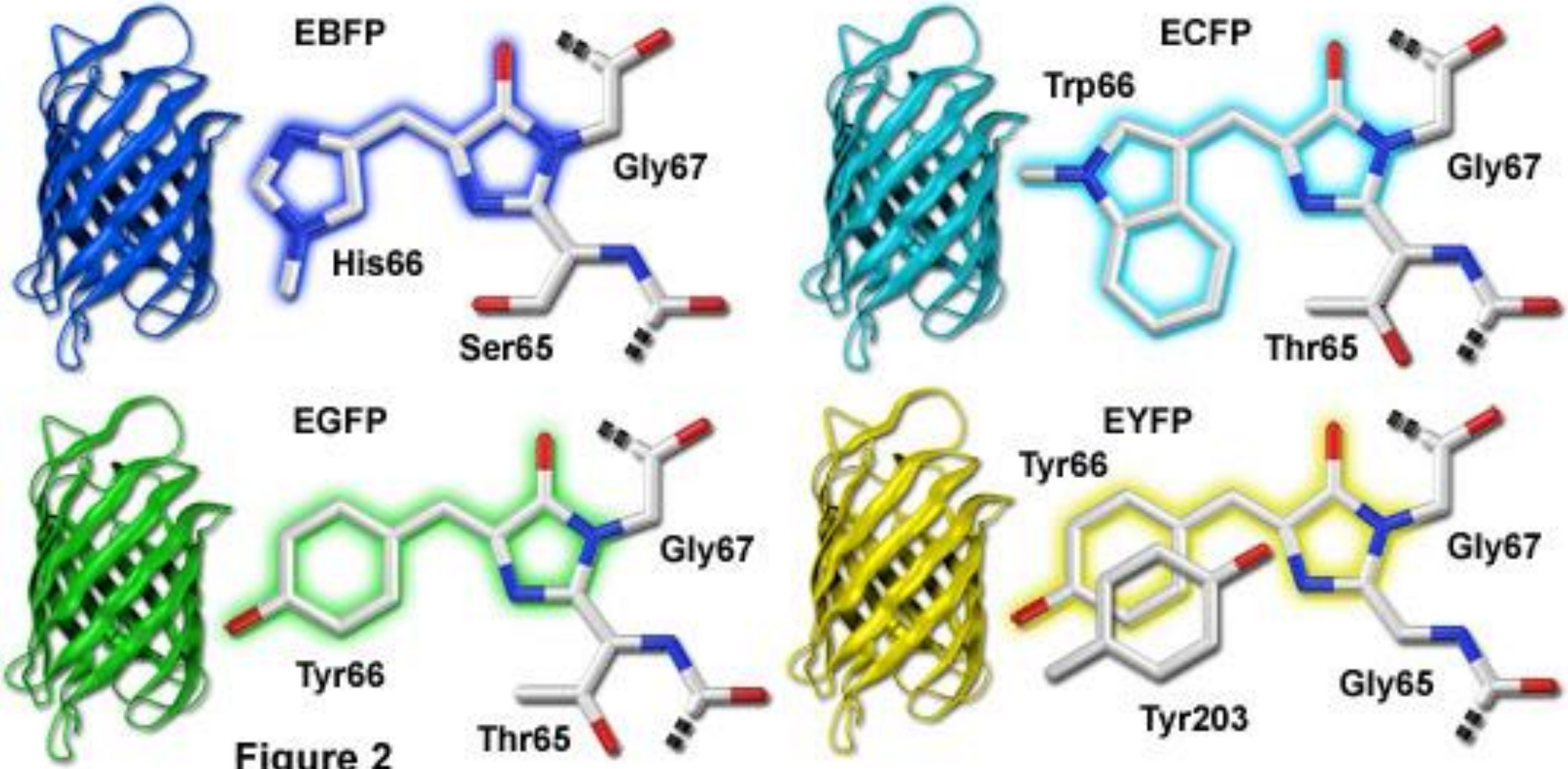


Figure 2

ABSORPČNÍ A EMISNÍ SPEKTRA

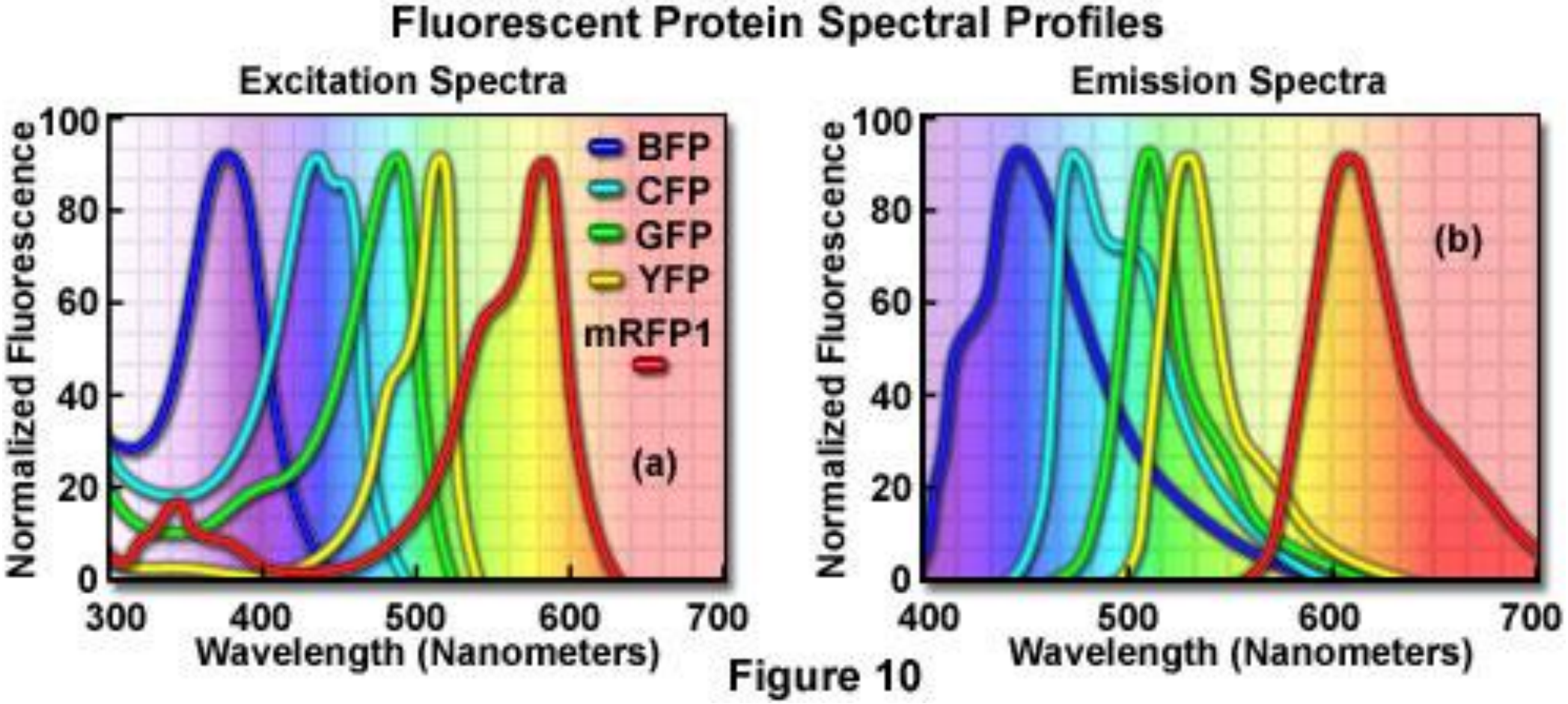
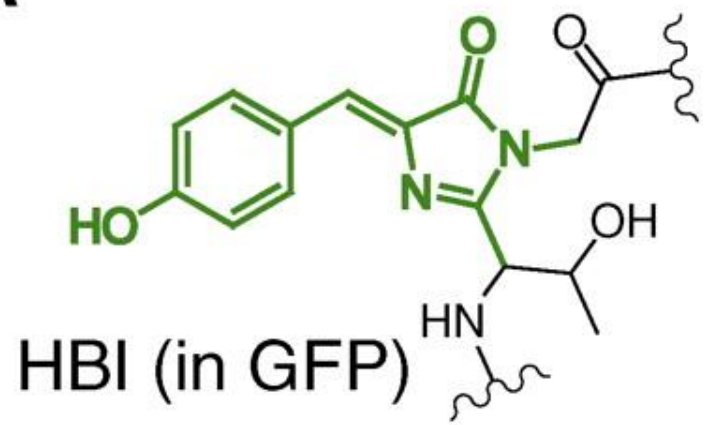


Figure 10

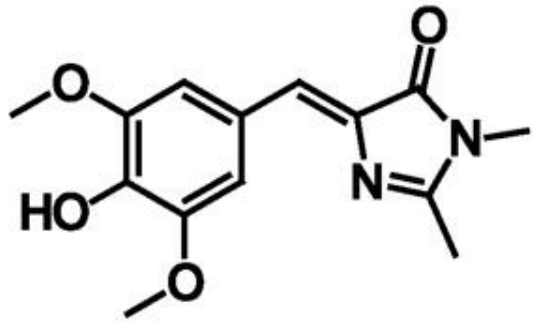
http://www.olympusmicro.com/primer/techniques/confocal/fluorophoresintro.html

Spinach – RNA mimic of GFP

A

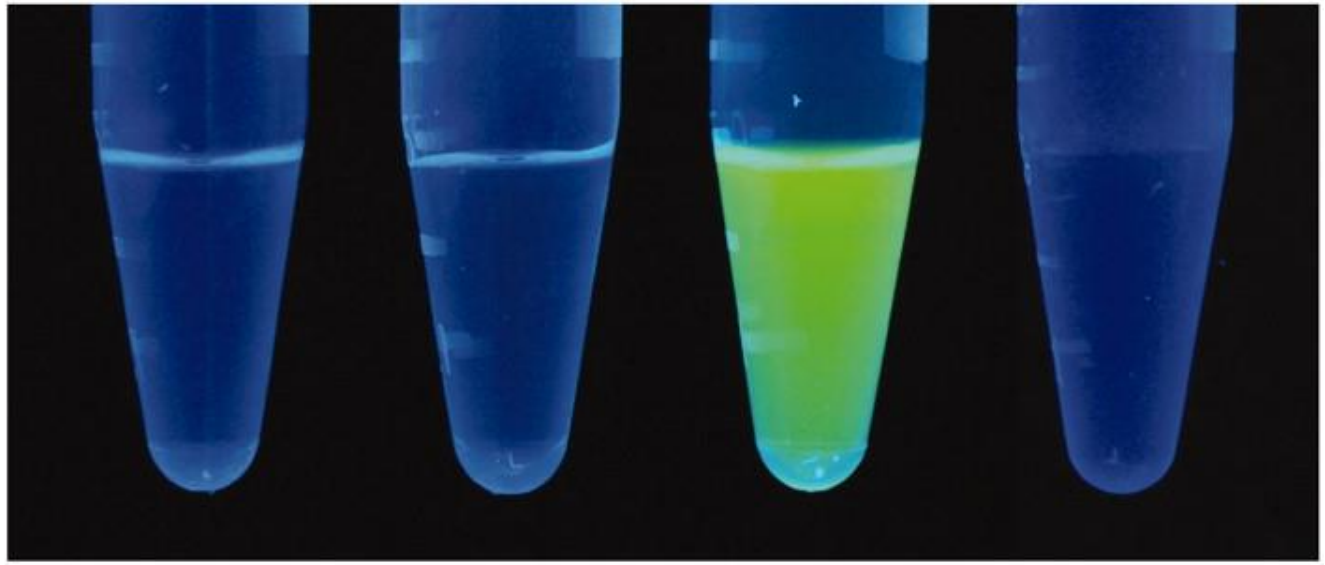


HBI (in GFP)



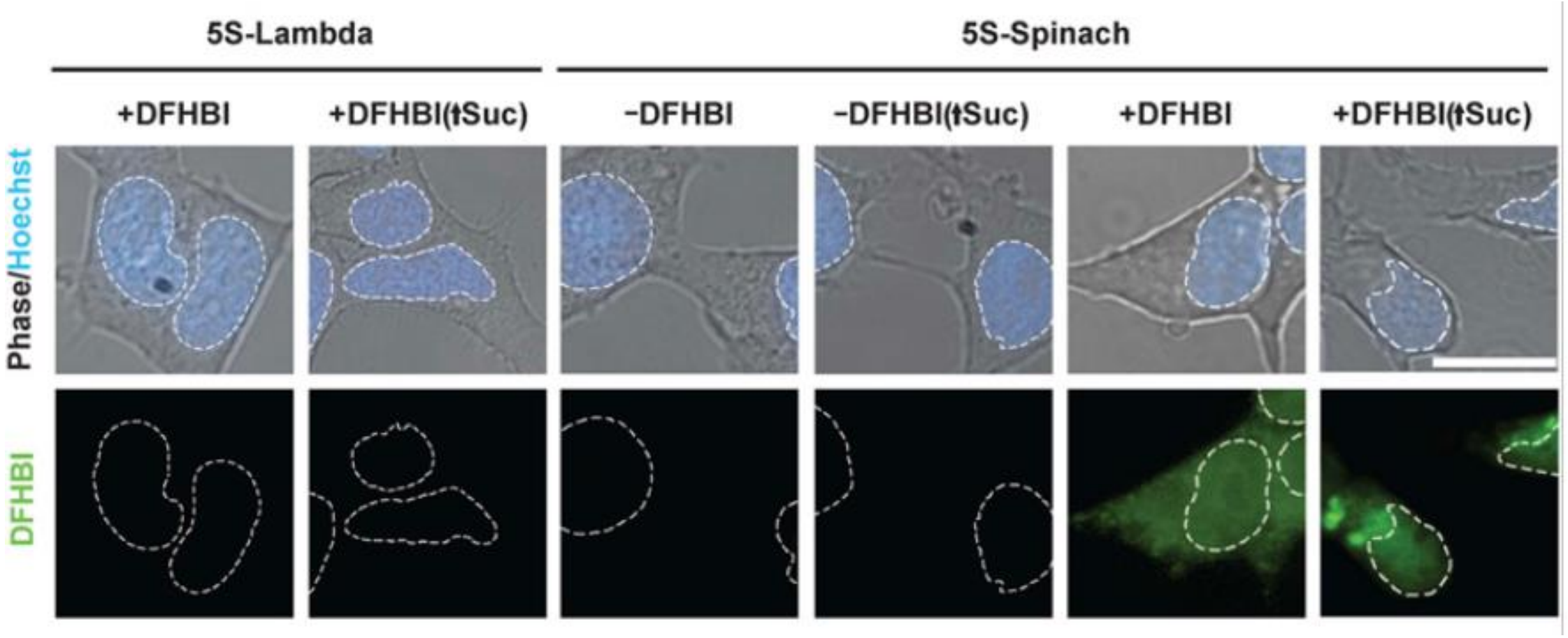
DMHBI

B



DMHBI | 13-2 RNA | DMHBI + 13-2 RNA | DMHBI + control RNA

Spinach – RNA mimic of GFP

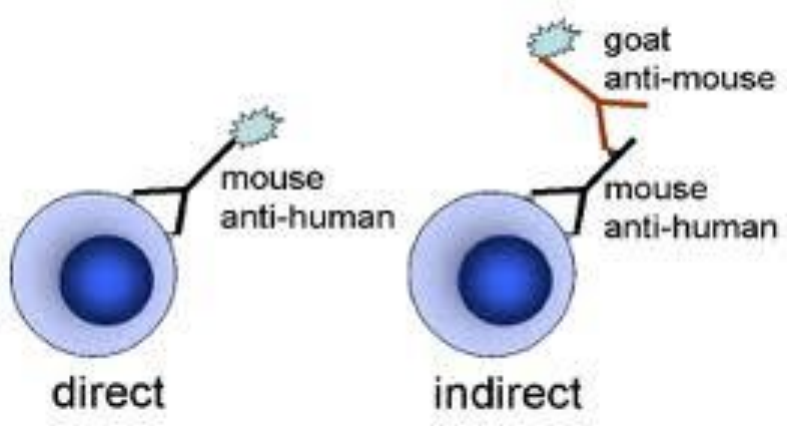


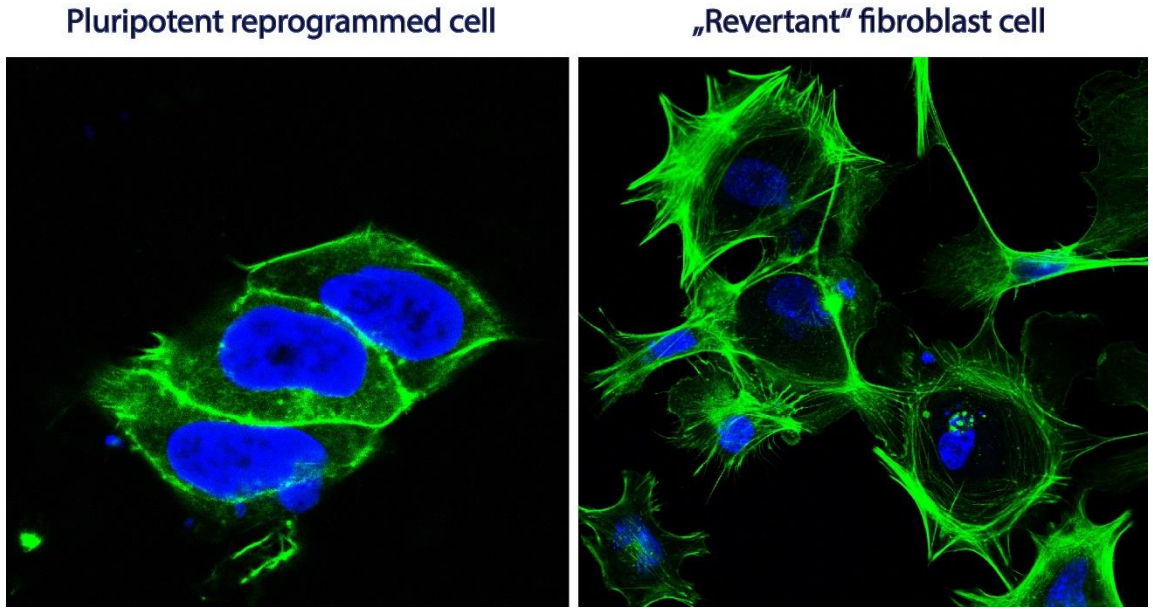
NĚKTERÉ TECHNIKY MOLEKULÁRNÍ CYTOLOGIE

1. Imunocytochemie
2. Transfekce buněk
3. Indukce poškození DNA (indukce dvouřetězcových zlomů po senzitizaci, vnesení oxidativního poškození a fotoproduktů)
4. Studium dynamiky proteinů (FRAP)
5. Studium interakce proteinů (FRET)
6. Hybridizace in situ
7. Studium živých buněk (Live-cell imaging)

IMUNOCYTOCHEMIE

Vizualizace proteinů pomocí primárních a sekundárních protilátek. Primární nebo sekundární protilátka je značená fluoroforem (Alexa 488, Alexa 594, Cy3, Cy5…)





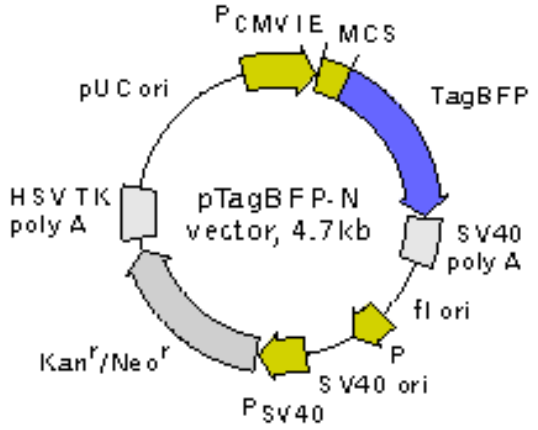
PŘECHODNÁ TRANSFEKCE EUKARYONTNÍCH BUNĚK

Různé komerční kity pro lipofekci

Možnost elektroporace / nukleofekce obtížně transfekovatelných buněčných typů (embryonální kmenové buňky, quiescentní buňky)

Optimální transfekce při 40–80% konfluenci

Exprese proteinu neseného plazmidem zpravidla 24h – 48h po transfekci (závisí na délce buněčného cyklu konkrétní buněčné linie)



Overview

1 **Terminology**

2 **Factors Affecting Transfection**

Host Cell

Cell health

Cell culture

Genetic Material

DNA quality and quantity

3 **Transfection Workflow**

4 **Common Transfection Methods**

Reagent-Based Methods

Lipids

Calcium phosphate

Cationic polymers

DEAE-dextran

Activated dendrimers

Magnetic beads

Instrument-Based Methods

Electroporation

Biolistic technology

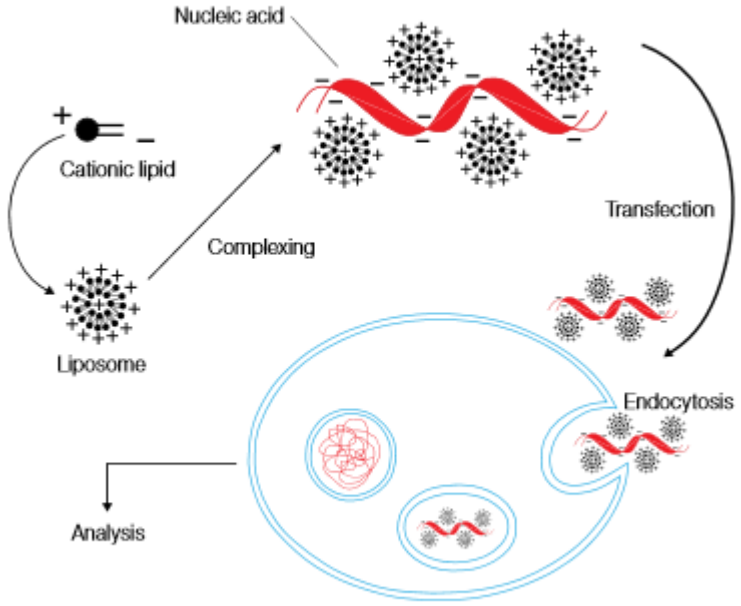
Microinjection

Laserfection/optoinjection

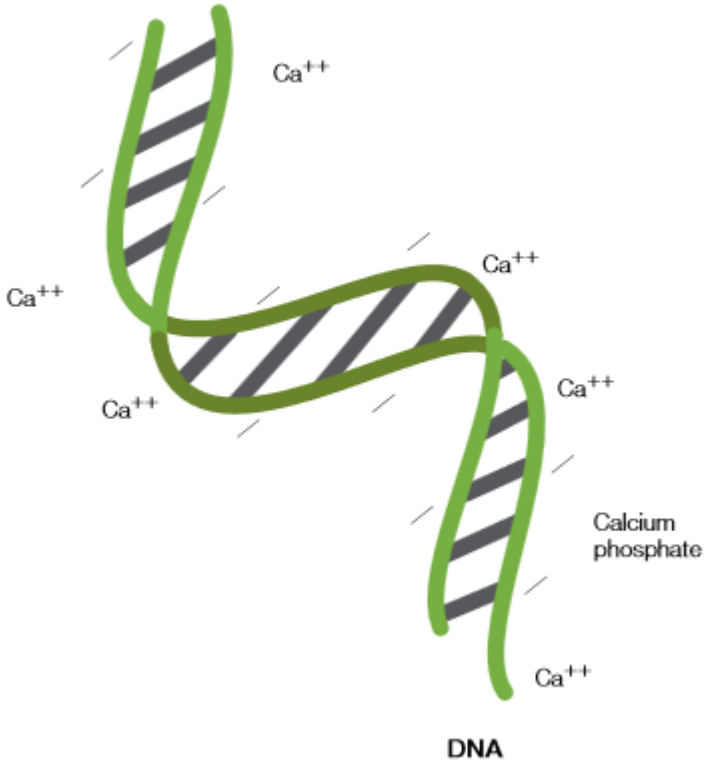
Virus-Based Methods

http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10-0826_transfection_tutorial_interactive.pdf

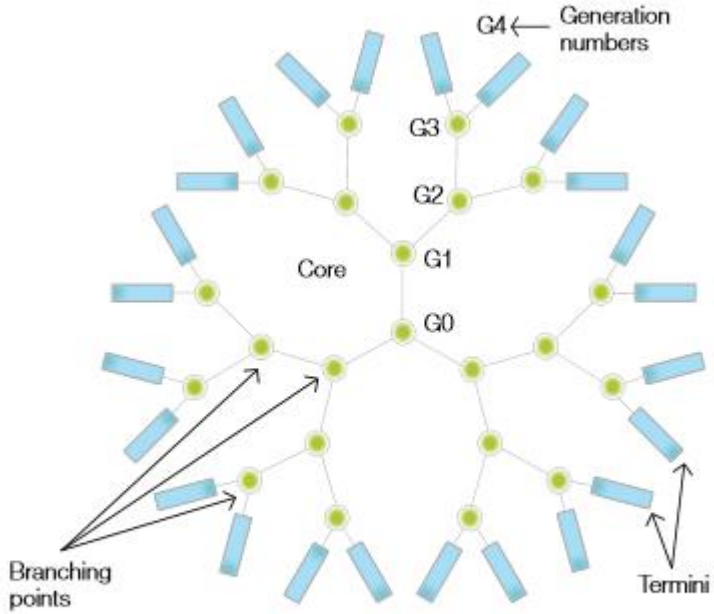
Lipofekce



Precipitace fosforečnanem vápenatým

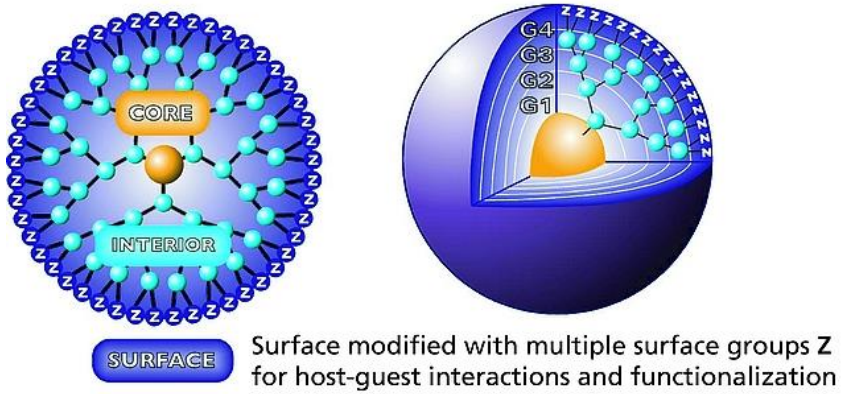


Transfekce dendrimery



Dendrimer

Interior with multiple branches (generations **G**), well-suited for encapsulation of drugs and nanomaterials



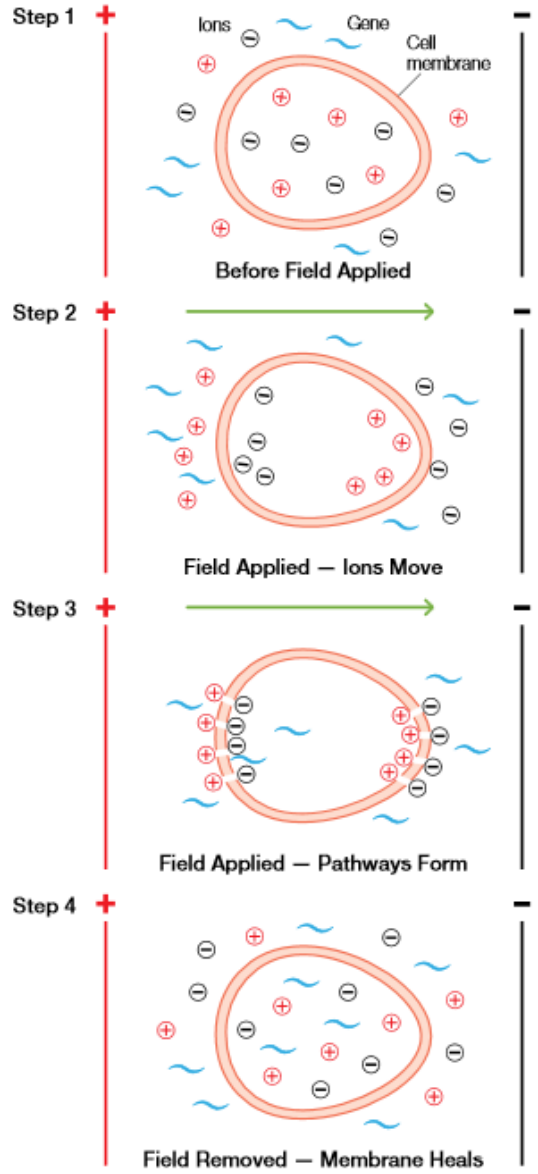
Surface modified with multiple surface groups **Z** for host-guest interactions and functionalization

http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10-0826_transfection_tutorial_interactive.pdf

http://www.sigmaaldrich.com/materials-science/material-science-products.html?TablePage=16375655

How Electroporation Works

1. Electroporation exposes a cell to a high-intensity electric field that temporarily destabilizes the membrane.
2. During this time the membrane is highly permeable to exogenous molecules present in the surrounding media.
3. DNA then moves into the cell through these holes.
4. When the field is turned off, the pores in the membrane reseal, enclosing the DNA inside.



http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10-0826_transfection_tutorial_interactive.pdf

Viral Attributes

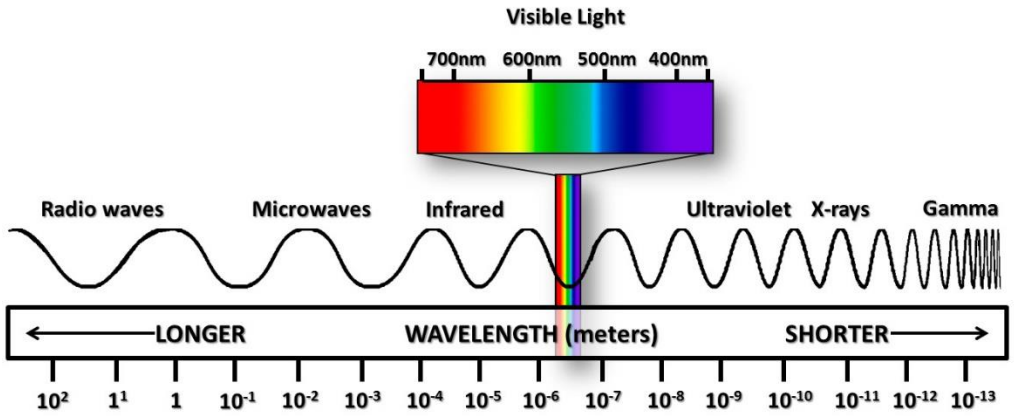
Viral Vector	DNA Insert Size	Maximum Titer	Cell Type	Expression	Pitfalls
Retroviral	8 kb	1×10^9	Dividing cells	Stable	Random insertion site
Lentivirus	9 kb	1×10^9	Dividing cells Nondividing cells	Stable	Random insertion site
Adenovirus	8 kb	1×10^{13}	Dividing cells Nondividing cells	Transient	Highly immunogenic
Adeno-associated virus (AAV)	5 kb	1×10^{11}	Dividing cells Nondividing cells	Stable, site-specific location	Requires helper virus to grow; difficult to remove helper virus
Herpes simplex virus	30–40 kb	1×10^9	Dividing cells Nondividing cells	Transient	No gene expression during latent infection
Vaccinia virus	25 kb	3×10^9	Dividing cells	Transient	Potential cytopathic effects

INDUKCE POŠKOZENÍ DNA

Metody fyzikální:

A) Ozáření buněk γ zářením

B) Ozáření buněk UV-lampou, UV-laserem

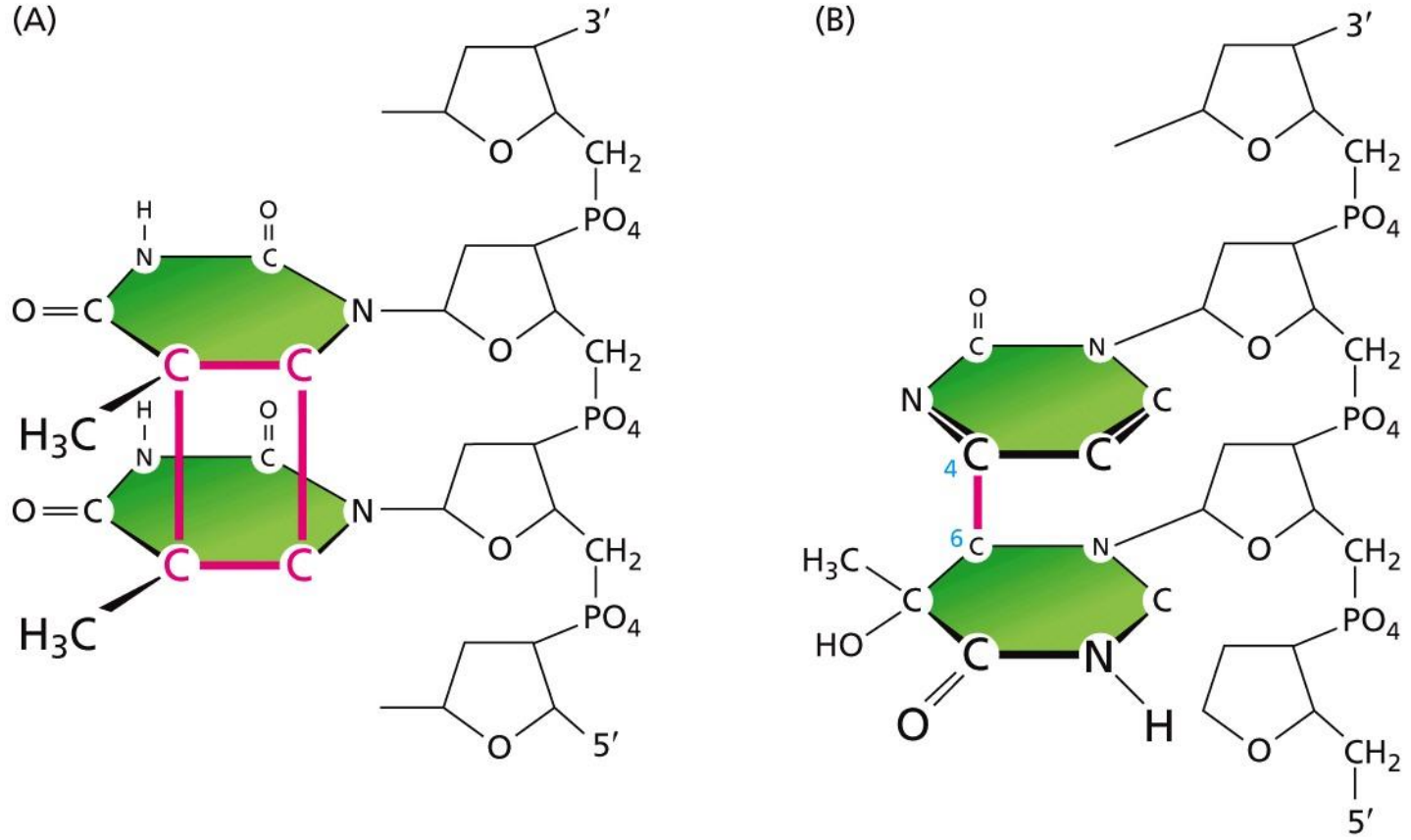


Metody chemické a biologické:

A) Využití mutagenů

B) Využití vzácně štěpících restrikčních endonukleáz

C) Generace cílených dsDNA zlomů pomocí CRISPR/Cas9 systému



Cyklobutanové pyrimidinové dimery a 6’–4’ fotoprodukty

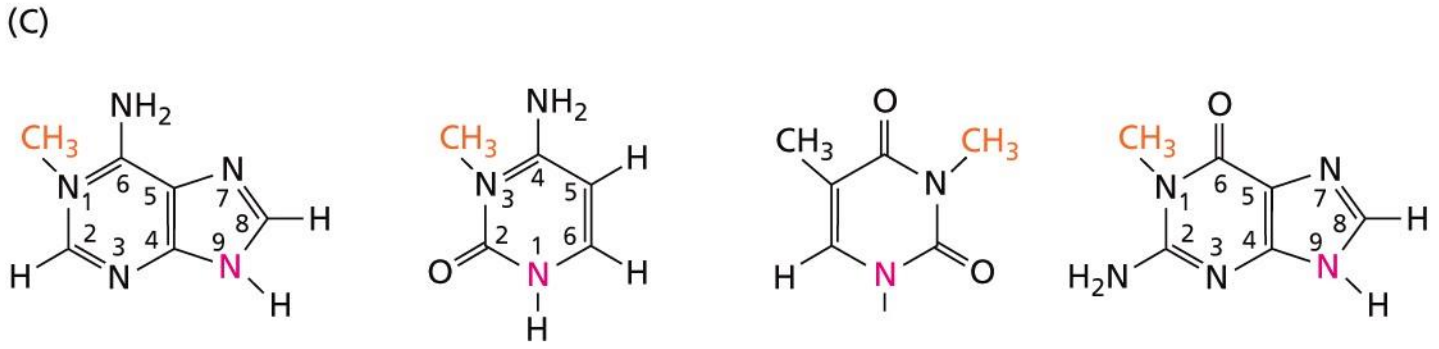
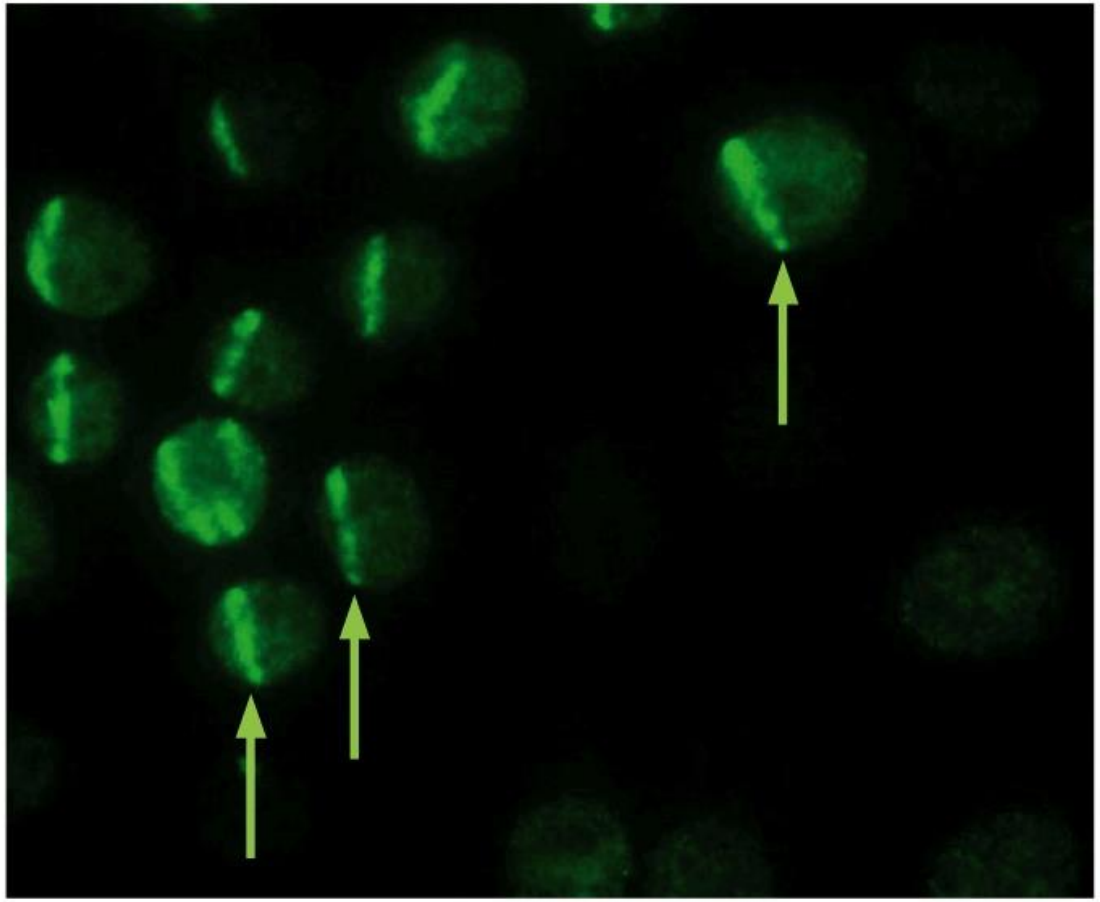


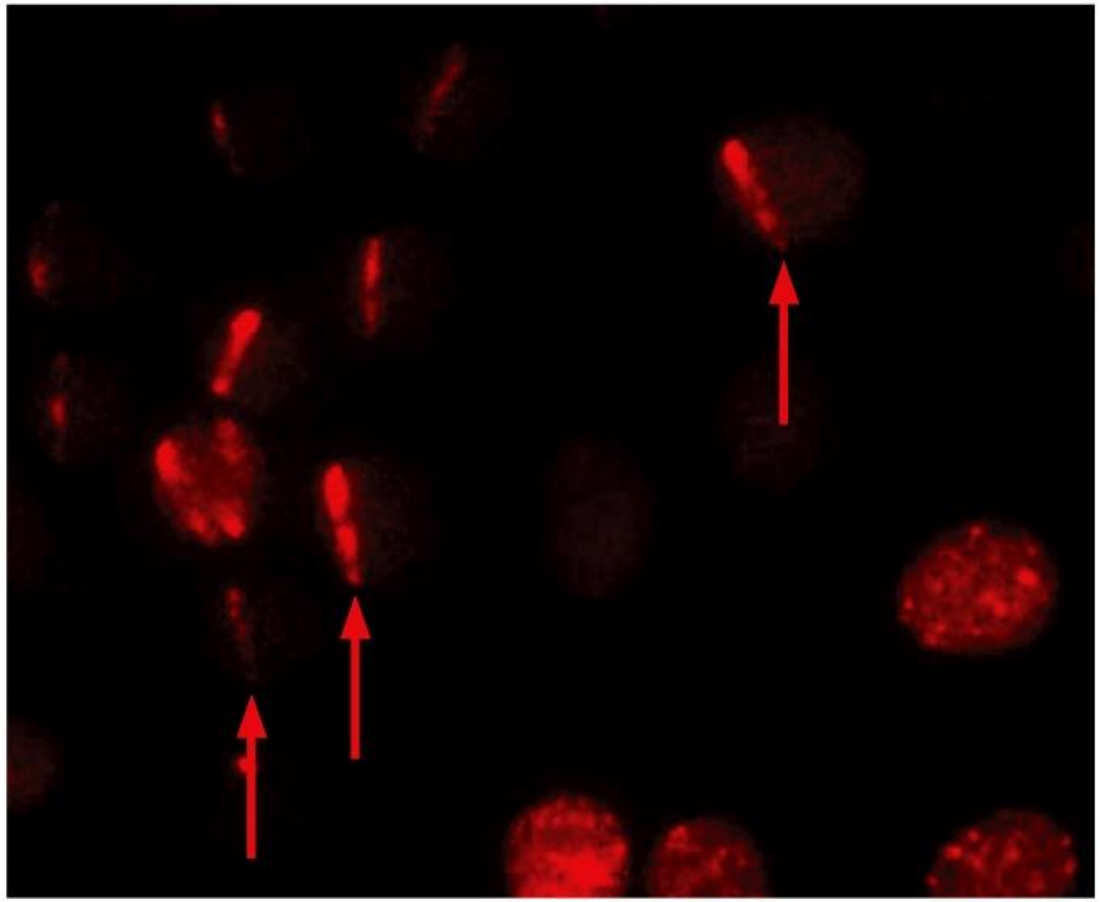
Figure 12.12 The Biology of Cancer (© Garland Science 2014)

Alkylované dusíkaté báze vzniklé působením alkylačních agens (EMS, MMS)

Indukce dsDNA zlomů UV-laserem (355 nm) po senzitizaci buněk přidáním Brd-U / Hoechst 33342



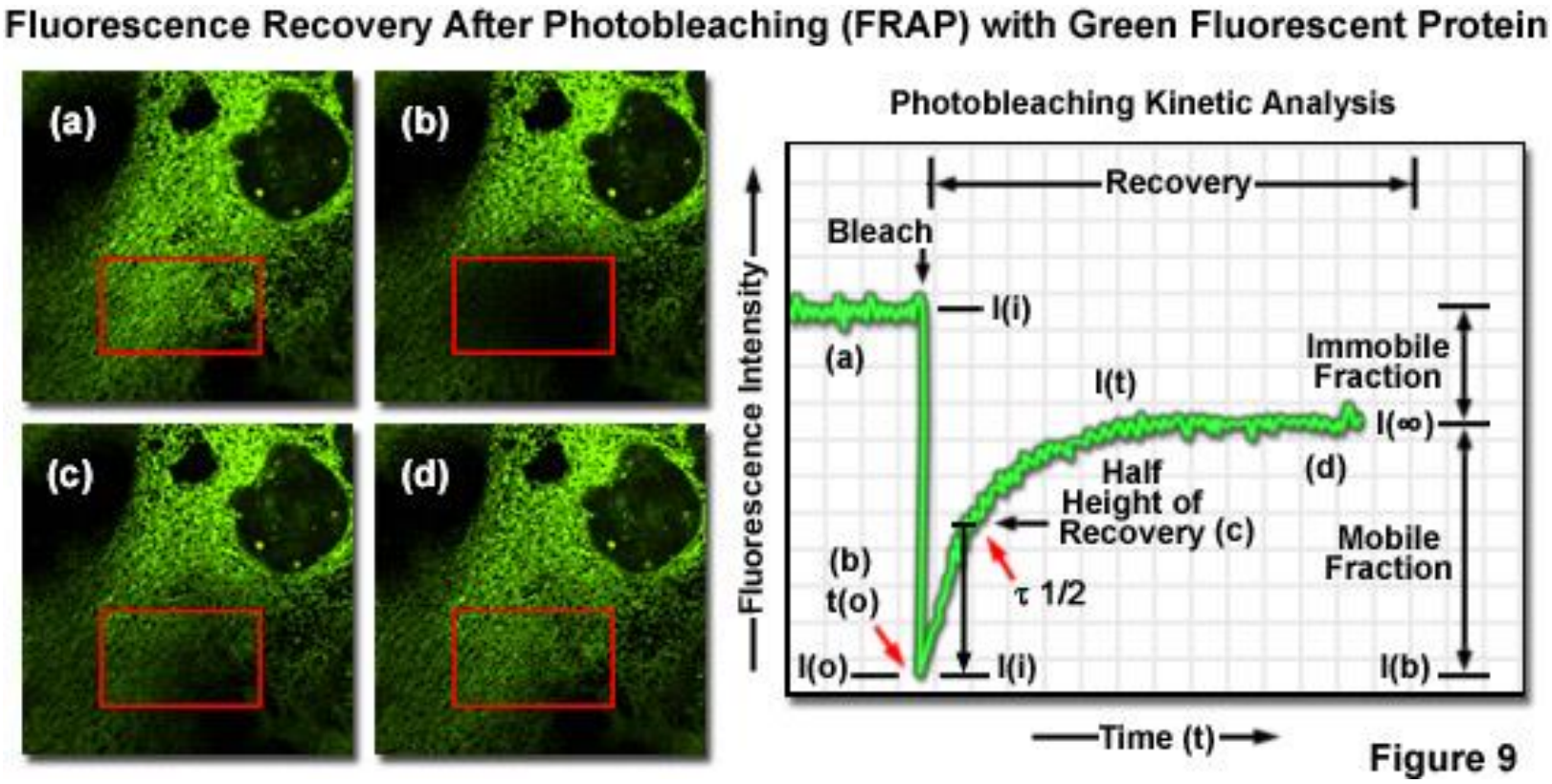
γ-H2AX



BRCA1

Figure 12.29b The Biology of Cancer (© Garland Science 2014)

FRAP – FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING



Photobleaching = fotochemická (ireverzibilní) destrukce fluoroforu

Slouží ke sledování dynamiky proteinů značených fluoroforem

FRET – FORSTER RESONANCE ENERGY TRANSFER

Jedná se o nezářivý přenos energie mezi párem fluoroforů, donorem a akceptorem.

Účinnost přenosu závisí na:

1. Překryvu emisního spektra donoru a absorpčního spektra akceptoru
2. Vzájemné orientaci dipólového momentu přechodu fluoroforů
3. Vzdálenosti fluoroforů ($E \sim 1/r^6$)

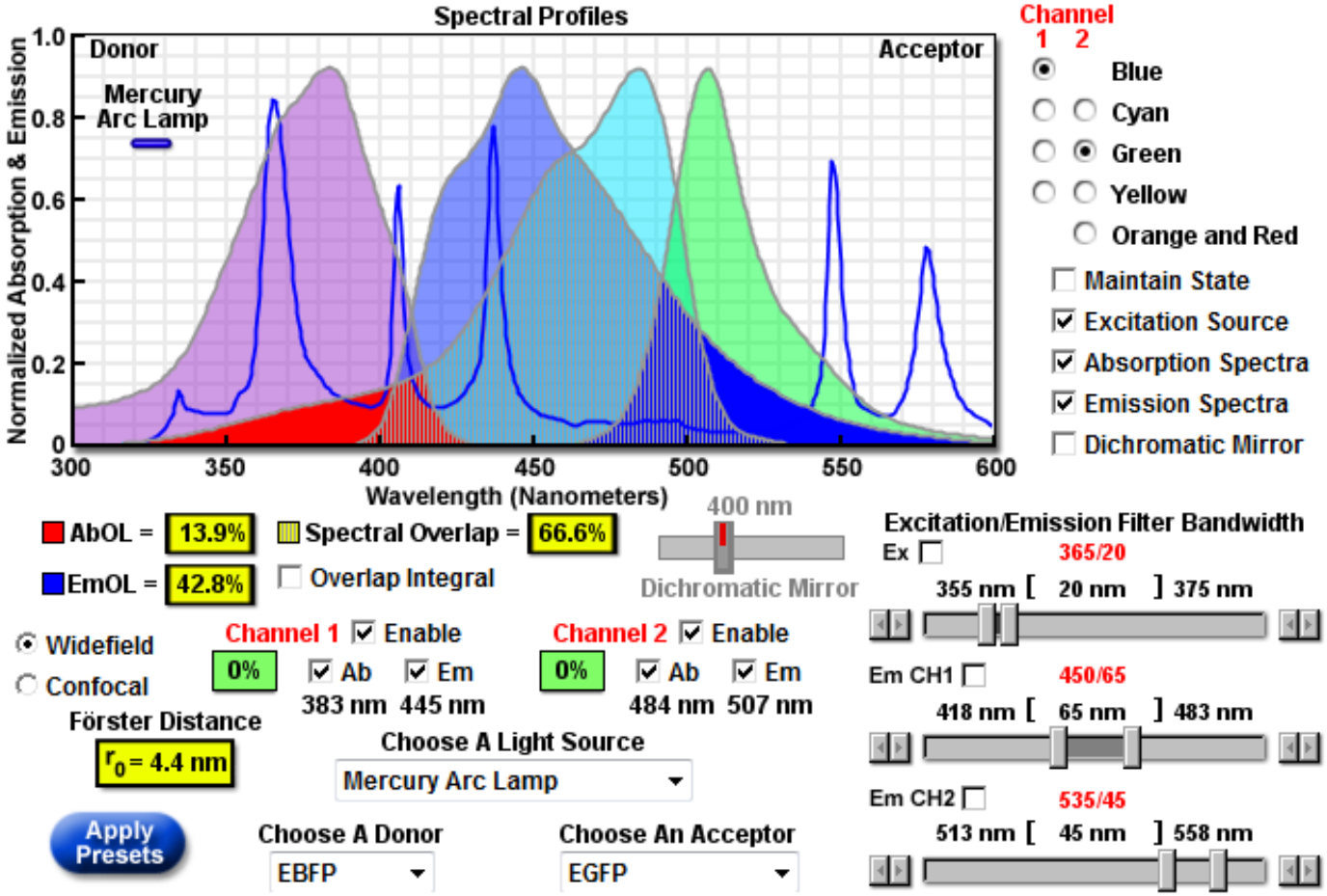
Účinnost přenosu (E) se vyjadřuje jako:

F_{DA} = Fluorescence donoru za přítomnosti akceptoru

F_D = Fluorescence donoru bez přítomnosti akceptoru

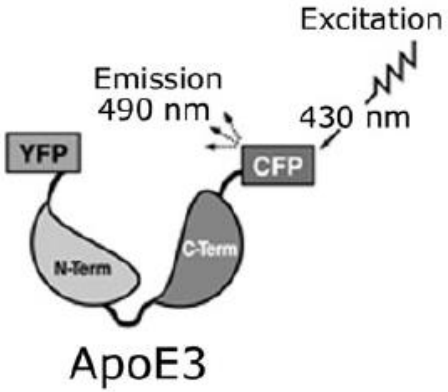
$$E = 1 - \frac{F_{DA}}{F_D}$$

VÝPOČET PŘEKRYVU SPEKTER DONORU A AKCEPTORU PRO FRET APLIKACE

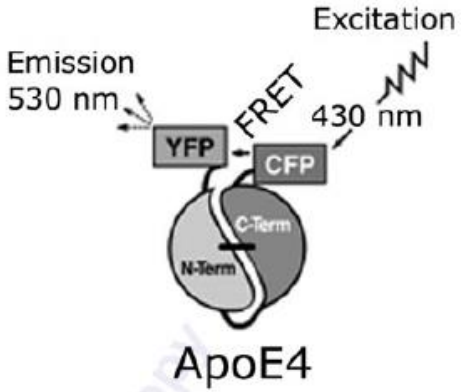


http://www.microscopyu.com/tutorials/java/fluorescence/fpfret/index.html

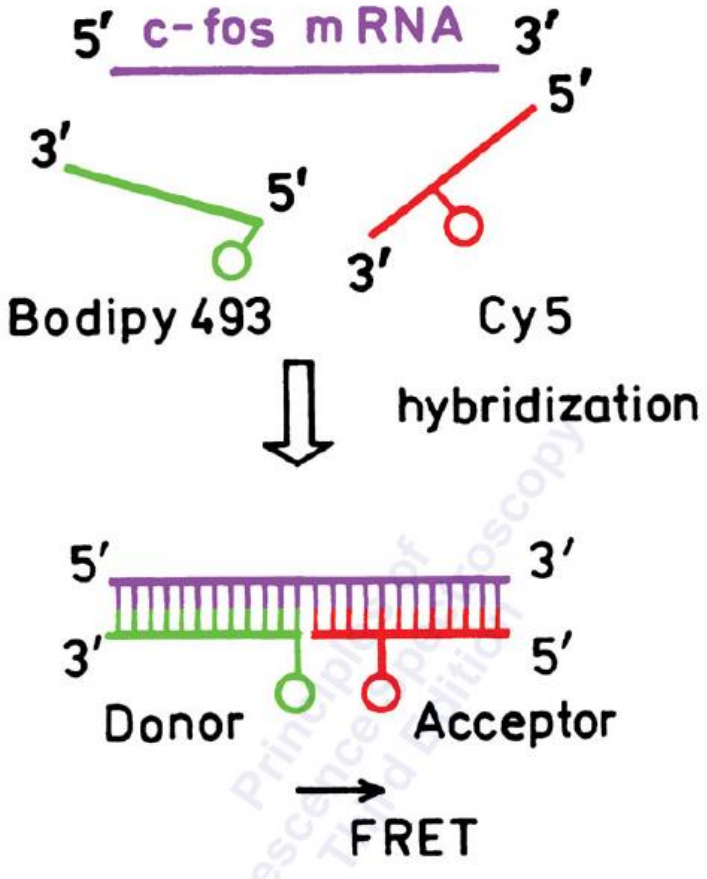
FRET – VYUŽITÍ

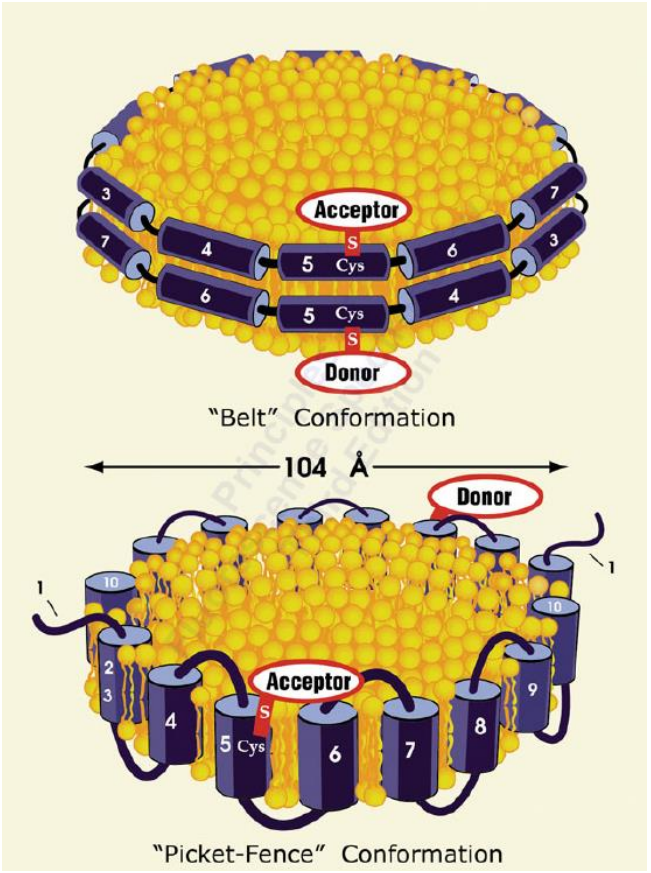


ApoE3

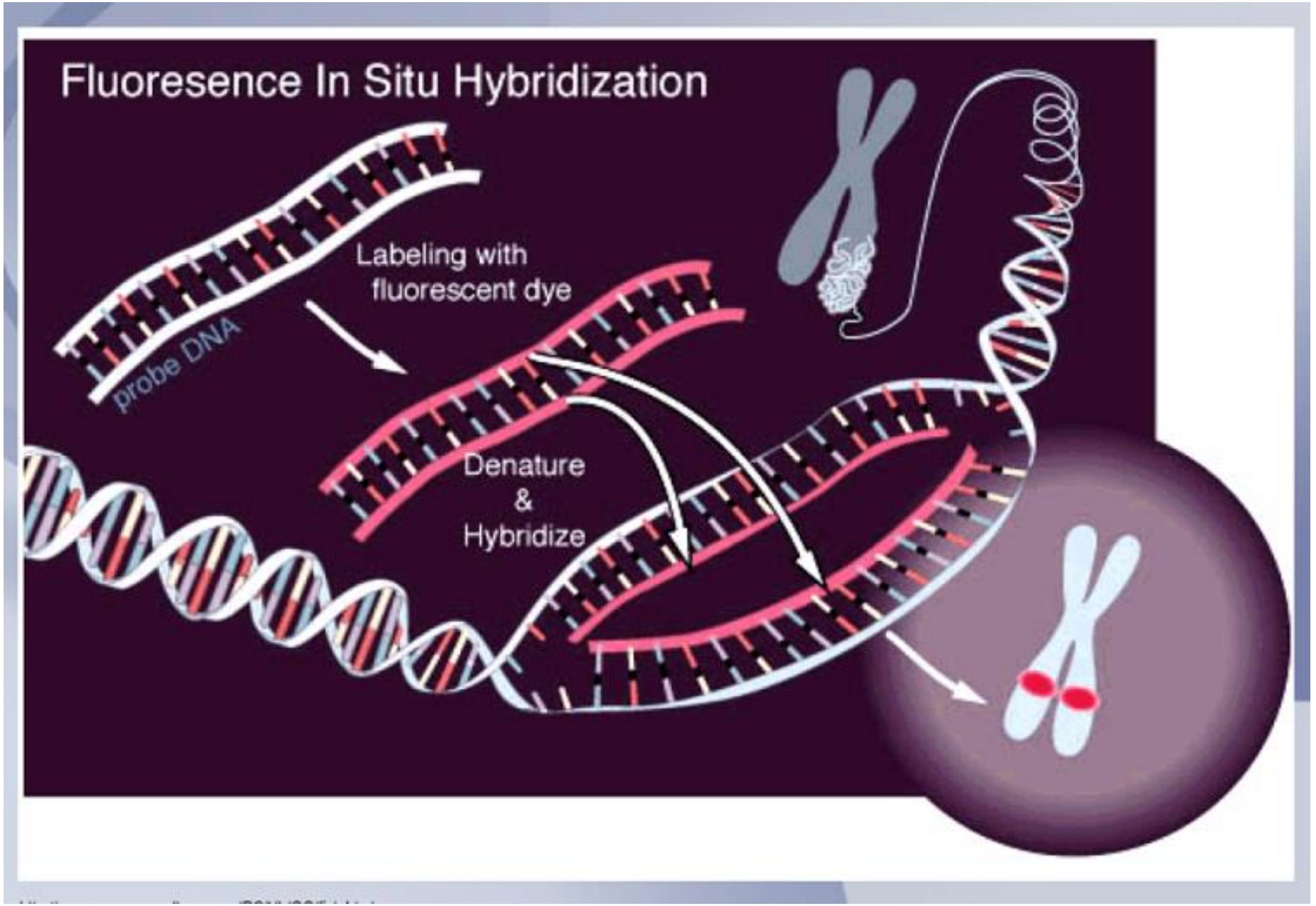


ApoE4

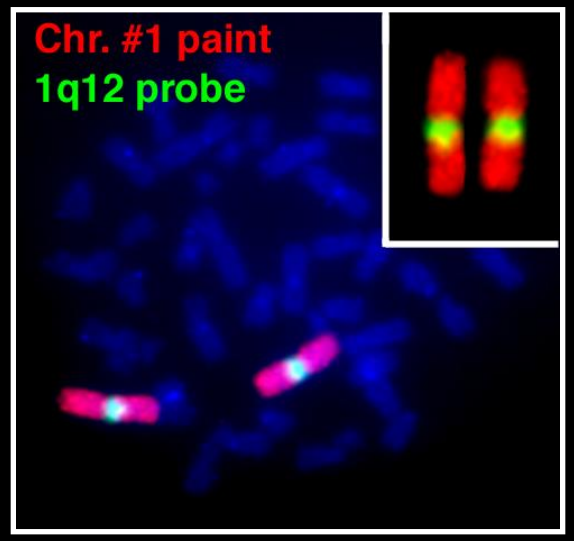


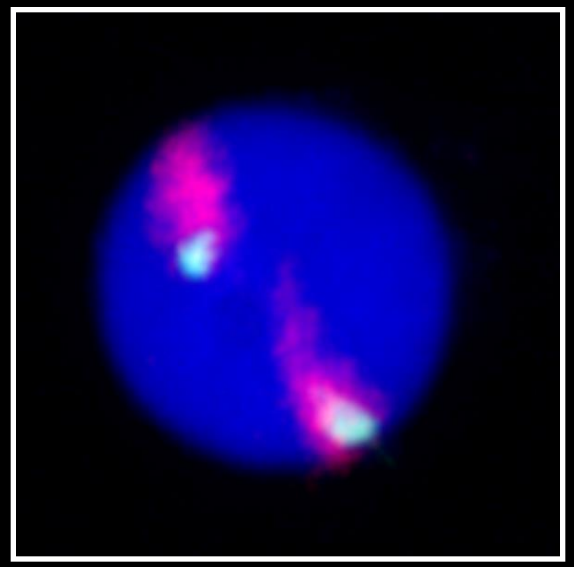


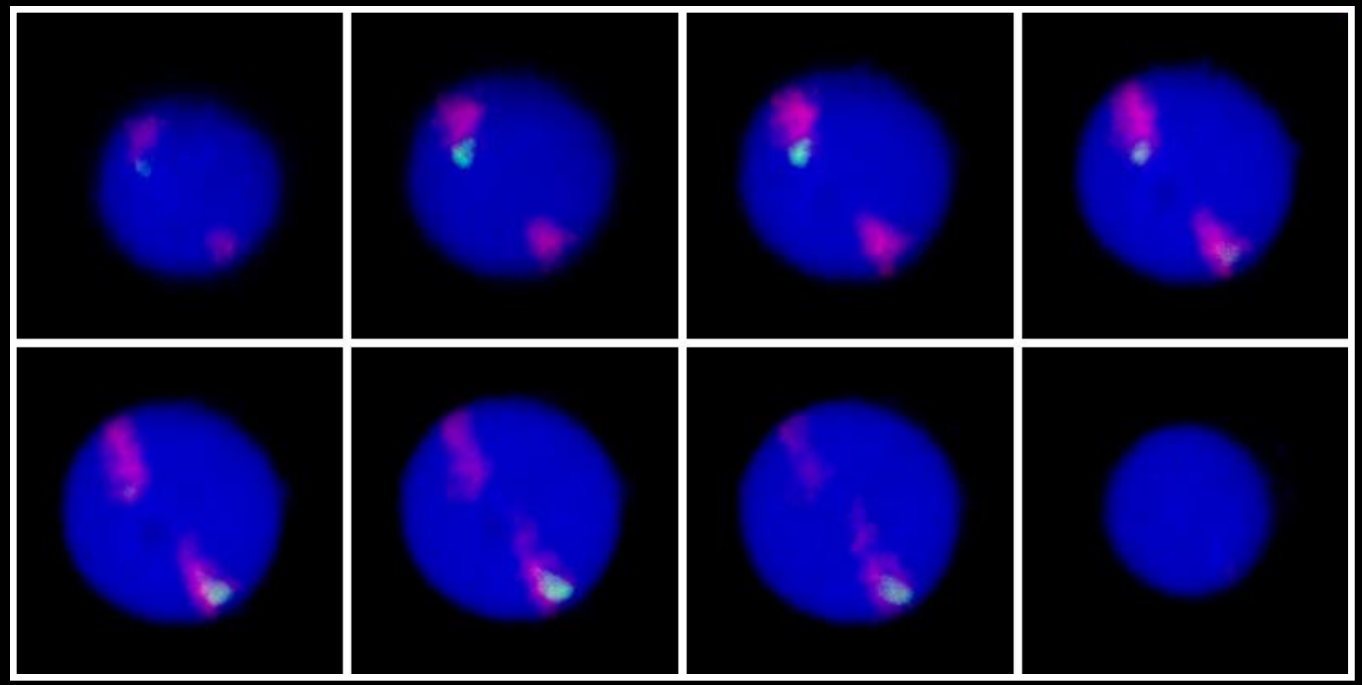
Principles of Fluorescence Spectroscopy, J. R. Lakowicz, 2010


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

FISH – Fluorescence *in situ* Hybridization



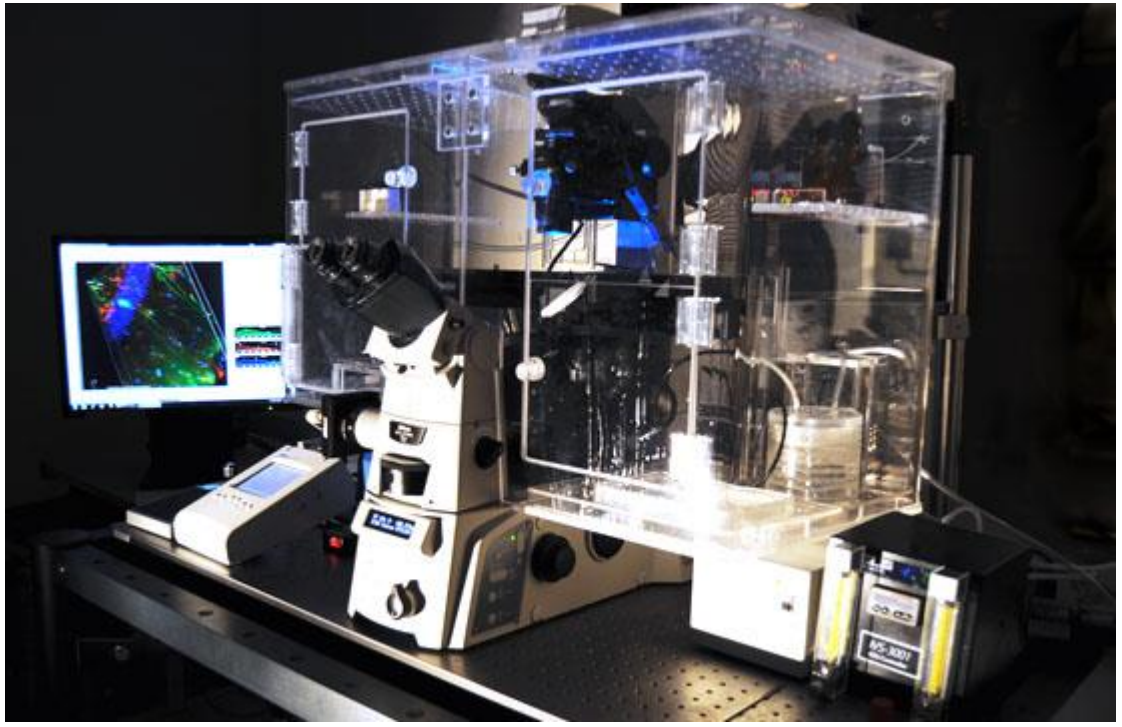




LIVE CELL IMAGING

Předpokladem pro vizualizaci živých buněk je inkubátor vestavěný do mikroskopu, který udržuje stabilní hladinu oxidu uhličitého (5 %) a teplotu (37 °C)

Kultivační miska s mřížkou



Nobelova cena za chemii 2014 : Objev superrezoluční fluorescenční mikroskopie

Eric Betzig

Stefan W. Hell

William E. Moerner

"The Nobel Prize in Chemistry 2014". *Nobelprize.org.* Nobel Media AB 2014. Web. 6 Nov 2014. <http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/>

PŘÍKLAD SUPERREZOLUČNÍ MIKROSKOPIE – STED (STIMULATED EMISSION DEPLETION)