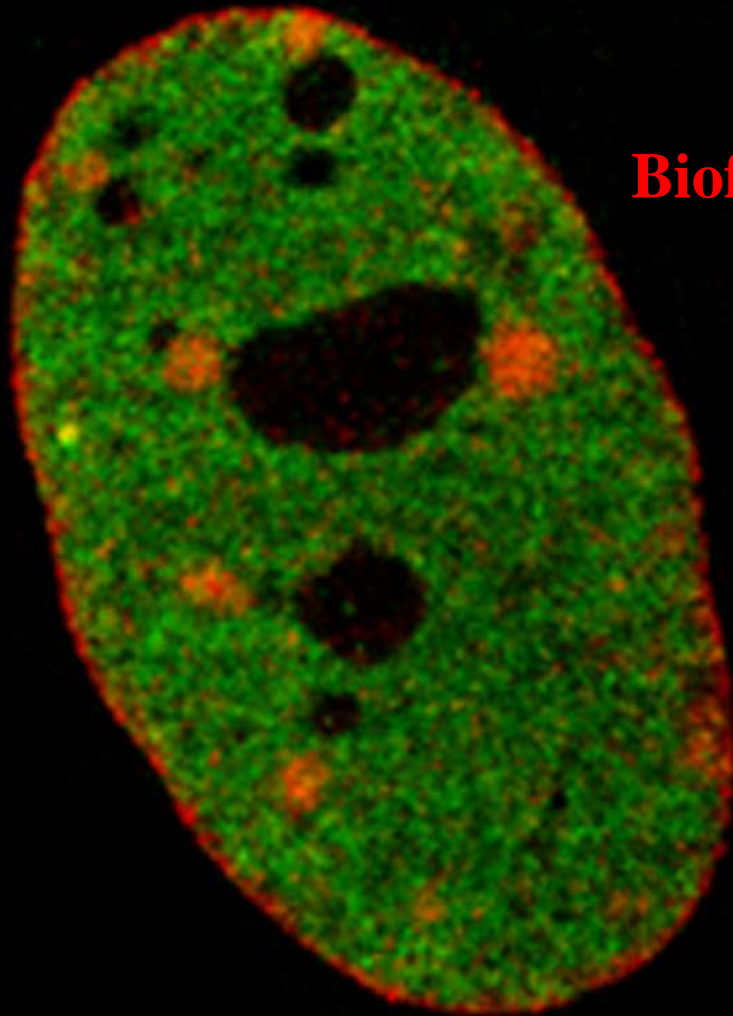
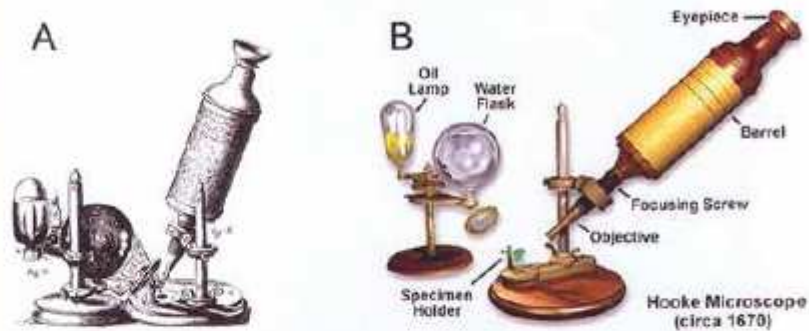


**Mikroskopické techniky a její
praktické využití**

**Eva Bártová
Biofyzikální ústav Akademie věd ČR
Brno**





1665 – Robert Hooke 1. mikroskop

1955 – Marvin Minsky (Harvard)
konfokální mikroskopie
1957-patentováno

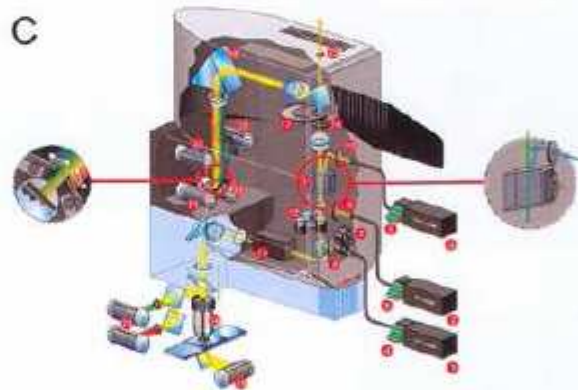
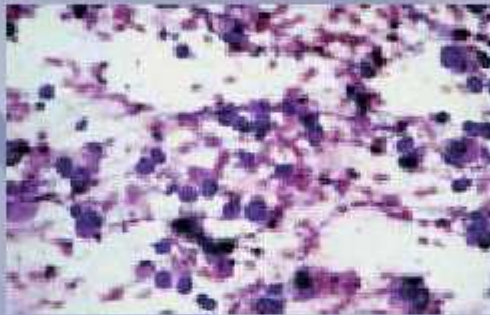


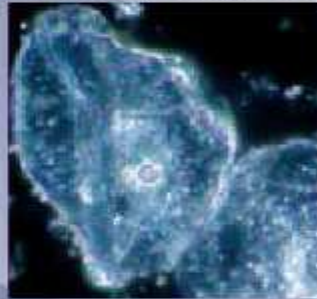
Figure 2. Microscopy from the 17th to the 21st centuries (see page 10).

(A) Robert Hooke's microscope as detailed in *Micrographia*, published in 1665 (23). Image reproduced with permission of the Charles Deering McCormick Library of Special Collections at Northwestern University, USA. (B) A schematic of Hooke's microscope showing the relevant components. Image reproduced with permission from the Molecular Expressions Microscopy Primer (www.microscopy.lsu.edu/primer/museum/hooke.html). (C) Leica Microsystems' TCS AOBs confocal scan head, commercially available from 2002 (www.leica-microsystems.com). The numbered components are as follows: 1, UV laser; 2, IR laser; 3, visible range laser; 4, UV acousto-optical tunable filter; 5, IR electro-optical modulator; 6, visible range acousto-optical tunable filter; 7, UV adaptation optics; 8, UV excitation pinhole; 9, IR excitation pinhole; 10, VIS excitation pinhole; 11, primary beam splitter; 12, adjustable pupil illumination; 13, 'K'-scanner with rotator; 14, microscope and objective; 15, transmitted light detector; 16, confocal detection pinhole; 17, analyzer wheel; 18, spectrophotometer prism; 19, photomultiplier channel 1; 20, photomultiplier channel 2; 21, photomultiplier channel 3; 22, photomultiplier channel 4; 23, external optical port; 24, nondescanned reflected light detectors.

- pozorování ve světlém poli (základní metoda, s níž se určitě setkal každý, kdo s mikroskopem někdy pracoval ve škole)



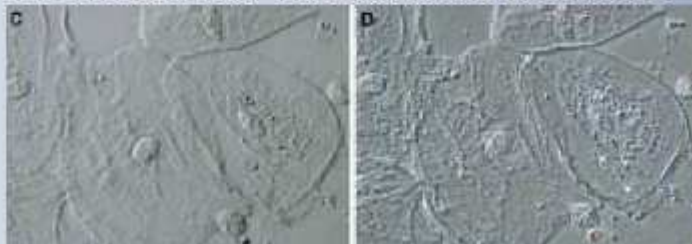
- pozorování v temném poli



- fázový kontrast



- Hoffmannův modulační kontrast

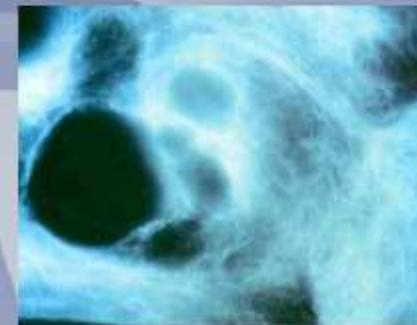


- diferenciální interferenční kontrast
- interferenční mikroskopie
- polarizační mikroskopie



Fig. 1: Vitamin C crystals observed in polarised light.

- fluorescenční mikroskopie



Fotoreceptorové buňky oka (ROZLIŠOVACÍ SCHOPNOST OKA)

- funkčně analogické k senzorům (picture elements, pixels) na CCD chipu televizní kamery
- absorbují světlo z obrázku, formují ho na retině pomocí rohovky a čočky
- posílají nervové impulsy do mozku
- 2 typy **tyčinky** – senzitivnější
 - noční vidění
- čípky** – barevné vidění při dostatečné intenzitě světla
 - s rozdílnou senzitivitou ke spektru – viditelnou část spektra rozdělují na 3 pruhy, které lidské oko vnímá jako red, green, blue (RGB)
 - 3 základní barvy lidského vidění

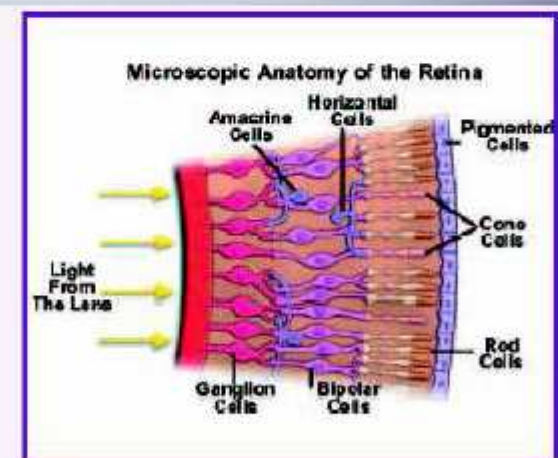
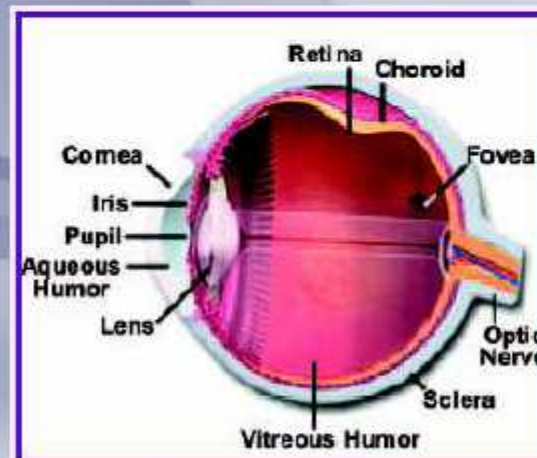
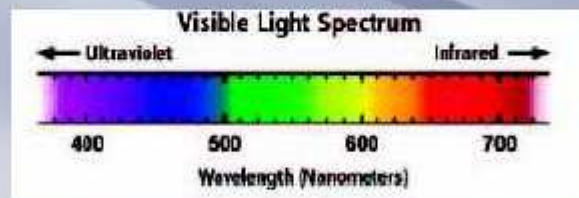
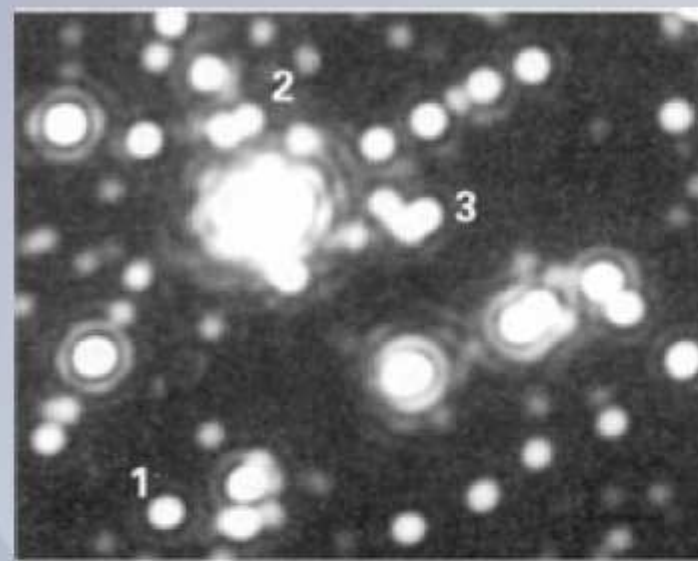


Fig. 1: Anatomy of the human eye and the retina.



Ilustrace vlivu numerické apertury objektivu na rozlišovací schopnost mikroskopu. Horní snímek – zrnka mikromletého vápence, pozorovaná v tzv. temném poli objektivem zvětšujícím 6,3krát, $NA = 0,20$.



Zrnka mikromletého vápence, pozorovaná v tzv. temném poli objektivem zvětšujícím 6,3krát, avšak o podstatně menší numerické apertuře ($NA \approx 0,03$);

- 1 – izolovaný bodový objekt (zrnko) se zřetelnými Airyho kroužky,
- 2 – dvě zrnka nacházející se ve vzdálenosti zhruba odpovídající teoretické rozlišovací schopnosti zvoleného objektivu,
- 3 – několik splývajících zrněk.

Numerická apertura nejkvalitnějších objektivů bývá ~ 1,3 až 1,4.

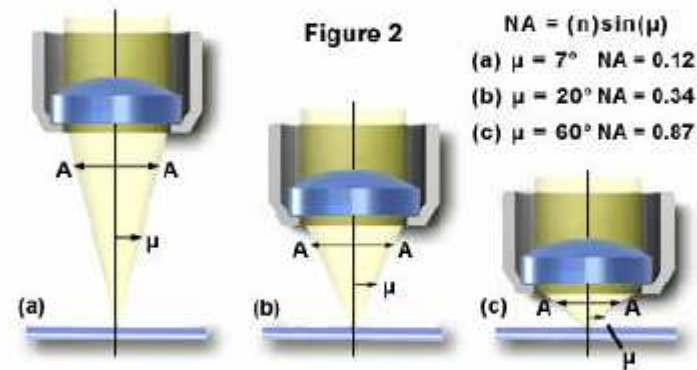
Numerická apertura objektivu (NA) je vyjádřena matematickým zápisem $n \sin \theta$, kde

n je index lomu prostředí před objektivem

θ je polovina vrcholového úhlu kužele paprsků vstupujících do objektivu.

Visible Light Microscopy: Objectives: numerical aperture

- NA=ability of lens to gather light and resolve detail at a fixed distance from object.
 - Dependent on ability of lens to capture diffracted light rays.
- n =Refractive index is limiting (air=1.0, oil=1.51)
 - Do not mix mediums when using a lens
- Theoretical resolution depends on NA and the wavelength of light. $NA=n \cdot \sin(\mu)$
 - Shorter wavelengths=higher resolution.
 - Resolution limit for green light (NA=1.4, 100X) is 0.2 μm .
 - $R=0.61\lambda/NA$



<http://www.micro.magnet.fsu.edu/primer/obja/microscopy/immersion/index.html>

Visible Light Microscopy

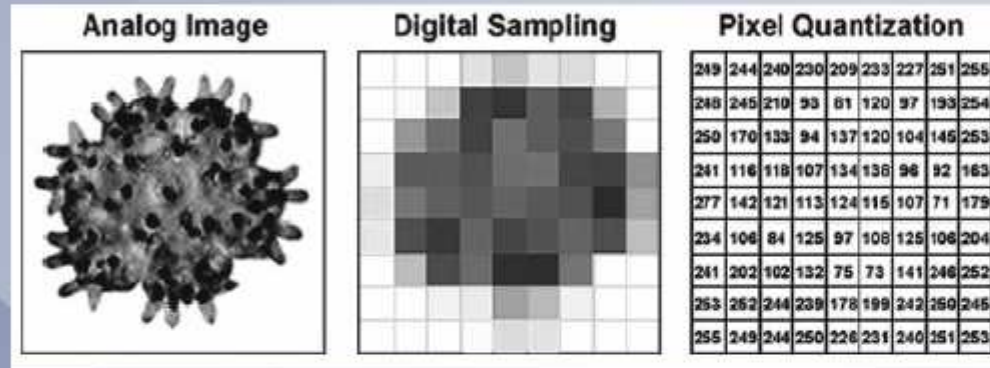
Objectives: Specifications and Identification

- Older lenses need to match oculars, now lenses are infinity-corrected.
- Information on objective barrel:
 - Linear magnification
 - Numerical aperture
 - Optical corrections
 - Achromatic: color (red/blue) corrected.
 - Fluorite: optical aberration corrected
 - Apochromatic: color (red, green, blue and spherical aberration corrected
 - Microscope tube length
 - Coverglass thickness (0.17mm)
 - Immersion medium (air, water, oil)



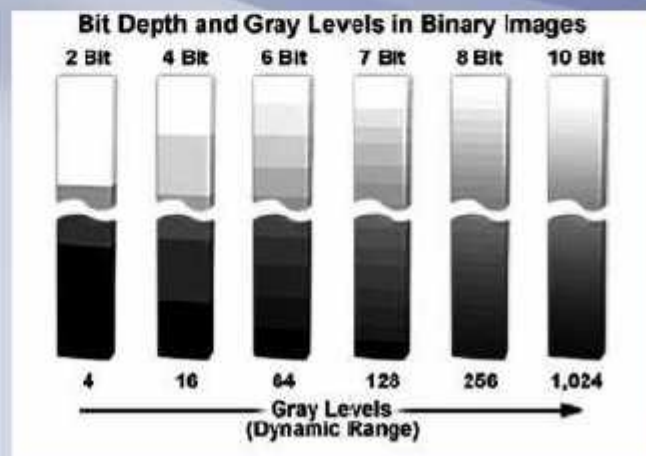
Figure 1

Vytváření digitálního obrazu



Světlo generuje elektronovou díru cestou fotoelektrického efektu. Náboje se sbírají, spojují se ve svazky a následně jsou transportovány přes CCD senzor, náboj se převádí na elektrické napětí. To je amplifikováno na samotném chipu i mimo něj.

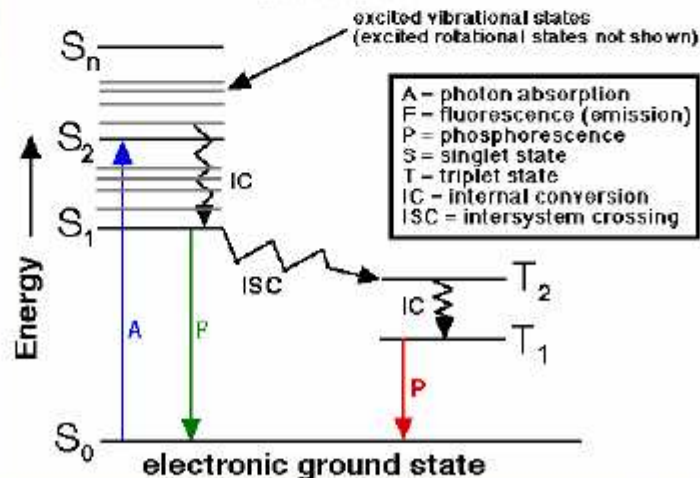
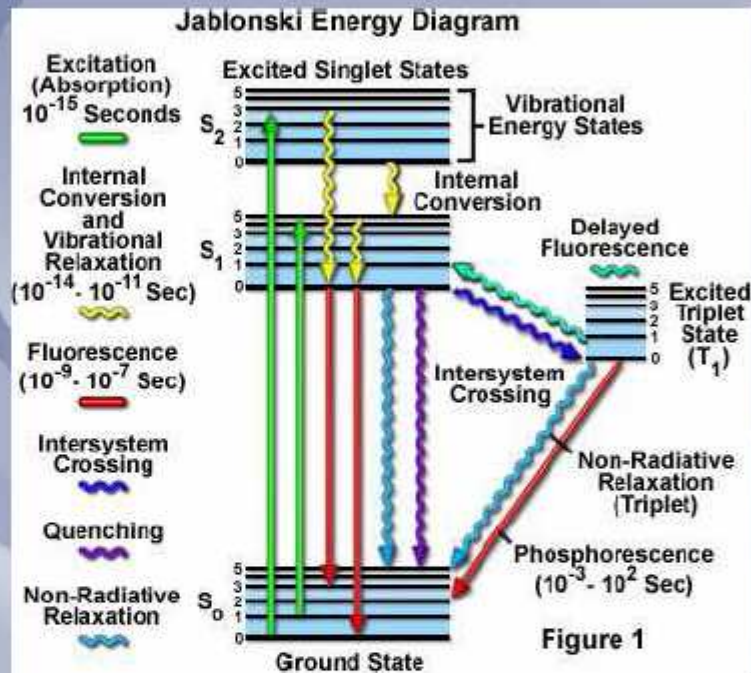
Analogově číslicový konvertor převádí napětí (signál) na binární formát o různé bitové hloubce.



Počet pixelů kamery závisí na použitém CCD chipu a na technologii vytvoření obrazu.

Fig. 2: Bit depth and gray levels in digital images

Fluorescenční mikroskopie



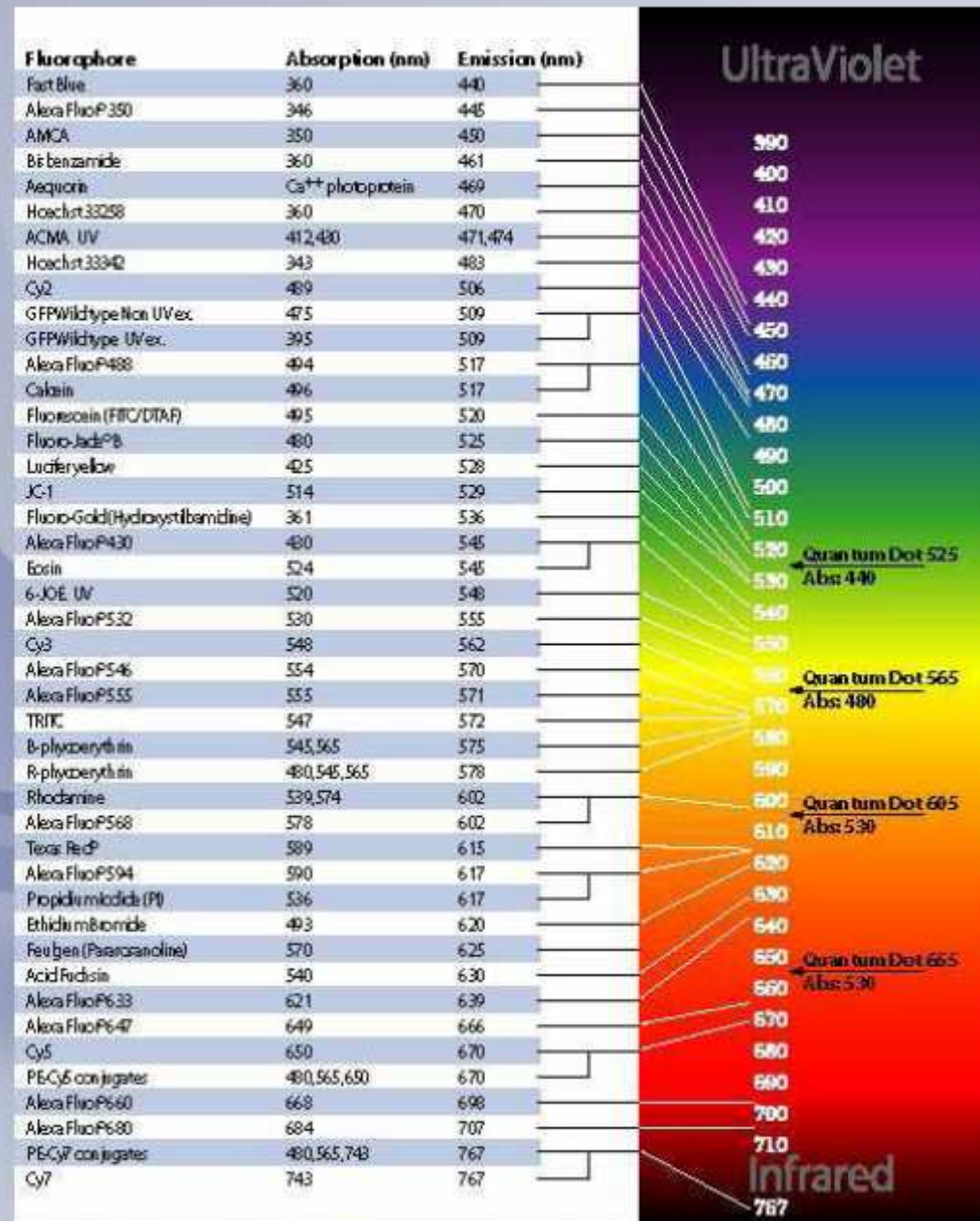
Polský prof. Alexander Jablonski vysvětlil, jak jsou elektrony v fluoroforech excitovány ze základního stavu do stavu energeticky vyššího a jak mohou tyto excitované elektrony emisi fotonů relaxovat nebo jinými mechanismy se nakonec vrátit zpět na základní hladinu.

Fluorescence je statisticky pravděpodobnější než fosforescence, doba existence fluorescenčního stavu je velmi krátká (1×10^{-5} to 10^{-8} s), zatímco fosforescence je poněkud delší (1×10^{-4} s až minuty nebo hodiny)

FLUOROCHROMY:

3 skupiny

1. fluorochromy nesoucí další molekulu, která je cílená na určitou strukturu (protilátky, lektiny, a tzv. quantum dots)
2. základní jejich vlastností je vazebná schopnost na nějakou strukturu (DAPI) nebo se mění jejich fluorescence, s měnícím se množstvím navázané molekuly
3. fluorochromy produkované samotnými organismy (GFP) – znázorňování živých buněk, vývojové studie a vůbec použití v molekulární biologii





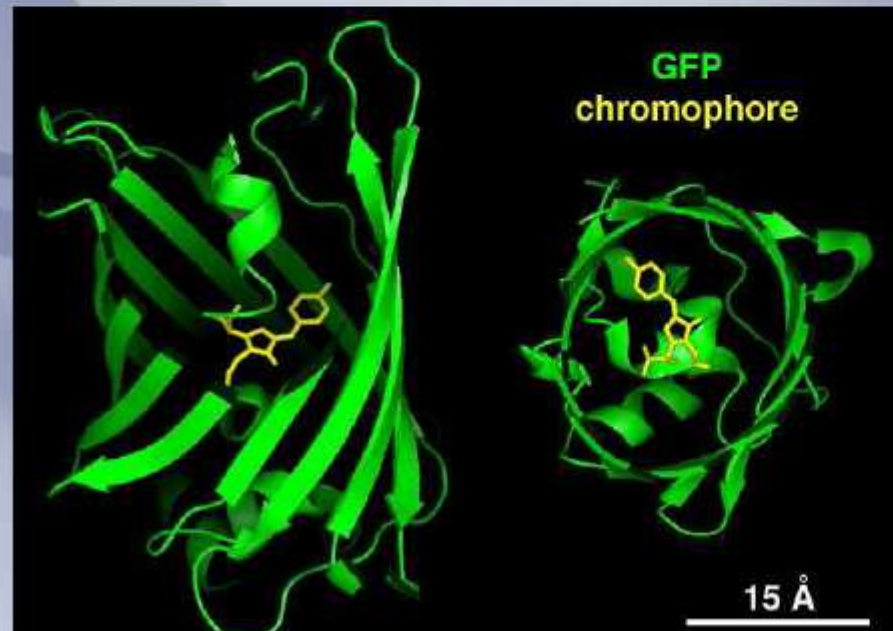
Aequorea victoria

Fenomén fluorescence první pozoroval Sir George Gabriel Stokes roku 1852

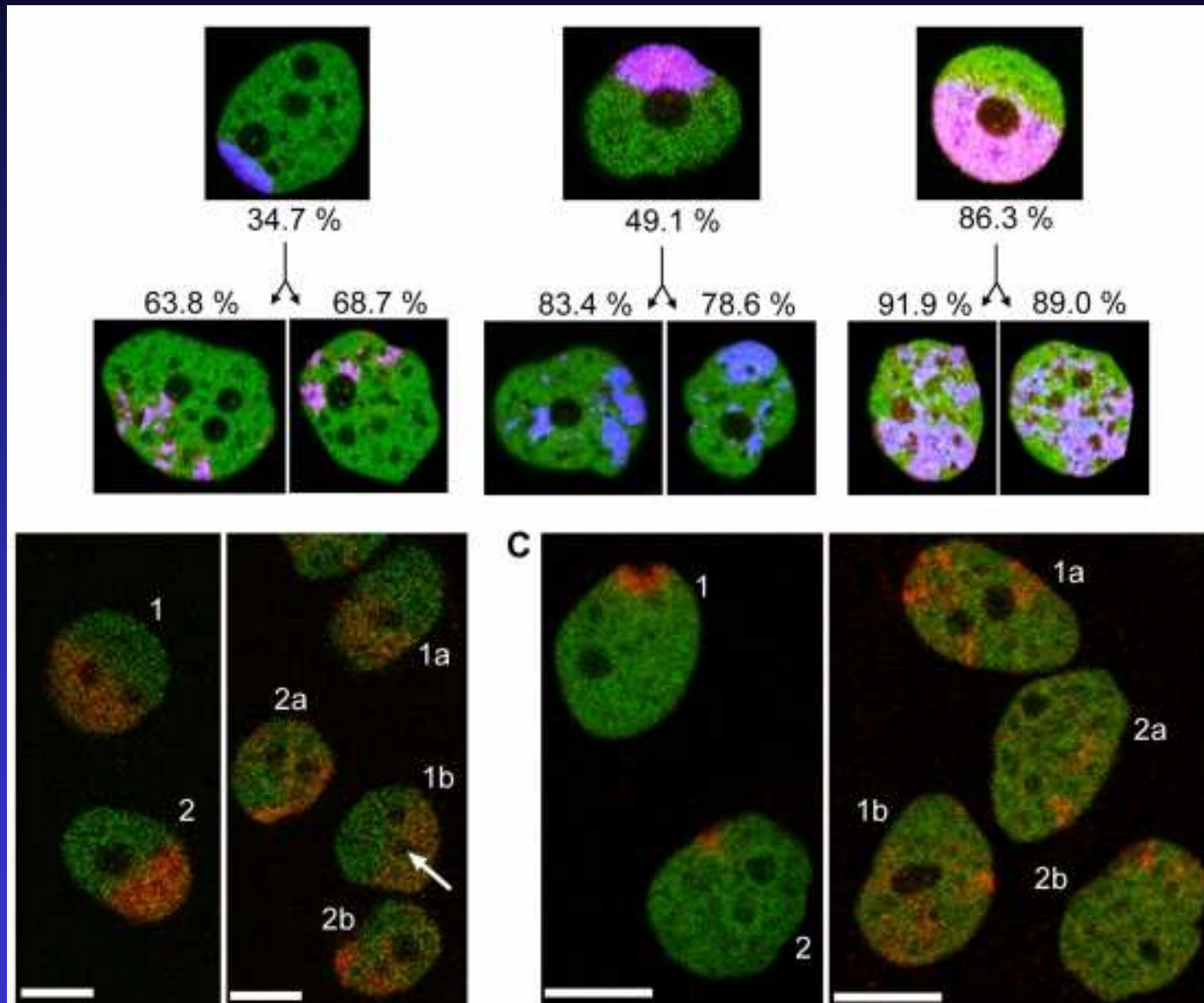
Jev byl fyzikálně popsán Alexanderem Jablonskim roku 1935

Počet užívaných fluorochromů se uzavřel v 90. letech 20. stol.

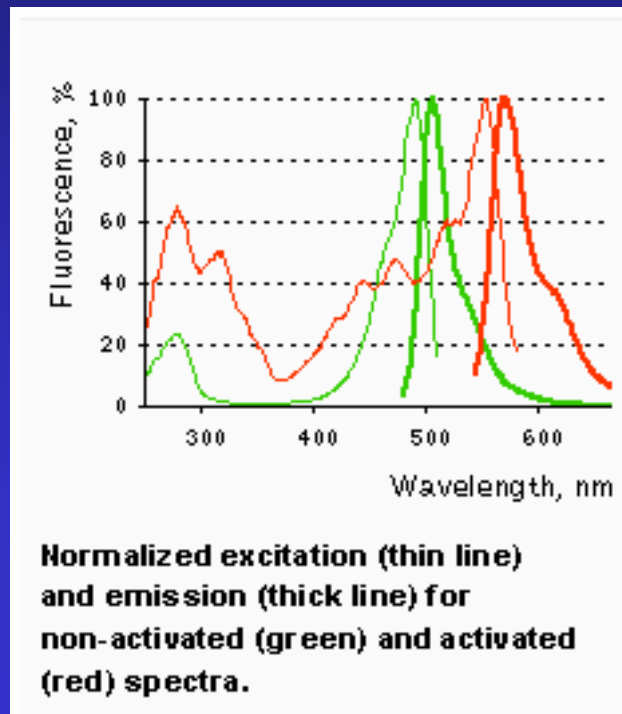
Shimomura izoloval v roce 1962 z medúzy *Aequorea victoria* žijící v Tichém oceánu green fluorescent protein - GFP



Dendra2 photo-conversion



Dendra2 is an improved version of a green-to-red photoswitchable fluorescent protein Dendra, derived from octocoral *Dendronephthya* sp. [Gurskaya *et al.*, 2006].

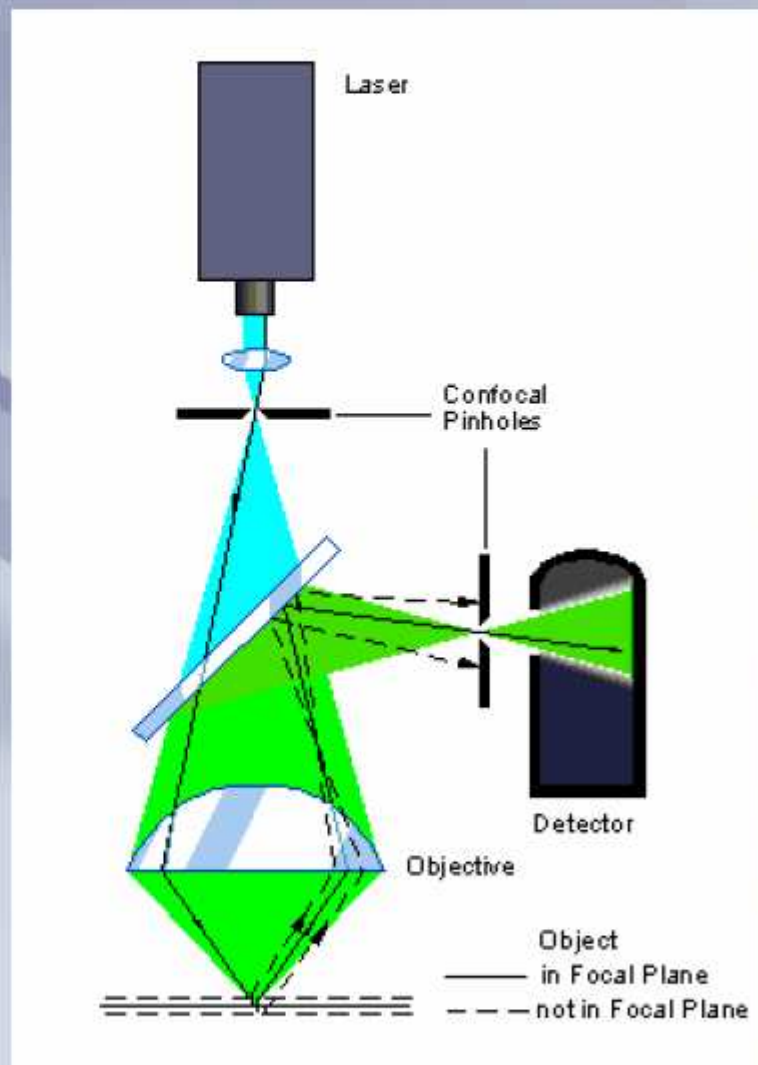


Faktory ovlivňující citlivost fluorescence

1. Intenzita zdroje
2. Účinnost optického systému
3. Štěrby monochromátoru
4. Citlivost detektoru

- vzorek sám o sobě obsahuje fluorescenční molekuly (**auto-fluorescence**)
- nutno fluorochrom ke vzorku přidat v závislosti na cílech pozorování

Principle of confocal microscopy



Kontrolovaná hloubka pole

Eliminace degradace obrazu mimo ohnisko pomocí prostorové filtrace

Eliminace Airy disc pattern

Série optických řezů o definované tloušťce

Vysoká kvalita obrazu

- Stage scanning
- Beam scanning
- Nipkow disk scanning
- LSCM

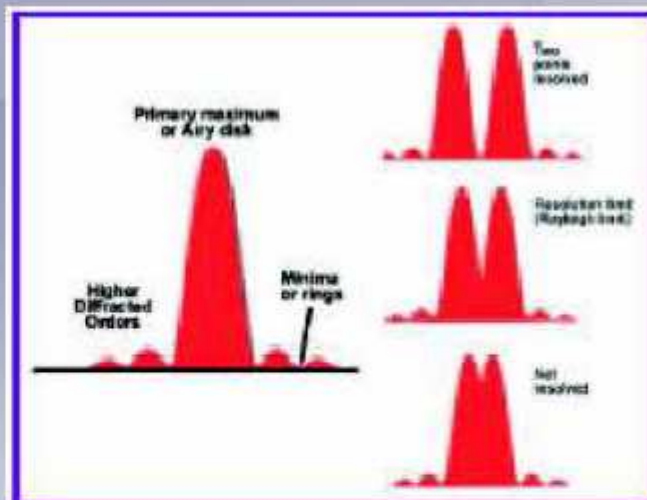


Fig. 4: Intensity profiles of the Airy disk patterns of one specimen detail and of two details at different distances.

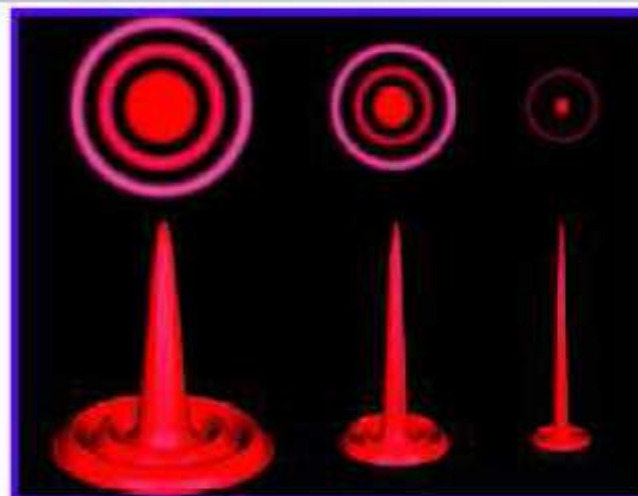
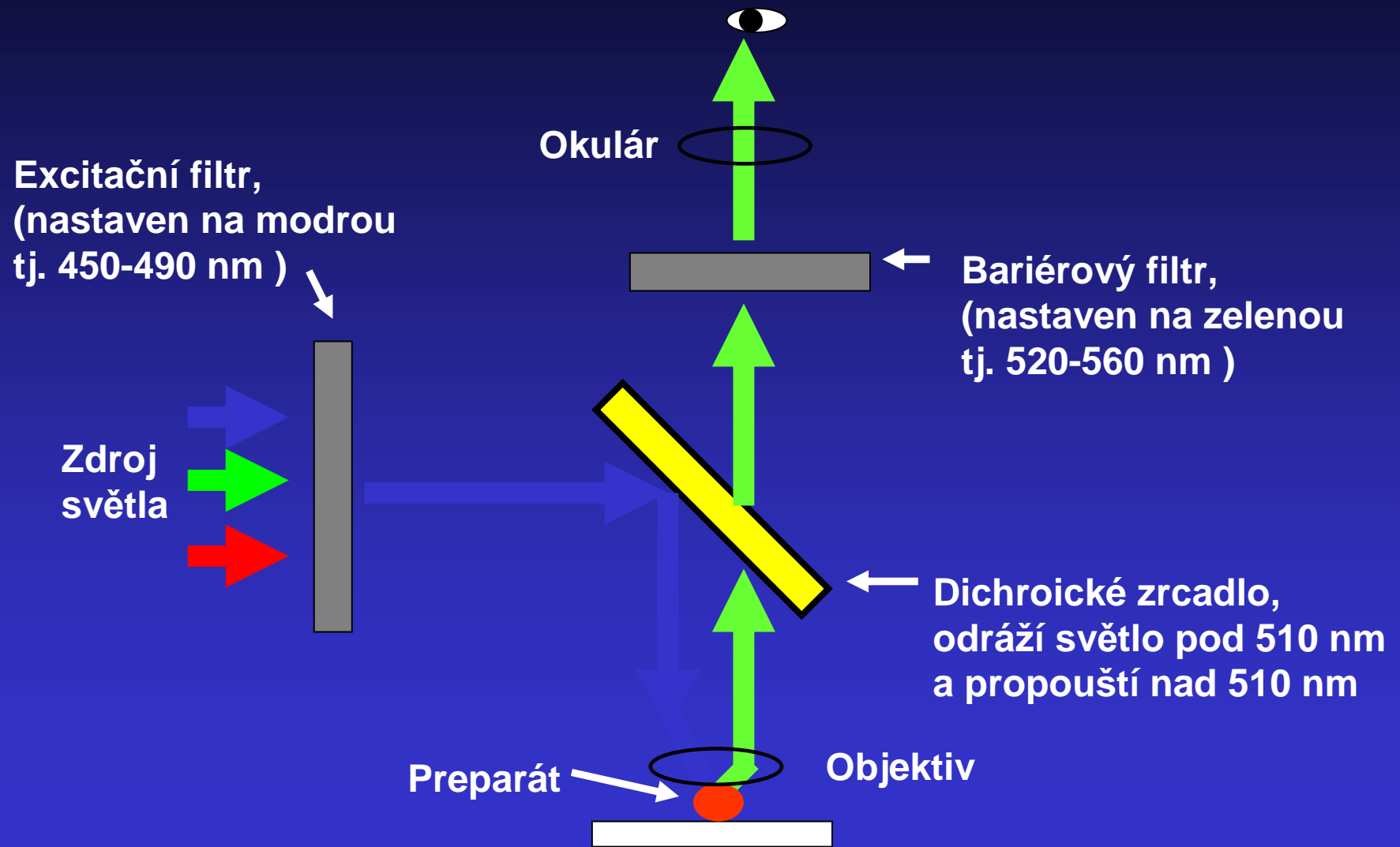


Fig.5: Airy disk patterns of different size as an example for the resolving power of low NA (left) and high NA (right) objectives.

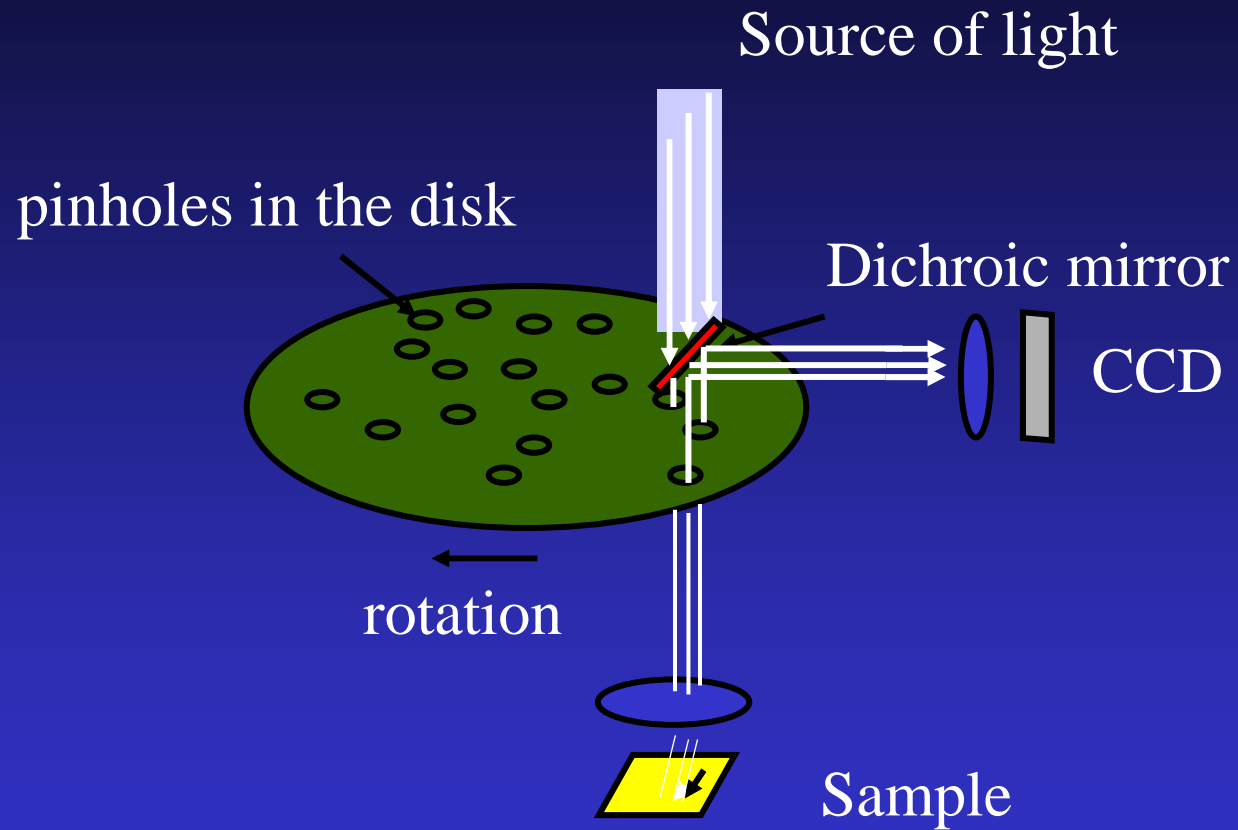
Pod rozlišovací schopností mikroskopu se chápá minimální vzdálenost dvou bodů objektu, které se ještě zobrazí jako navzájem oddělené. Žádný objektiv nemůže zobrazit bodový objekt opět jako bod. I při dokonalé korekci všech možných vad zobrazení, které souvisí s technologií výroby objektivů, jsou obrazem bodu Airyho kroužky. Tak se nazývá difrakční obrazec vznikající ohybem zobrazujícího se světla na čočkách objektivu. Při zobrazení dvou blízkých bodů se mohou jejich Airyho kroužky překrývat, až se při jisté minimální vzdálenosti stanou téměř nerozlišitelnými. Tato mez se běžně odhaduje dle Rayleighova kritéria (1879), které v podstatě vychází ze skutečnosti, že lidský zrak zaznamená pozvolný předěl mezi dvěma difrakčními kroužky teprve tehdy, poklesne-li intenzita mezi nimi alespoň o 20 % oproti přilehlým maximům. Pro modré světlo (viditelné záření nejkratších vlnových délek) se teoretická rozlišovací schopnost blíží hodnotě kolem 0,17 μm . Praktická rozlišovací schopnost reálných objektivů závisí ovšem také na tom, jak dokonale se při výrobě podaří zkorigovat různé vady zobrazení.

Princip fluorescenčního mikroskopu



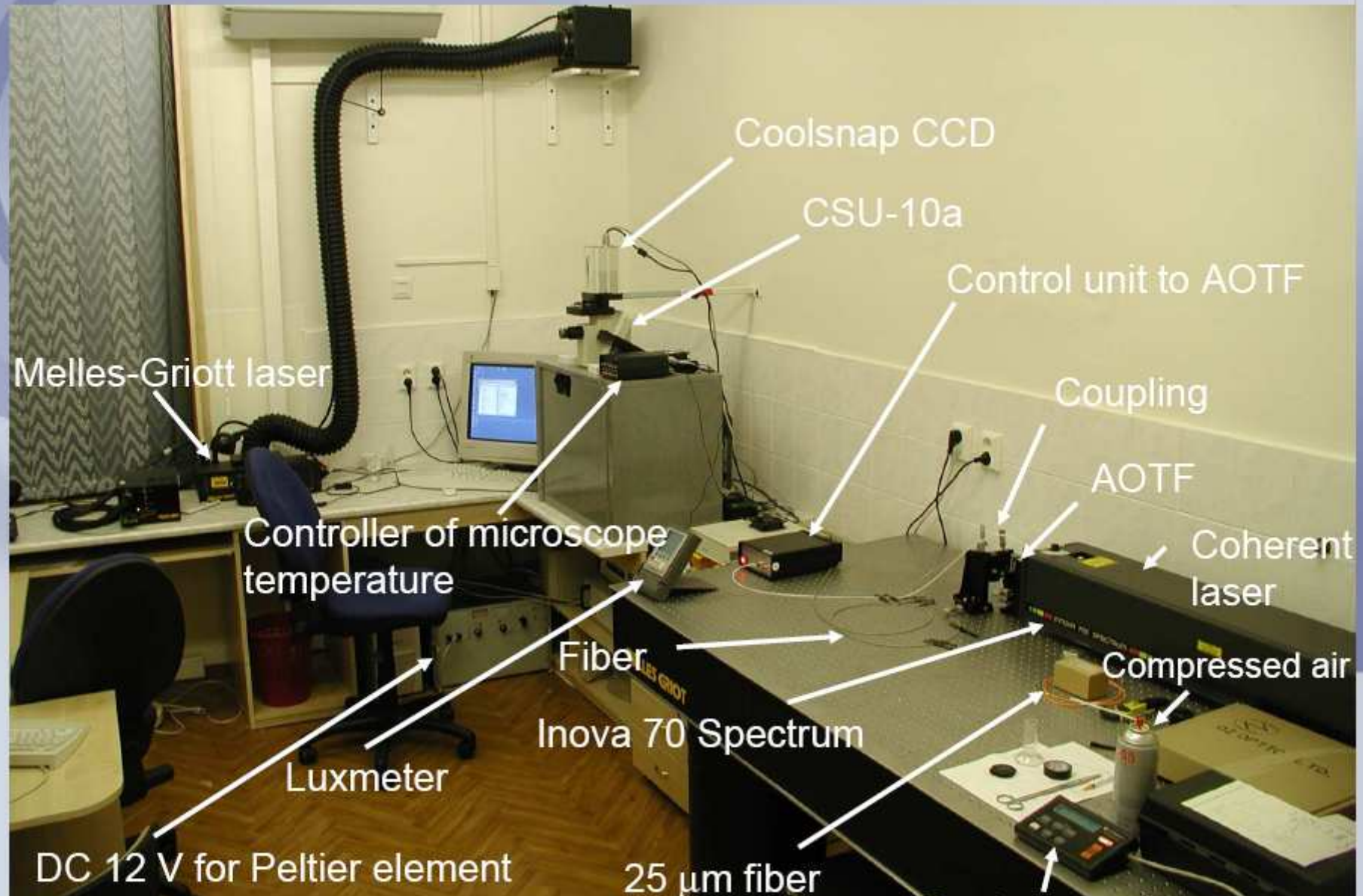
Vstupní štěrbinu, dělič svazků, dichroické zrcadlo, AOTF (akusto-optický dělič svazků)

Tandem scanning microscopes based on Nipkow disk

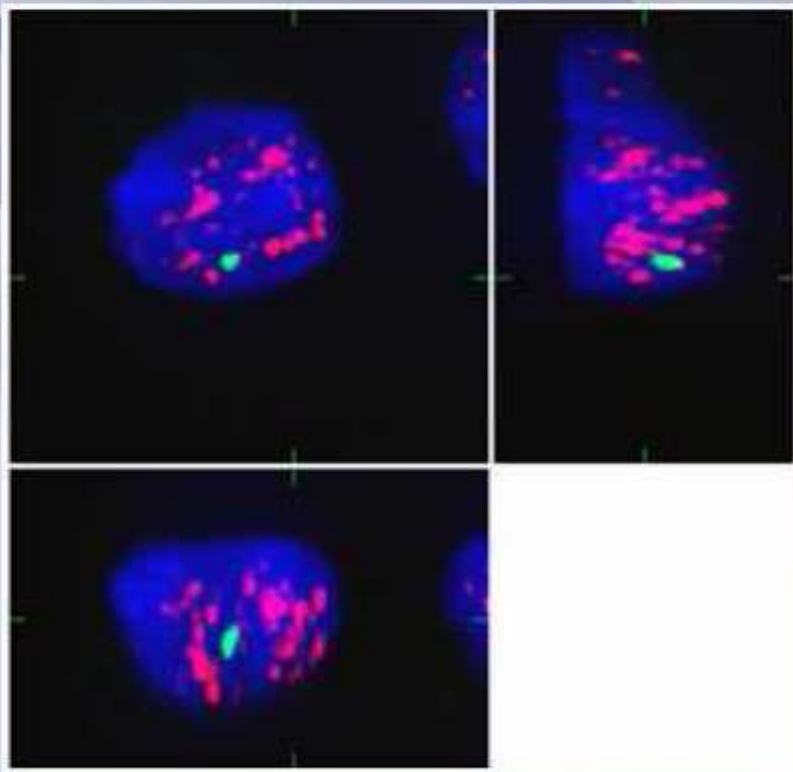
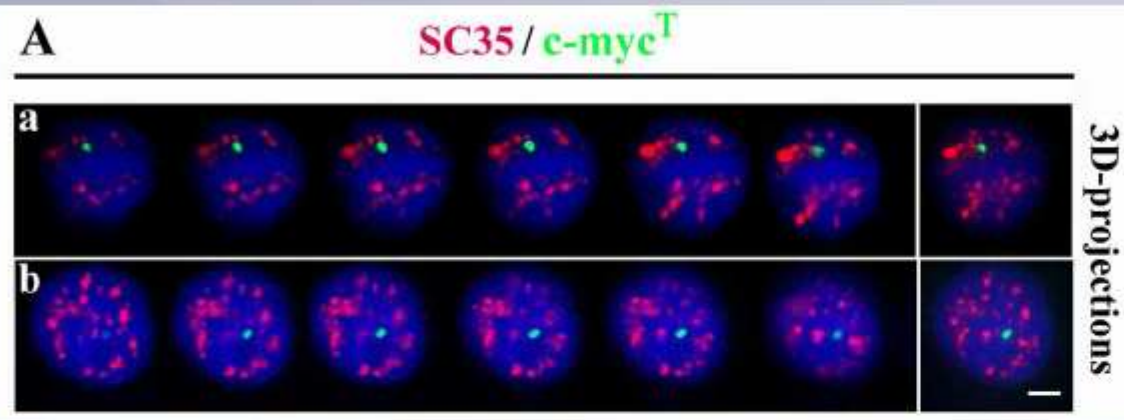




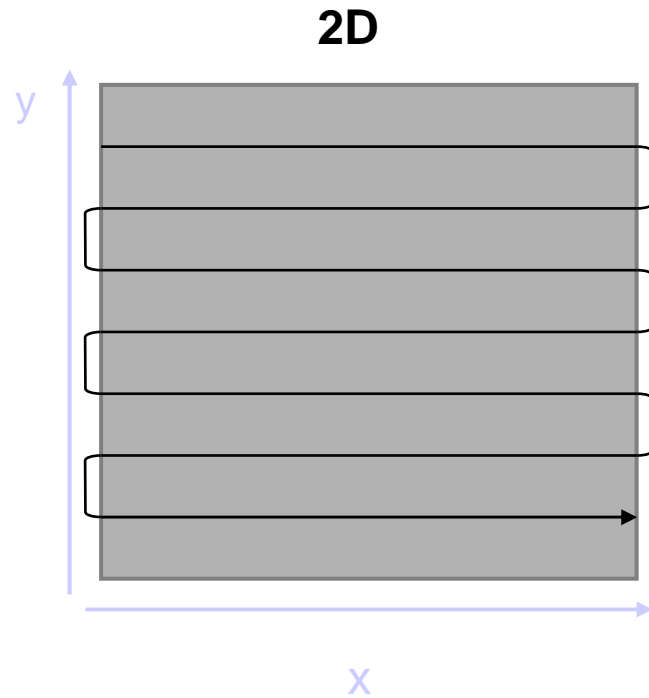
Mikroskopovna



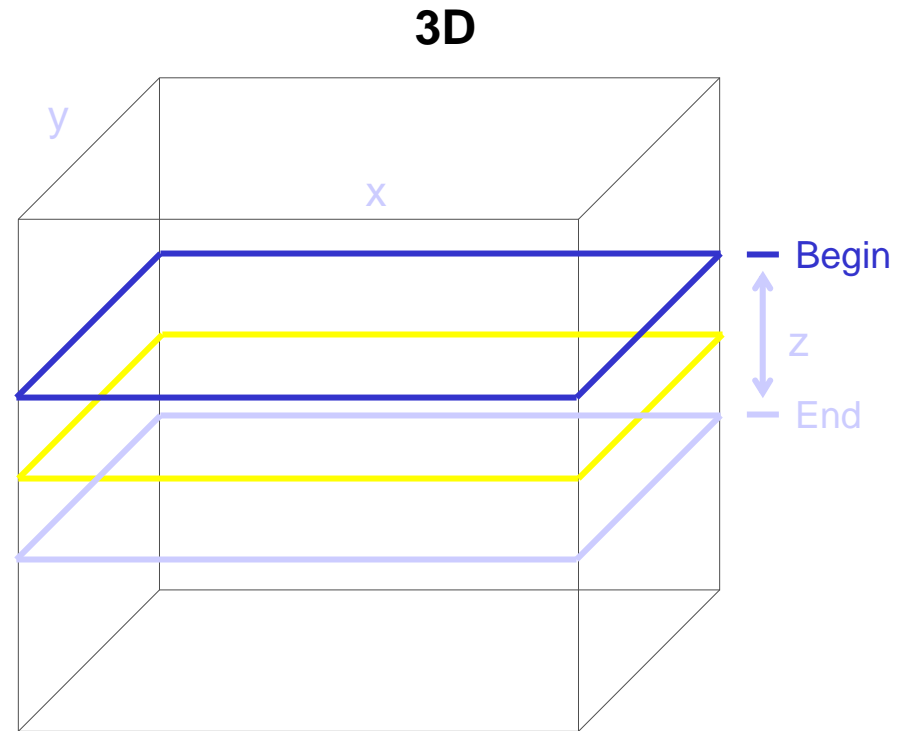
3D-projection



Scanning in 2D and 3D by confocal microscope

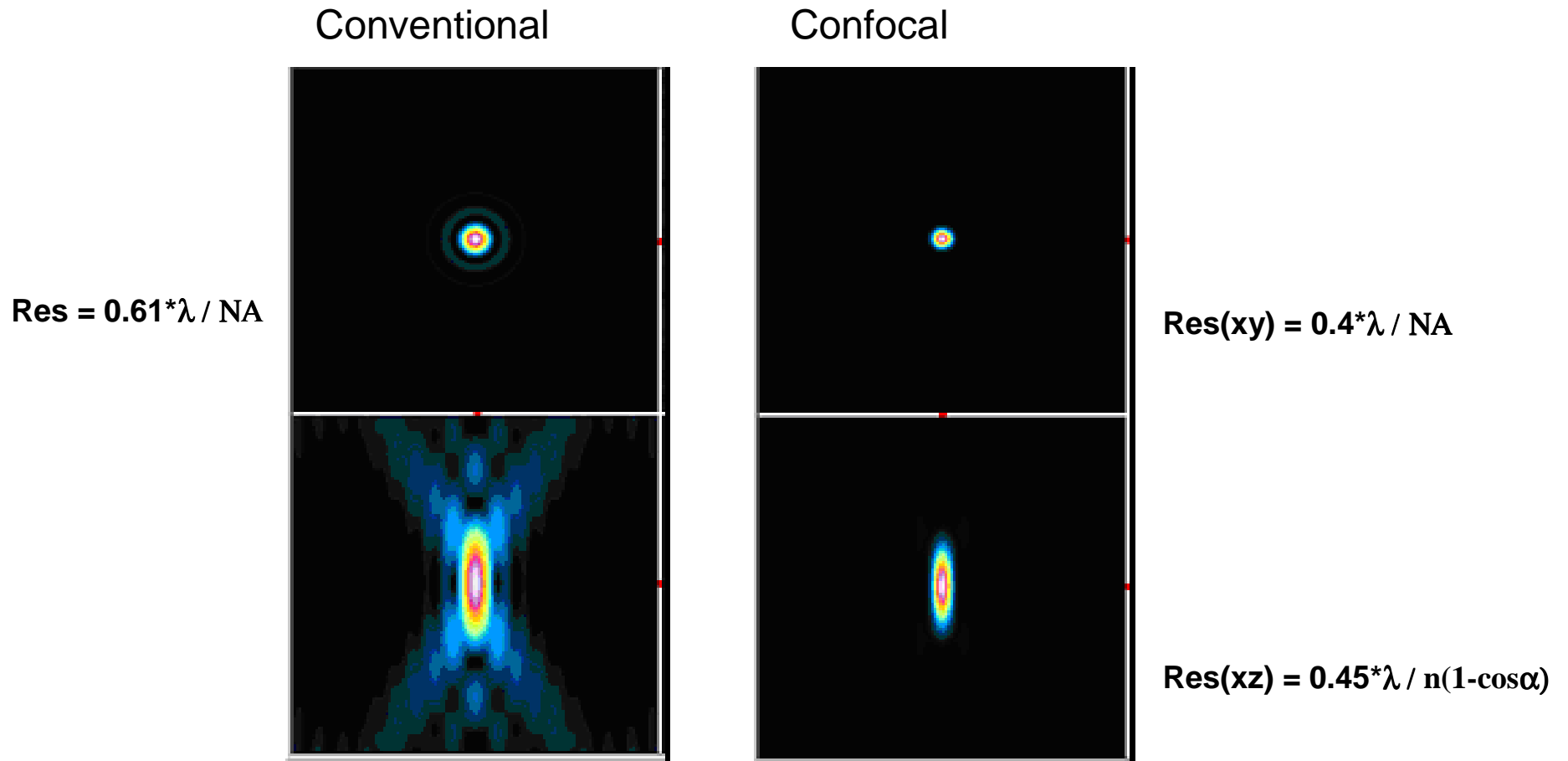


Laser beam moves firstly along x axis and then starts with new line in y axis.



Finishing scanning of one thin optical slice in xy plane, the scanning plane is moved in z axis to other slice

Optical resolution: conventional versus confocal



Formulas by Kino

4PI and STED resolution are much higher...

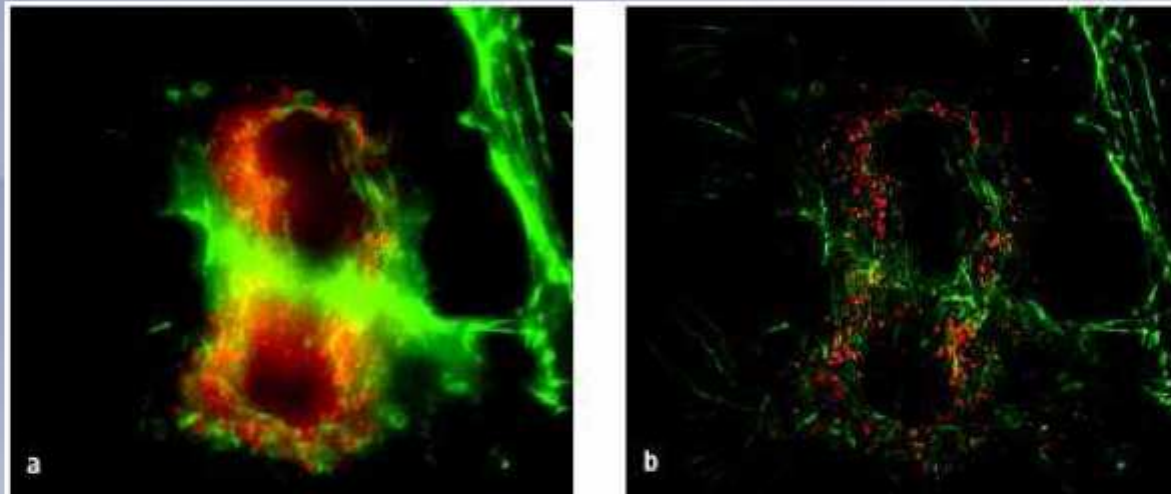
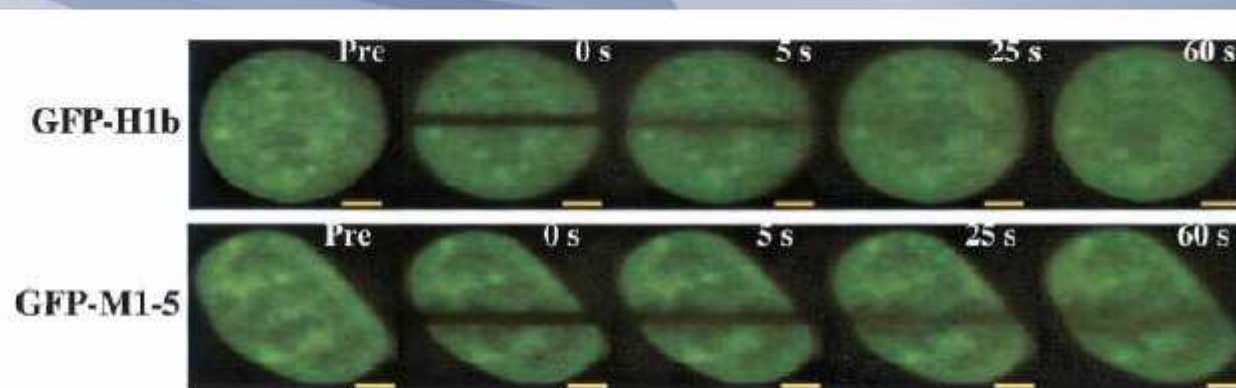
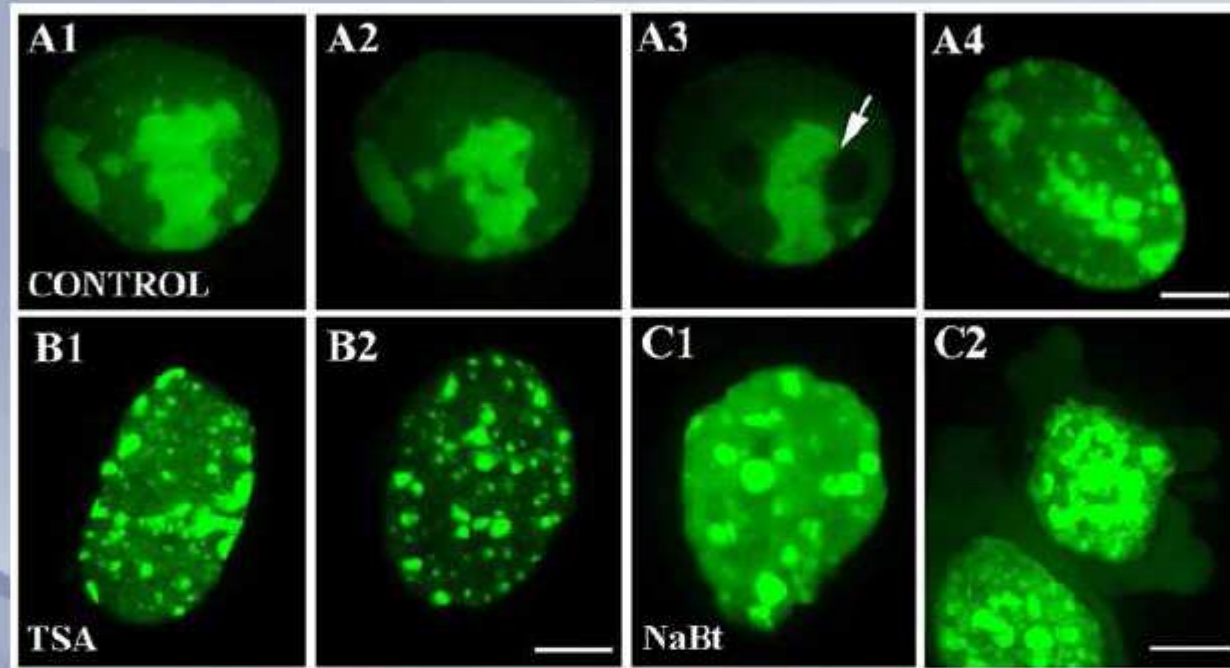


Fig. 3: Via deconvolution, artefacts can be computed out of fluorescence images. a). These artefacts are caused by the stray light from non-focused areas above and below the focus level. These phenomena, referred to as convolution, result in glare, distortion and blurriness. b). Deconvolution is a recognised mathematical procedure for eliminating such artefacts. The resulting image displayed is sharper with less noise and thus at higher resolution. This is also advantageous for more extensive analyses.

KONVOLUCE – artefakty obrázku
 – nezaostřená místa pod nebo nad rovinou zobrazení (u fluorescenceční mikroskopie nebo u histologických řezů - záře, deformace obrazu, zastření).
 – omezuje přesnost a rychlost analýzy obrazu

DEKONVOLUCE – matematická metoda sloužící k eliminaci artefaktů obrázku
 – výsledný obrázek má méně šumu a větší rozlišení

GFP-HP1 β

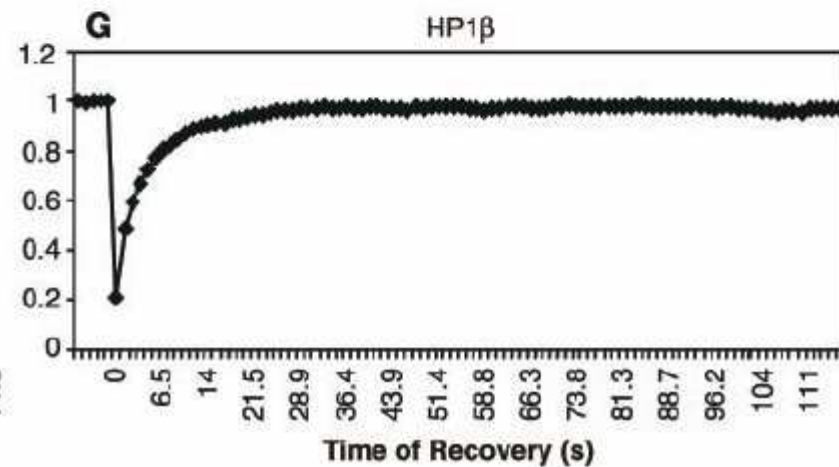
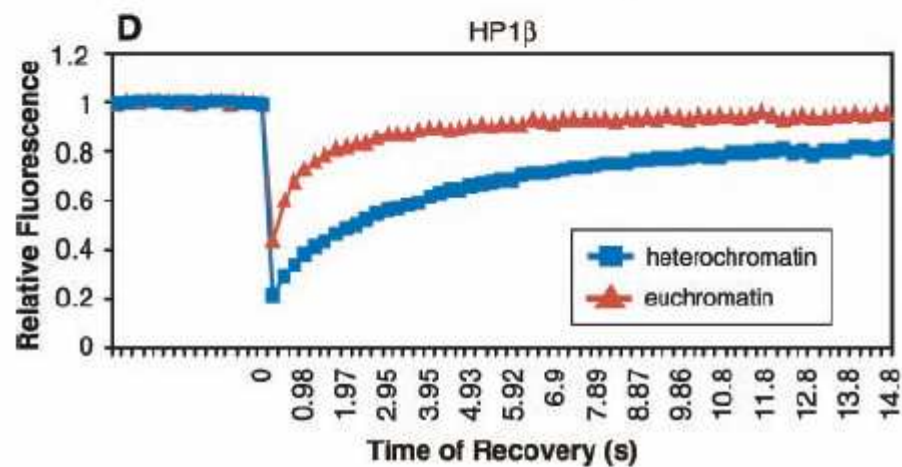
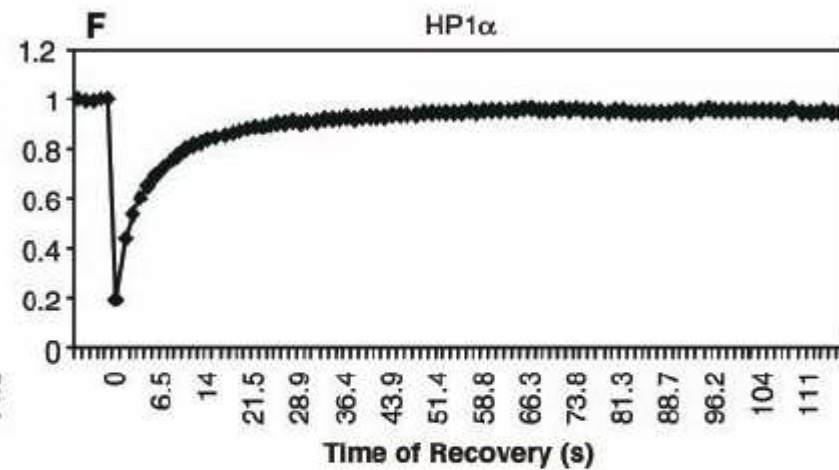
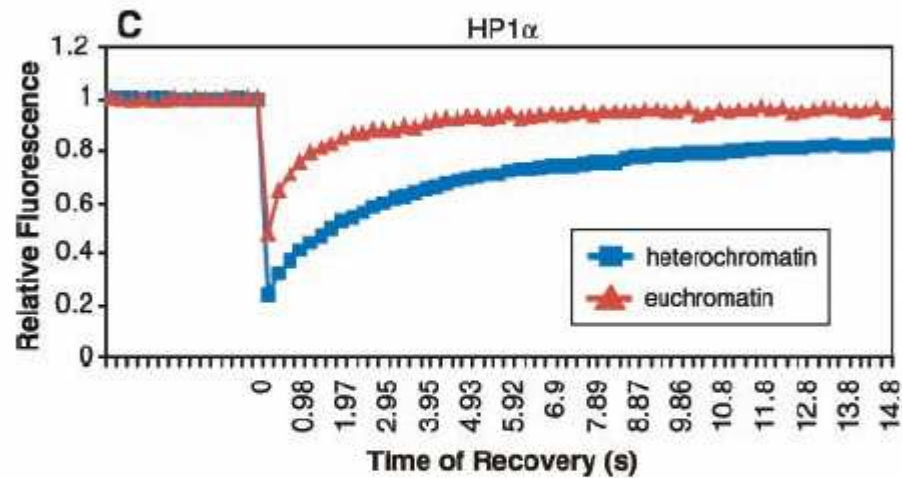
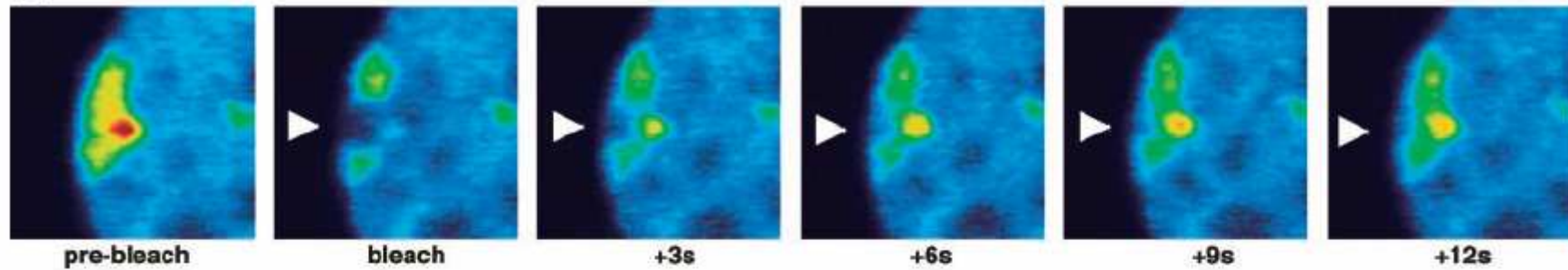


Co je to FRAP?

FRAP (Fluorescence Recovery after Photobleaching)

- návrat fluorescence v definované oblasti vzorku po vysvícení 'bleaching procesu'.
- výsledkem je pohyb nevysvícených fluoroforů z okolních míst vysvícené oblasti
- je využíván k měření dynamiky 2D nebo 3D mobility molekul, např. difuze, transport nebo další typy pohybů fluorescenčně značených molekul membrán nebo živých buněk



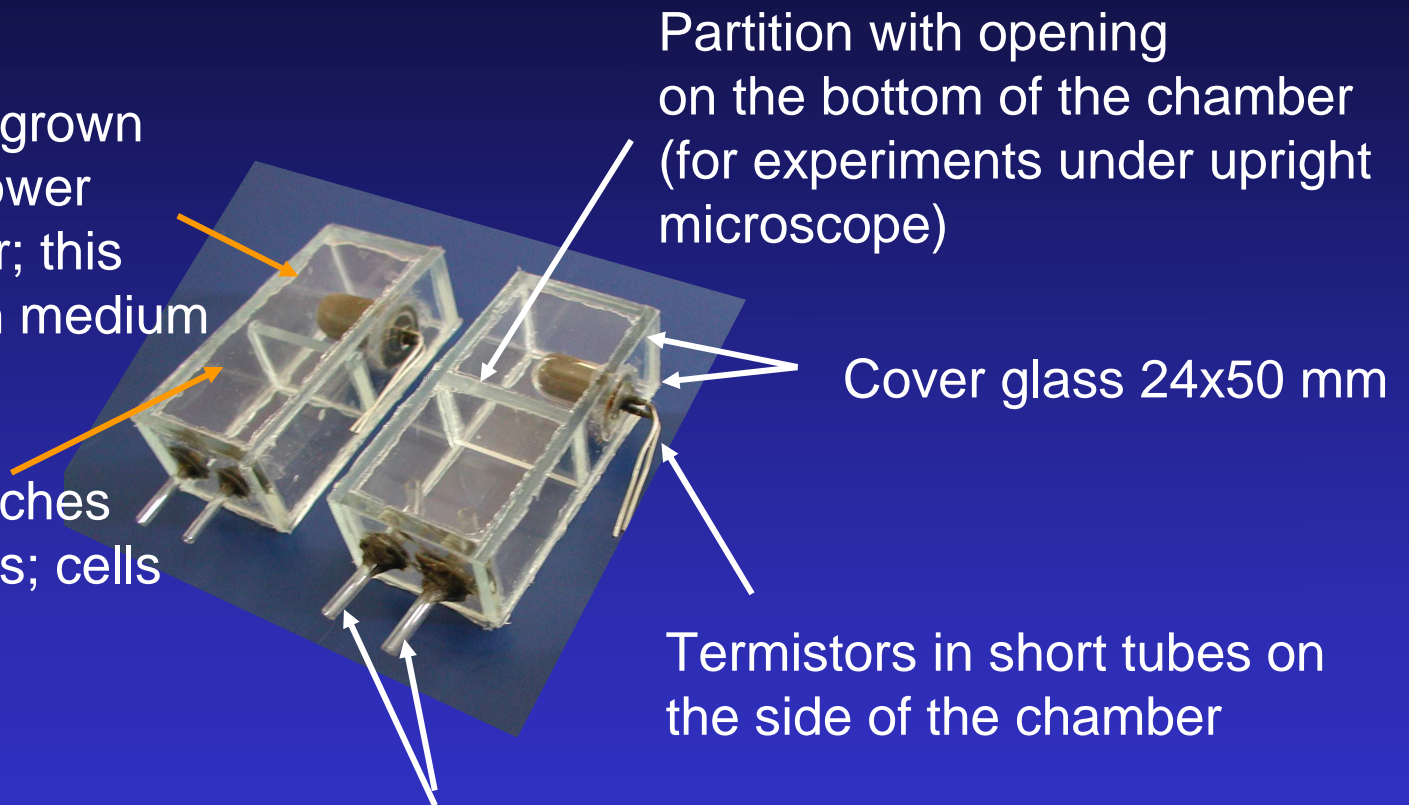
B



Inkubační komůrky pro přímý mikroskop

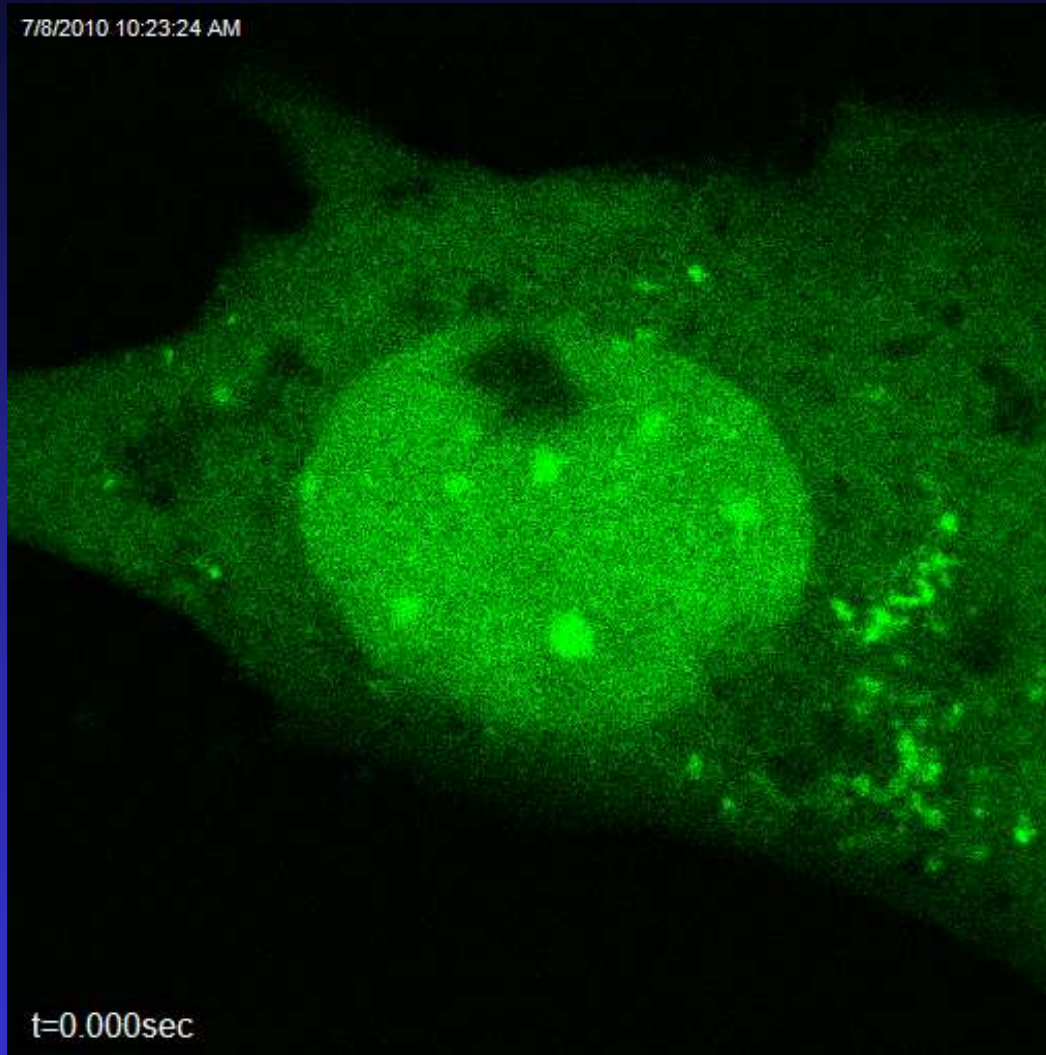
Part I – cells can be grown on both upper and lower sides of the chamber; this part is filled fully with medium

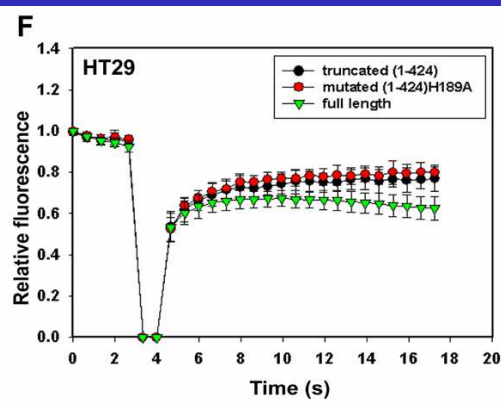
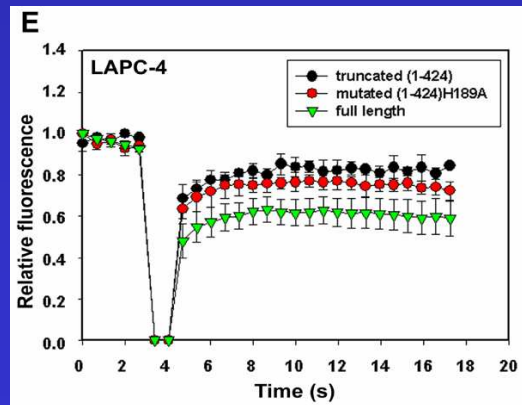
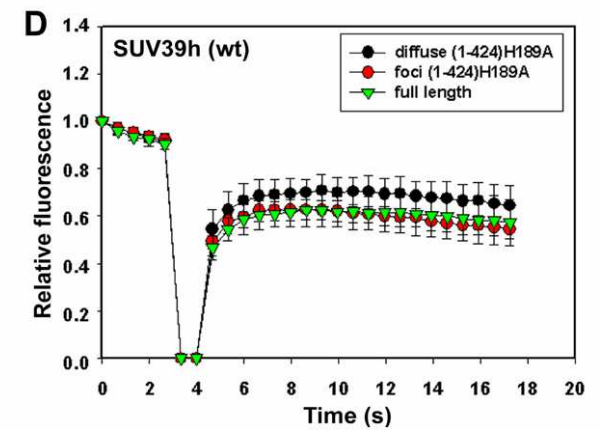
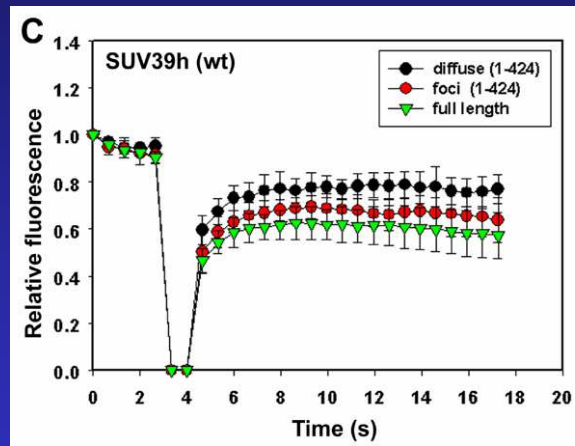
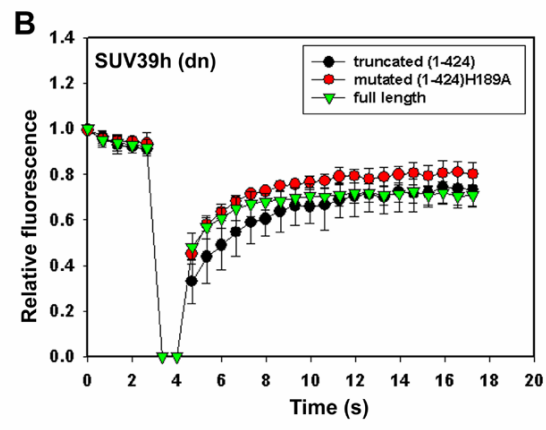
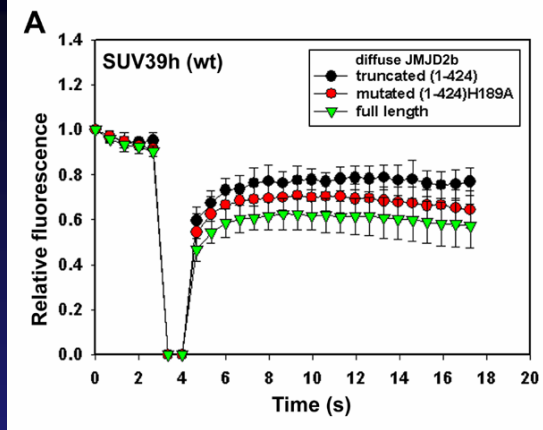
Part II – medium reaches under the glass tubes; cells are not grown here



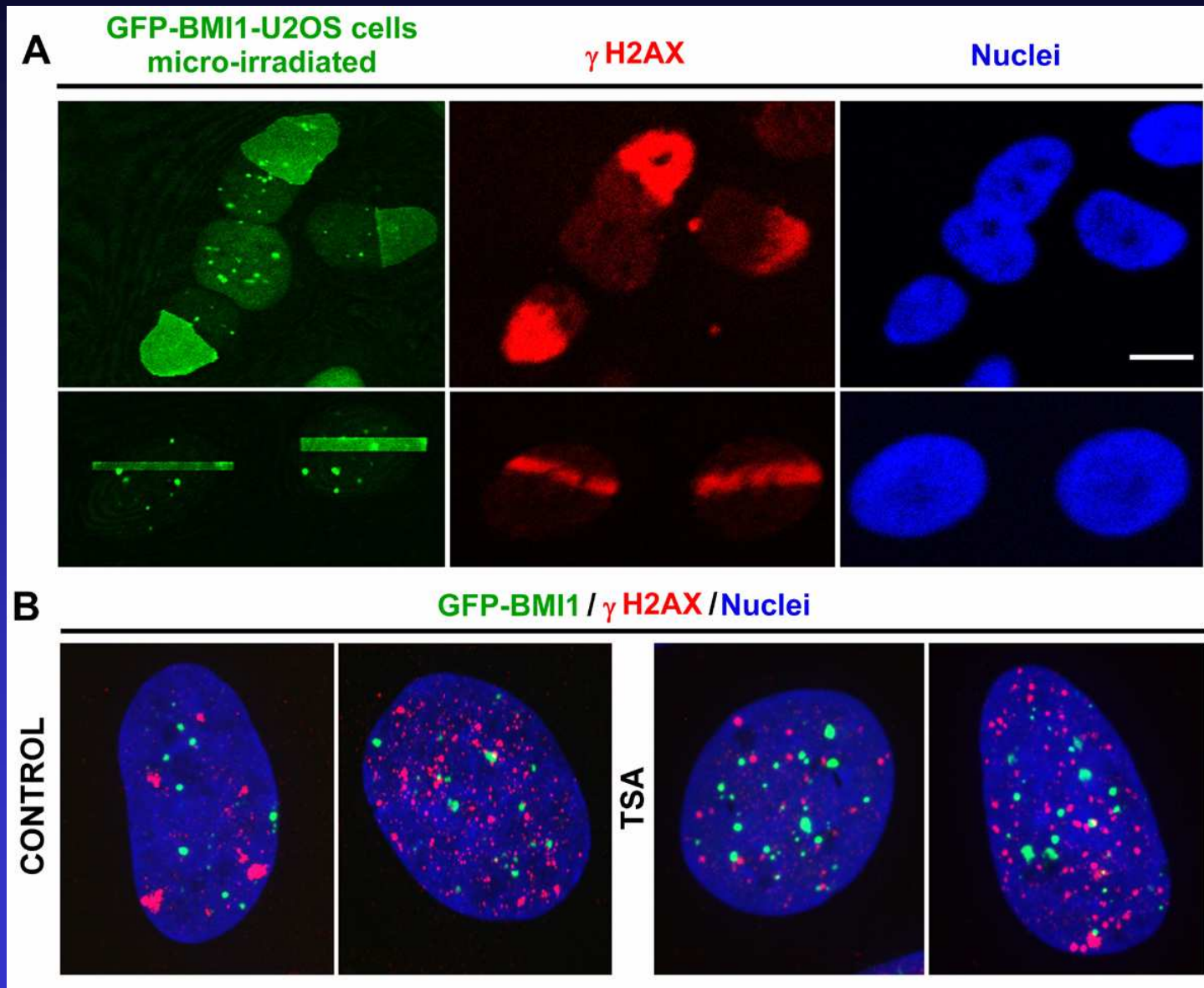
Glass tubes bent under the upper glass (to enable CO₂ to flow above medium in the part II of the chamber)

JMJD2b(1-424)-GFP

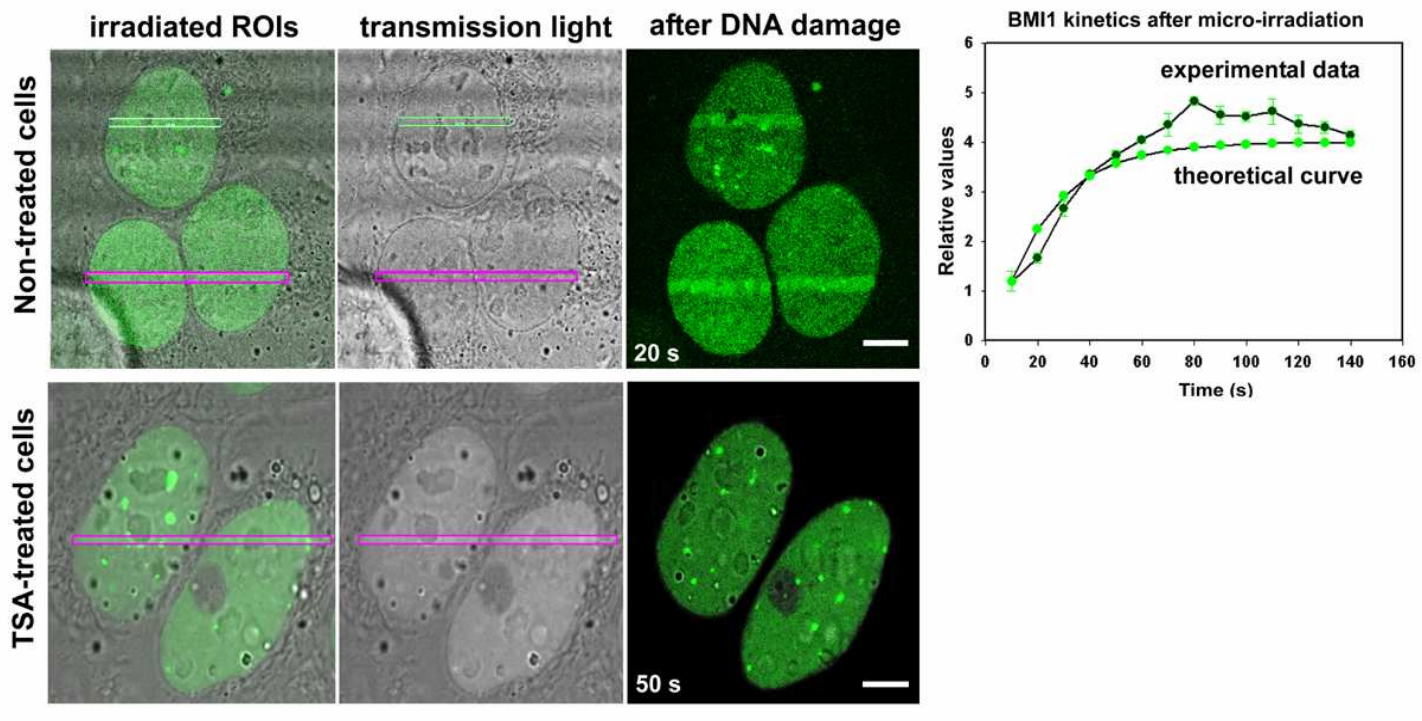




Využití UV laseru 355 nm ke studiu DNA reparace

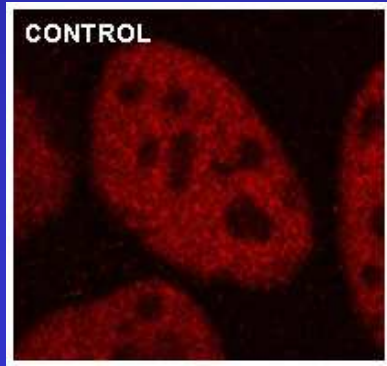
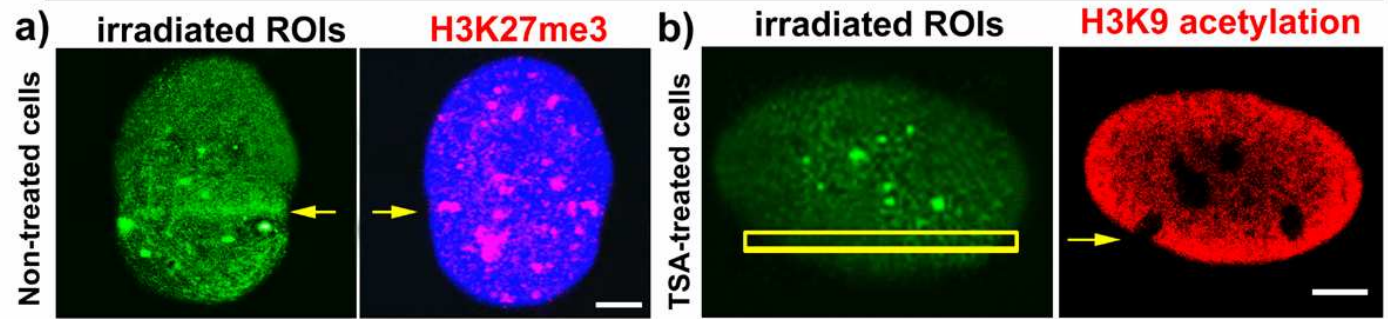


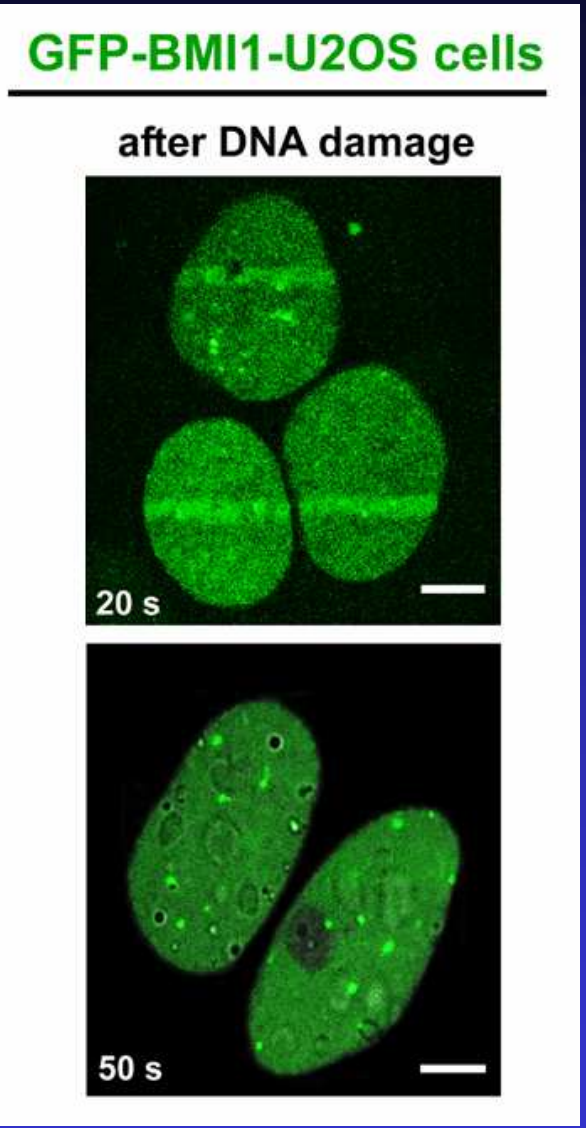
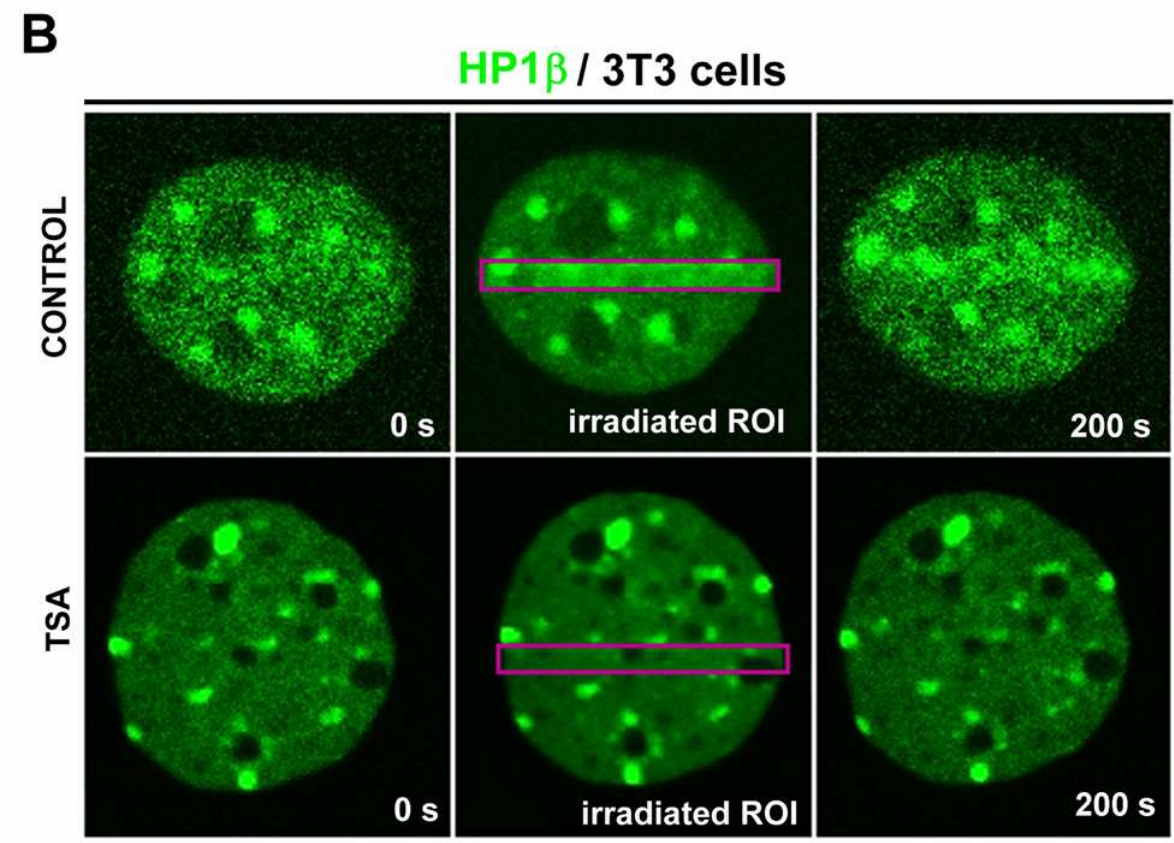
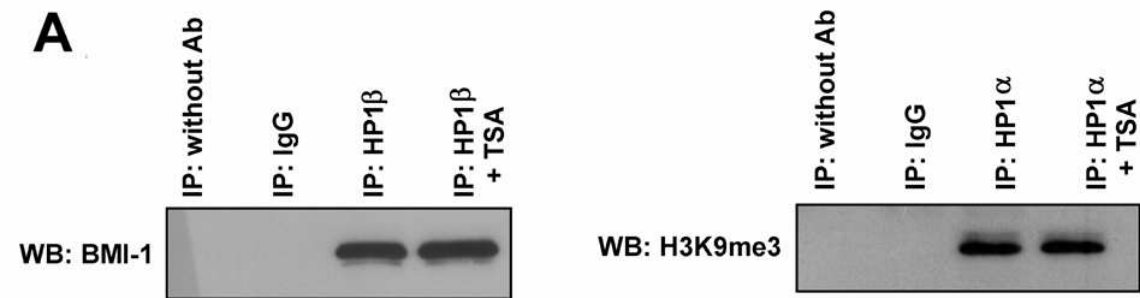
GFP-BMI1-U2OS cells



D

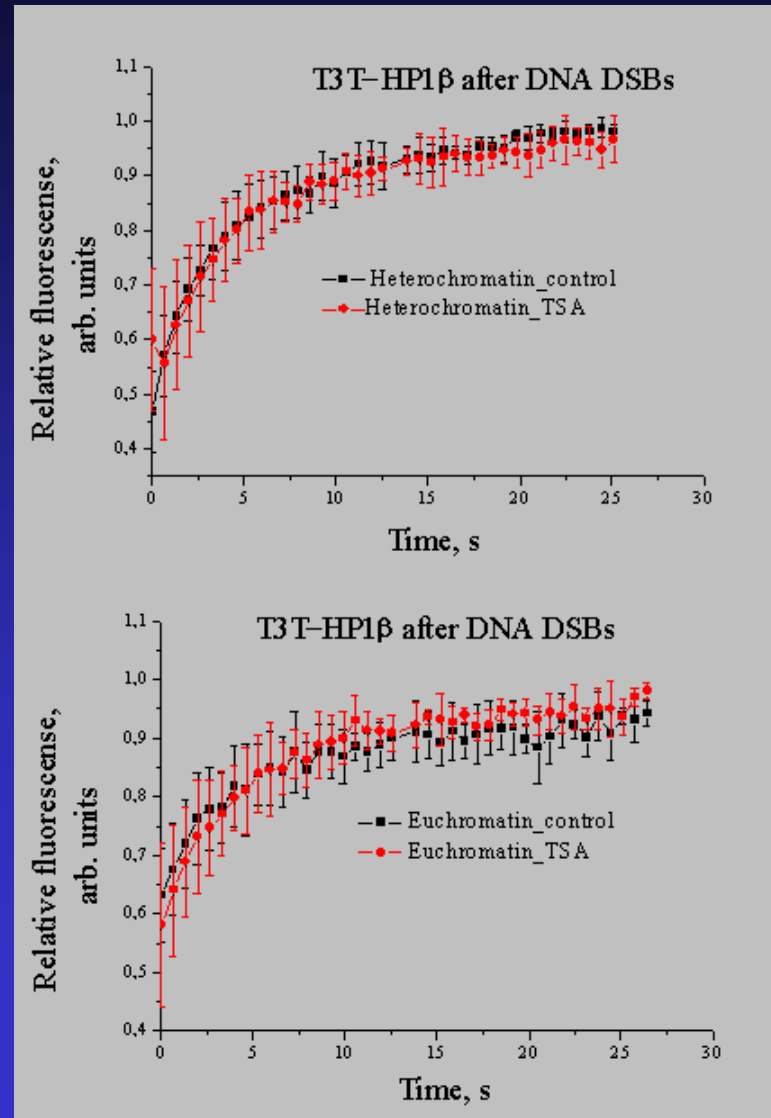
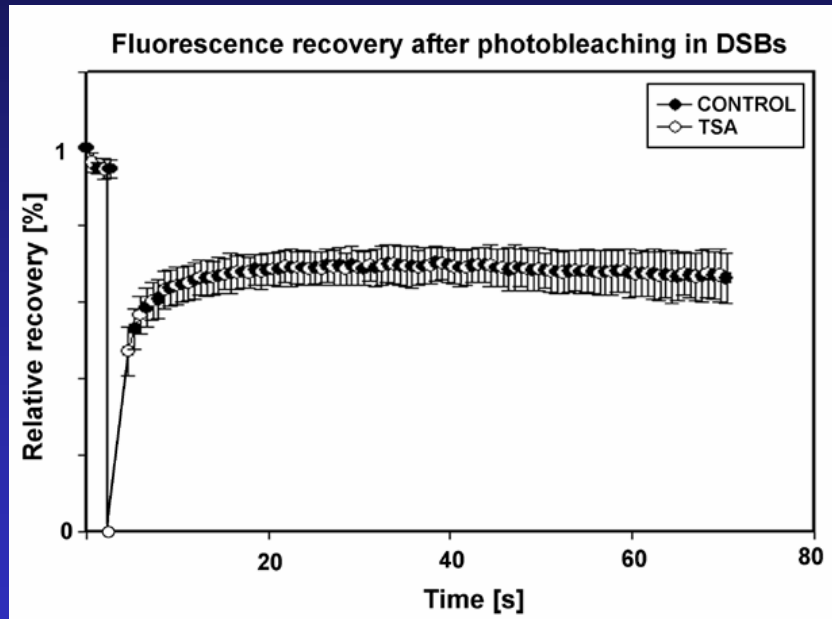
GFP-BMI1-U2OS cells



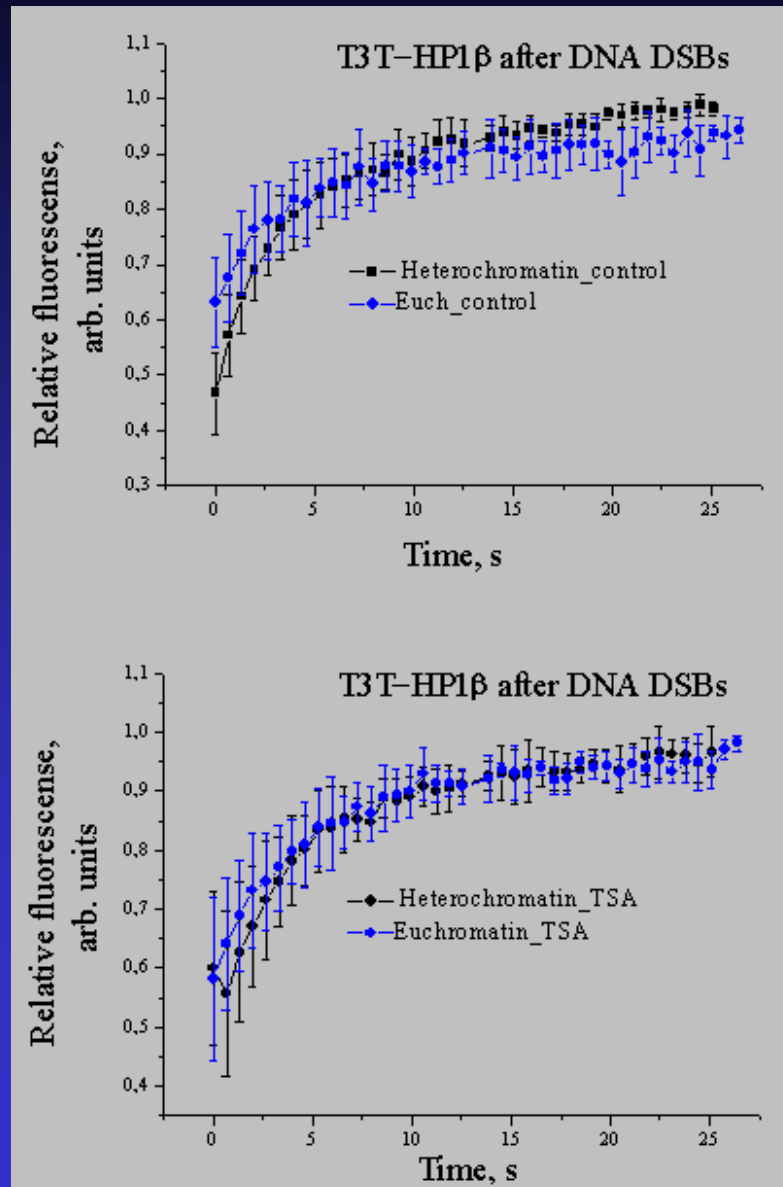


FRAP in UV-damaged chromatin with accumulated BMI1 and HP1 β

BMI1



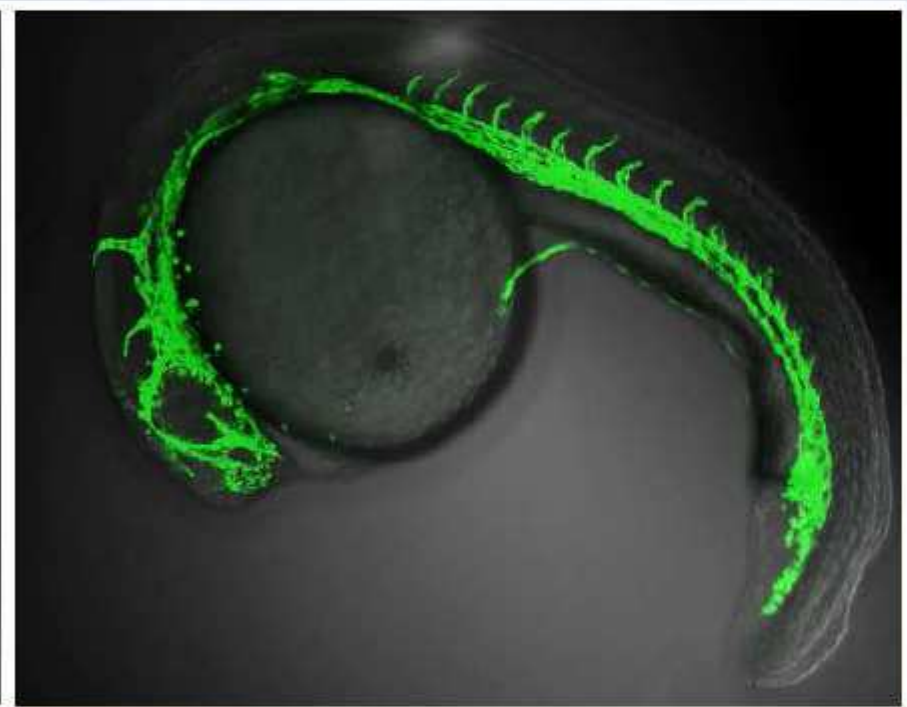
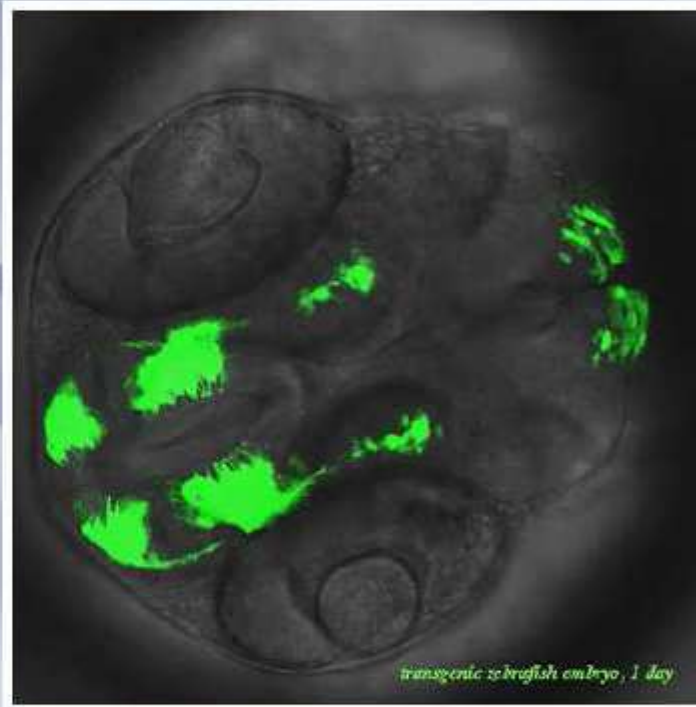
FRAP in UV-damaged chromatin with accumulated BMI1 and HP1 β



Experiments of Darya Orlova

GFP in zebrafish (*Danio rerio*)

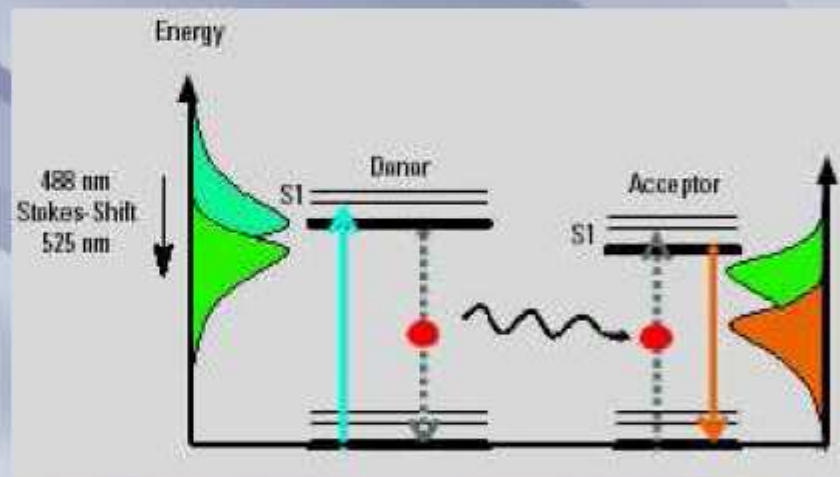
(www.ifom-ieo-campus.it/research/mione.php; Marina Mione)



Förster Resonance Energy Transfer

Fluorescence Resonance Energy Transfer (FRET)

studium interakcí proteinů
nebo blízkých molekul



$$E_A(i) = \frac{B - A \times b - C \times (c - a \times b)}{C}$$

- popisuje mechanismus přenosu energie mezi dvěma fluorescenčními molekulami

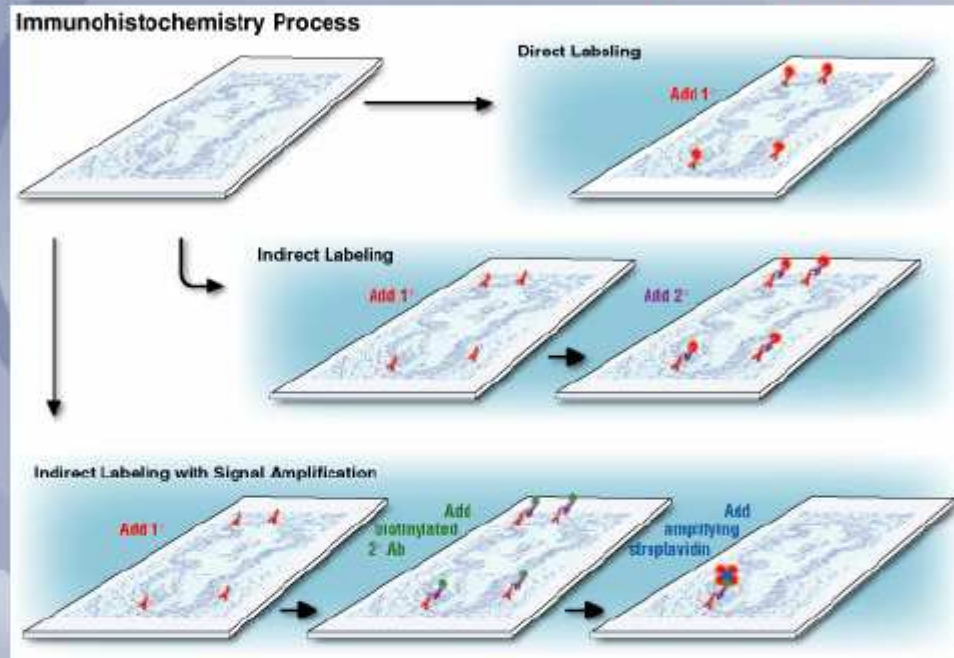
- fluorescenční **donor** je excitován svojí specifickou fluorescencí o excitační vlnové délce

- dalekonosným dipol-dipole vazebným mechanismem nezářivým přenosem excitována druhá molekula - **akceptor**

- donor se vrací do elektronově základního stavu

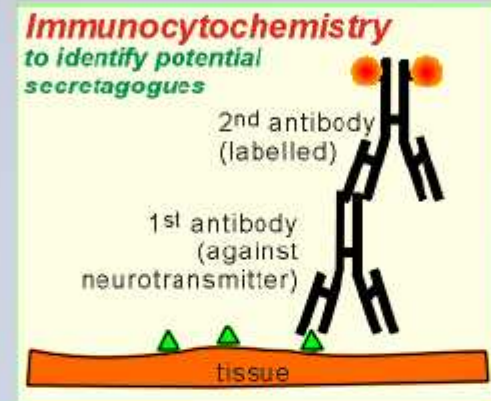
- popsaný mechanismus přenosu energie je nazýván "**Förster resonance energy transfer**" (FRET), pojmenováno podle německého vědce **Theodora Förstera**. Pokud jsou obě molekuly fluorescenční, je často užíván název "fluorescence resonance energy transfer", ačkoliv **energie není**

Příprava preparátu



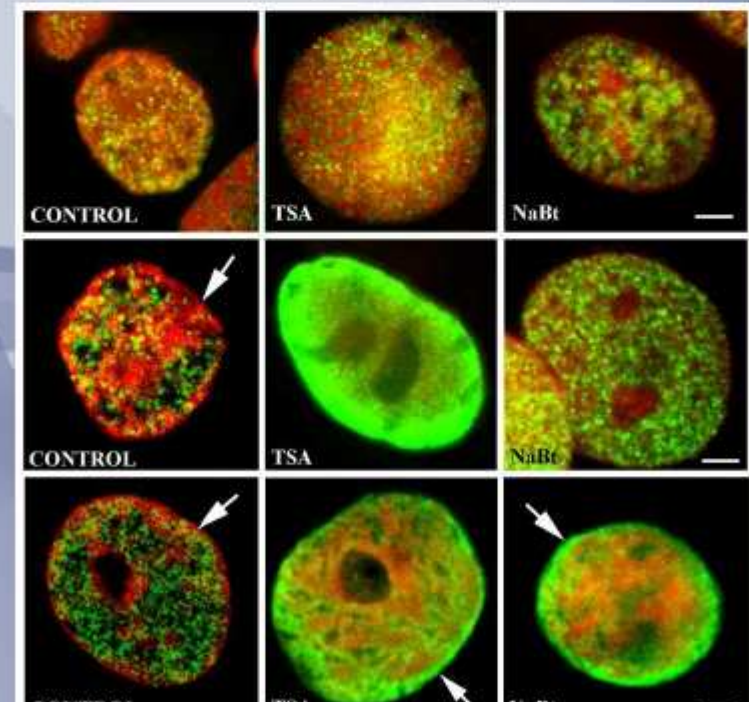
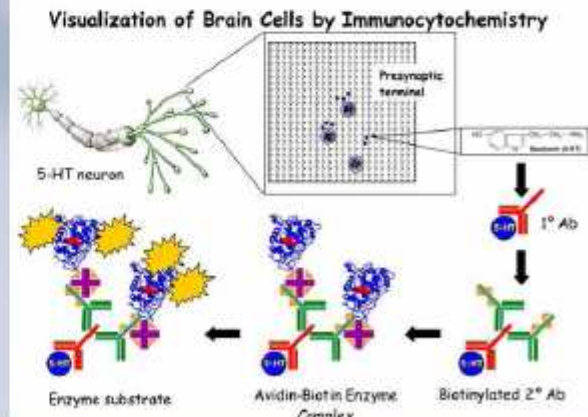
<http://www.chemicon.com>

Imunocytobarvení

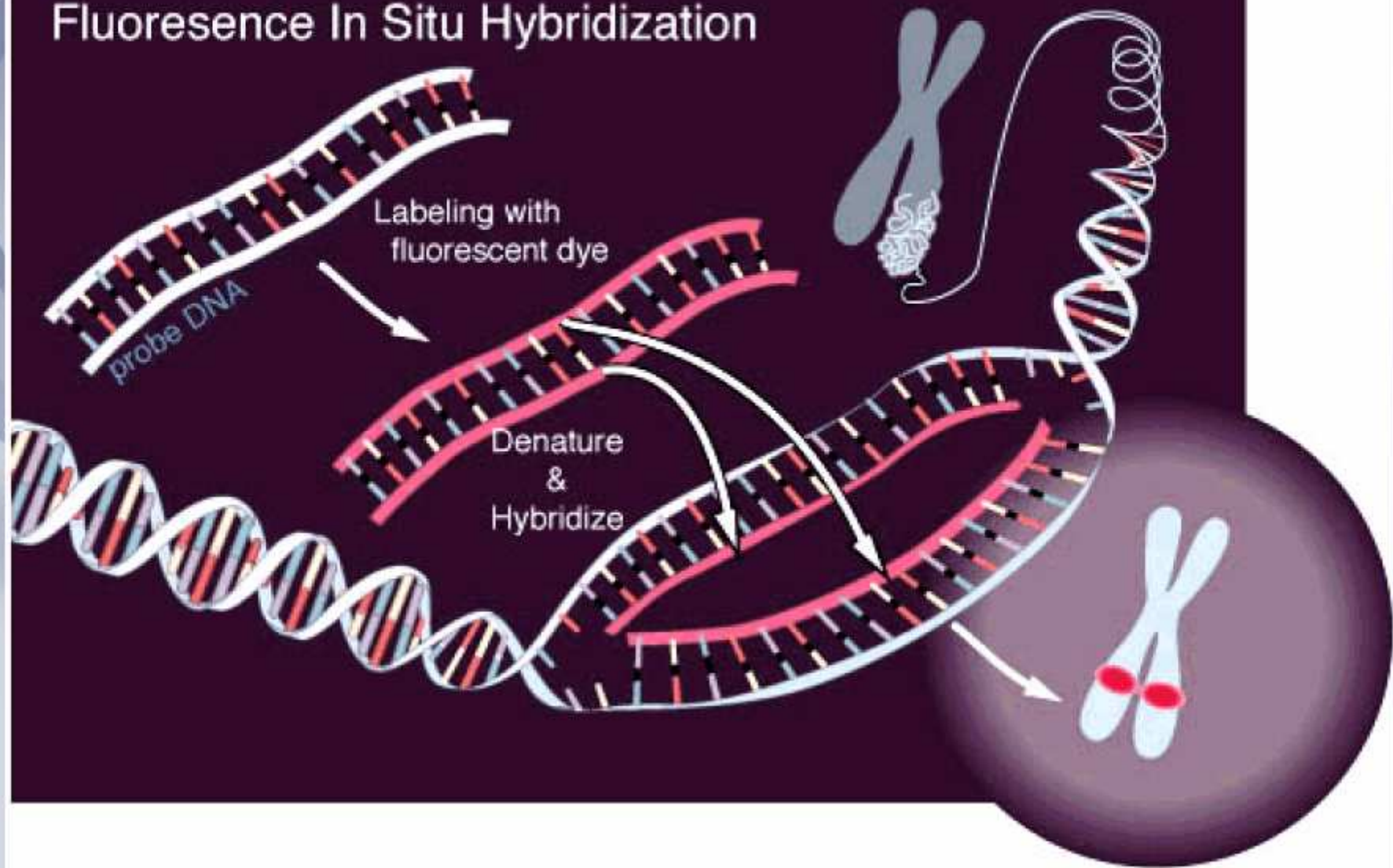


<http://www.celanphy.science.ru.nl>

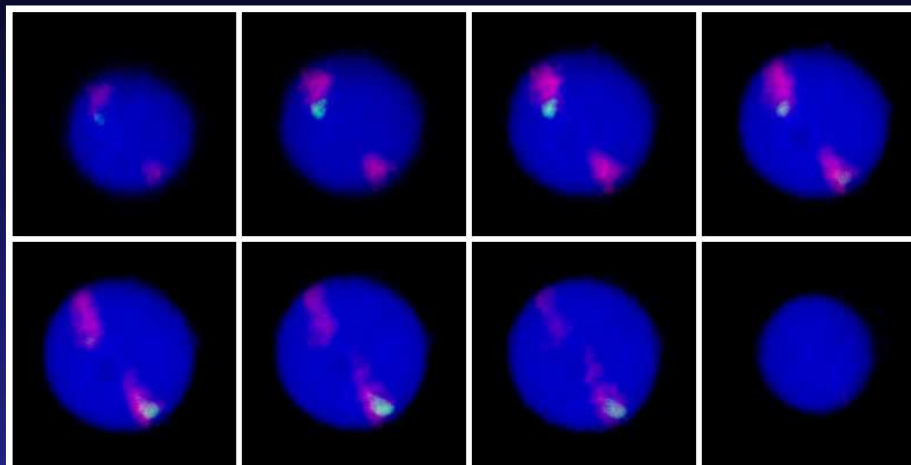
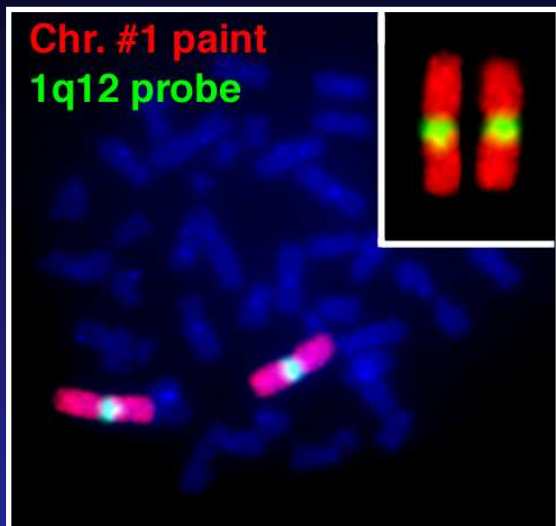
Immunocytochemistry



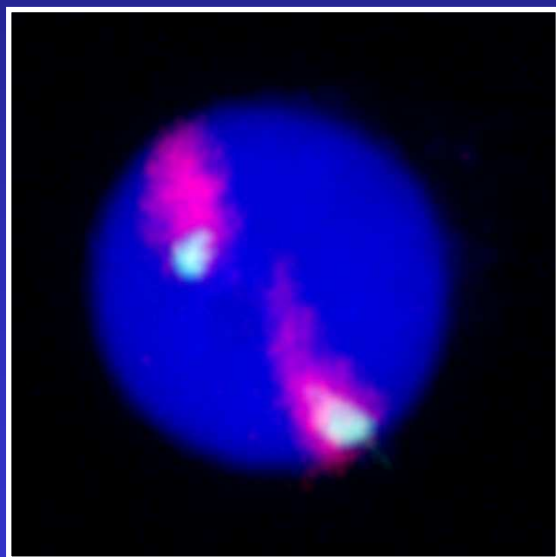
Fluorescence In Situ Hybridization



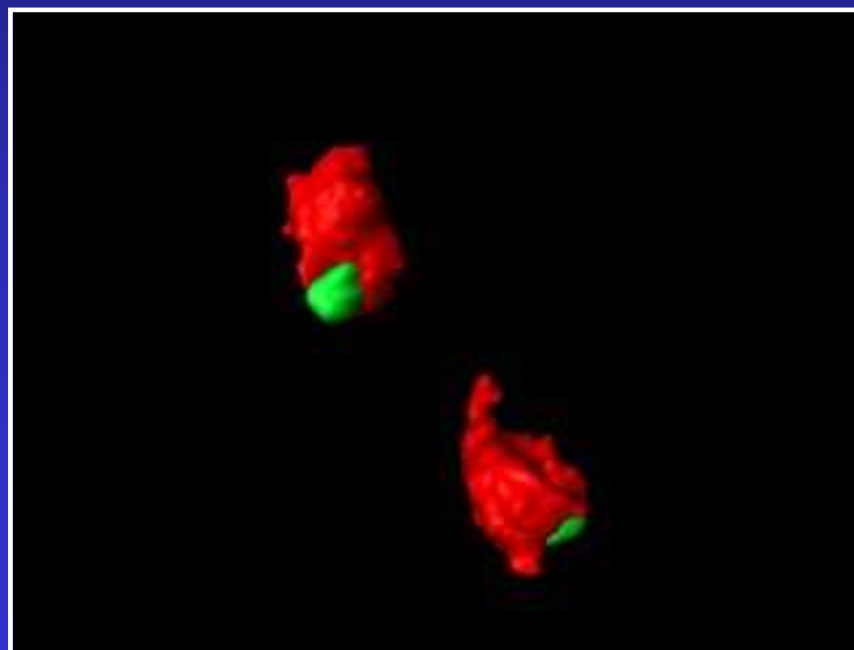
3D-FISH a konfokální mikroskopie



Galerie optických řezů

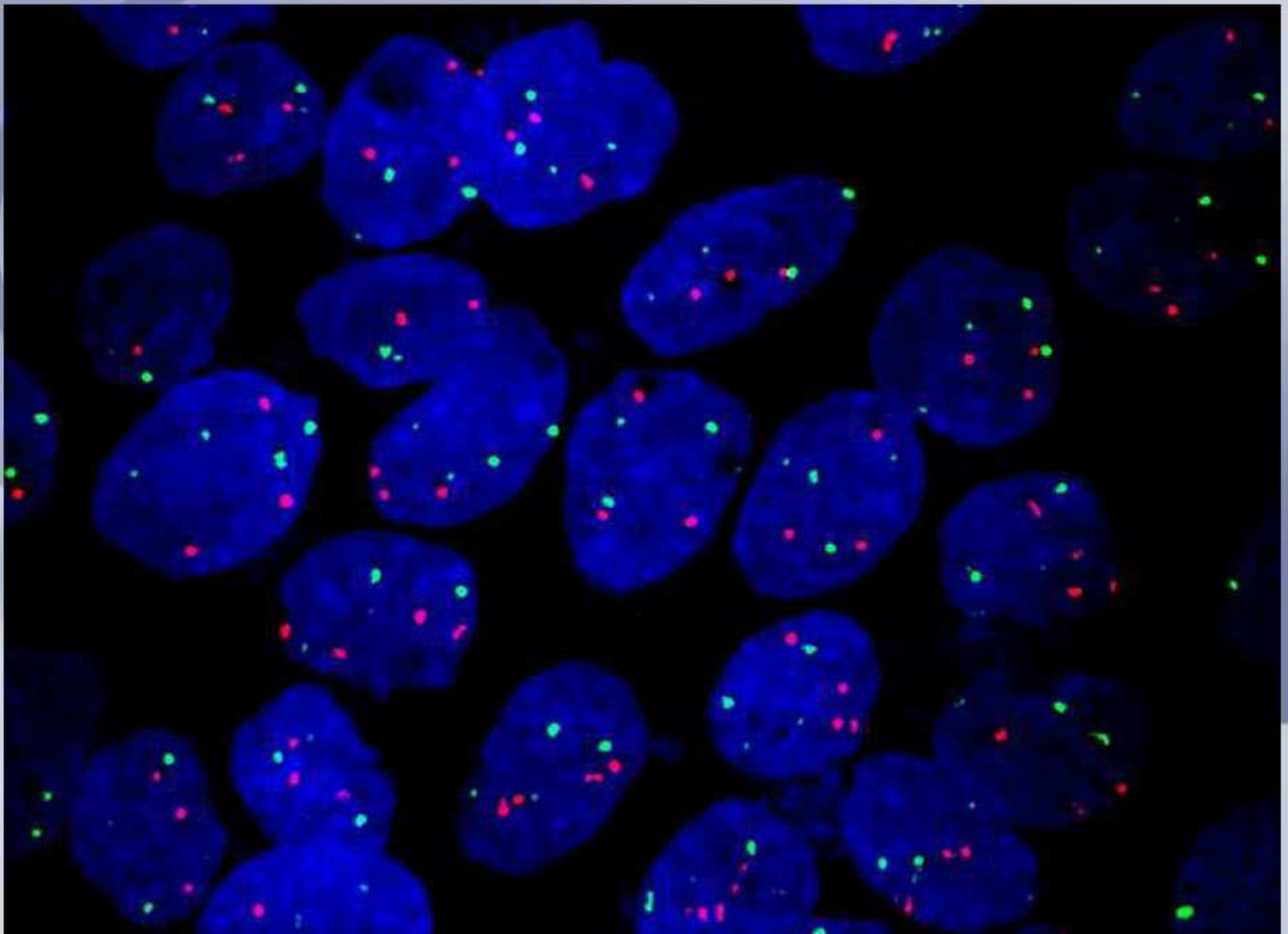


Maximální obraz
Všech řezů

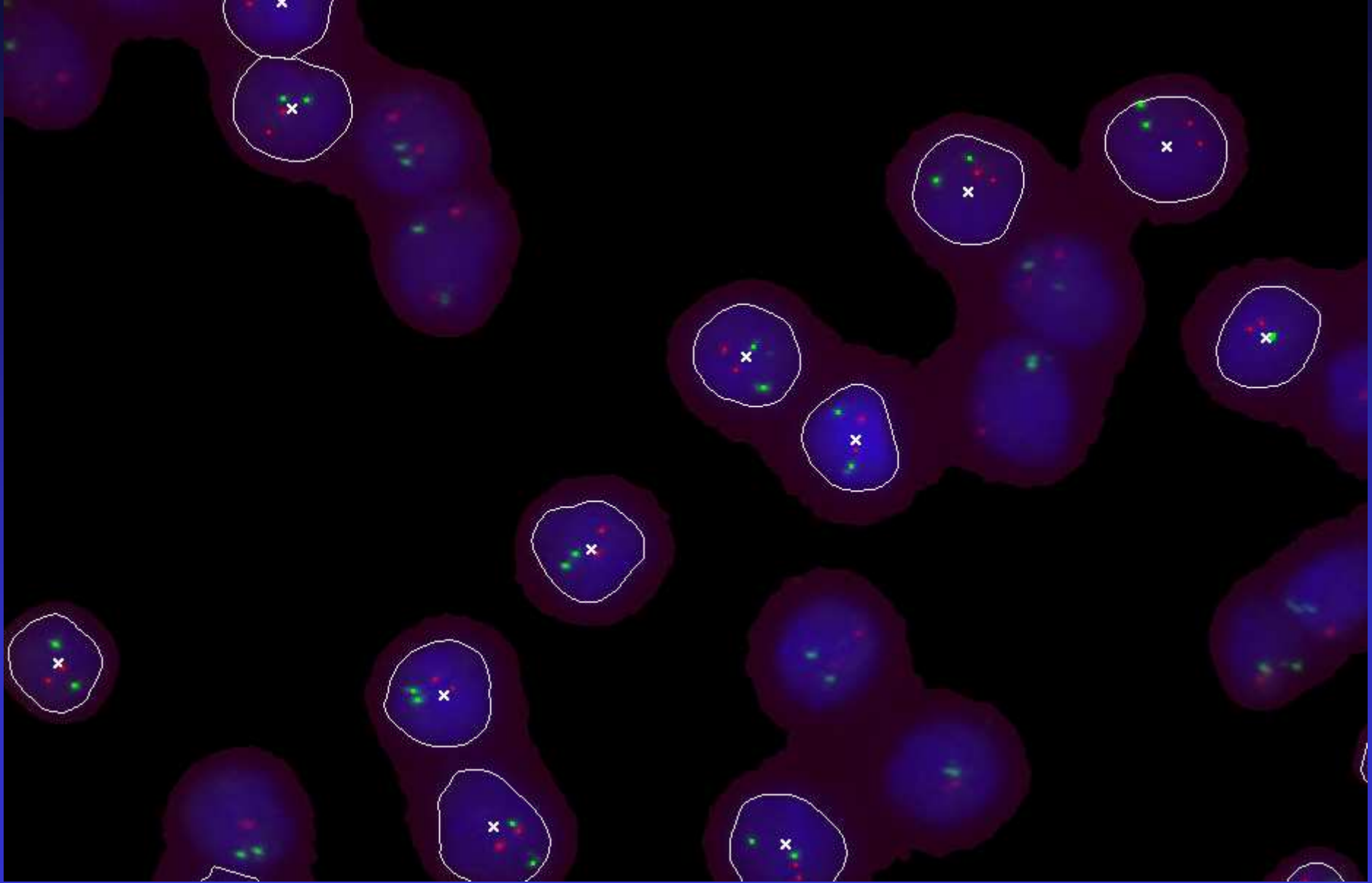


3D rekonstrukce CT

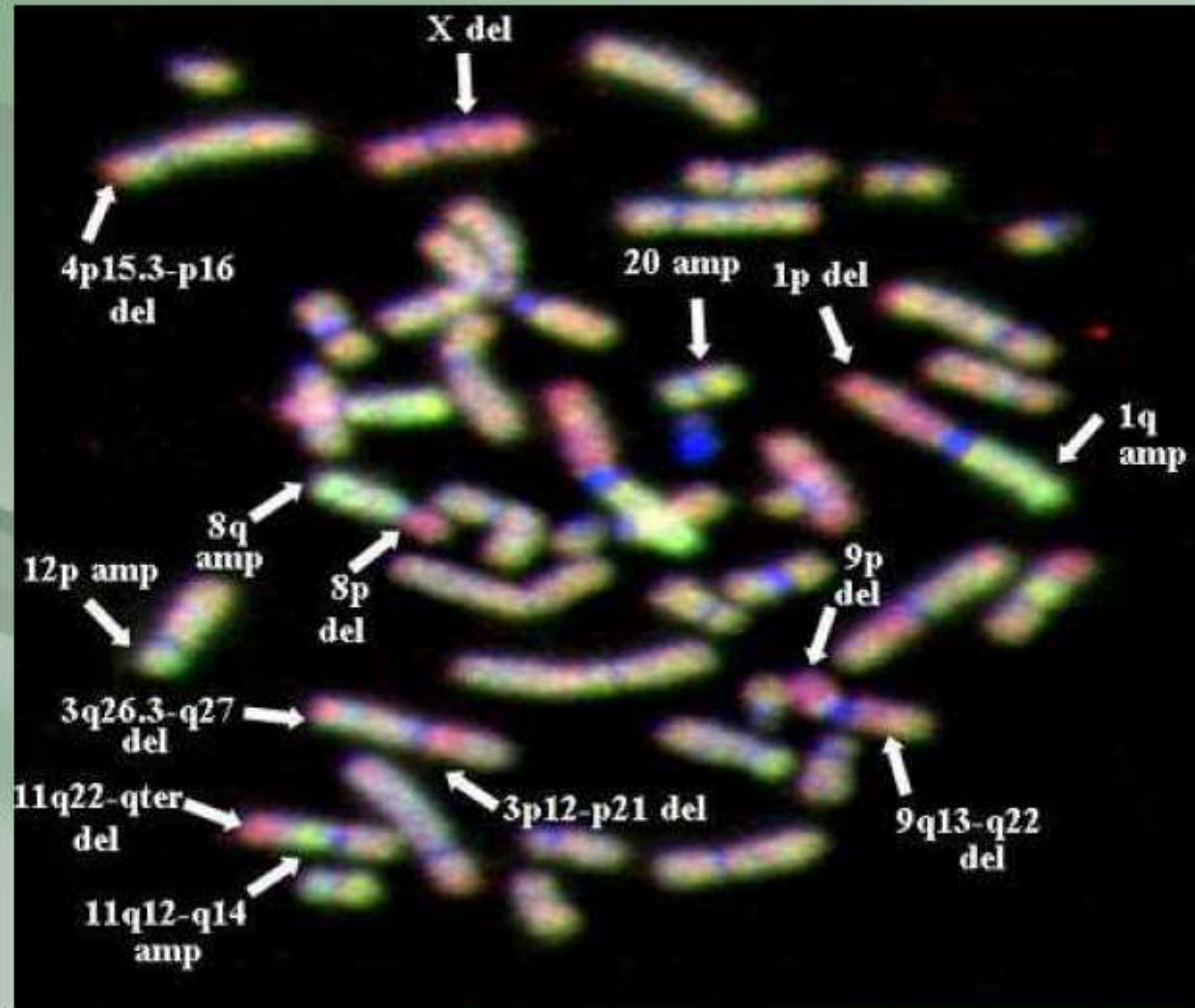
Weierich et al., (2003) in press



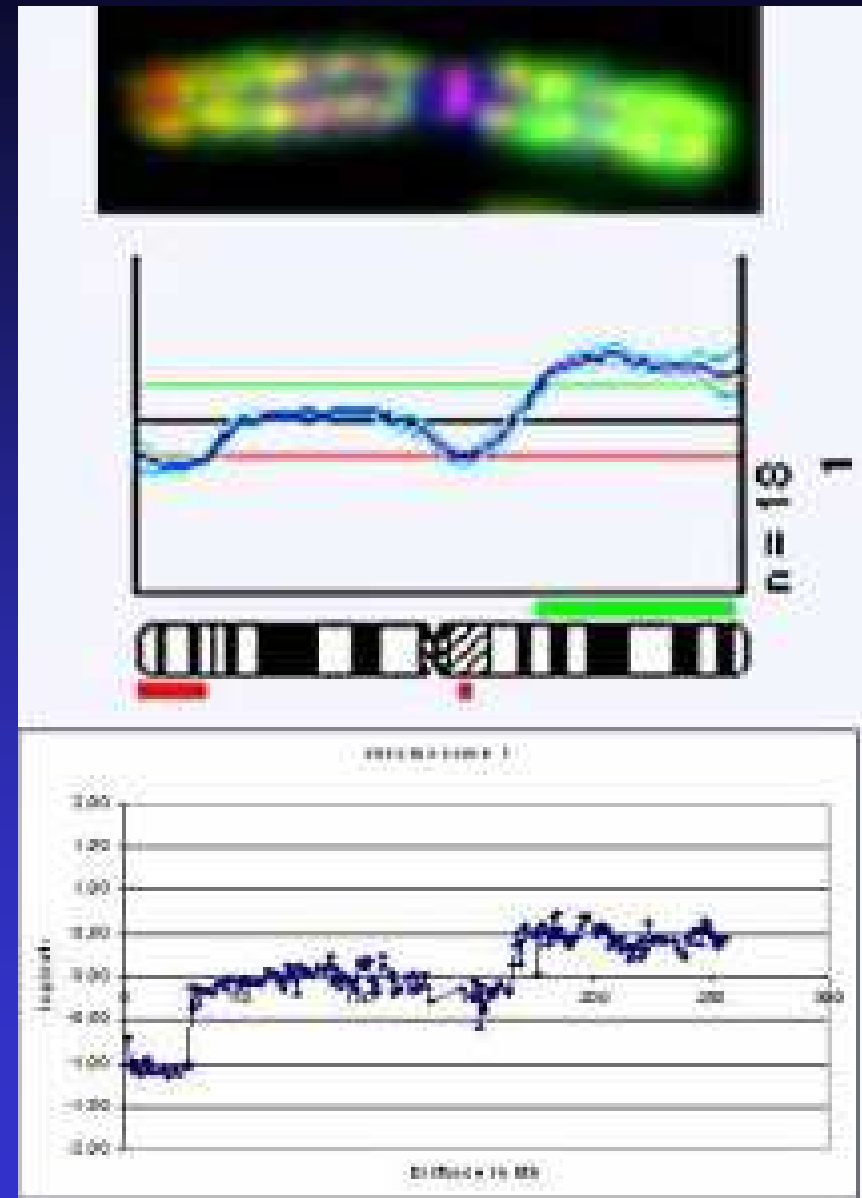
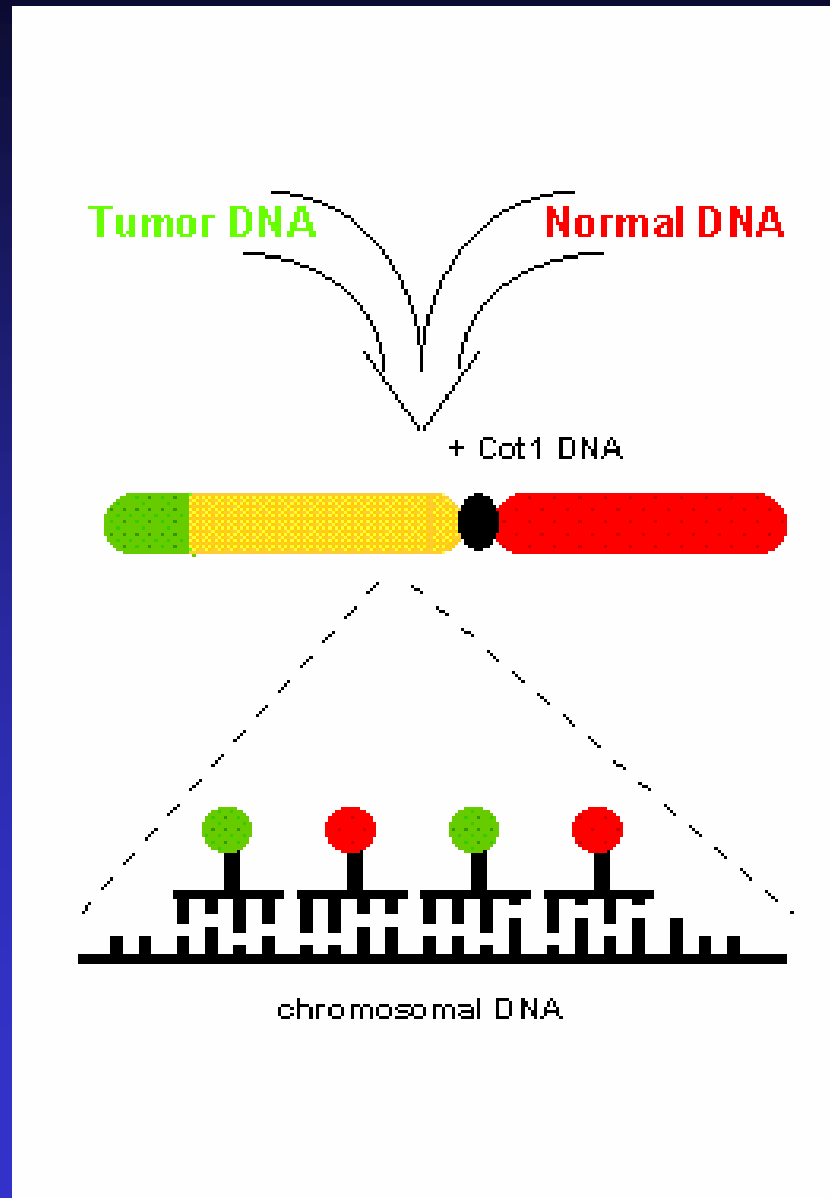
Automatická analýza obrazu



Comparative genome hybridization



CGH on metaphase spreads



ELEKTRONOVÝ MIKROSKOP

Transmisní nebo rastrovací (skenovací)

Na každé místo vzorku je zaměřen úzký paprsek elektronů (prochází jej po řádcích – odtud řádkovací). Interakcí dopadajících elektronů s materiálem vzorku vznikají různě detekovatelné složky. Jak paprsek putuje po vzorku, mění se podle charakteru povrchu úroveň signálu v detektoru. Z těchto signálů je pak sestavován výsledný obraz. Získaný obraz je standardně monochromatický.

Zdrojem elektronů je elektronová tryska, nejčastěji wolframové žhavené vlákno, umístěné v tzv. Wehneltově válci.

Elektrony jsou urychlovány směrem k vzorku urychlovacím napětím (typicky 0,1-30kV).

Svazek elektronů (paprsek) je upravován, zaostřován elektromagnetickými čočkami. Tubus obsahuje zpravidla jednu nebo více kondenzorových čoček, objektivovou čočku, vychylovací cívky rastrů a cívky stigmátorů pro korekci astigmatismu.

Introduction to Electron Microscopy

- Electron Microscopes were not developed until the Twentieth Century
- The first Transmission Electron Microscope (TEM) was built in 1932
- The first Scanning Electron Microscope (SEM) was built in 1942
- The Scanning Tunnelling Microscope was developed in 1982.
- The Atomic Force Microscope was developed in 1985

Transmission Electron Microscope



Scanning Electron Microscope

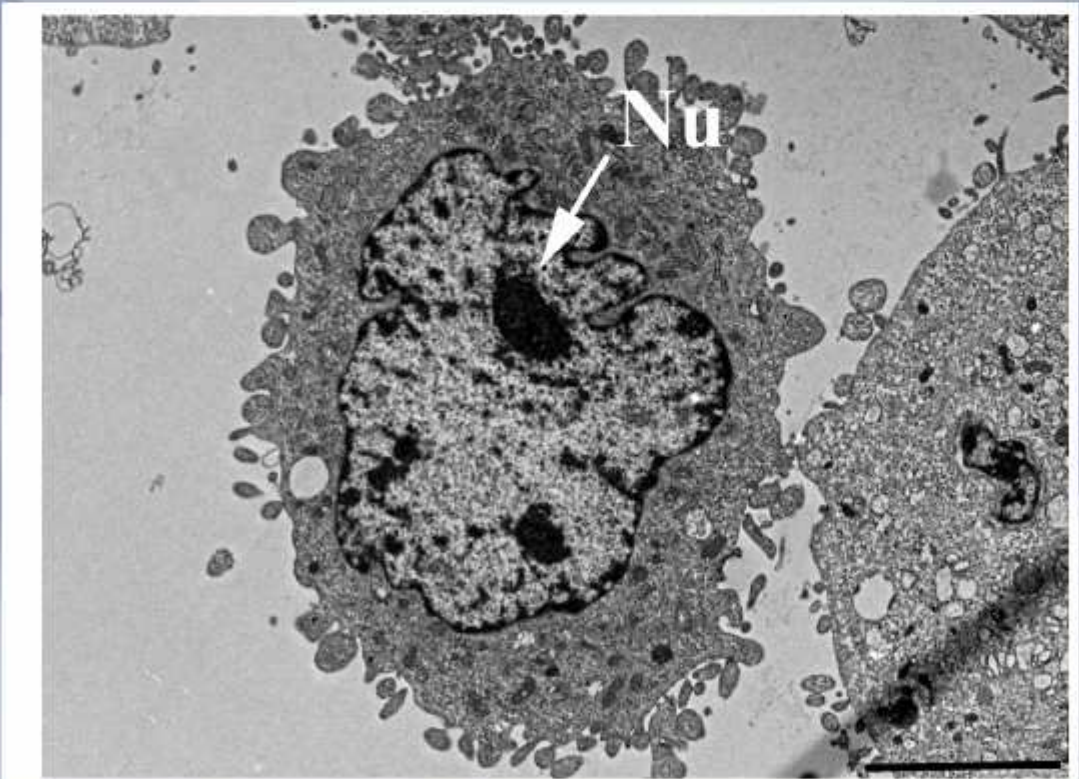
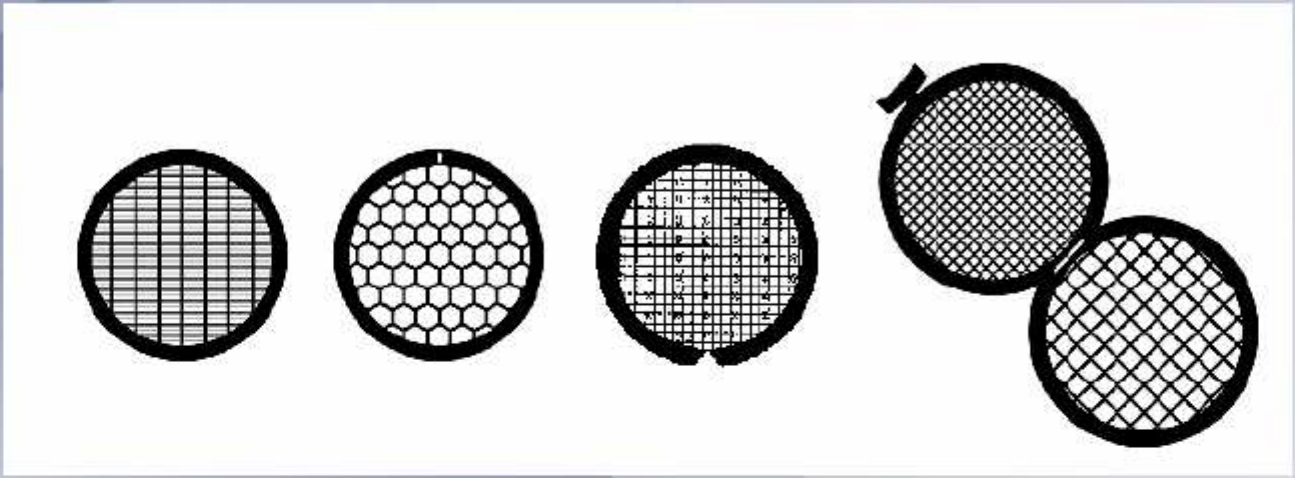


Scanning Tunneling Microscope

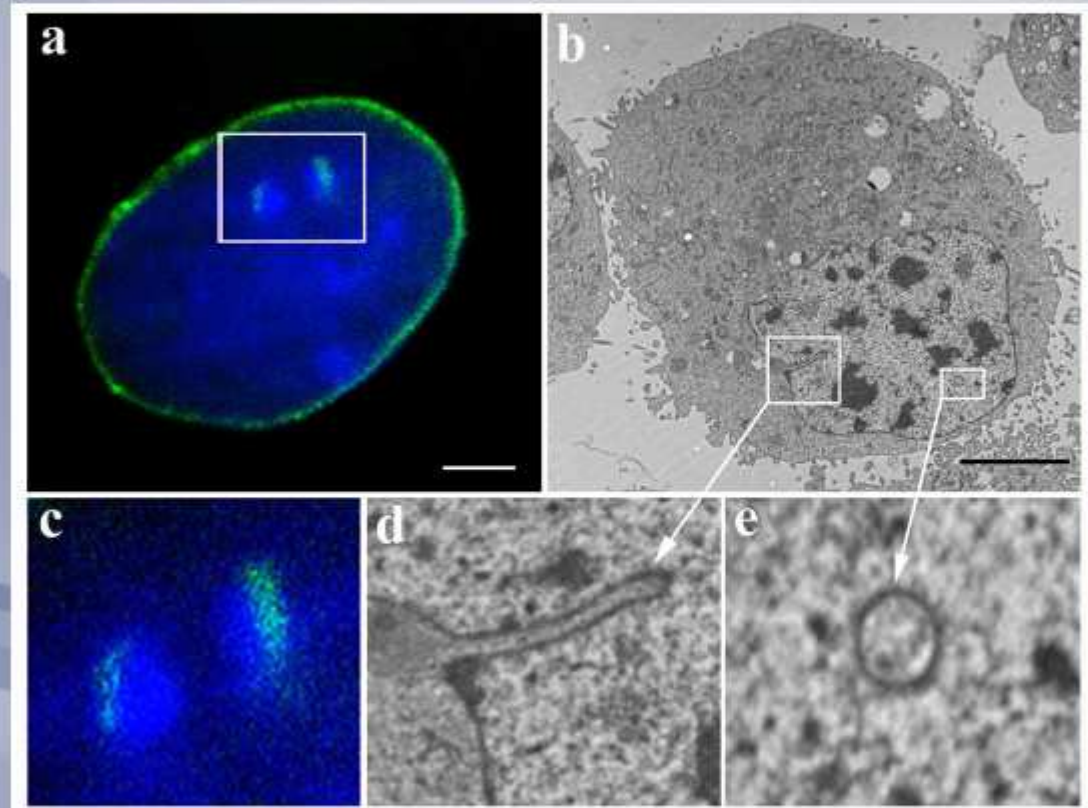


Atomic Force Microscope

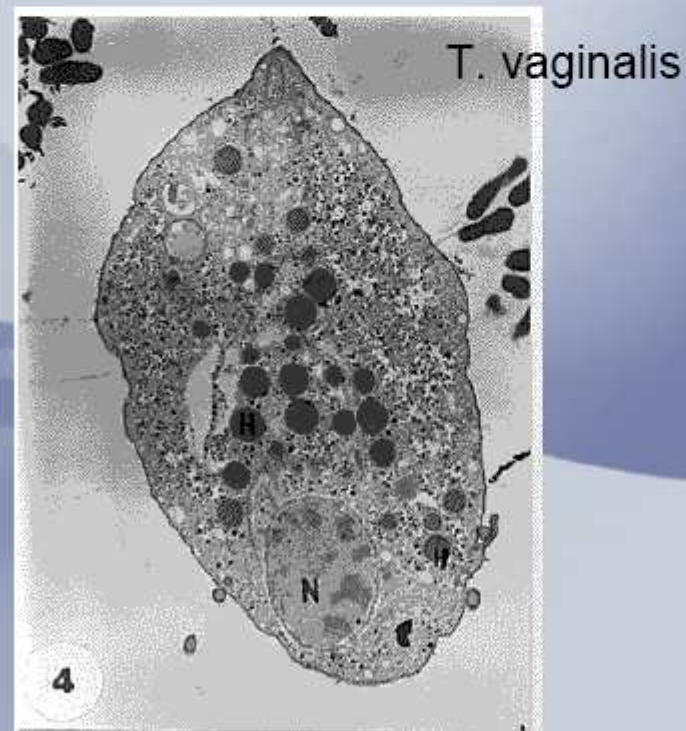
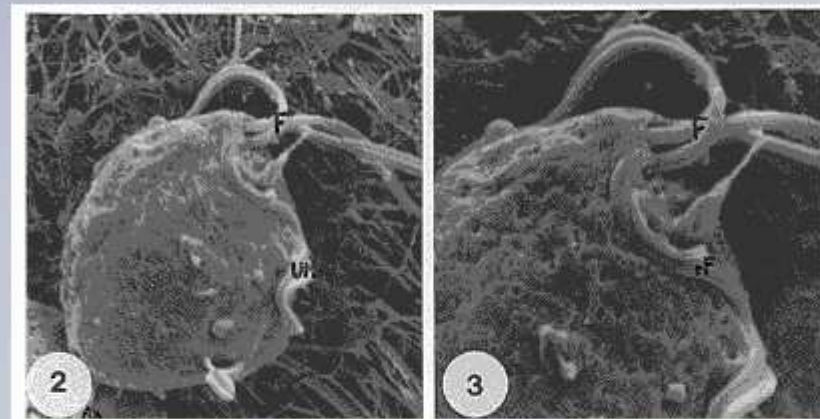




Transmisní Elektronový Mikroskop (TEM)



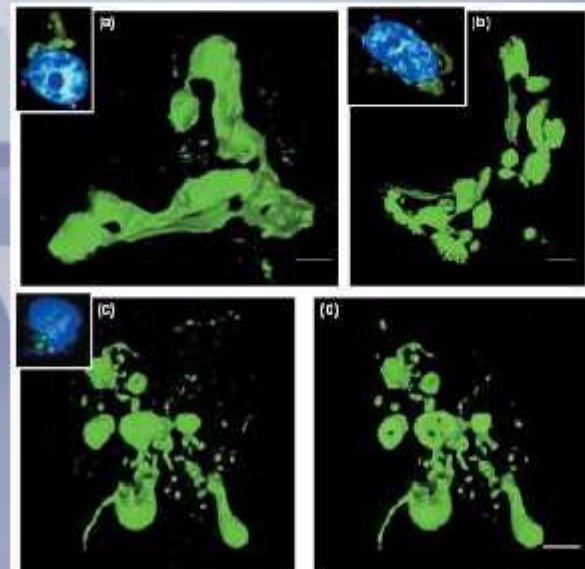
Skenovací (Rastrovací) Elektronový Mikroskop



atomic force microscopy - 4Pi

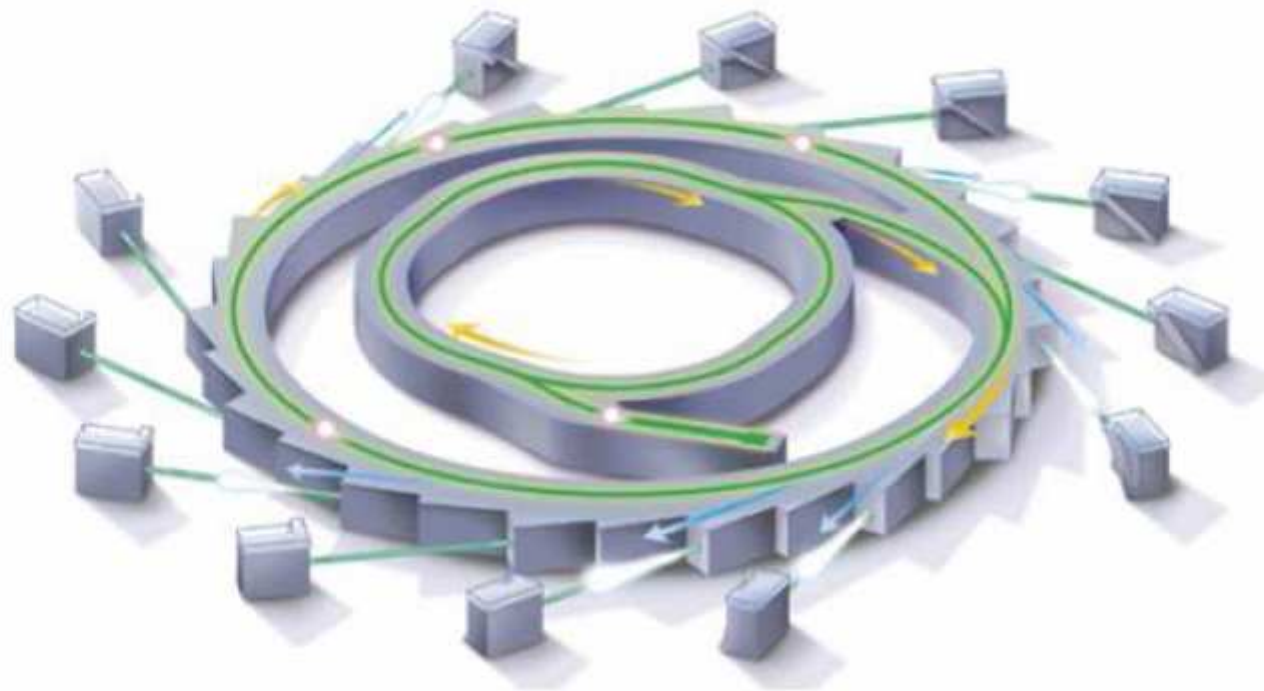


- rozlišení převyšuje možnosti konvenční mikroskopie pomocí double-objective imaging systému podobnému CLSM – nutná zvláštní skla (drahá)
- 4až7krát vyšší axiální rozlišení než CLSM/MP
- aplikace limitovaná hloubkou 10 mikronů
- technicky náročný
- nutnost precizně sesazených čoček a velmi precizně nastavený laser
- komerčně dostupný, ale velice drahý



WHAT IS A SYNCHROTRON

A synchrotron is a device that accelerates electrons to almost the speed of light. As the electrons are deflected through magnetic fields they create extremely bright light. The light is channelled down beamlines to experimental workstations where it is used for research.



X-RAY MICROSCOPY

Soft X-ray microscopes can be used to study hydrated cells up to 10 μm thick and produce images of 30 nm resolutions. X-ray microscopy, that has the more pronounced properties of laser scanning confocal microscopy (LSCM), has been a long-standing goal for experimental science (Seres et al., 2005). Since the cells are imaged in the X-ray transmissive "water window", where organic material absorbs approximately an order of magnitude more strongly than water, chemical contrast enhancement agents are not required to view the distribution of cellular structures (Meyer-Ilse et al., 2001). In such experiments, cells must be rapidly frozen to be studied on a cryostage, showing information which is closely similar to 4D-living cell observation by LSCM.

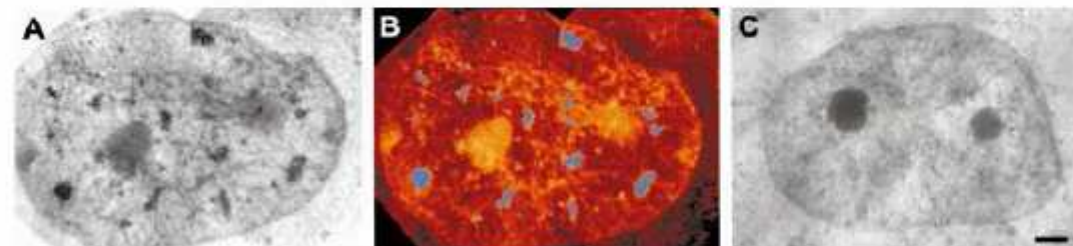
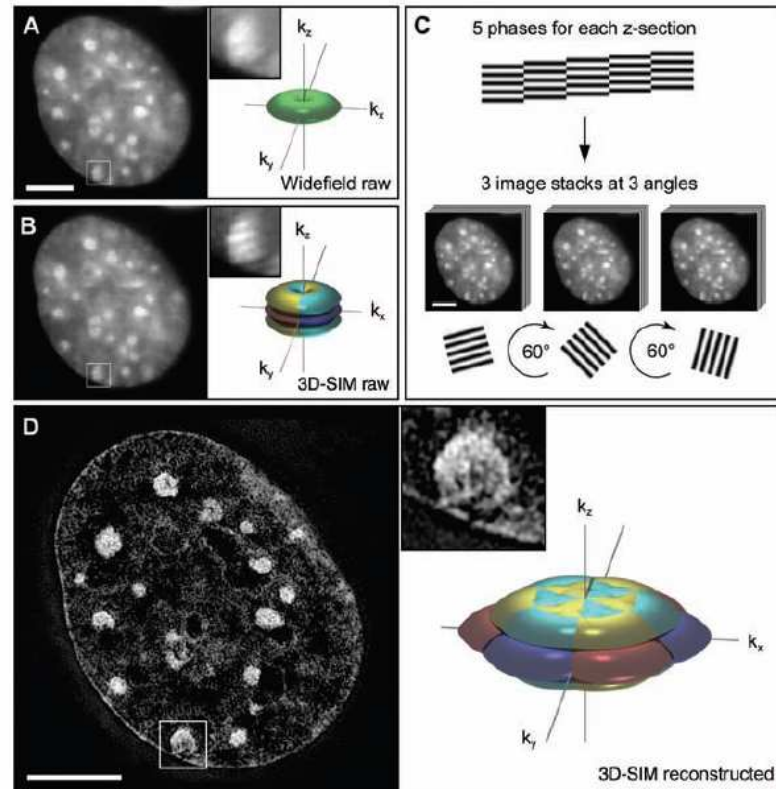


Fig. 4. Nuclei of human mammary epithelial cells (T4) labelled for RNA splicing factor (SRm300). (A) X-ray micrograph of a single nucleus after silver enhancement. This image is a montage compiled from two individual X-ray microscope images. (B) Same nucleus after colour coding to emphasize the label. (C) Control; single nucleus that was exposed to secondary antibodies and silver enhancement but not primary antibodies. This image is a montage compiled from two individual X-ray microscope images. Magnification = 2400 \times , 0.034 NA with 20 nm pixel size at 517 eV ($\lambda = 2.4$ nm).

(Meyer-Ilse et al., 2000)

Fig. 1. Subdiffraction resolution imaging with 3D-SIM. **(A and B)** Cross section through a DAPI-stained C2C12 cell nucleus acquired with conventional wide-field illumination **(A)** and with structured illumination **(B)**, showing the striped interference pattern (inset). The renderings to the right illustrate the respective support of detection in frequency space. The axes k_x , k_y , and k_z indicate spatial frequencies along the x , y , and z directions. The surfaces of the renderings represent the corresponding resolution limit. The depression of the frequency support ("missing cone") in z direction in **(A)** indicates the restriction in axial resolution of conventional wide-field microscopy. With 3D-SIM, the axial support is extended but remains within the resolution limit. **(C)** Five phases of the sine wave pattern are recorded at each z position, allowing the shifted components to be separated and returned to their proper location in frequency space. Three image stacks are recorded with the diffraction grating sequentially rotated into three positions 60° apart, resulting in nearly rotationally symmetric support over a larger region of frequency space. **(D)** The same cross section of the reconstructed 3D-SIM image shows enhanced image details compared with the original image (insets). The increase in resolution is shown in frequency space on the right, with the coverage extending two times farther from the origin. Scale bars indicate $5 \mu\text{m}$.



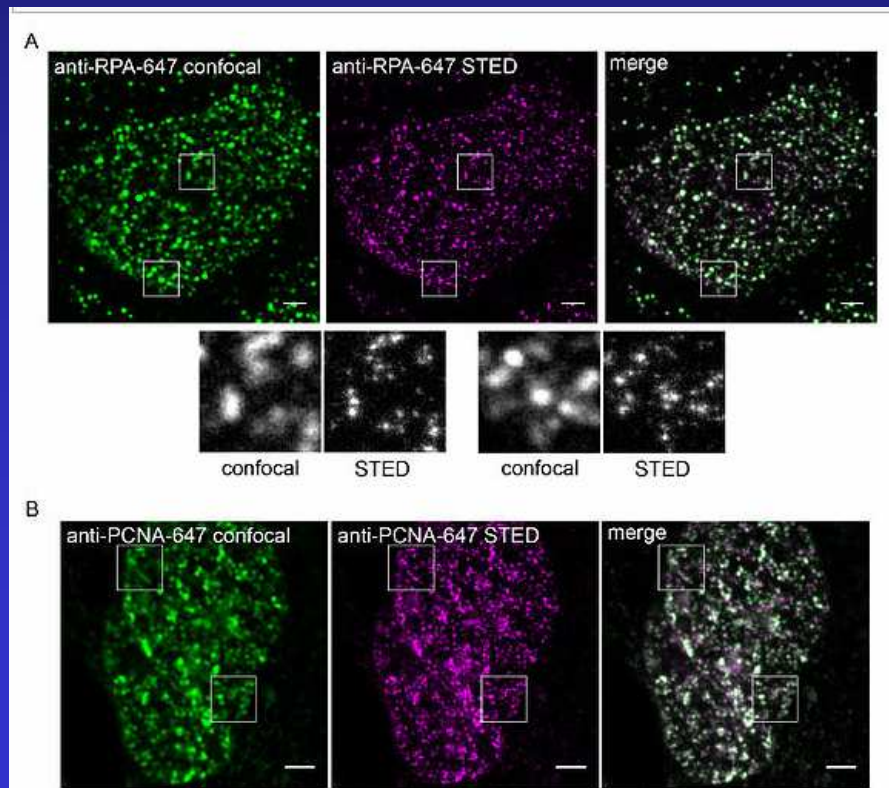
6 JUNE 2008 VOL 320 SCIENCE www.sciencemag.org

Subdiffraction Multicolor Imaging of the Nuclear Periphery with 3D Structured Illumination Microscopy

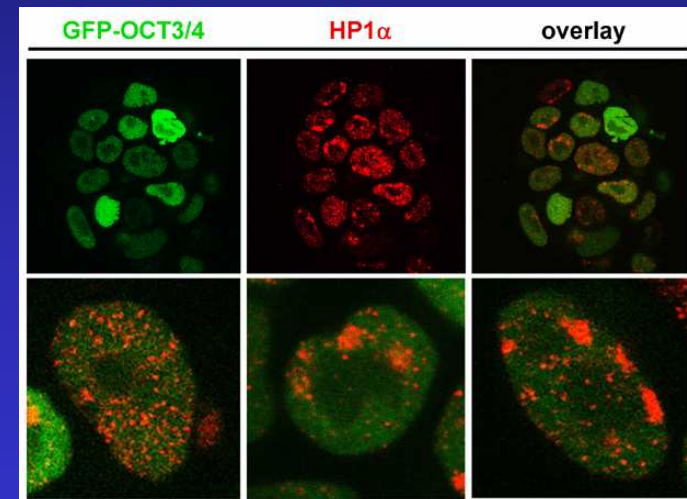
Lothar Schermelleh,^{1*} Peter M. Carlton,^{2*} Sebastian Haase,^{2,4} Lin Shao,²
 Lukman Winoto,² Peter Kner,² Brian Burke,³ M. Cristina Cardoso,⁴ David A. Agard,²
 Mats G. L. Gustafsson,⁵ Heinrich Leonhardt,^{1*†} John W. Sedat^{2*†}

Stimulated Emission Depletion microscopy, or STED microscopy, is a fluorescence microscopy technique that uses the non-linear de-excitation of fluorescent dyes to overcome the resolution limit imposed by diffraction with standard confocal laser scanning microscopes and conventional far-field optical microscopes.

STED



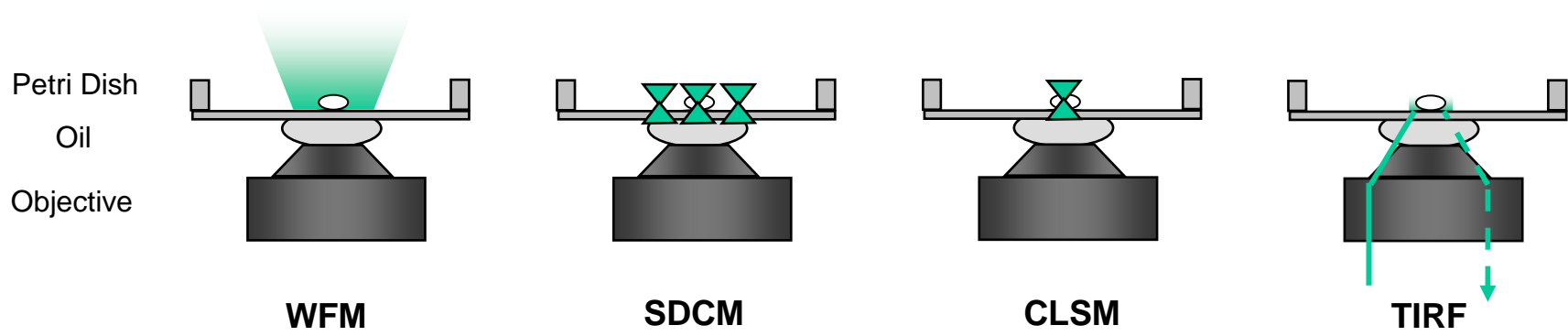
SP-5 LSCM



**Superresolution microscope systems from Carl Zeiss
ELYRA product family combines PAL-M (Photo-activated
localization microscopy) and SR-SIM technology**



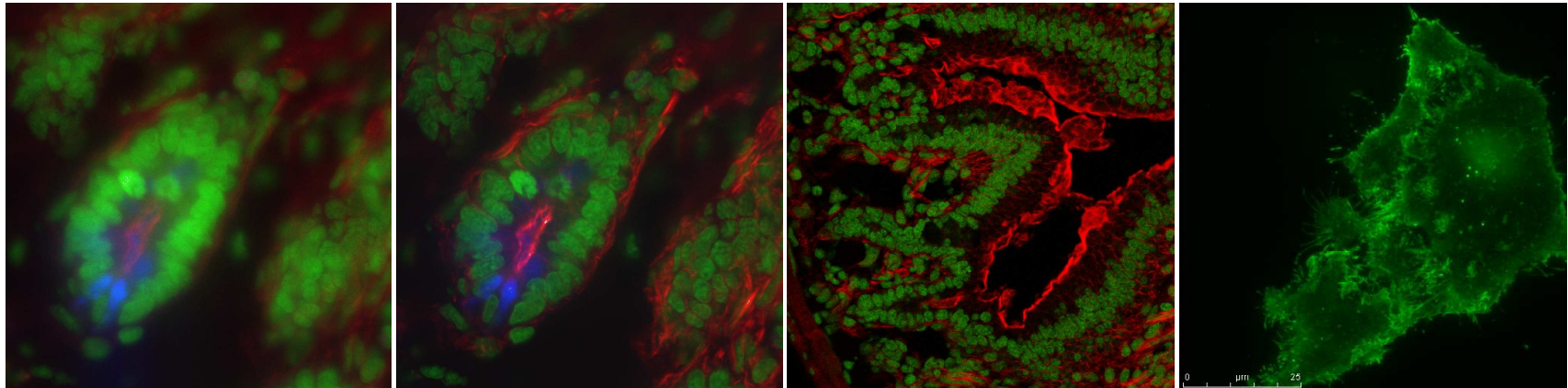
Fluorescence microscopy technique comparison



Z resolution measured

widefield: $\sim 1.13 \mu\text{m}$ SD6000: $\sim 0.84 \mu\text{m}$ **4PI: $0.1 - 0.12 \mu\text{m}$!** **TIRF: $0.1 - 0.3 \mu\text{m}$**

SP5: $\sim 0.50 \mu\text{m}$



samples thin samples $< 5 \mu\text{m}$
moderately thick + deconvolution

moderately thick
 $5 \mu\text{m} - 30 \mu\text{m}$

thick $> 30 \mu\text{m}$

! does not matter !
Only evanescent waves

Confocal Laser Scanning Microscopy – advanced systems

Leica TCS SP5 – universal system for everything!

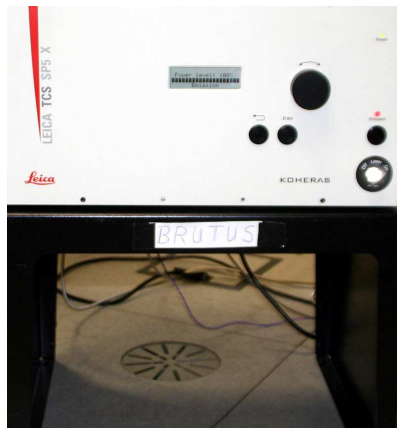


- FRAP
- FRET AB, SE
- Live Data Mode
- ROI spectrophotometer
- APD
- SMD – FCS, FLIM, FCCS
- Spectral FLIM
- High Content Screening Auto
- 2-photon, 3-photon

Leica DM6000 CFS – Confocal Fixed Stage



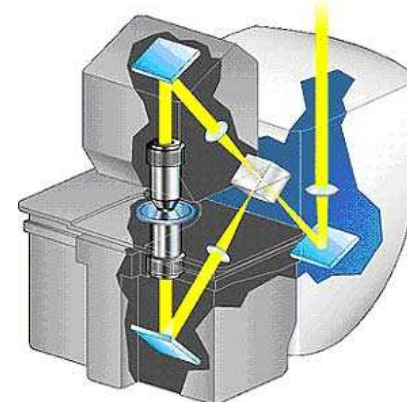
Leica TCS SP5-X WLL



Leica TCS SP5 STED



Leica TCS 4PI



Leica TCS SP5: the only broadband confocal



Separate UNIQUE systems

- Leica DM6000 B CFS - electrophysiology
- Leica 4PI – high resolution in z axis

Leica TCS SP5 basic features

- full range of lasers: 355, 405, VIS, IR up to 1300 nm
- conventional scanner up to 8192x8192 pxls
- resonant scanner up to 29 f/s for 512x512pxls
- AOBS – Acousto-Optical Beam Splitter
- Up to 5 confocal spectral detectors
- SuperZ Galvo and Pifoc

Wide range of UNIQUE upgrades:

- White Light Laser
- Spectral FLIM
- online ROI spectrometer
- STED – superresolution in xy plane

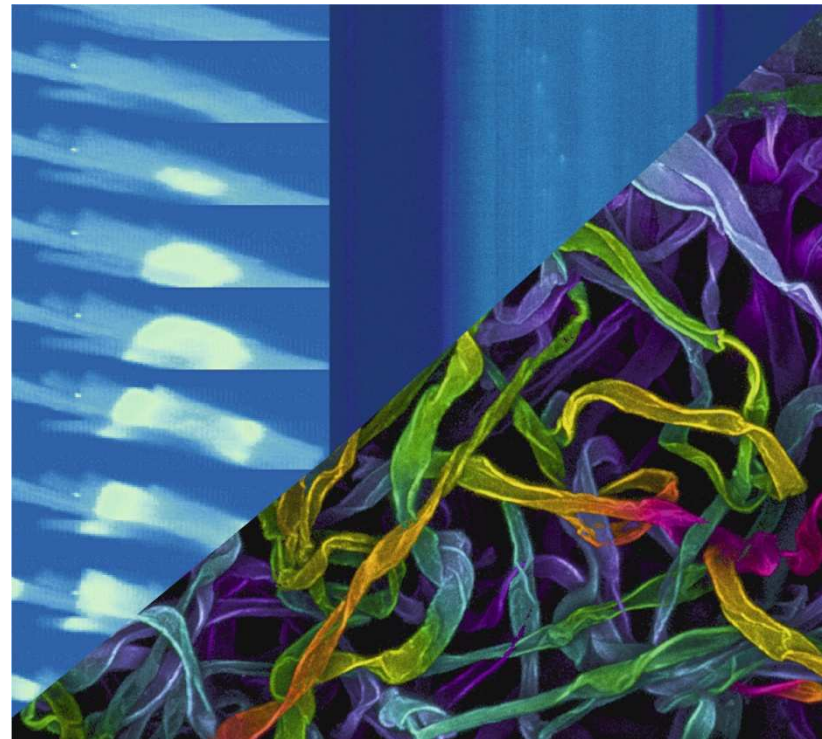
Unique

Unique

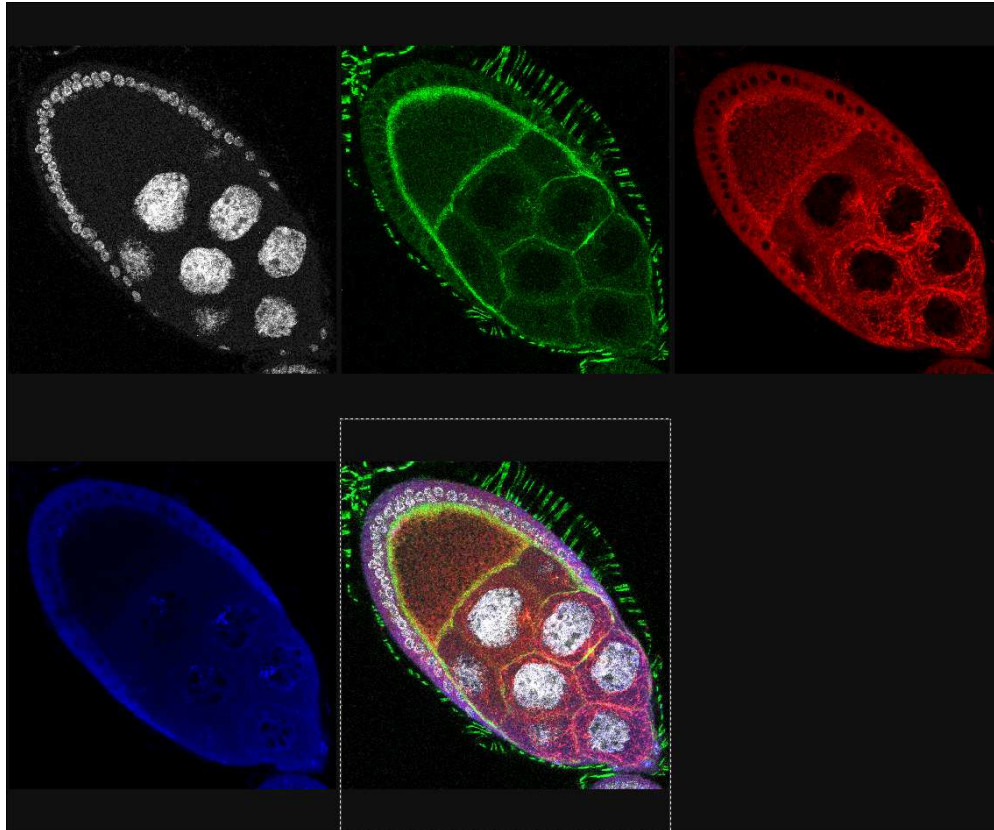
Leica TCS SP5: The Only Broadband Confocal

Resolution and speed in one system

- **Structure**
 - High resolution optical sections
 - 3D Structures
 - Correlation analysis
 - Single Point Illumination
- **Dynamic**
 - Movement analysis
 - Kinetic measurements



Leica TCS SP5 – Leading in Multispectral imaging

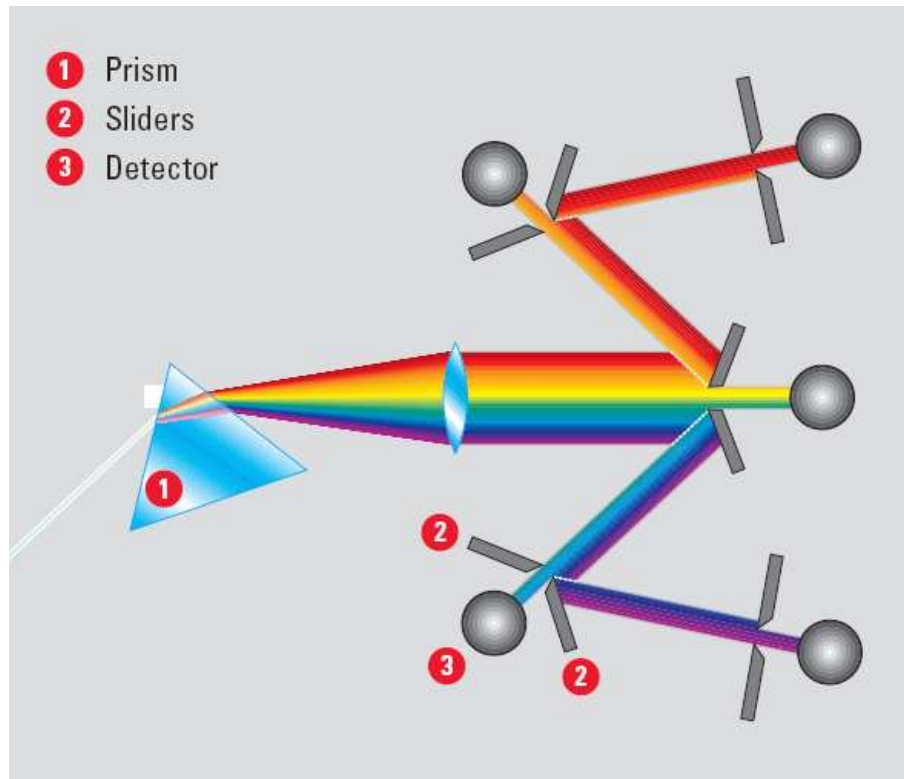


Ideal for multi-labelled specimens and fast events:

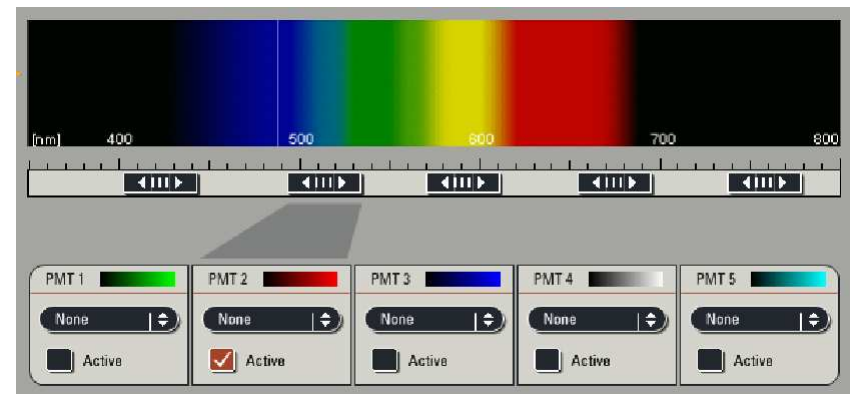
- **Up to 5 SP Prism Spectrophotometer channels** for sensitivity and flexibility
- **AOBS – the dynamic beam splitter** for sensitivity, selectivity and flexibility
- **Spectral FLIM** - a new dimension of experiments
- **ROI-spectrometer** captures dynamic spectral events

Leica TCS SP5 – Leading in Multispectral imaging

Leica SP – Leica Spectral detection (1997)



- 5 spectral confocal detectors simultaneously
- Arbitrary settings of spectra
- Low photobleaching, high efficiency
- Intuitive operation

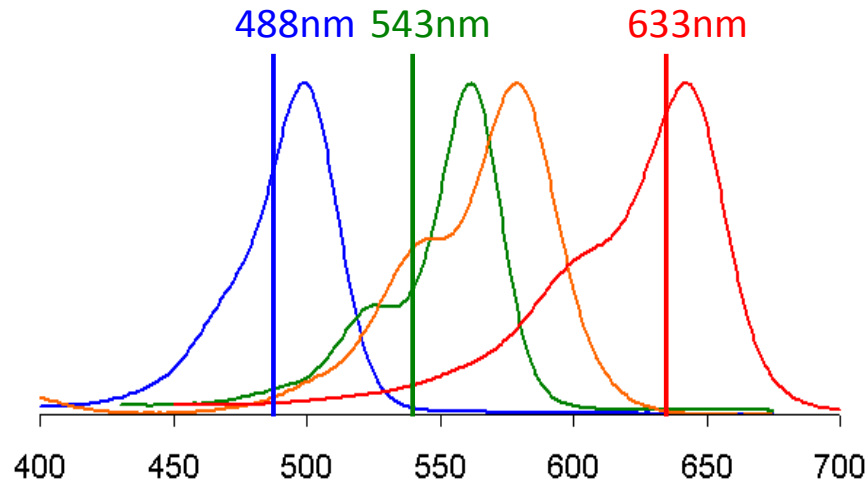


Unique

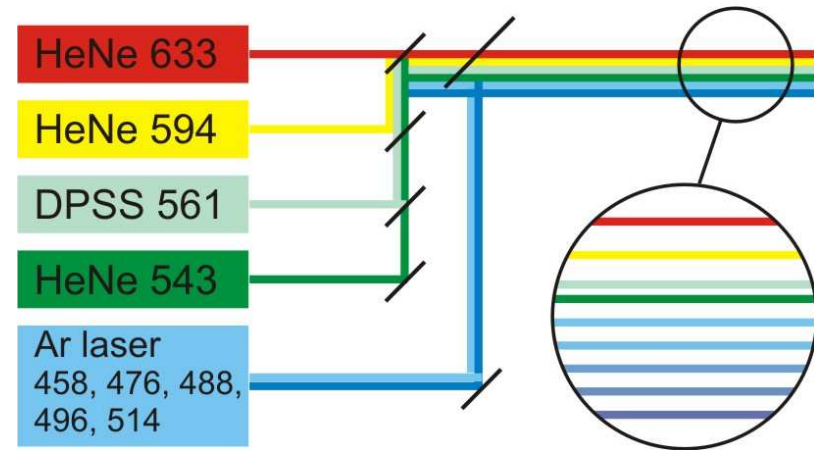
White Light Laser – set of lasers or just one tuneable source?

Excitation Spectra

(Alexa 488, Alexa 546, Alexa 568, Toto-3)



- No tunability
- Sub-optimal excitation
- Cross-excitation fixed



- Set of gas, DPSS or DL lasers
- Sophisticated merge module
- Expensive solution
- Only several combinations of wavelengths

White Light Laser – new lambda scan, new wavelengths

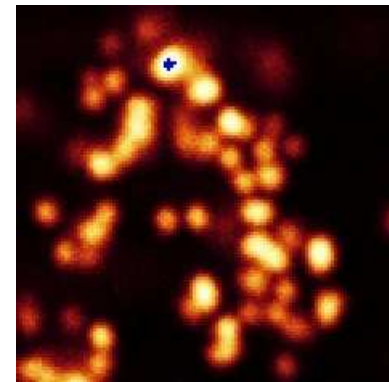
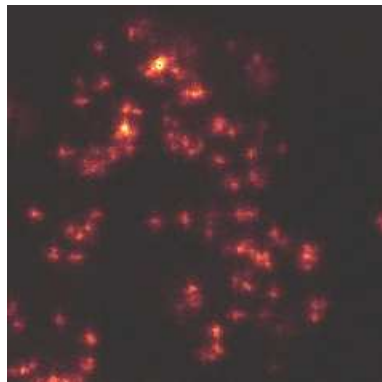
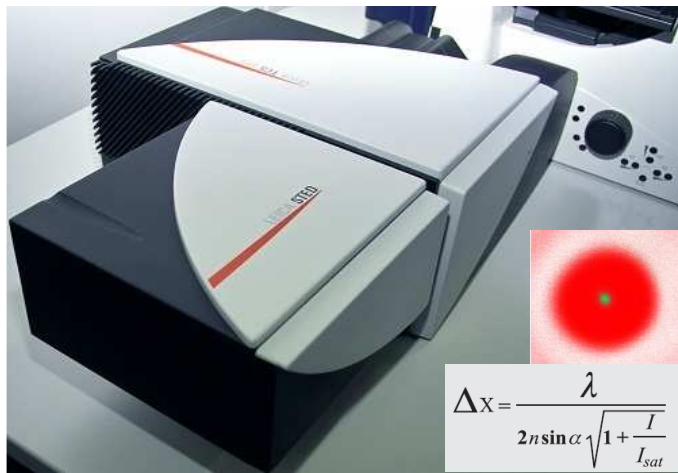
- setting of excitation wavelength and intensity in software or at Panel Box „Smart Wavelength“ and „Smart Intensity“
- Lambda Square Scan: lambda excitation and emission over whole visible spectra

The screenshot displays the software interface for a microscope system, divided into several functional areas:

- Experiments / Acquisition Panel (Left):**
 - Acquisition Mode: xyz (with a 'seq.' icon)
 - XY: 512 x 32 | 1400 Hz | 64 | 12.11 μm * 756.84 nm
 - Z-Stack: z - Galvo (with 'Set Plane' and 'Go to' buttons)
 - A 3D schematic of a specimen volume with a yellow plane at the center. 'Begin [μm]' and 'End [μm]' sliders are positioned on either side.
 - z-Position [μm]: 0
 - Nr. of steps: 1; z-step size: 0 μm; z-Volume: 0 μm
 - system optimized: Compensation
- Conventional Lasers Panel (Middle-Left):**
 - Load/Save single setting: 3wll (with 'Delete' and 'Save' buttons)
 - ROI Scan: ROI (checked), SPOT (unchecked), Set Background (unchecked)
- White Laser Panel (Middle-Right):**
 - WL Shutter: (checked)
 - A color gradient bar representing the visible spectrum from 470 nm (blue) to 670 nm (red). Tick marks are at 470, 490, 548, 600, and 670 nm. Below the bar, checkboxes are present for 490, 548, and 600 nm.
- Bottom Panel (Spectral Control):**
 - Control Panel: (text label)
 - Substrate: (text label)
 - Objective: 20x 0.7 (text label)
 - Specimen: (text label)
 - AOBS: (text label with a green arrow pointing to the spectral bar)
 - A spectral bar with a wavelength scale from 400 to 800 nm and navigation controls.
 - Five PMT channels (PMT 1 to PMT 5) with color-coded filters and 'None' dropdown menus. Checkboxes for 'Active' are present for PMT 1 through PMT 4.
 - Additional Channels: (checked)

Leica STED – STimulated Emission Depletion SUPERRESOLUTION (subdiffraction) in xy plane

Hell, S. W. and J. Wichmann (1994). *Opt. Lett.*
"Breaking the diffraction resolution limit by stimulated emission"



neurobiology
membrane biology
membrane rafts
intracellular transport

Willig KI et al. *Nature* 2006

Sieber JJ et al. *Biophys J* 2006

Kittel RJ et al. *Science* 2006

Fitzner D et al. *EMBO J* 2006

Kellner RR et al. *Neuroscience* 2007

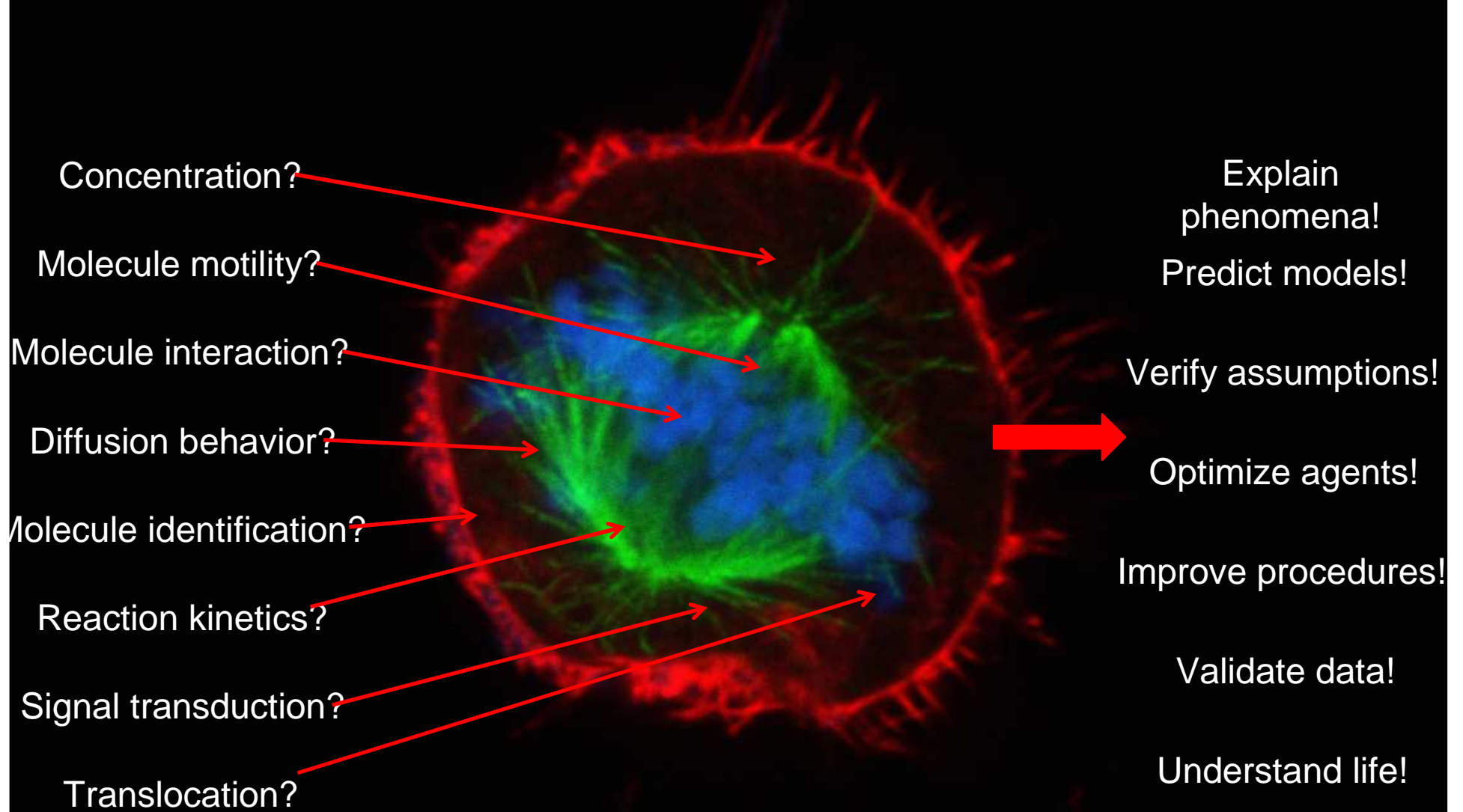
Lin W et al. *PNAS* 2007

Seebach J *Cardiovas. Res.* 2007

Sieber JJ *Science* 2007

Unique

Quantify Life! – The Challenge



Possibilities: FCS - FCCS - FLIM – FRET - FLCS - gated FCS