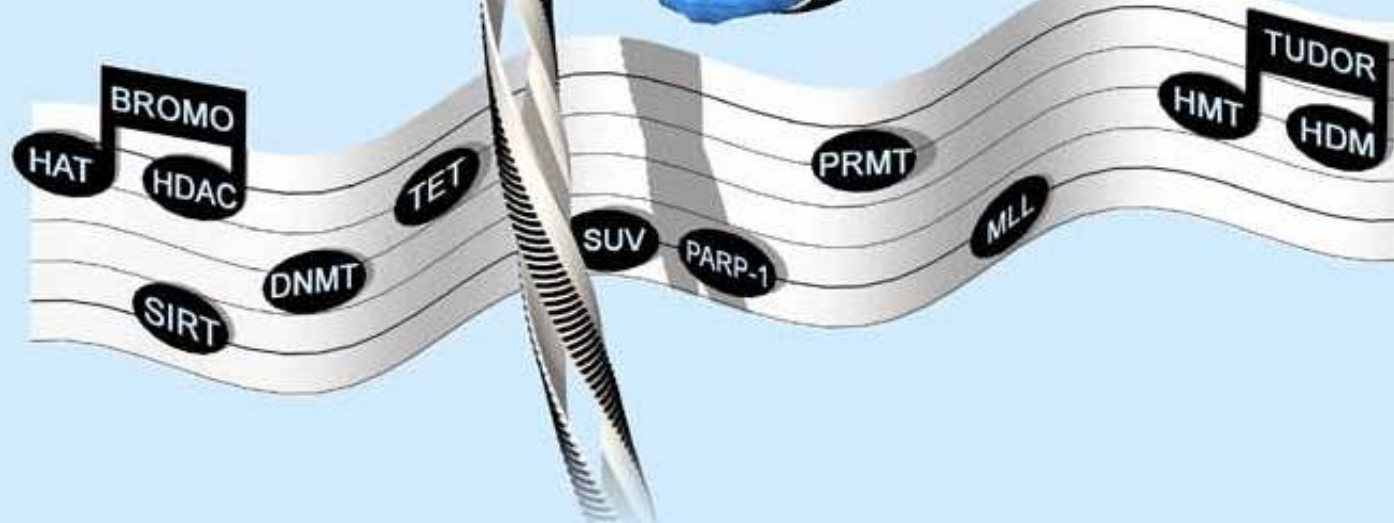A Training School from COST Action TD0905
Epigenetics: bench to bedside
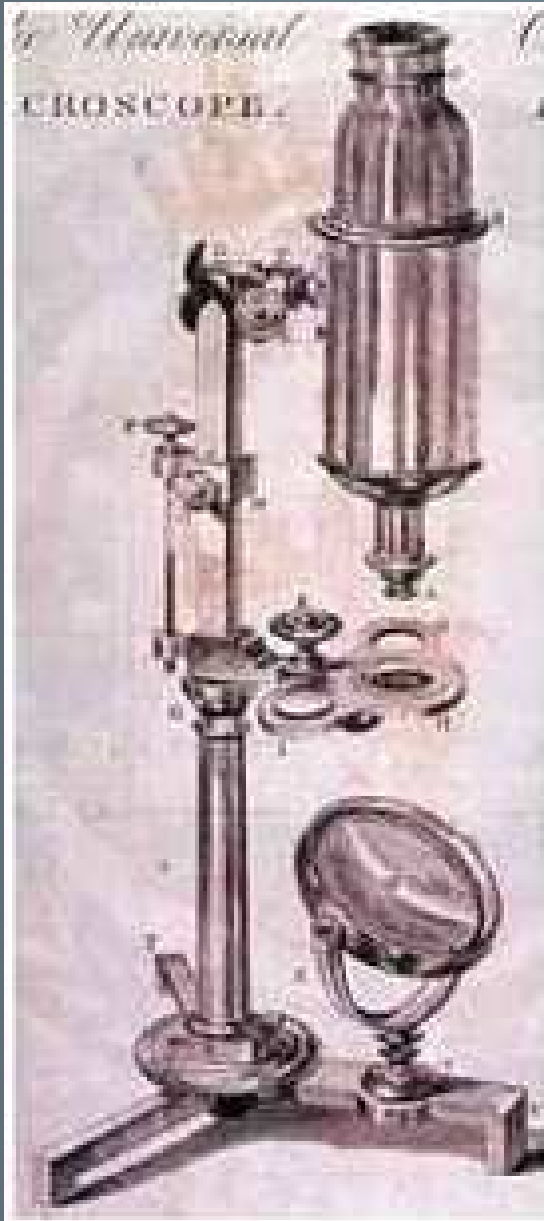
**Confocal Microscopy and Living Cell Studies**

**Eva Bártová**
*Institute of Biophysics*
*Academy of Sciences of the Czech Republic*

*History of microscopy:*

the light microscope (discovered by Robert Hook, 1665): an instrument that enables the human eye, by means of a lens or combinations of lenses, to observe enlarged images of tiny objects. It made visible the fascinating details of naked eye invisible worlds.
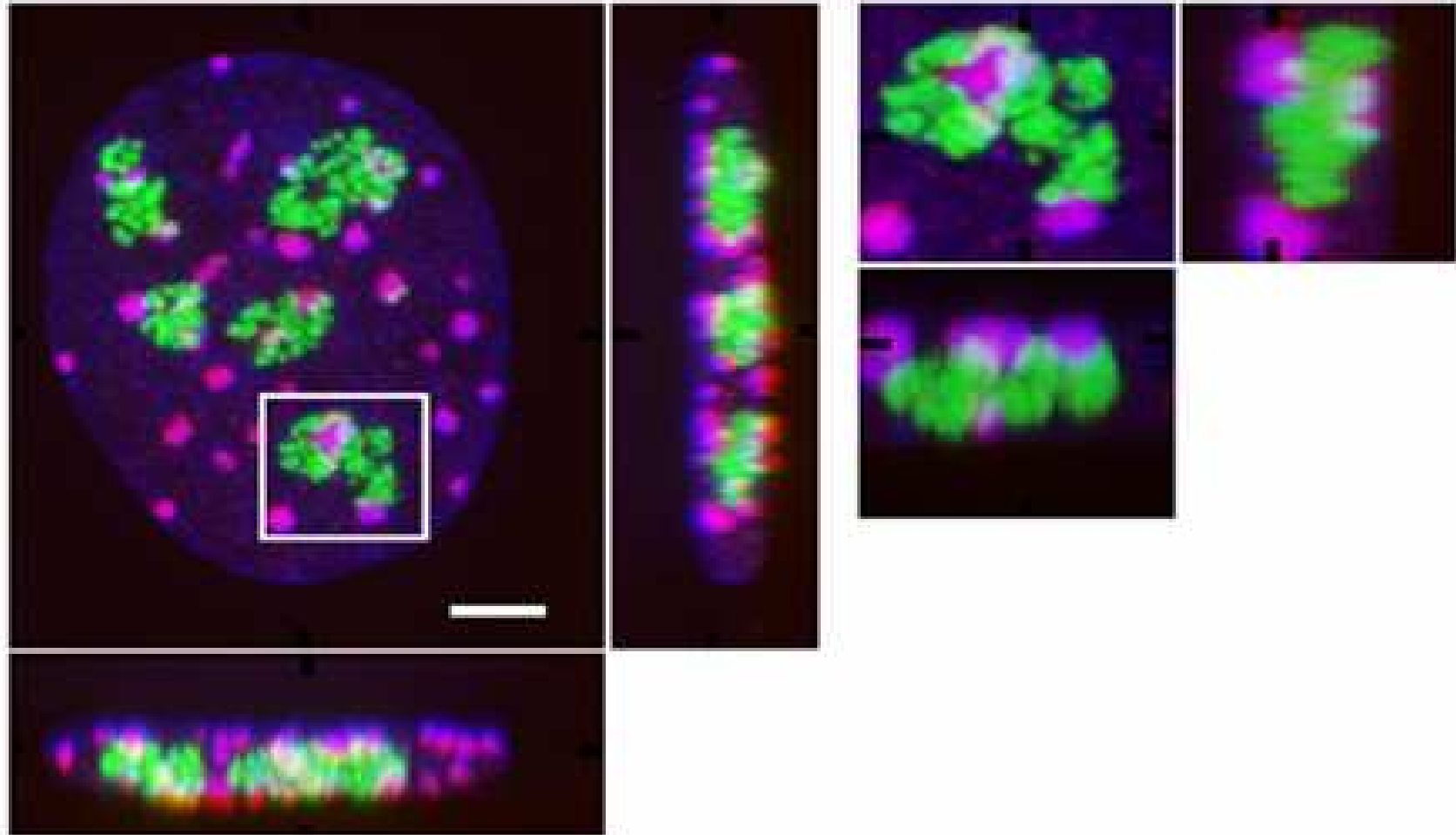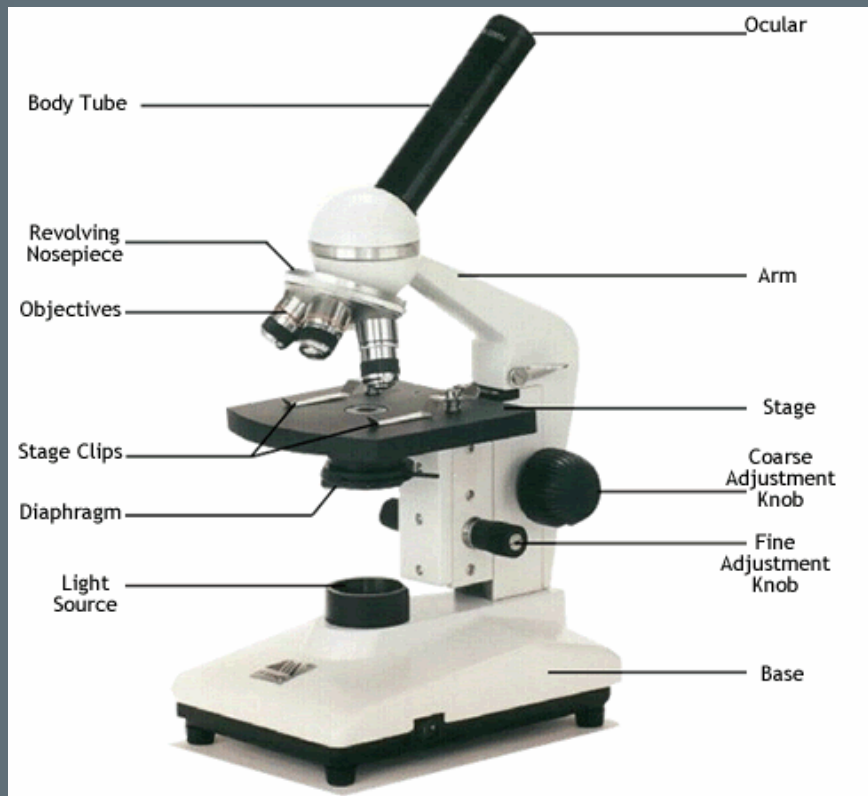
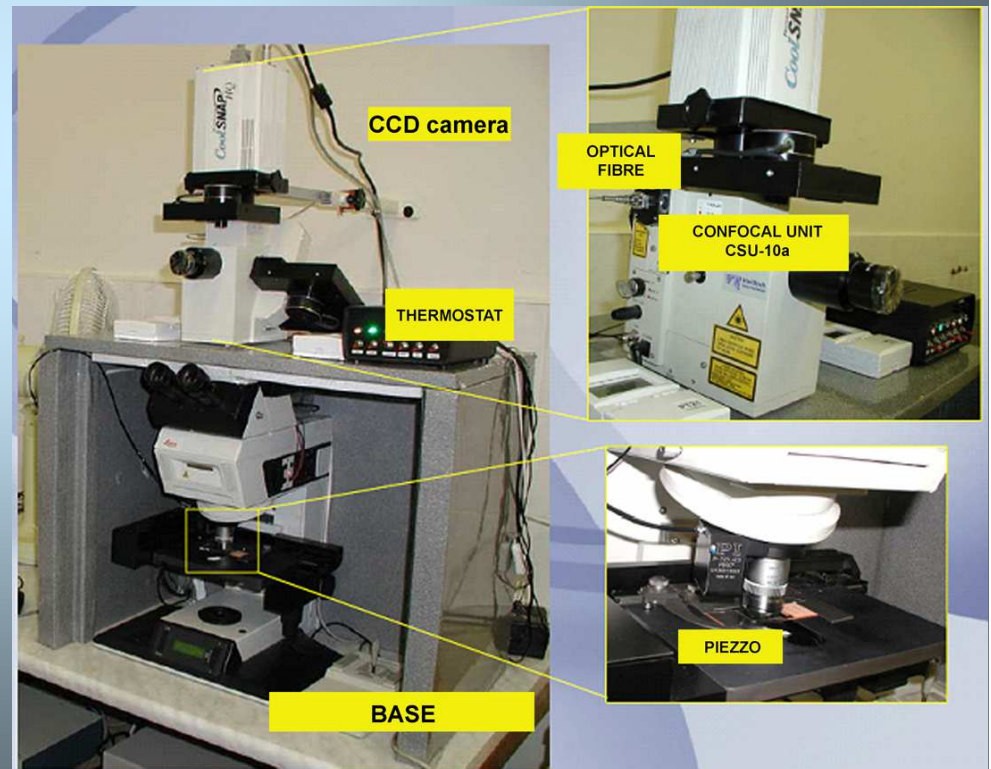1957: Marvin Minsky patented the fist confocal microscope

Fibrilarin / HP1α / DNA

Ocular

Body Tube

Revolving Nosepiece

Objectives

Arm

Stage

Stage Clips

Coarse Adjustment Knob

Diaphragm

Fine Adjustment Knob

Light Source

Base

http://www.google.cz/imgres?imgurl=http://
www.microscopehelp.com/

CCD camera

OPTICAL FIBRE

CONFOCAL UNIT CSU-10a
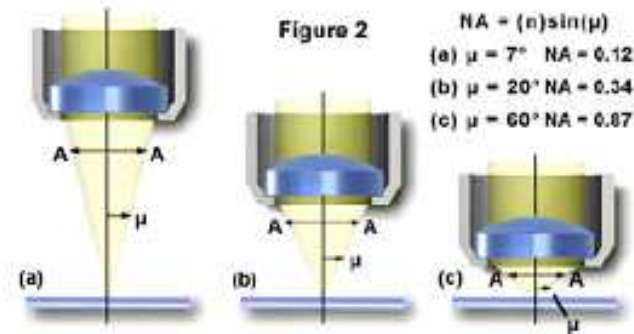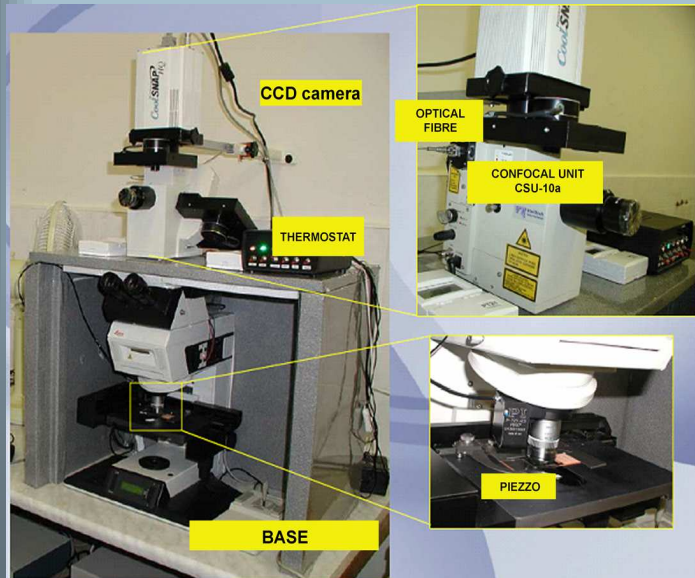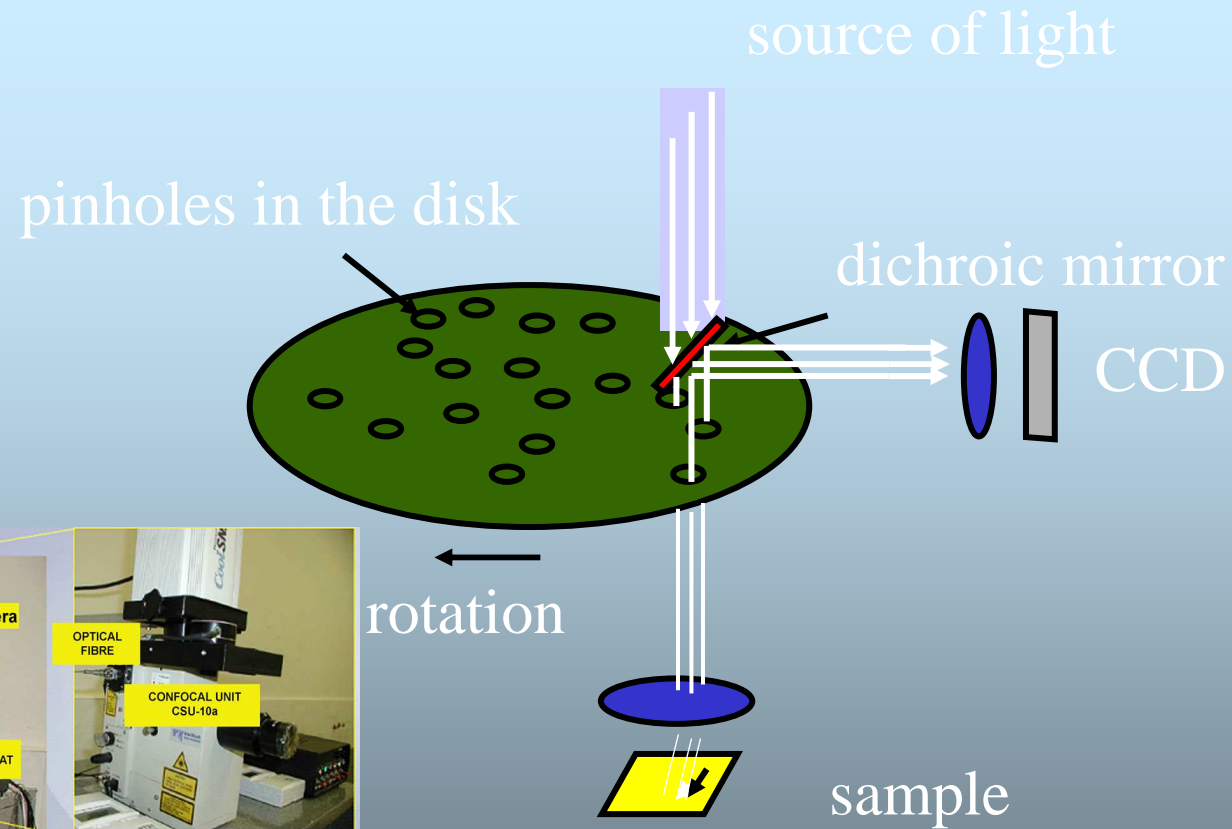
THERMOSTAT

PIEZZO

BASE

Brno laboratory at IBP

# Visible Light Microscopy:
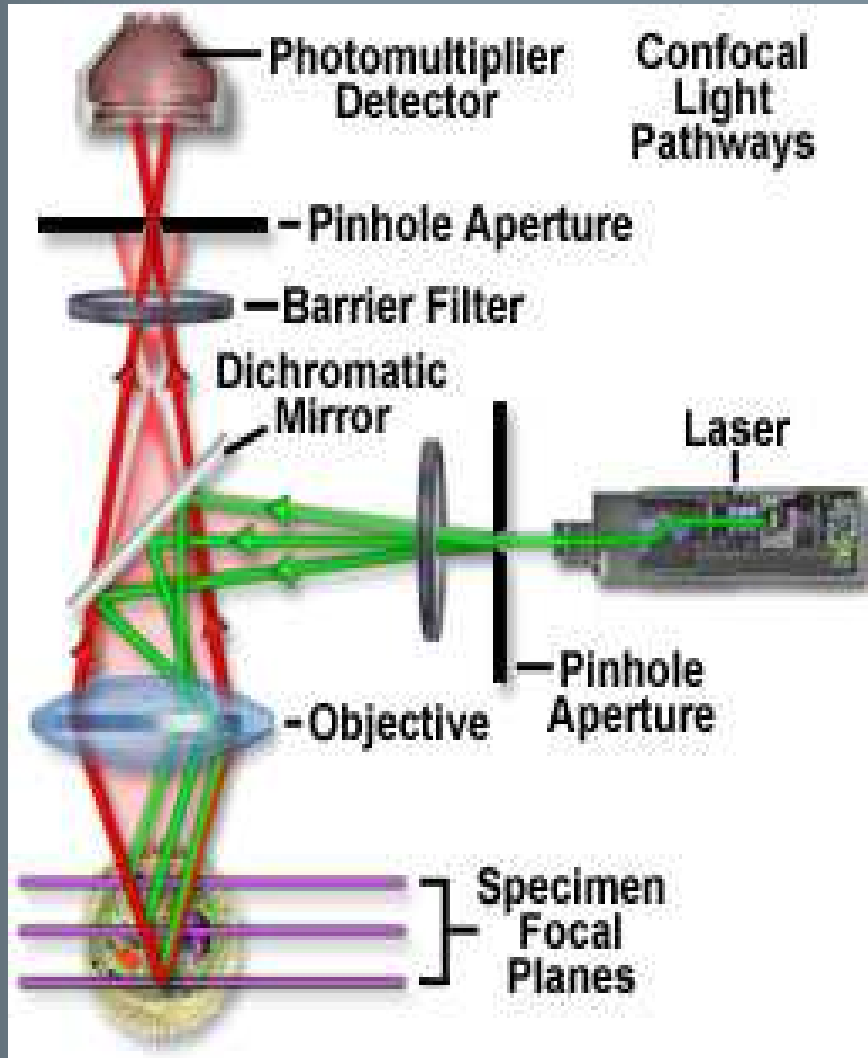## Objectives: numerical aperture

- 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·sin($\mu$)
  - Shorter wavelengths=higher resolution.
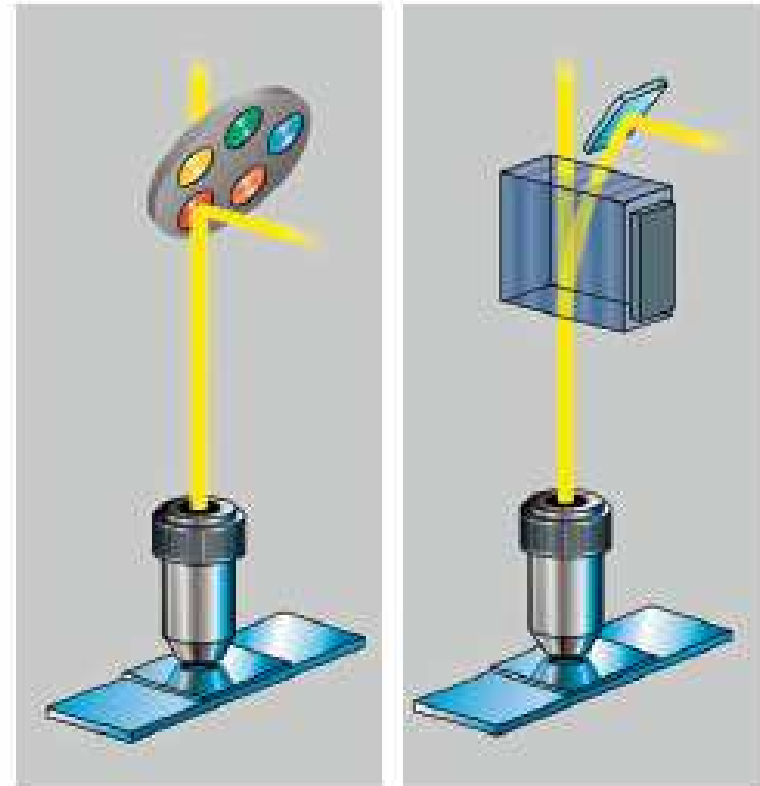  - Resolution limit for green light (NA=1.4, 100X) is 0.2 $\mu$m.
    - R=0.61$\lambda$/NA



Figure 2

$NA = (n)\sin(\mu)$
(a) $\mu = 7°$   NA = 0.12
(b) $\mu = 20°$ NA = 0.34
(c) $\mu = 60°$ NA = 0.87

60x Plan Apochromat Objective

Manufacturer
Flat-Field Correction
Linear Magnification
Specialized Optical Properties
Tube Length
Cover Slip Thickness

Screw Thread
Aberration Correction
Numerical Aperture
Immersion Medium
Working Distance
Color Code
Spring-Loaded Front Lens

Figure 1

# Tandem scanning microscopes based on Nipkow disk

source of light

pinholes in the disk

dichroic mirror

CCD

rotation

sample

CCD camera

OPTICAL FIBRE

CONFOCAL UNIT CSU-10a

THERMOSTAT

PIEZZO
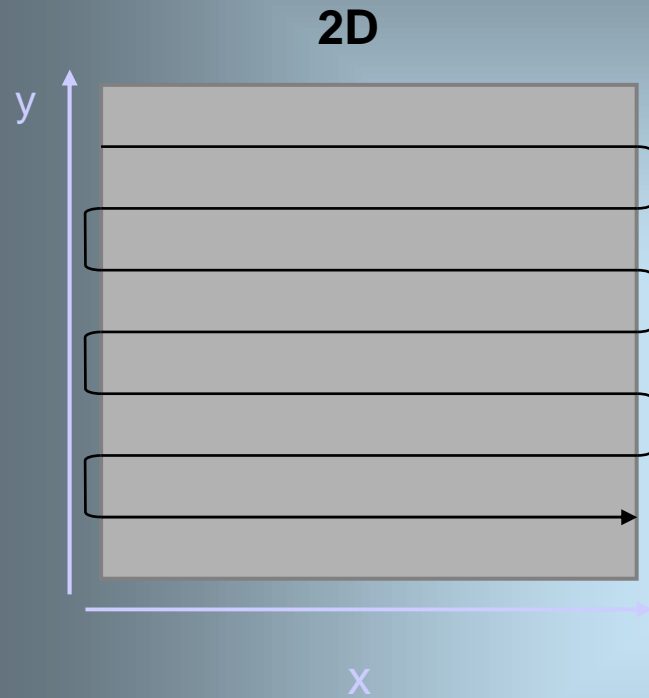
BASE

# Confocal microscopy - principles
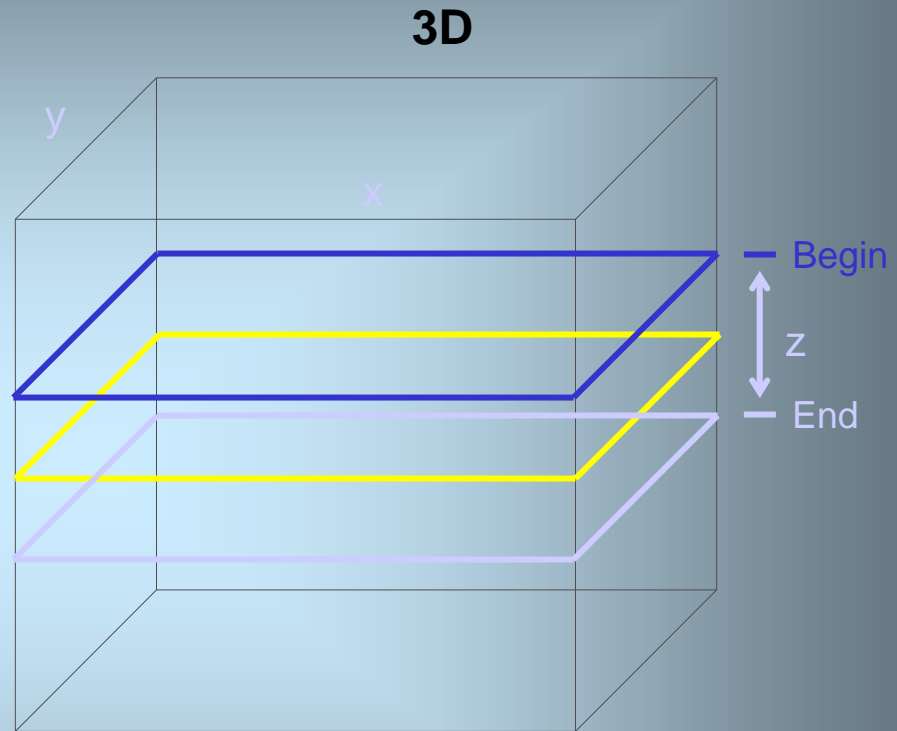
# AOTF and AOBS



Left: conventional beam splitting by dichroic mirrors requires many optical elements with fixed properties.
Right: the AOBS* is electronically adaptable to all tasks.

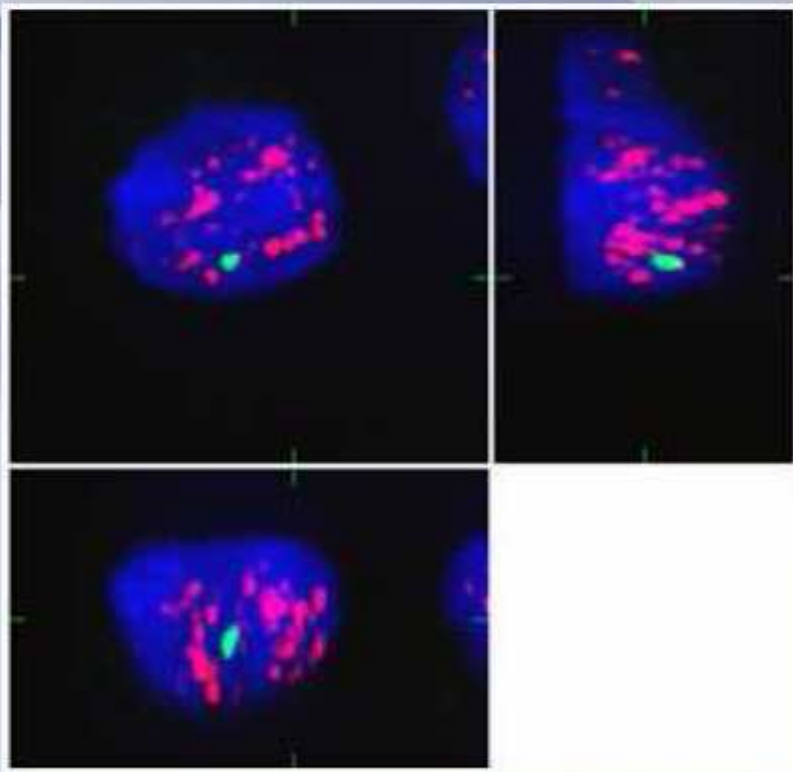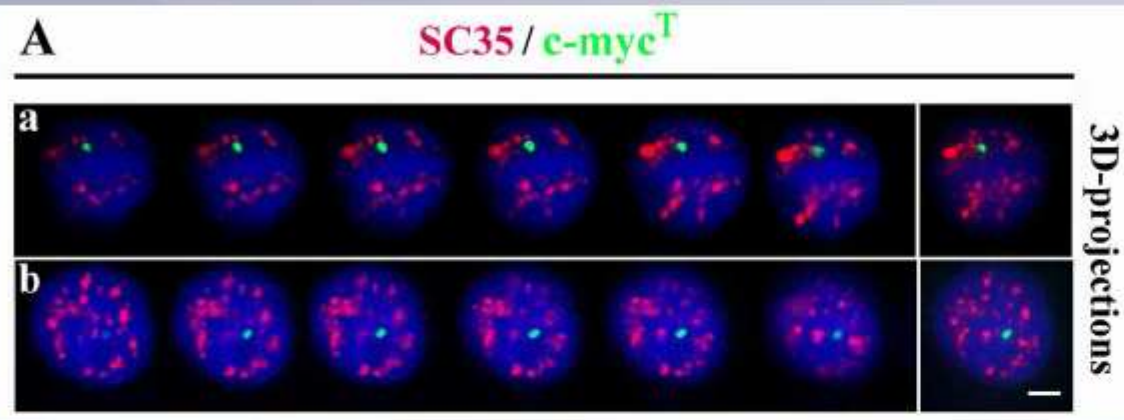# Scanning in 2D and 3D by confocal microscope

**2D**

**3D**

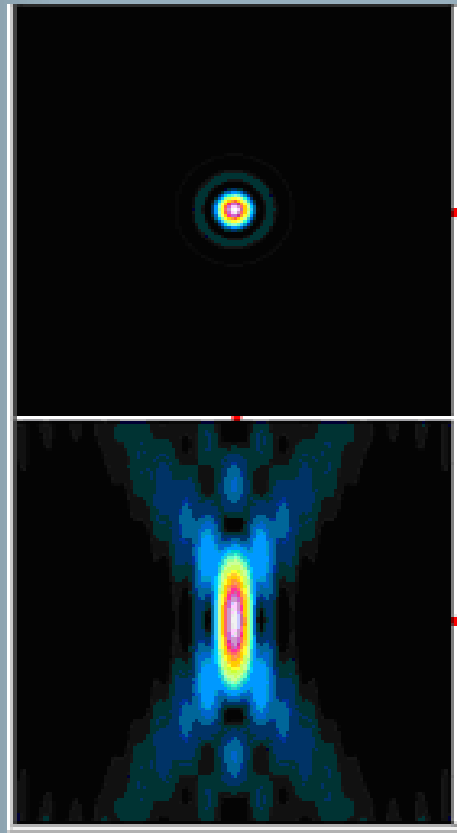Laser beam moves firstly along *x* axis and then starts with new line in *y* axis.

Finishing scanning of one thin optical slice in *xy* plane, the scanning plane is moved in *z* axis to other slice
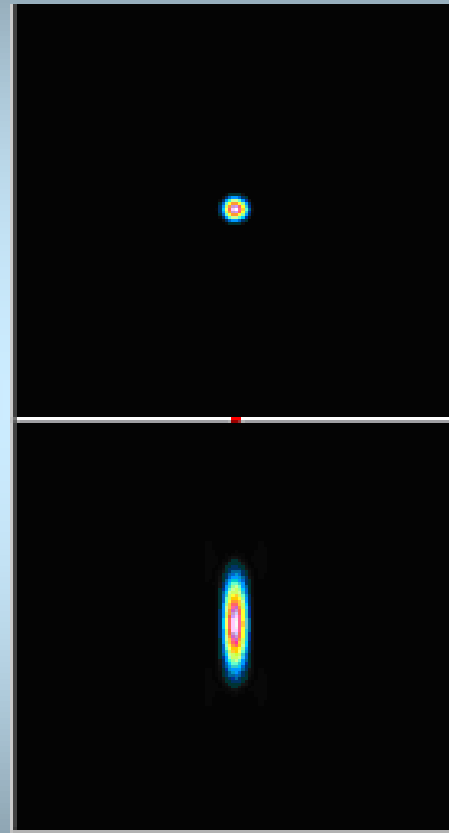
# 3D-projection



**A**      SC35 / c-myc$^T$

3D-projections

# Optical resolution: conventional versus confocal

**Conventional**

**Confocal**

Res = 0.61*$\lambda$ / NA

Res(xy) = 0.4*$\lambda$ / NA
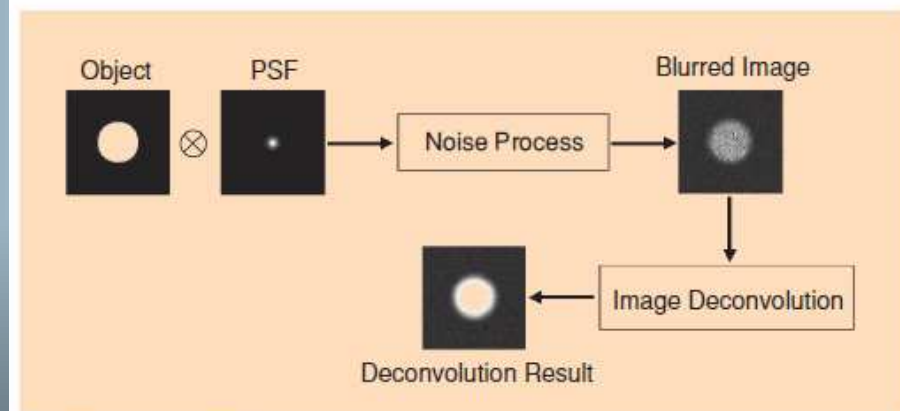
Res(xz) = 0.45*$\lambda$ / n(1-cos$\alpha$)

Formulas by Kino

4PI and STED resolution are much higher…
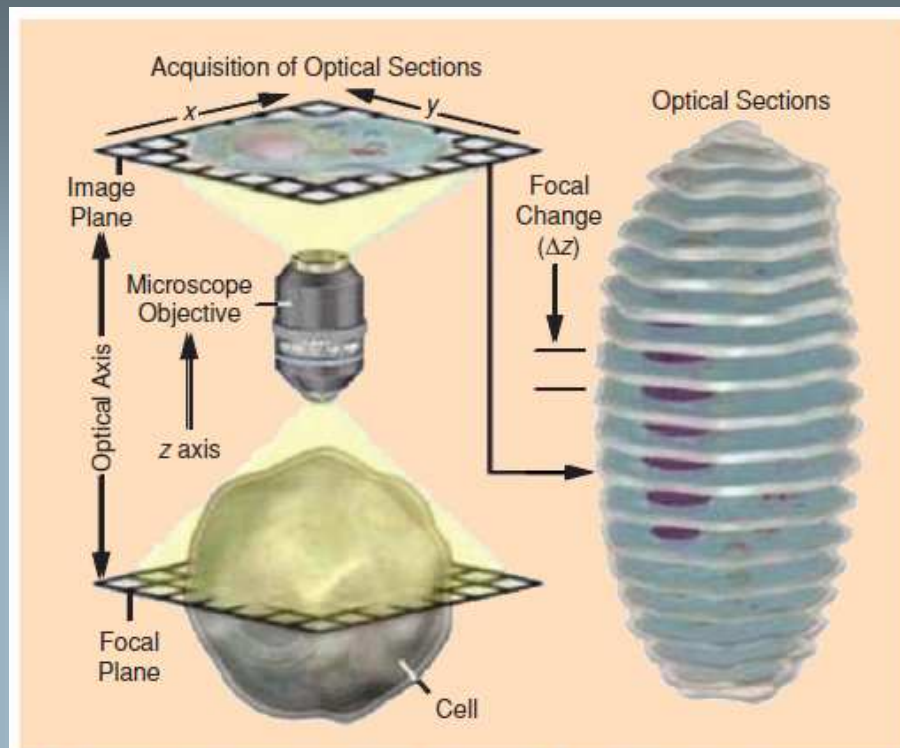
[FIG1] An example of the acquired 3-D image of a cell, captured by a fluorescence microscope.



[FIG2] Schematic of a general deconvolution procedure.

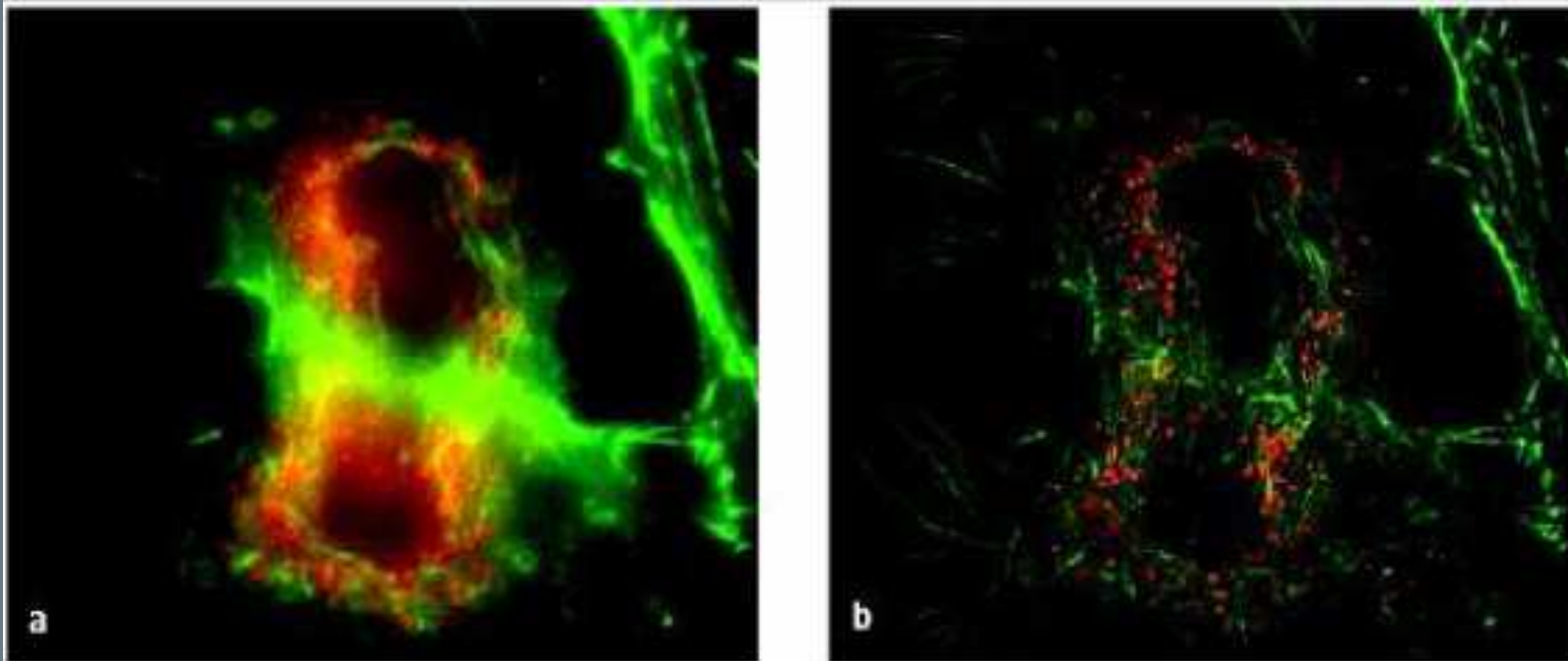**Pinaki Sarder and Arye Nehorai (2006)**

# Deconvolution



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.

Assuming linearity, convolution of the object and the imaging system PSF is affected by noise and produces a blurred image. Deconvolution restores the original object to an improved resolution and higher signal-to-noise ratio (SNR) level.
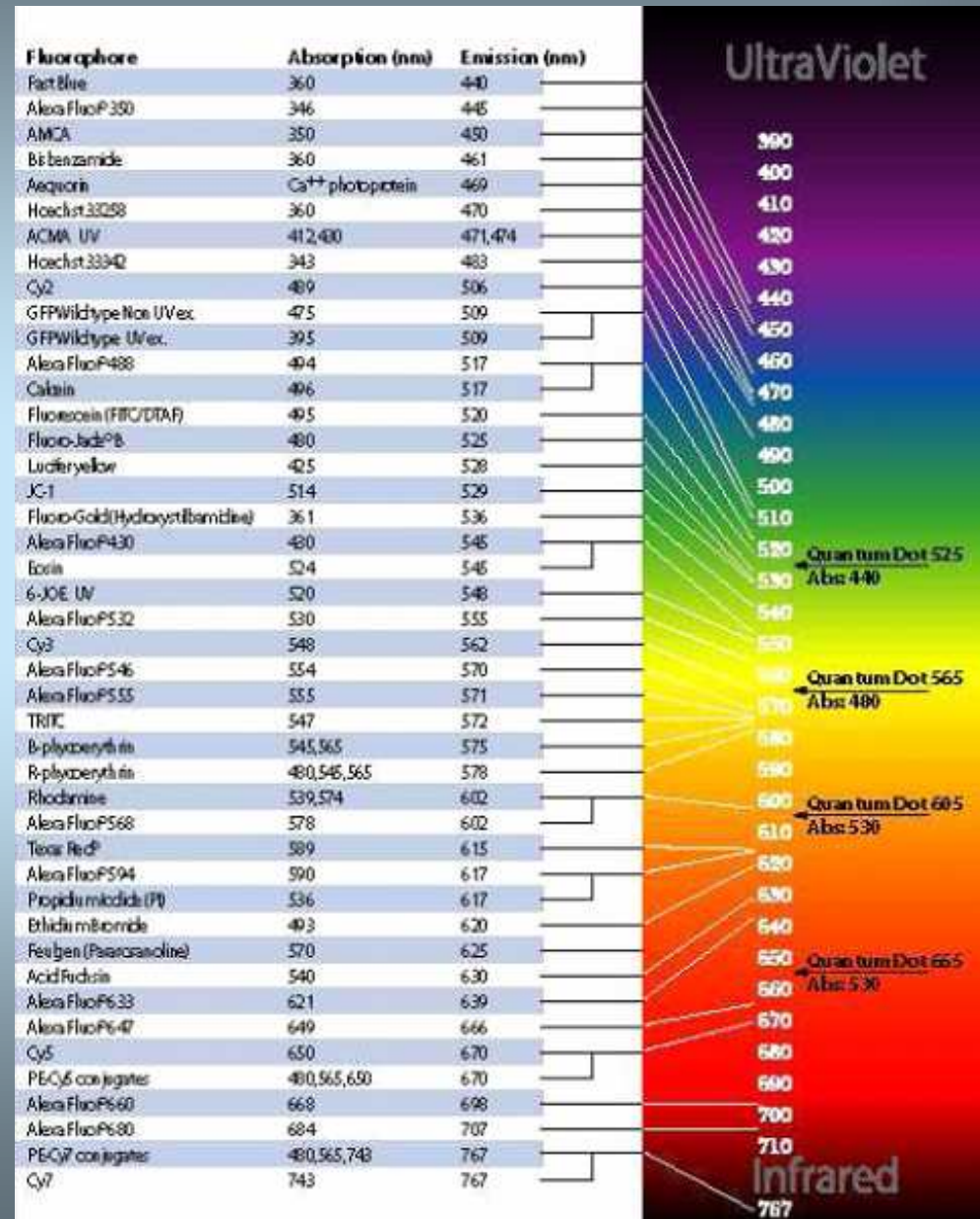
# Types of fluorochromes

Fluorochromes are essentially dyes, which accept light energy (e.g. from a laser) at a given wavelength and re-emit it at a longer wavelength. These two processes are called excitation and emission.
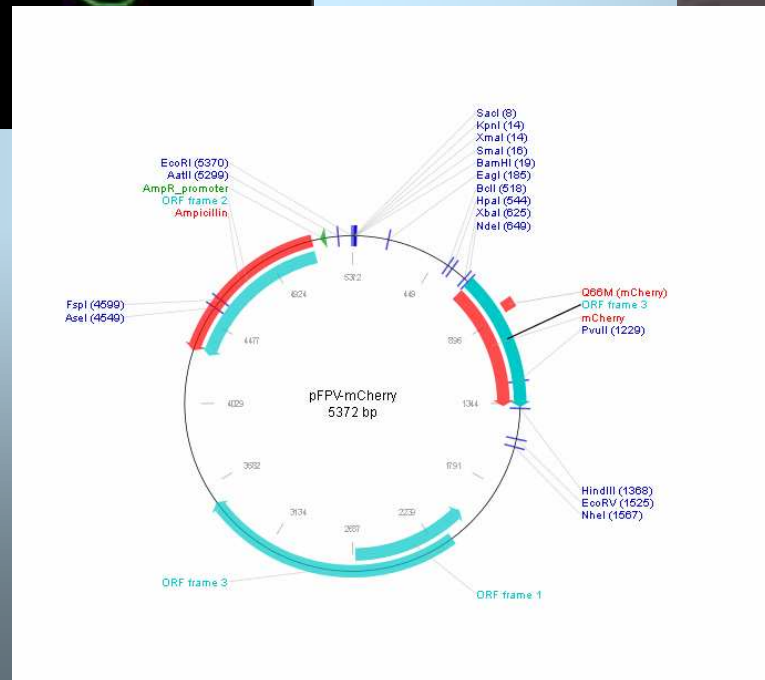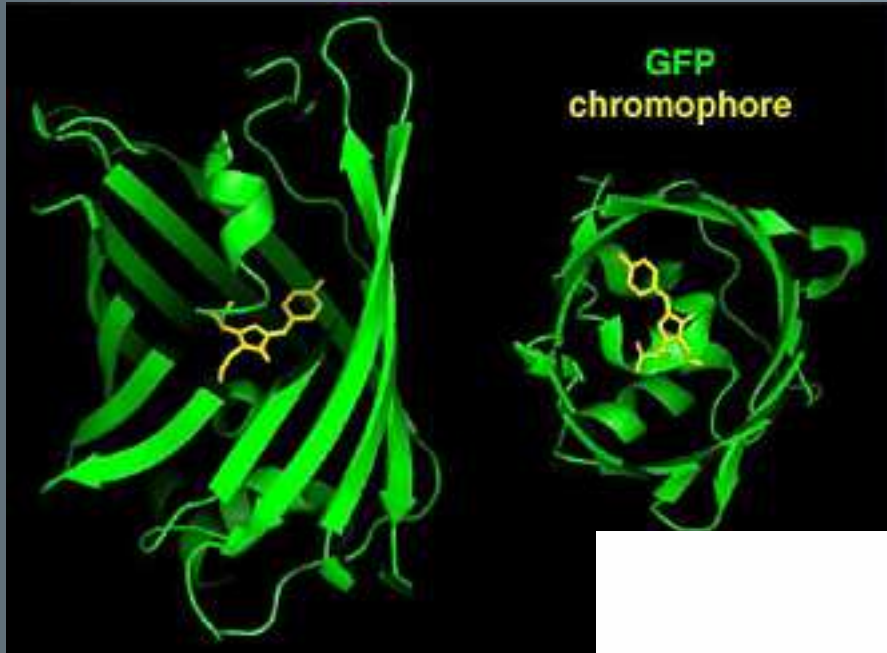
1. Fluorochromes conjugated with other molecule. Example represents quantum dots, used for for ultra-sensitive nonisotopic detection.

2. Fluorochormes that binds directly to some structure. For example, DAPI or PI binds to DNA
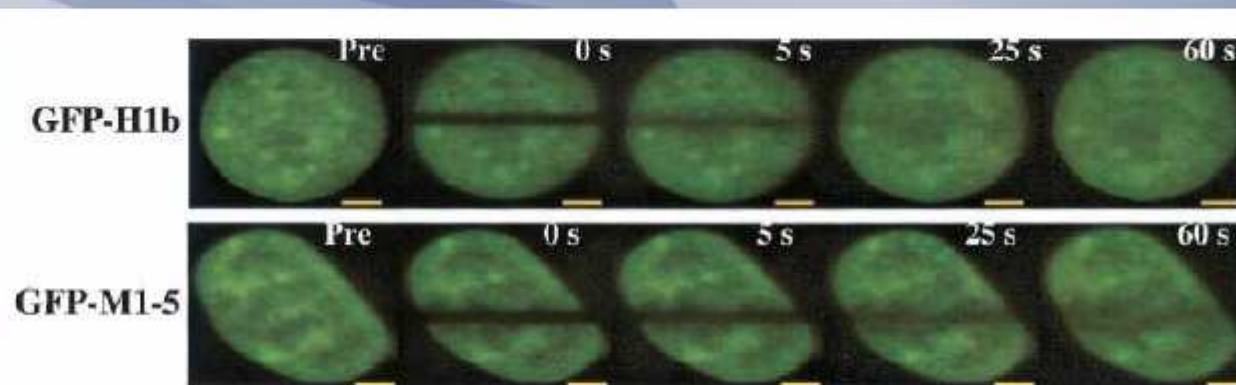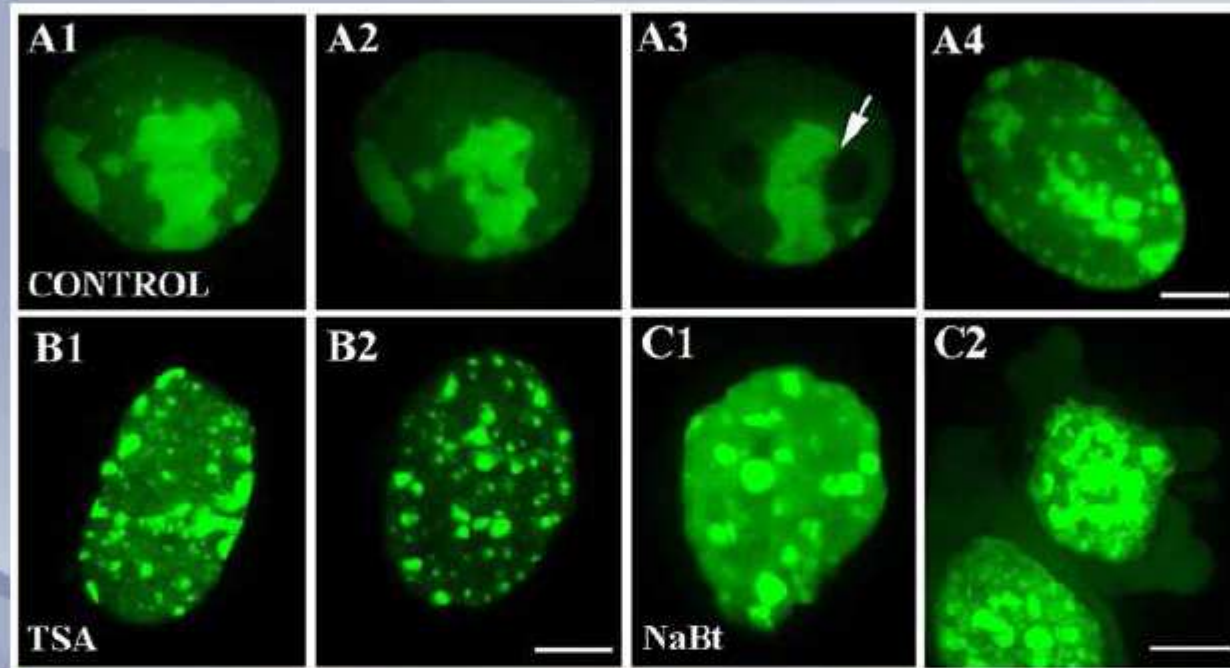
3. Fluorochromes produced by organism like *Aequorea victoria* (GFP) or octocoral *Dendronephthya sp.* (Dendra2)
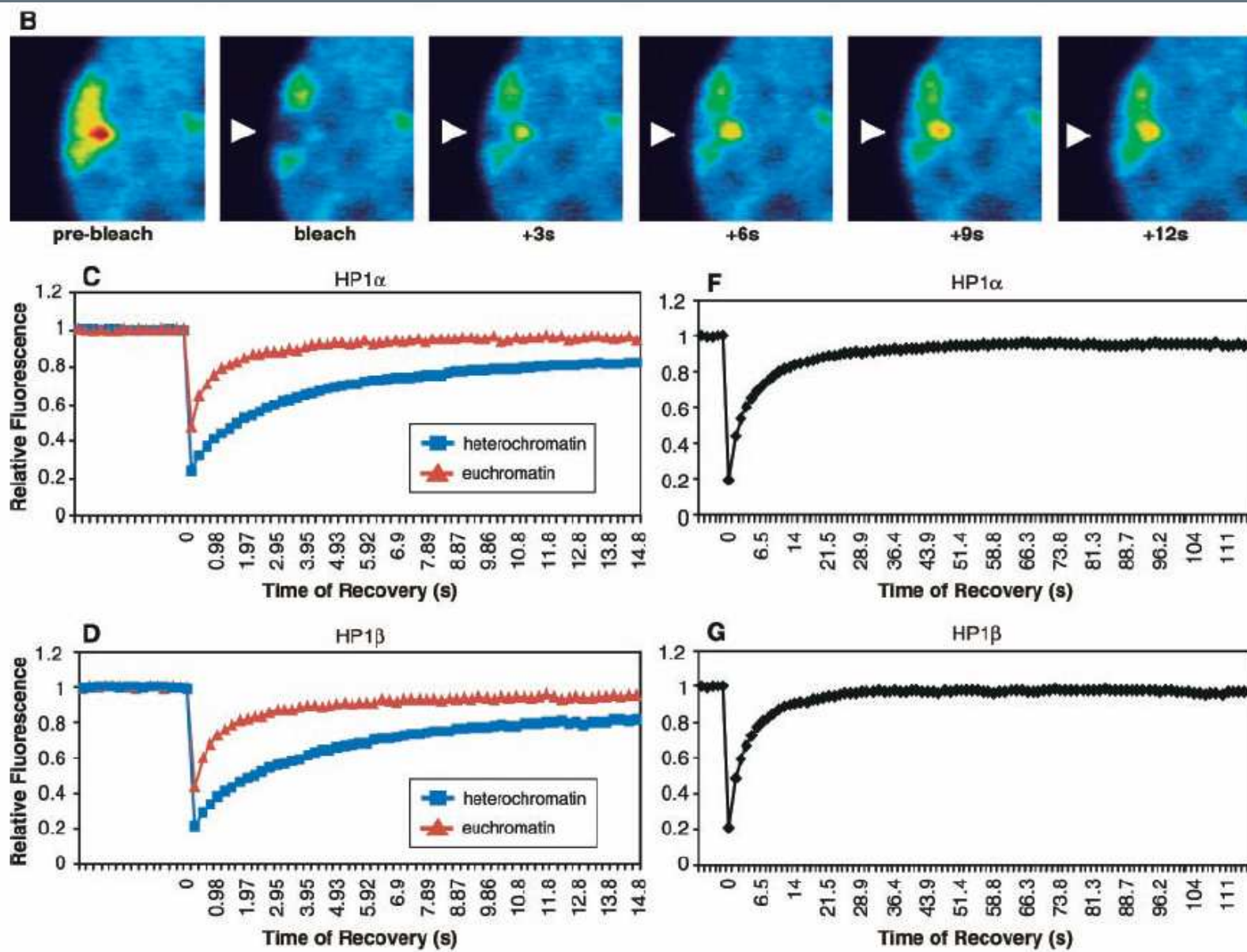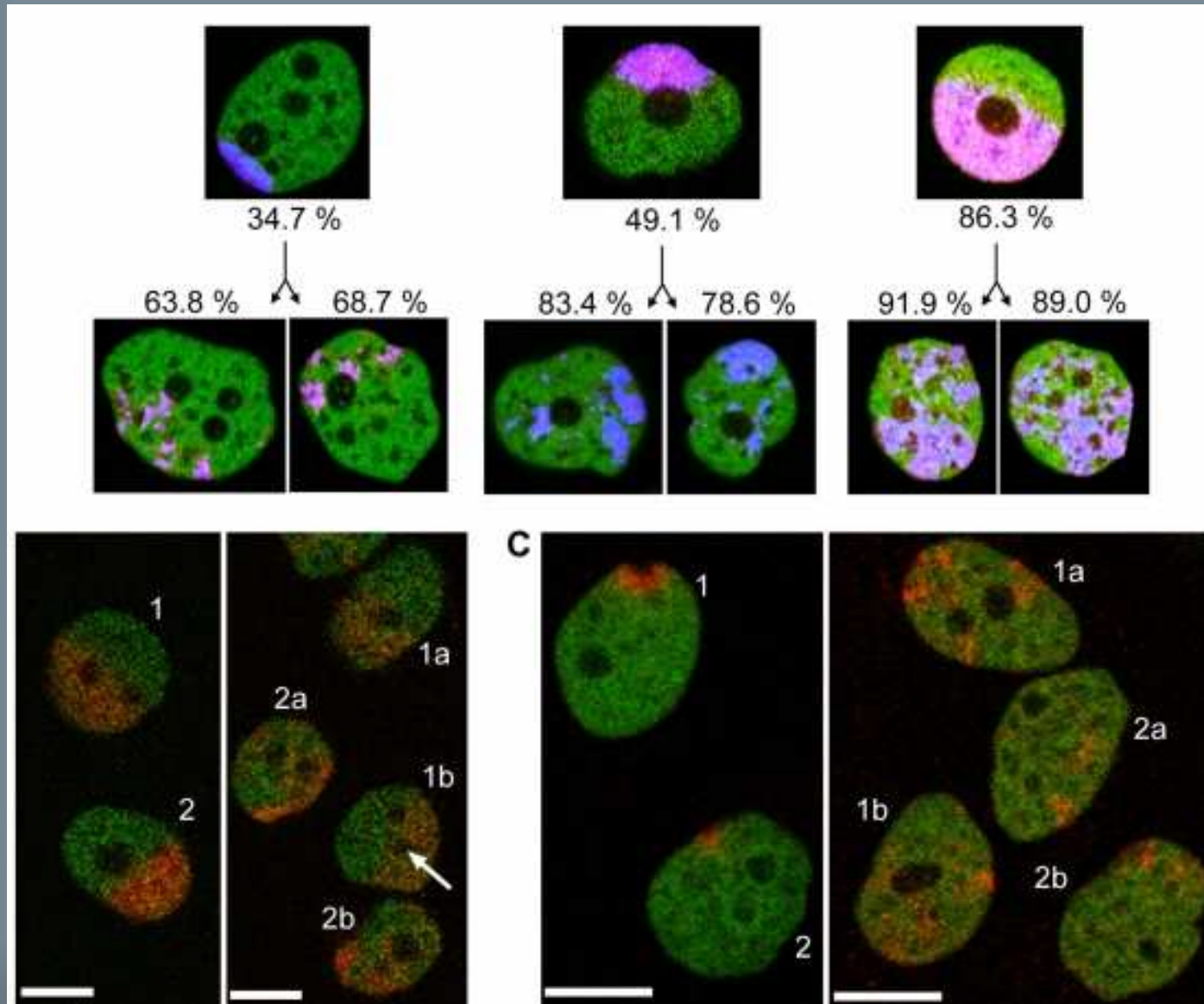
| Fluorophore | Absorption (nm) | Emission (nm) |
|---|---|---|
| Fast Blue | 360 | 440 |
| Alexa Fluor 350 | 346 | 445 |
| AMCA | 350 | 450 |
| Bisbenzamide | 360 | 461 |
| Aequorin | Ca++ photoprotein | 469 |
| Hoechst 33258 | 360 | 470 |
| ACMA UV | 412,430 | 471,474 |
| Hoechst 33342 | 343 | 483 |
| Cy2 | 489 | 506 |
| GFP Wildtype Non UV ex. | 475 | 509 |
| GFP Wildtype UV ex. | 395 | 509 |
| Alexa Fluor 488 | 494 | 517 |
| Calcein | 496 | 517 |
| Fluorescein (FITC/DTAF) | 495 | 520 |
| Fluoro-Jade B | 480 | 525 |
| Lucifer yellow | 425 | 528 |
| JC-1 | 514 | 529 |
| Fluoro-Gold (Hydroxystilbamidine) | 361 | 536 |
| Alexa Fluor 430 | 430 | 545 |
| Eosin | 524 | 545 |
| 6-JOE UV | 520 | 548 |
| Alexa Fluor 532 | 530 | 555 |
| Cy3 | 548 | 562 |
| Alexa Fluor 546 | 554 | 570 |
| Alexa Fluor 555 | 555 | 571 |
| TRITC | 547 | 572 |
| B-phycoerythrin | 545,565 | 575 |
| R-phycoerythrin | 480,546,565 | 578 |
| Rhodamine | 539,574 | 602 |
| Alexa Fluor 568 | 578 | 602 |
| Texas Red | 589 | 615 |
| Alexa Fluor 594 | 590 | 617 |
| Propidium iodide (PI) | 536 | 617 |
| Ethidium Bromide | 493 | 620 |
| Feulgen (Pararosaniline) | 570 | 625 |
| Acid Fuchsin | 540 | 630 |
| Alexa Fluor 633 | 621 | 639 |
| Alexa Fluor 647 | 649 | 666 |
| Cy5 | 650 | 670 |
| PE-Cy5 conjugates | 480,565,650 | 670 |
| Alexa Fluor 660 | 668 | 698 |
| Alexa Fluor 680 | 684 | 707 |
| PE-Cy7 conjugates | 480,565,743 | 767 |
| Cy7 | 743 | 767 |

UltraViolet

390
400
410
420
430
440
450
460
470
480
490
500
510
520 — Quantum Dot 525 / Abs: 440
530
540
550
560 — Quantum Dot 565 / Abs: 480
570
580
590
600 — Quantum Dot 605 / Abs: 530
610
620
630
640
650 — Quantum Dot 665 / Abs: 530
660
670
680
690
700
710
Infrared
767

# Living Cell Studies - GFP technology



GFP chromophore





pFPV-mCherry
5372 bp

# GFP-HP1 β

**B**

pre-bleach   bleach   +3s   +6s   +9s   +12s

**C** HP1α

Relative Fluorescence

Time of Recovery (s)

- heterochromatin
- euchromatin

**D** HP1β

Relative Fluorescence

Time of Recovery (s)

- heterochromatin
- euchromatin

**F** HP1α

Time of Recovery (s)

**G** HP1β

Time of Recovery (s)

**Cheutin et al. Science (2003)**

# 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).
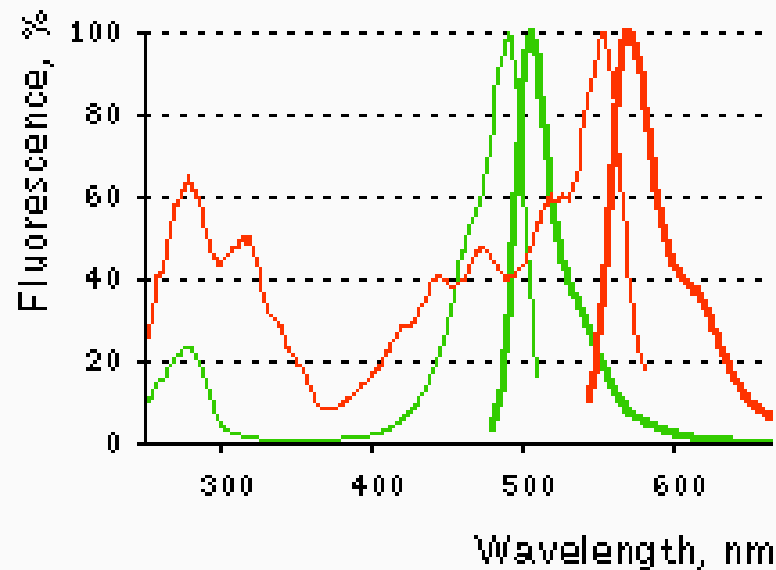


Normalized excitation (thin line) and emission (thick line) for non-activated (green) and activated (red) spectra.



Photoconversion by UV laser
H4-Dendra2   CPDs / Nucleus
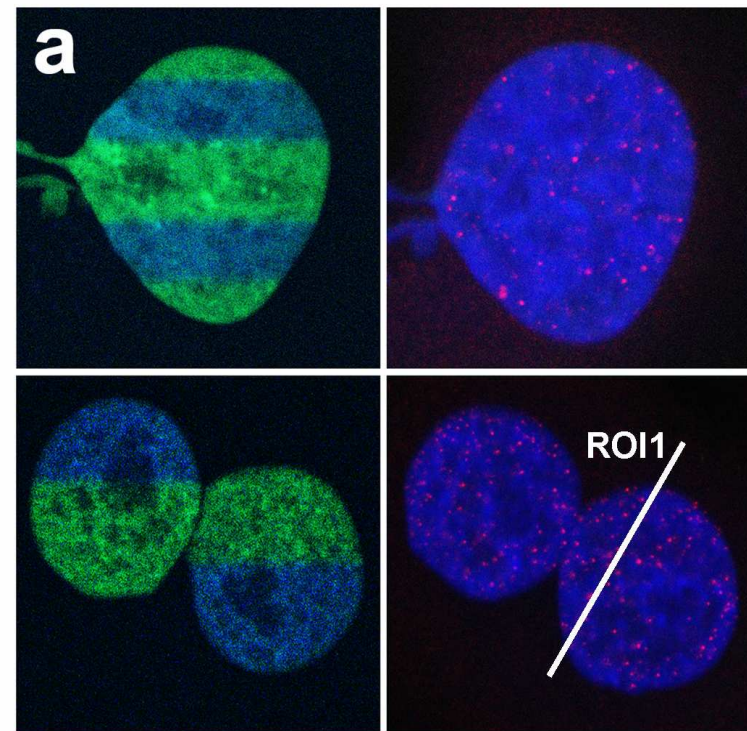
# H4-Dendra2 / H4-Dendra2

**Actinomycin-D
(0 min. after photoconversion)**
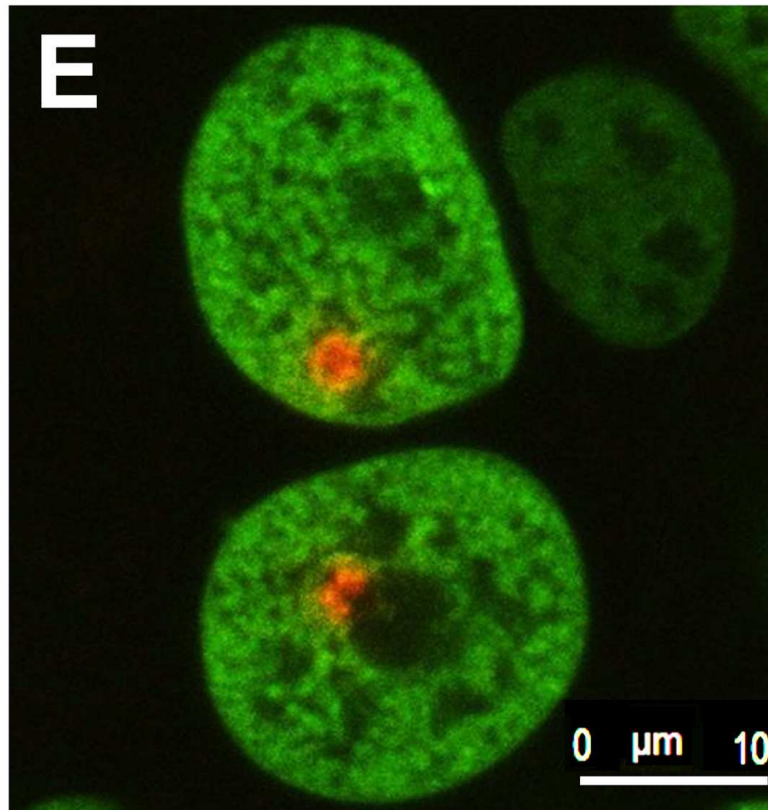
**Actinomycin-D (90 min.)**

E

10.5 μm

3.0 μm

0  μm  10

0  μm  10
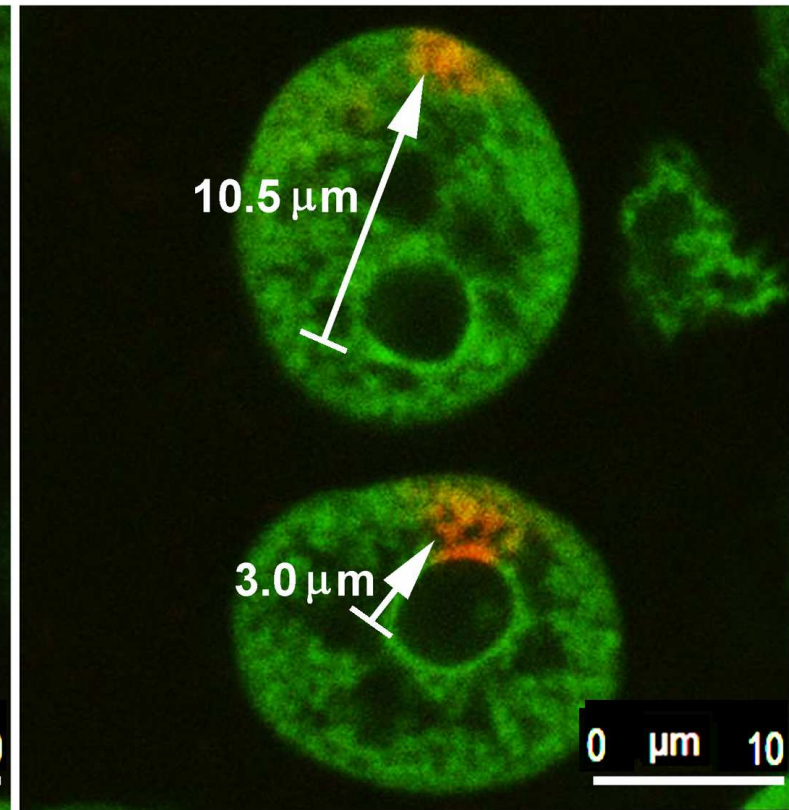
Leica TCS SP-5 X

# Incubation chambers for living cell studies



**Partition with opening on the bottom of the chamber (for experiments under upright microscope)**

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

**Cover glass 24x50 mm**

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

**Termistors in short tubes on the side of the chamber**

**Glass tubes bent under the upper glass (to enable $CO_2$ to flow above medium in the part II of the chamber)**
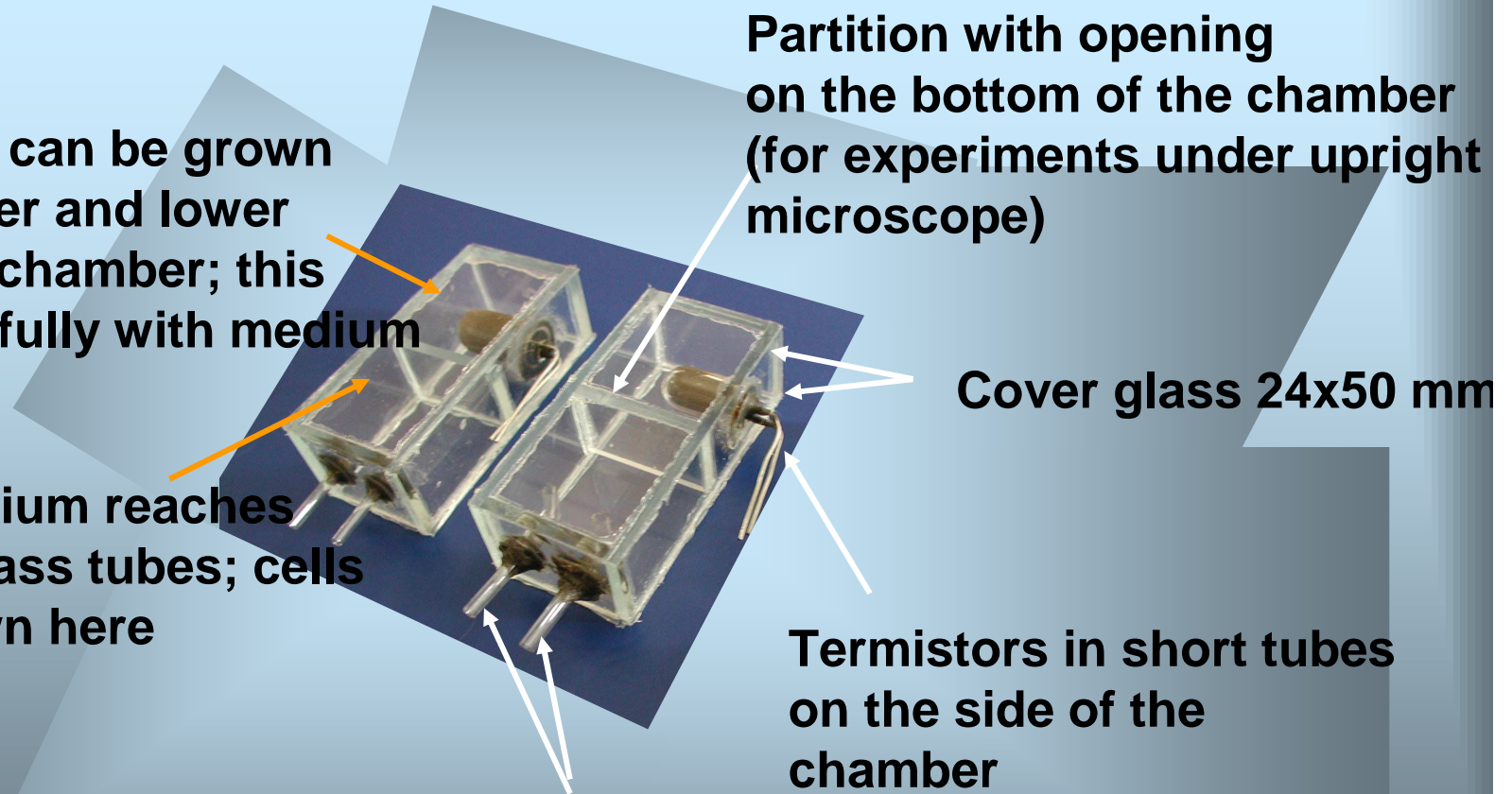
**Application of UV–laser (355 nm) in experimental studies on DNA repair**

# DNA repair
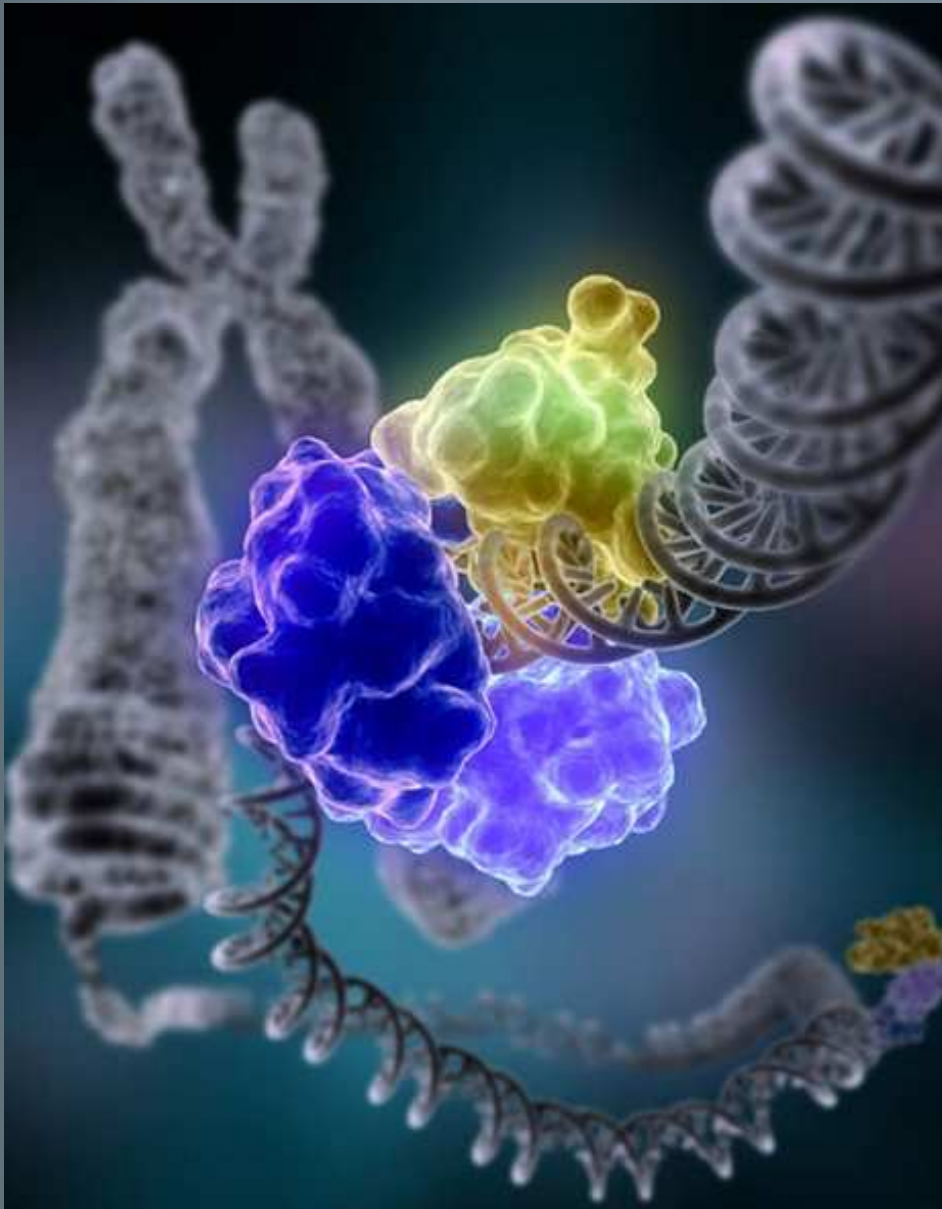


## Single-strand damage

❖**Base excision repair (BER), which repairs damage to a single base caused by oxidation, alkylation, hydrolysis, or deamination.**

❖**Nucleotide excision repair (NER), which recognizes bulky, helix-distorting lesions such as pyrimidine dimers and 6,4 photoproducts.**

❖**Mismatch repair (MMR), which corrects errors of DNA replication and recombination that result in mispaired (but undamaged) nucleotides.**

## Double-strand breaks

❖**non-homologous end joining (NHEJ)**

❖**microhomology-mediated end joining (MMEJ)**

❖**homologous recombination (HR)**

# DNA repair

## Box 1 | The two main types of double-stranded DNA-break repair

**Non-homologous end joining**
A DNA lesion (a double-stranded DNA break (DSB)) is sensed by the Ku80–Ku70 heterodimer, which in turn recruits the DNA-dependent protein kinase catalytic subunit DNAPKcs, resulting in assembly of the DNAPK complex and activation of its kinase activity (see the figure; left panel). Increasing evidence suggests that DNAPK functions as a regulatory component of non-homologous end joining (NHEJ), potentially facilitating and regulating the processing of DNA ends. DNAPK also increases the recruitment of XRCC4, DNA ligase IV, XLF and Artemis, which carry out the final rejoining reaction.

**Homologous recombination repair**
A DNA lesion is recognized by the MRN (MRE11–RAD50–NBS1) complex, which is recruited to the DSB to generate single-stranded DNA by resection (see the figure; right panel). The single-stranded ends are bound by replication protein A (RPA), RAD51 and RAD52 and can subsequently invade the homologous template, creating a D-loop and a Holliday junction, to prime DNA synthesis and to copy and ultimately restore genetic information that was disrupted by the DSB.
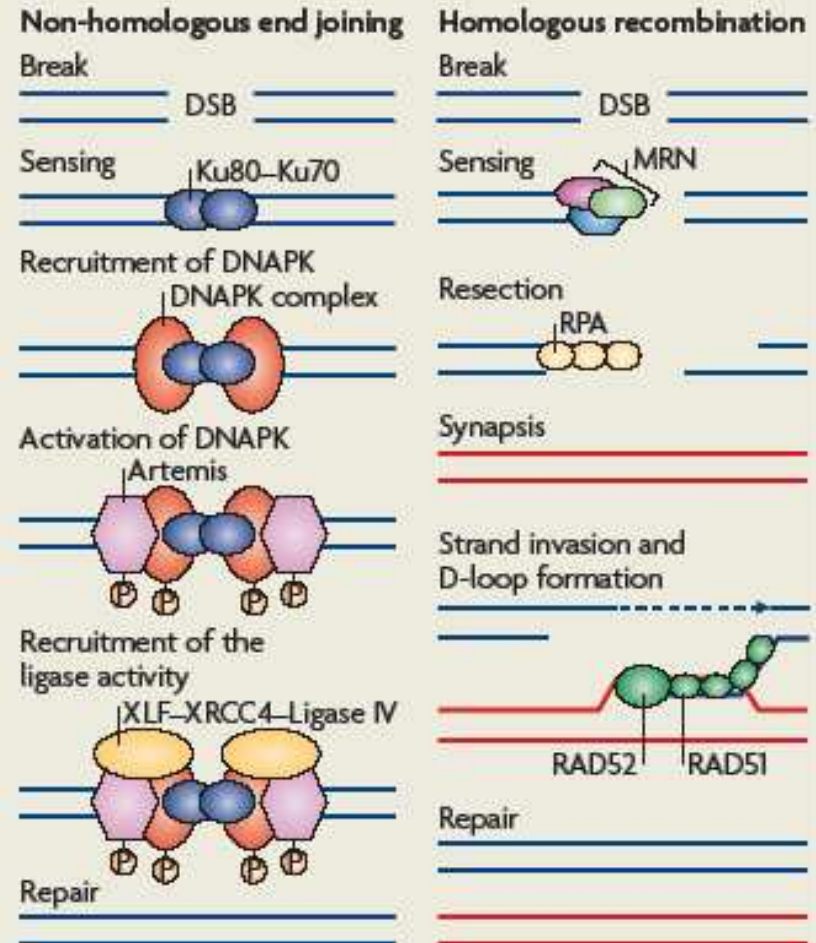
# DNA repair foci
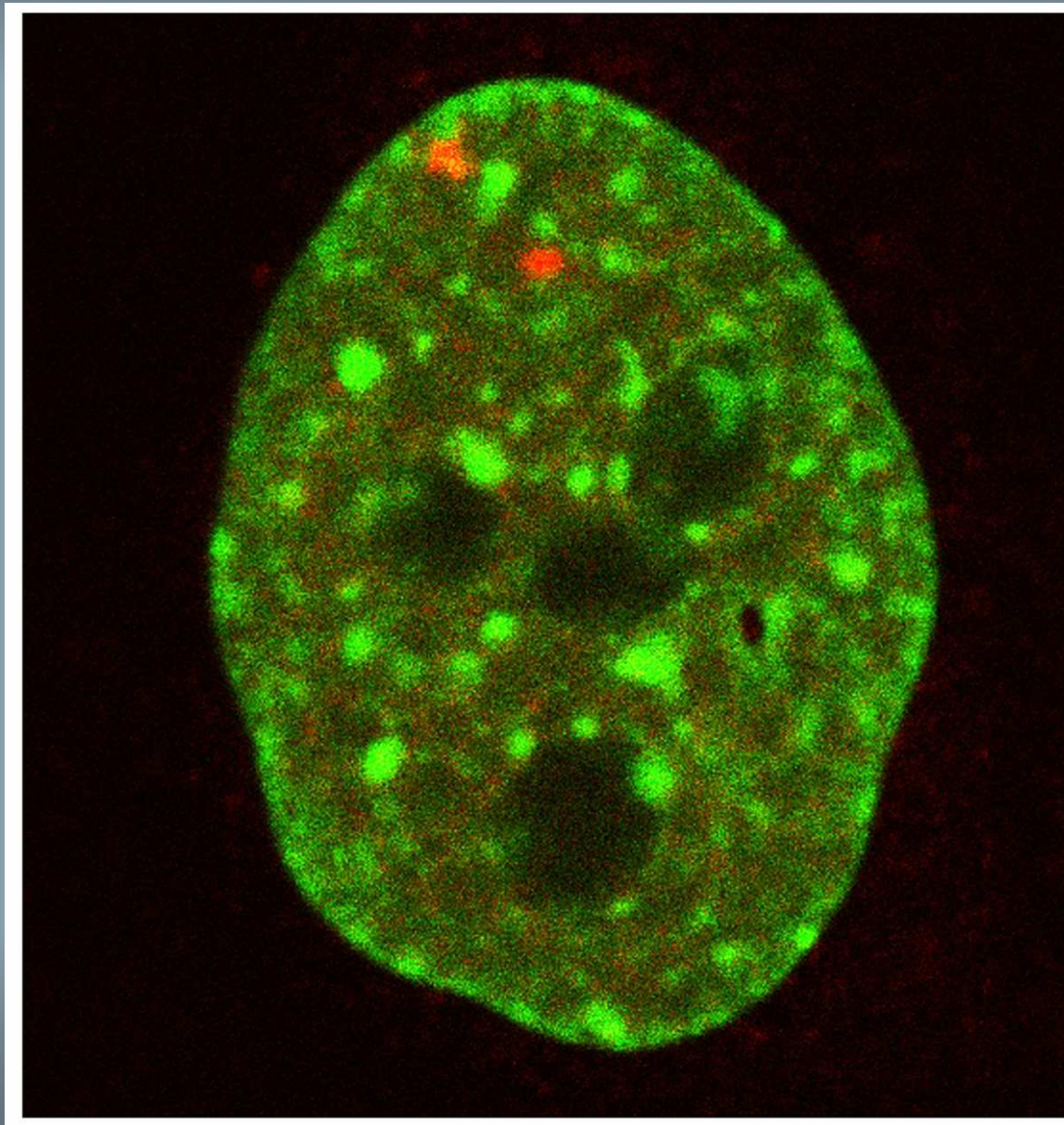
# Histone signature
## Brno nomenclature for histone modifications
### (Turner, 2005)

**A**

WB: BMI-1

IP: without Ab | IP: IgG | IP: HP1β | IP: HP1β + TSA

WB: H3K9me3

IP: without Ab | IP: IgG | IP: HP1α | IP: HP1α + TSA

**B**

HP1β / 3T3 cells

CONTROL

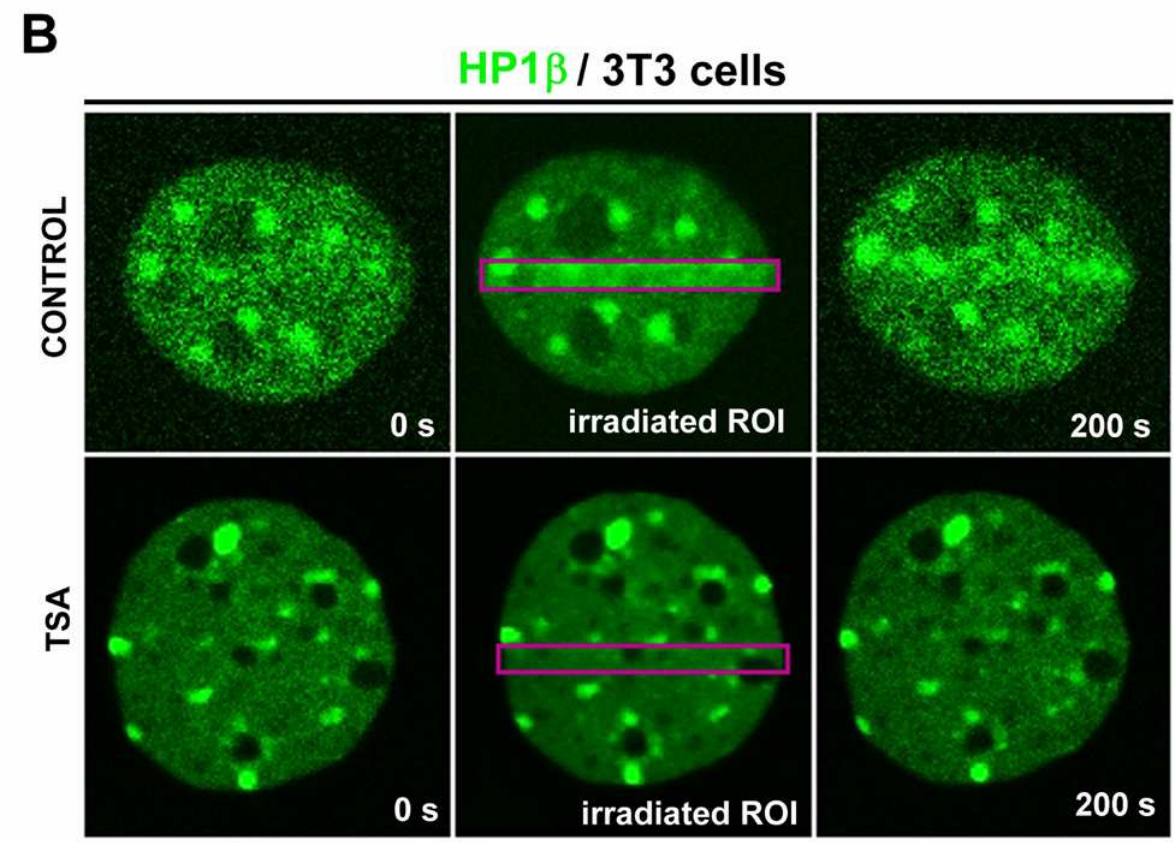0 s | irradiated ROI | 200 s

HP1β / BMI1

CONTROL | TSA

BrdU | BrdU
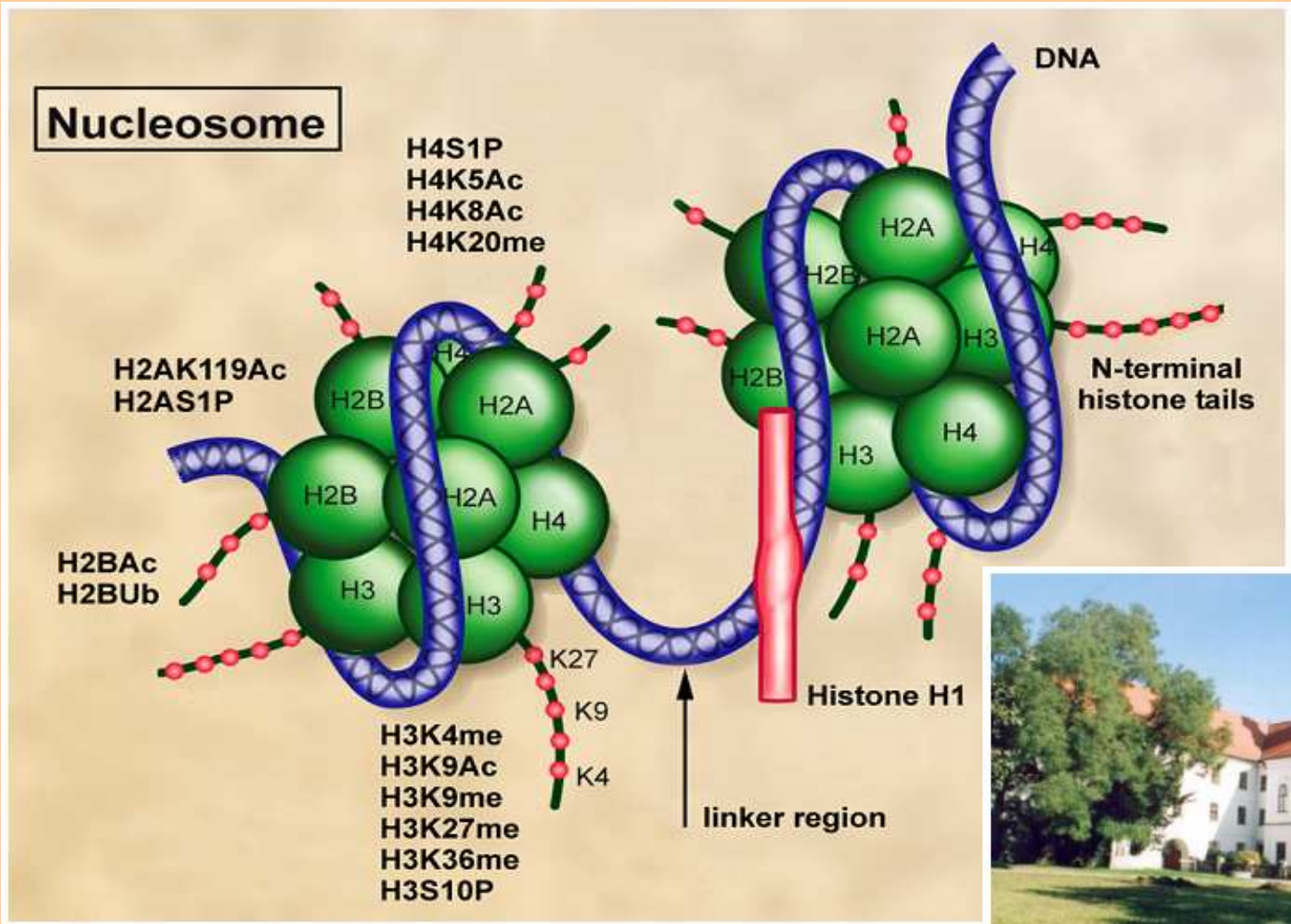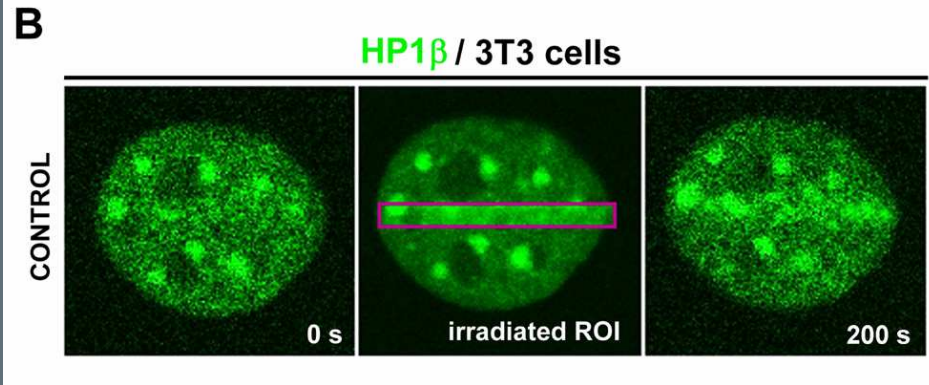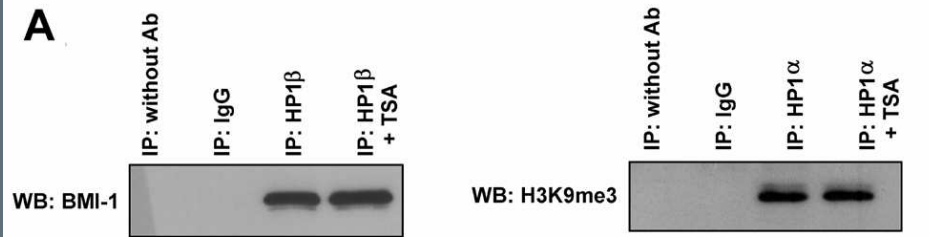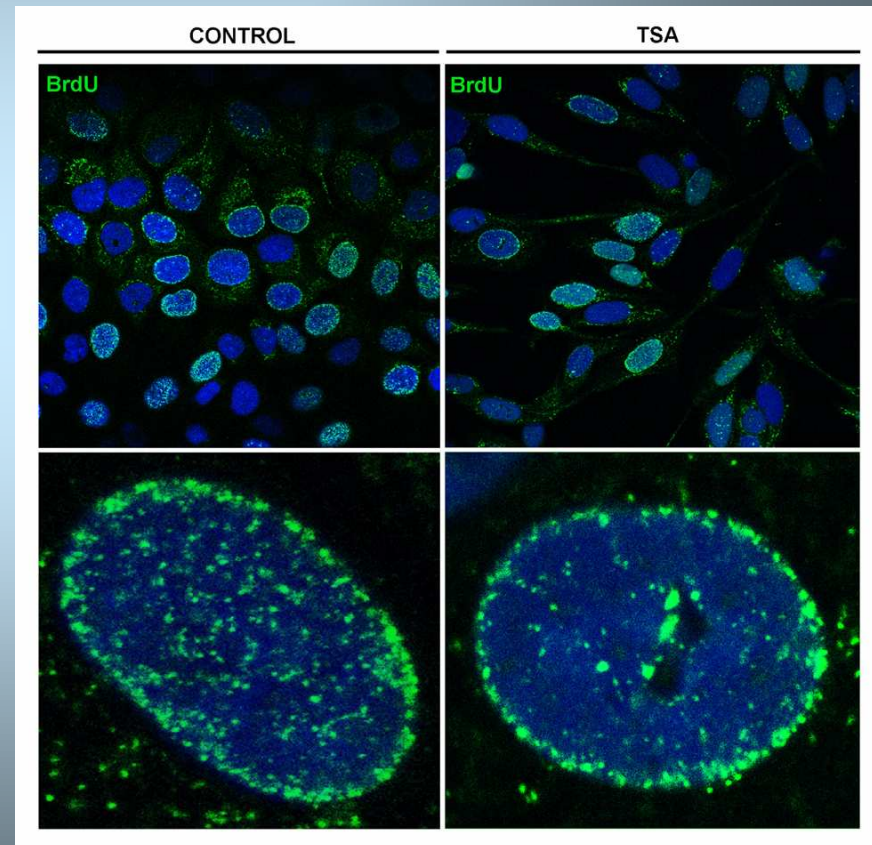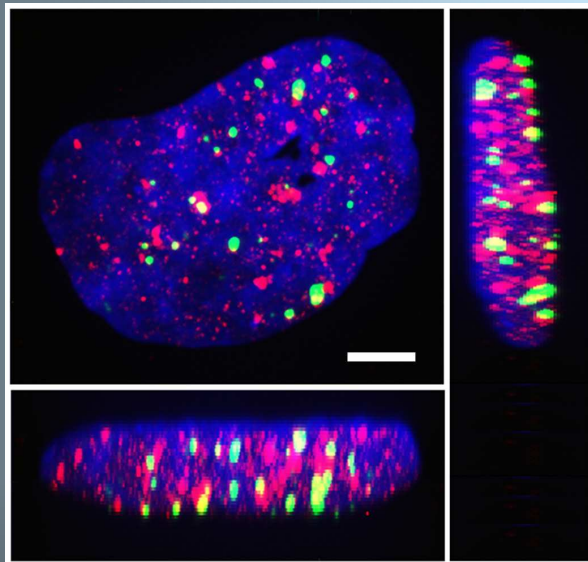
**Experiments of Gabriela Šustáčková, Eva Bártová and Lenka Stixová**

# Využití UV laseru 355 nm ke studiu DNA reparace

**Experiments of Gabriela Galiová**

GFP-BMI1-U2OS cells

**Experiments of Gabriela Galiová**

**A**

WB: BMI-1 — IP: without Ab | IP: IgG | IP: HP1β | IP: HP1β + TSA

WB: H3K9me3 — IP: without Ab | IP: IgG | IP: HP1α | IP: HP1α + TSA

**B**

HP1β / 3T3 cells

CONTROL — 0 s | irradiated ROI | 200 s

TSA — 0 s | irradiated ROI | 200 s

GFP-BMI1-U2OS cells

after DNA damage

20 s

50 s

**Experiments of Gabriela Galiová and Lenka Stixová**

# ATP depletion



Šustáčková et al., JCP, 2011

GFP-HP1β / γH2AX / Nucleus

irradiated ROI    after irradiation

ATP-depletion

Experiments of Gabriela Šustáčková

C EZH2 γ-H2AX DAPI

D H3K27me3 RPA32 MERGE+DAPI

E MEL-18 γ-H2AX MERGE+DAPI

BMI1 γ-H2AX MERGE+DAPI

F MEL-18 γ-H2AX MERGE+DAPI

DMSO

PARP inhibitor

Chou et al., PNAS (2010)

ICAD  CAD  Caspases  CAD  Histones  DNA

Nucleosome

DNA fragment

PARP  Caspase 3  p53

**DNA repair**

GFP-BMI1-U2OS cell · acetylated lysines · overlay · nucleus

t=8.480sec  0 μm 7.5
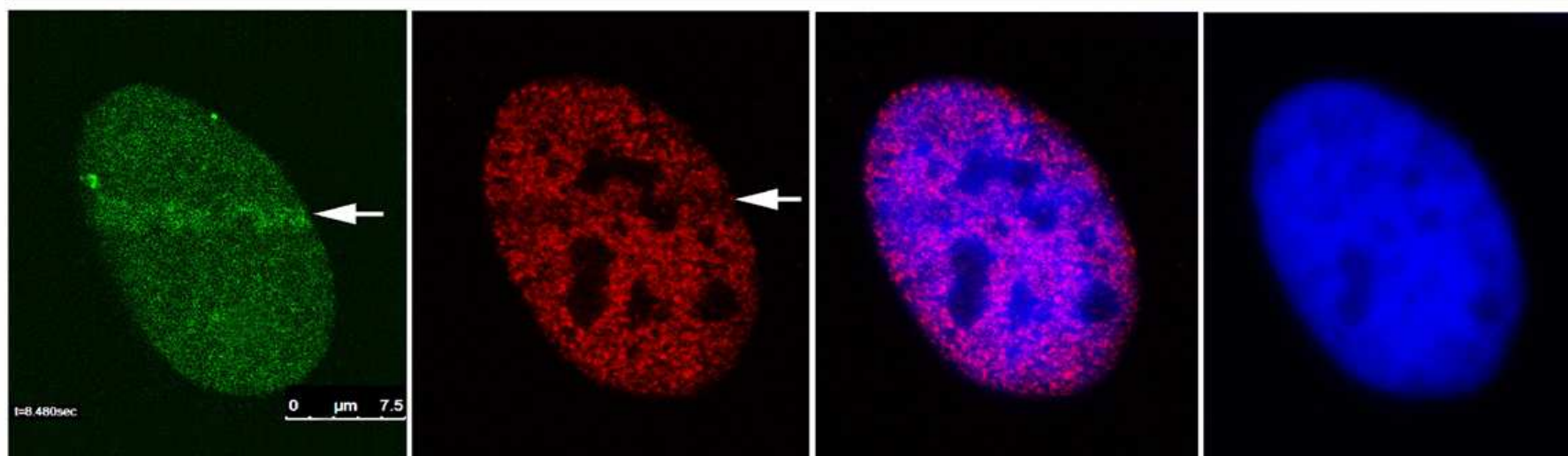
**Experiments of Gabriela Šustáčková and Soňa Legartová**

# FRAP in UV-damaged chromatin with accumulated BMI1 and HP1β





A  GFP-HP1β / γ H2AX / 3T3 cell nuclei

irradiated ROI    after DNA damage

Non-treated cell

TSA

B  HP1β in DSBs / 3T3 cells

HP1β - Foci
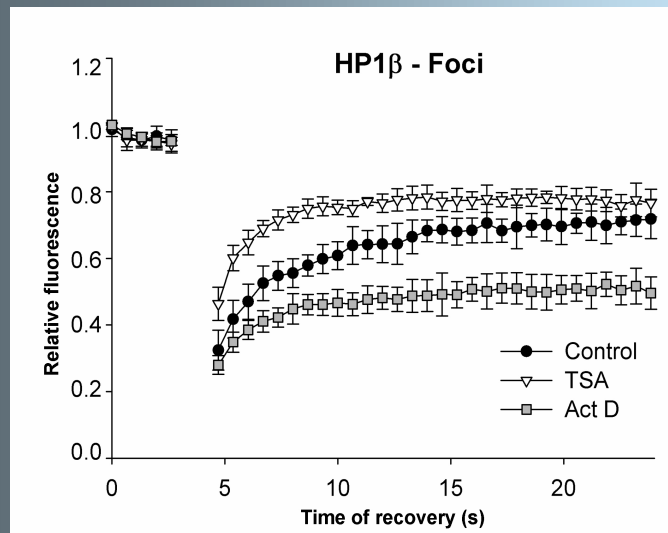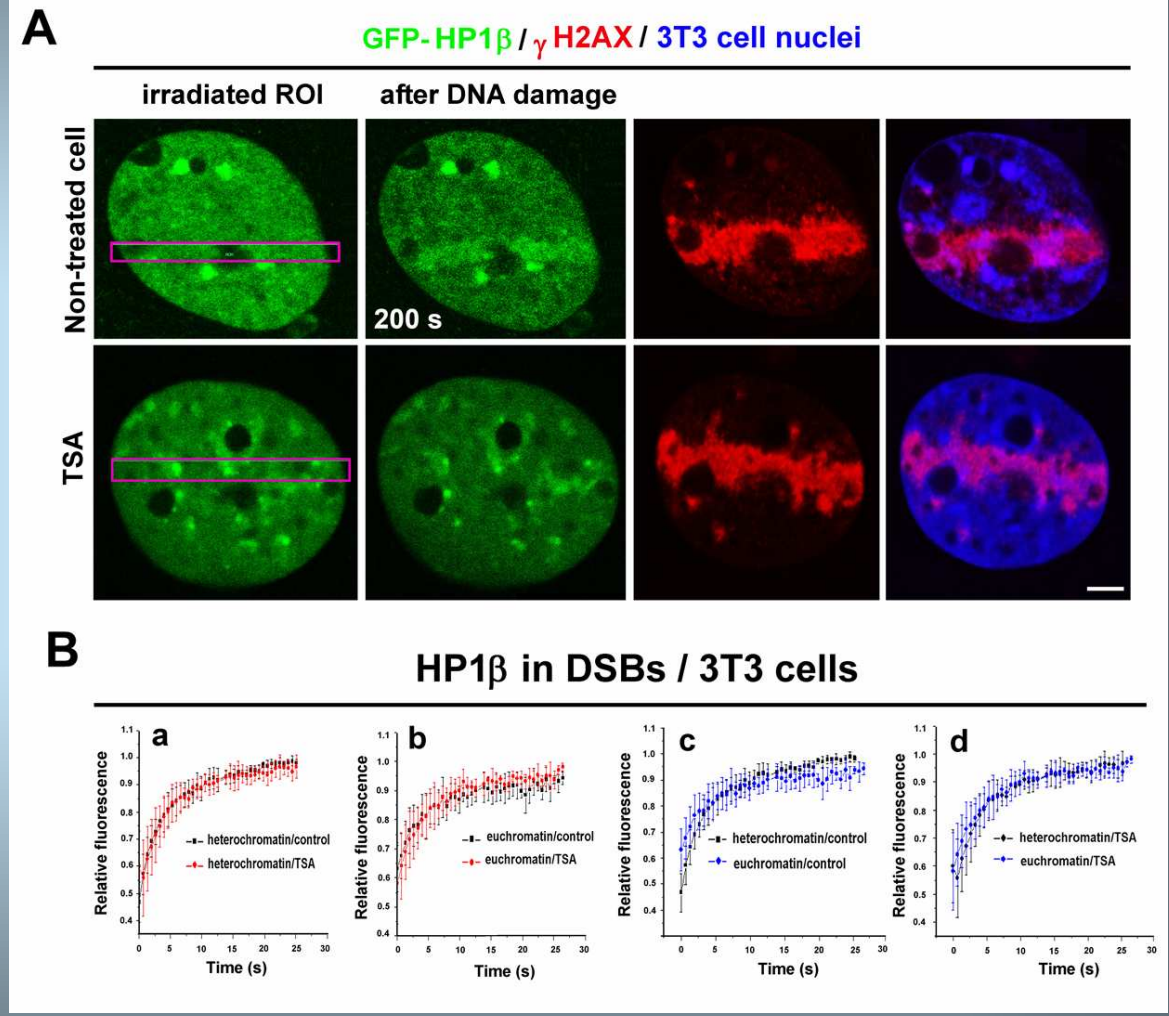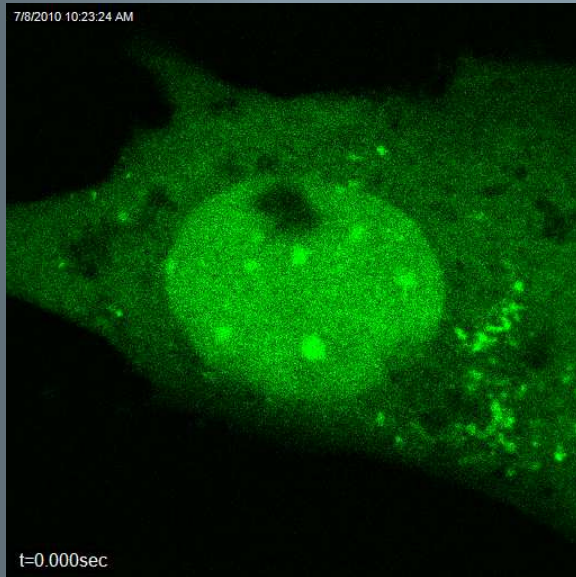
**Experiments of Gabriela Šustáčková and Darya Orlova**

# FRAP – BMI1



PcG bodies in living cell

PcG body
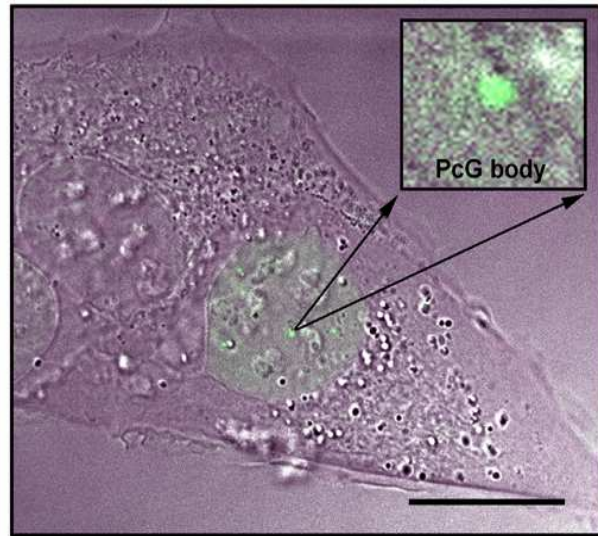


Fluorescence recovery after photobleaching - BMI1 protein

Control, $\tau_{50}$= 75-90 s

TSA, 24 h, $\tau_{50}$= 179-250 s

$\tau_{50}$
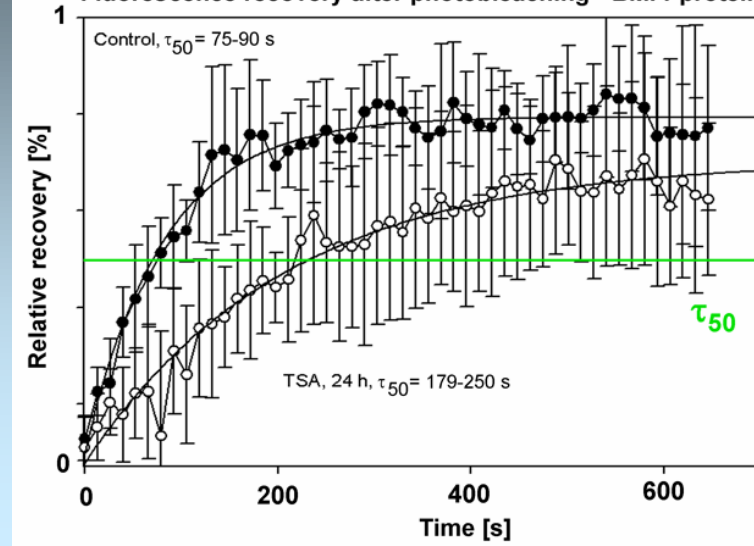


Fluorescence recovery after photobleaching in DSBs

CONTROL
TSA
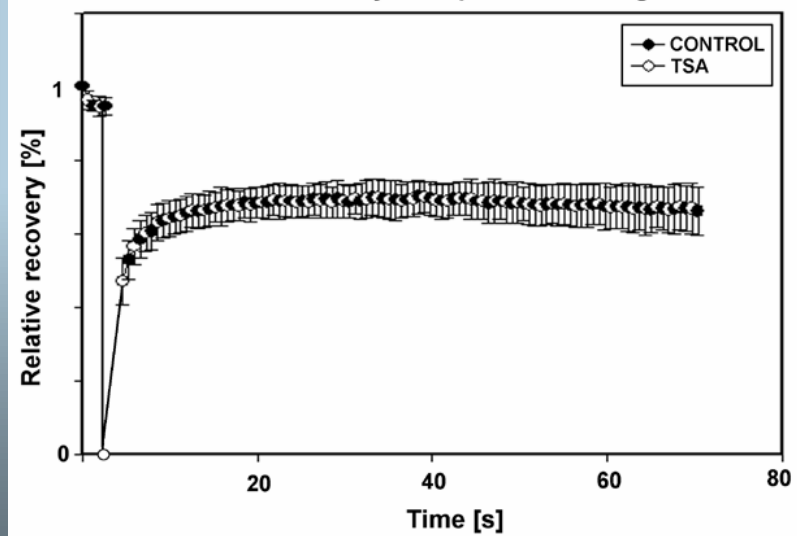
# Additional molecular-biology methods with application for confocal microscopy

**FRET (Fluorescence Resonance Energy Transfer) is a technique for measuring interactions between two proteins in vivo. In this technique, two different fluorescent molecules (fluorophores) are genetically fused the two proteins of interest.**
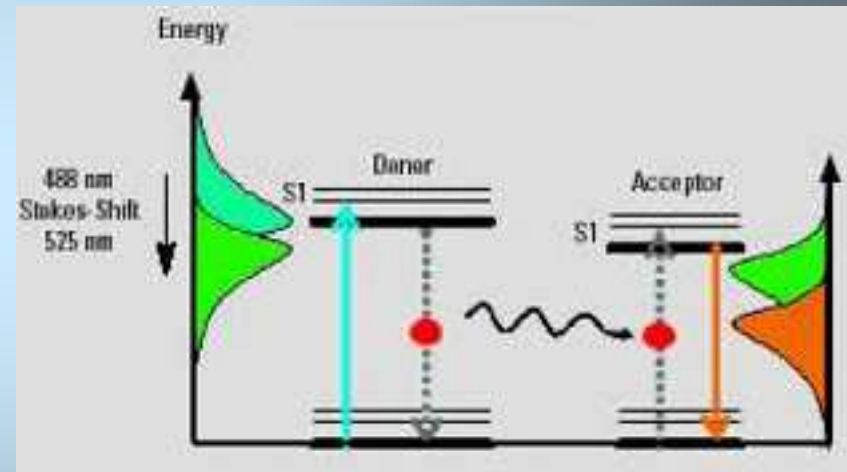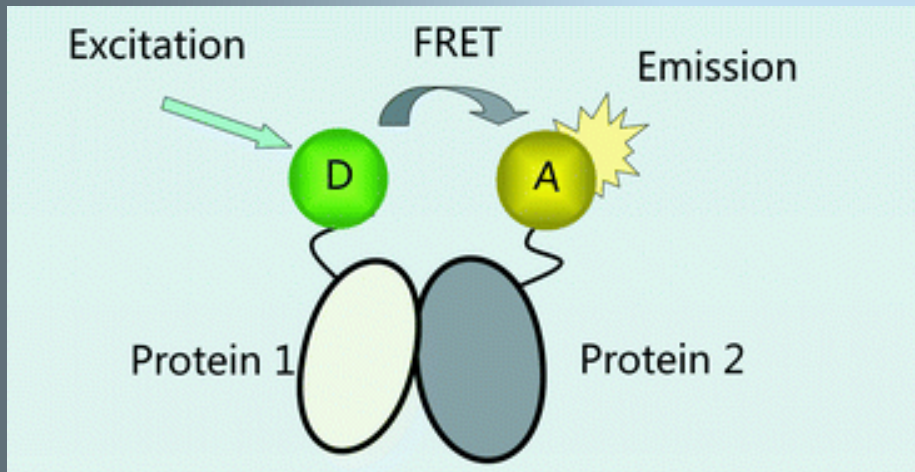
$$E_A(i) = \frac{B - A \times b - C \times (c - a \times b)}{C}$$
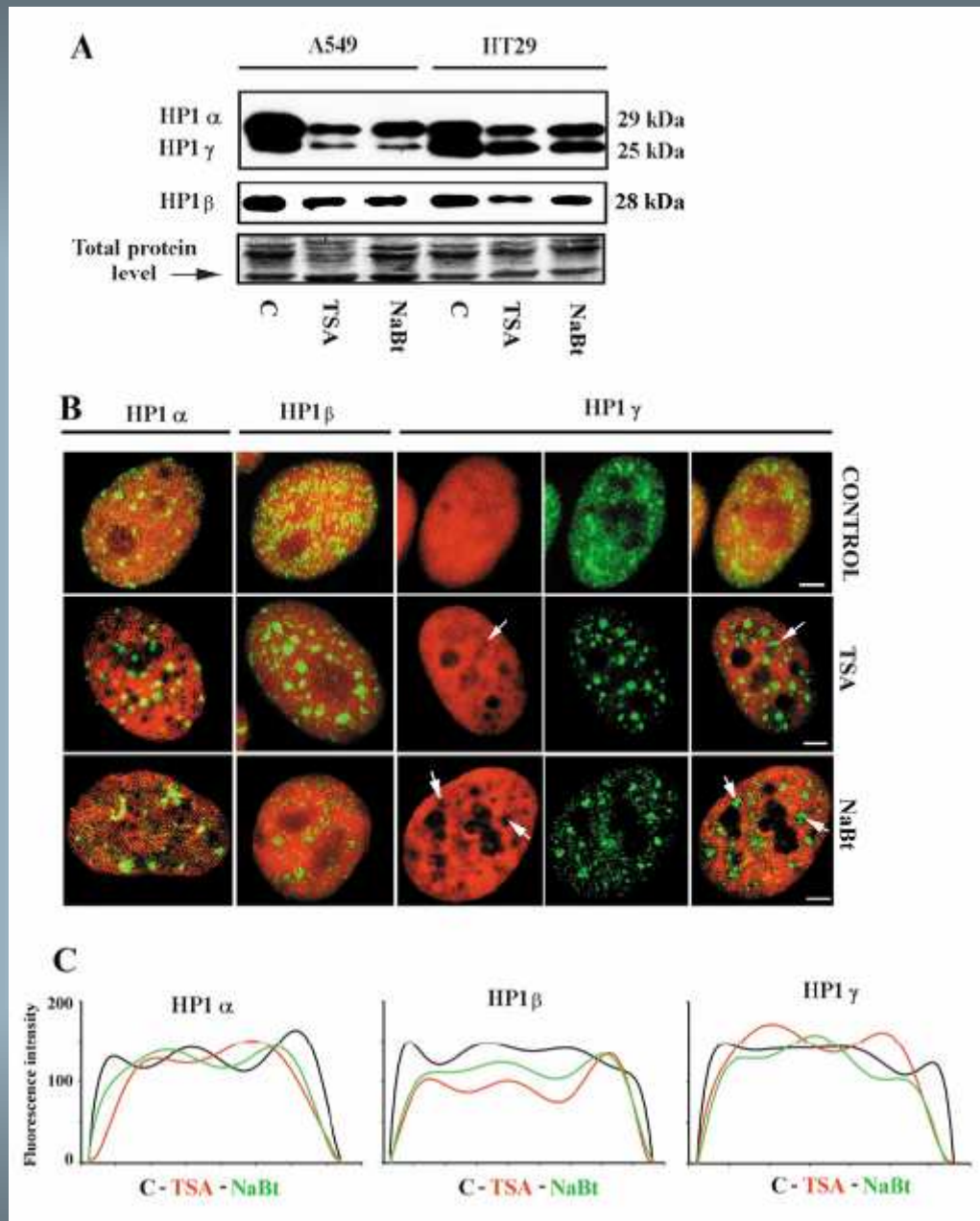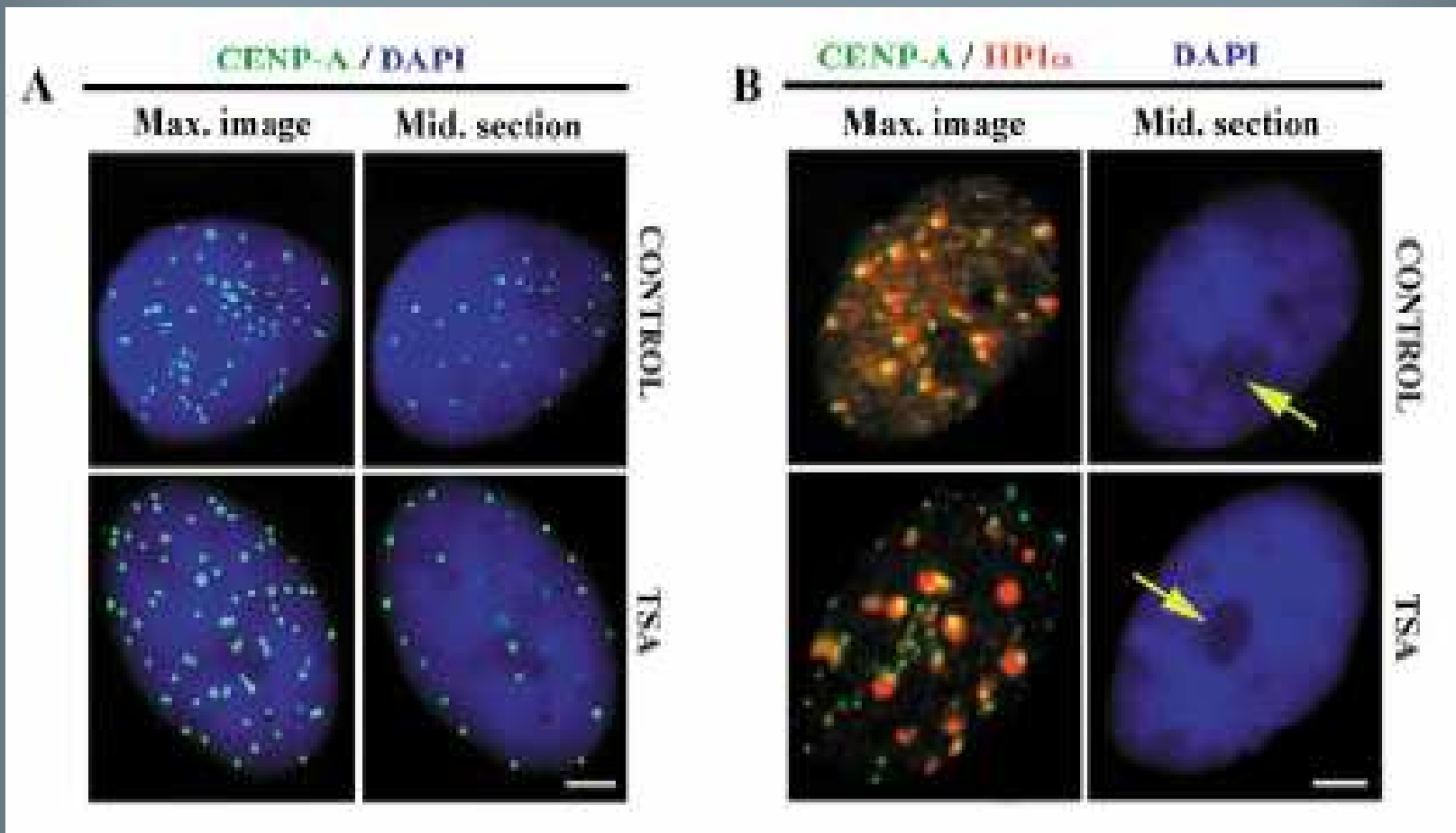
Immunocytochemistry
to identify potential
secretagogues

2nd antibody
(labelled)

1st antibody
(against
neurotransmitter)

tissue
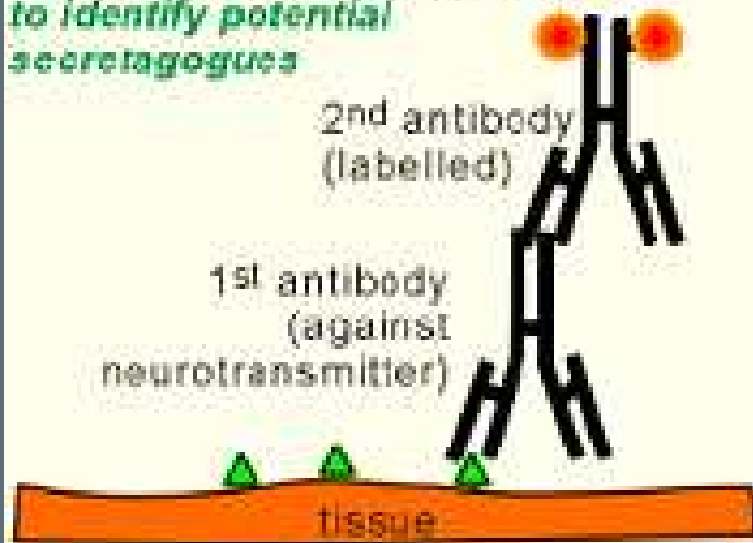
http://www.celanphy.science.ru.nl/
Bruce%20web/construction.htm
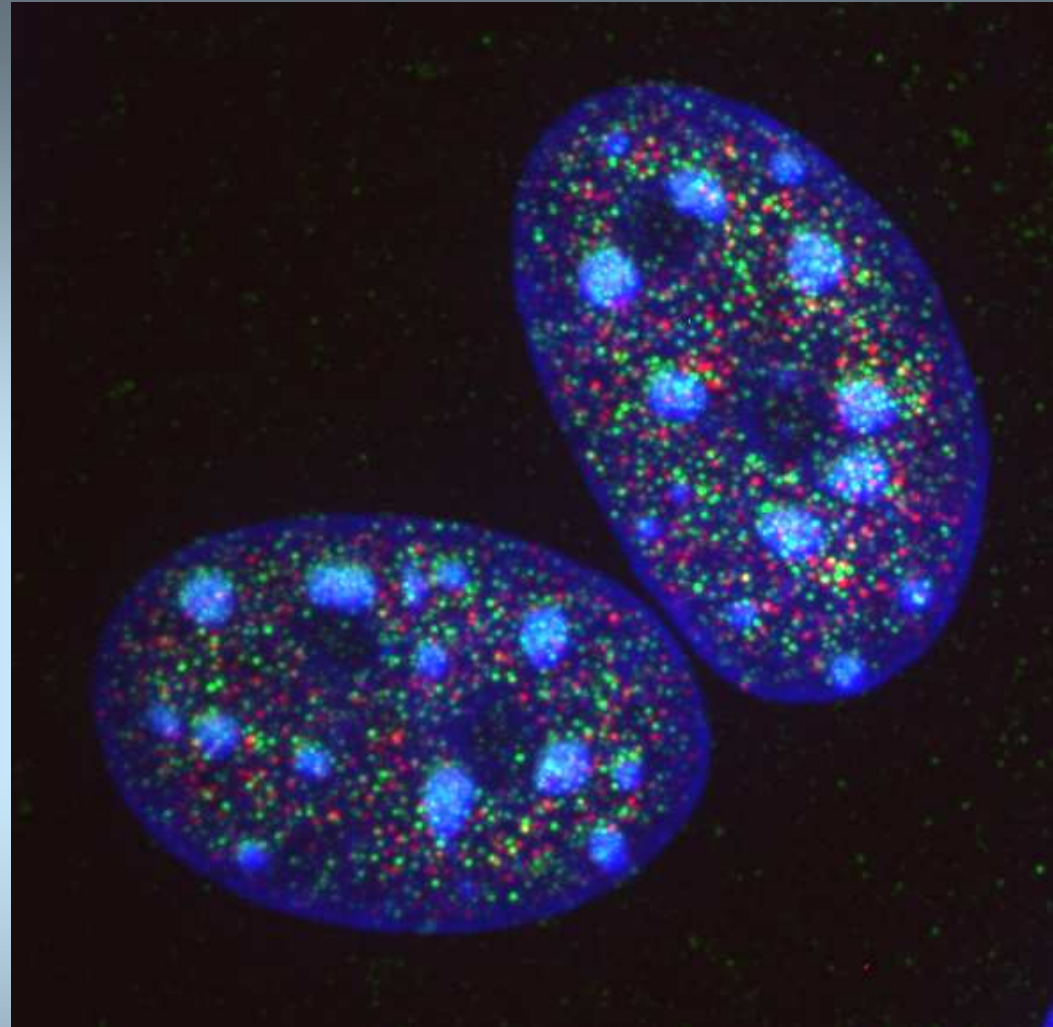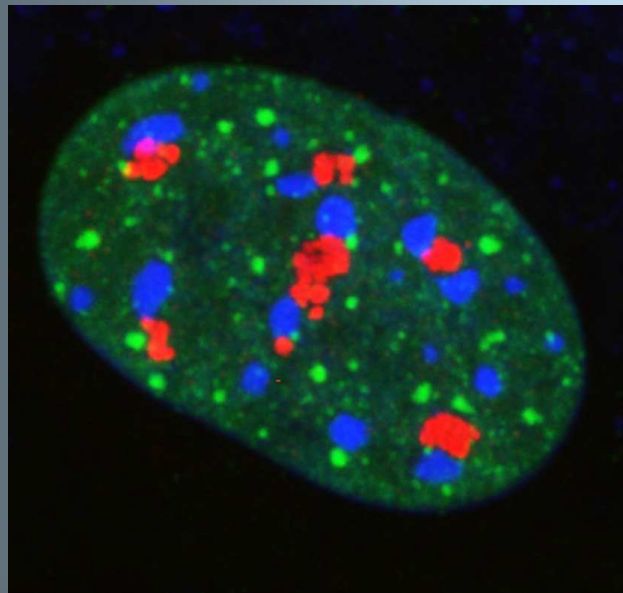
EB group, IBP, Brno

Bártová et al., JCS (2005)

**Bártová et al., JCS (2005)**

A  CENP-A / DAPI
Max. image    Mid. section
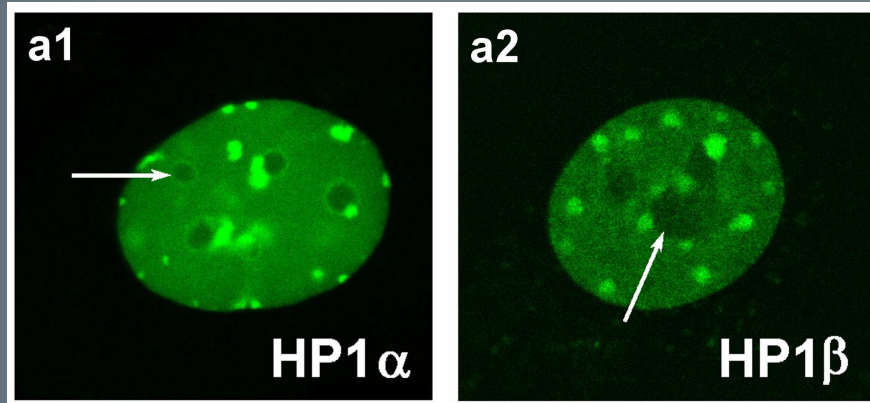CONTROL
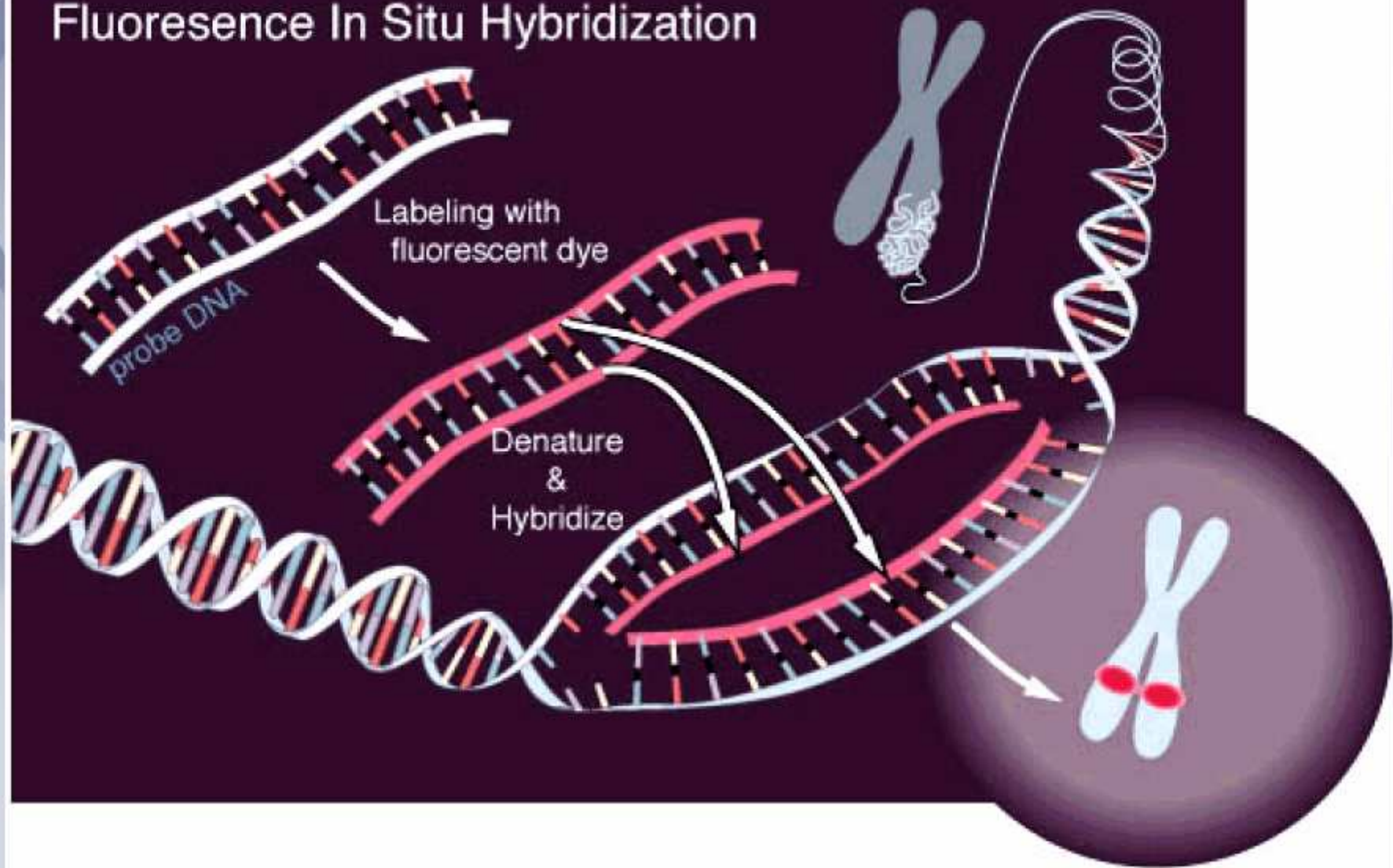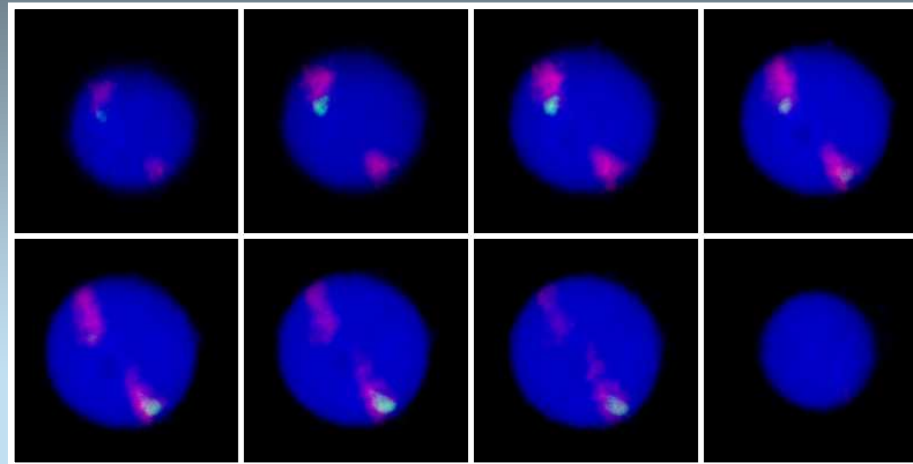TSA

B  CENP-A / HP1α    DAPI
Max. image    Mid. section
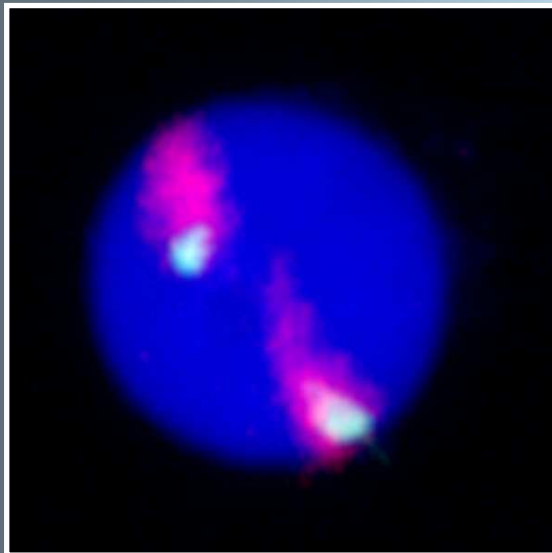CONTROL
TSA
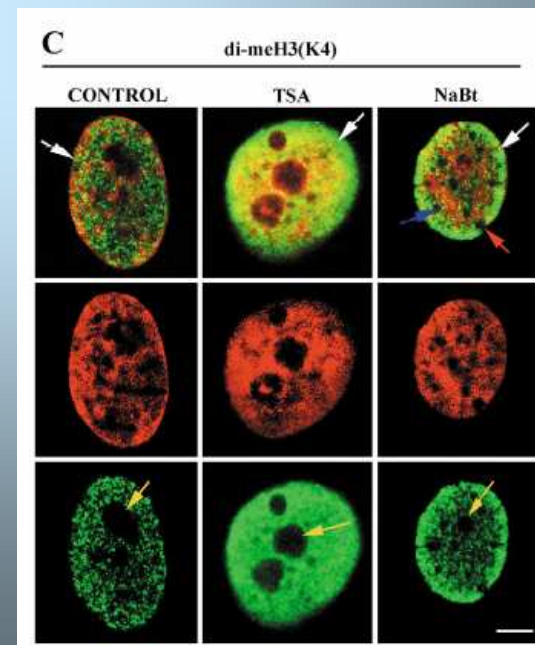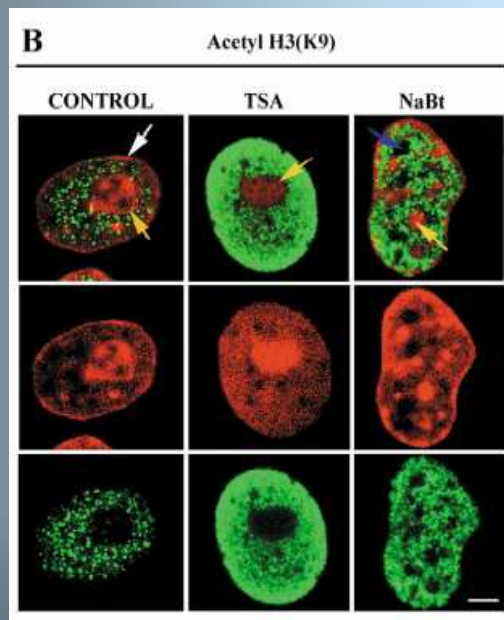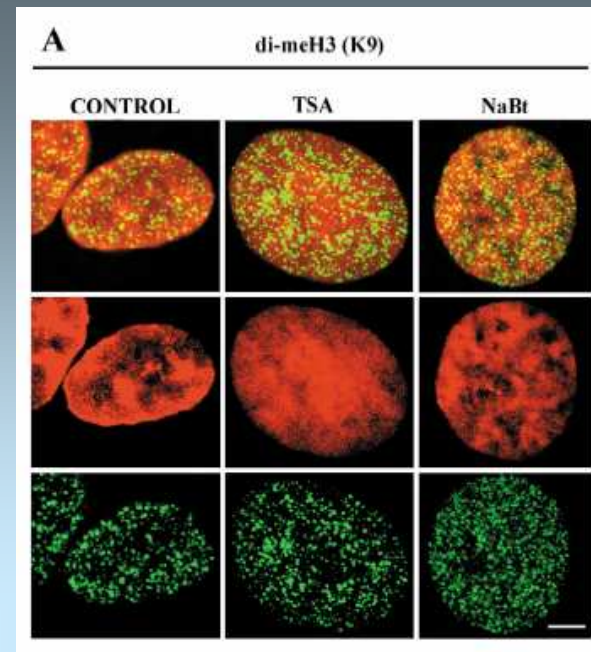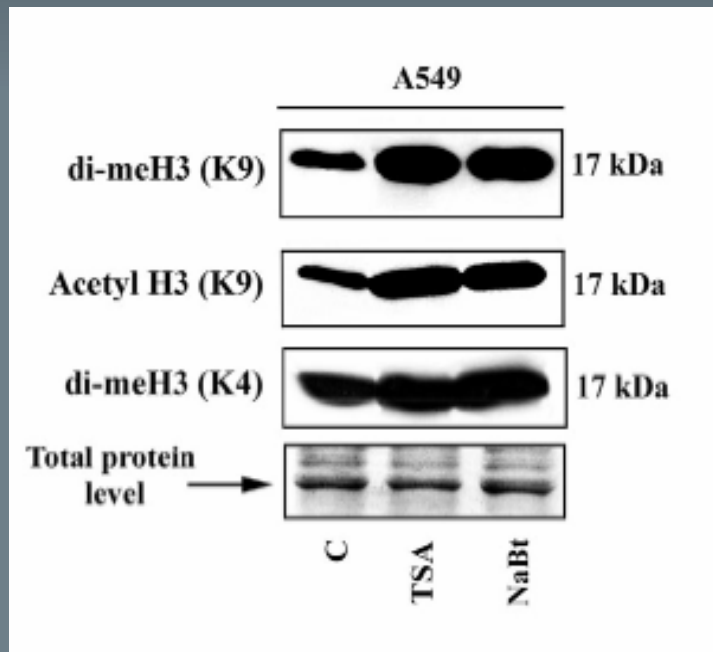
**Bártová et al., JCS (2005)**
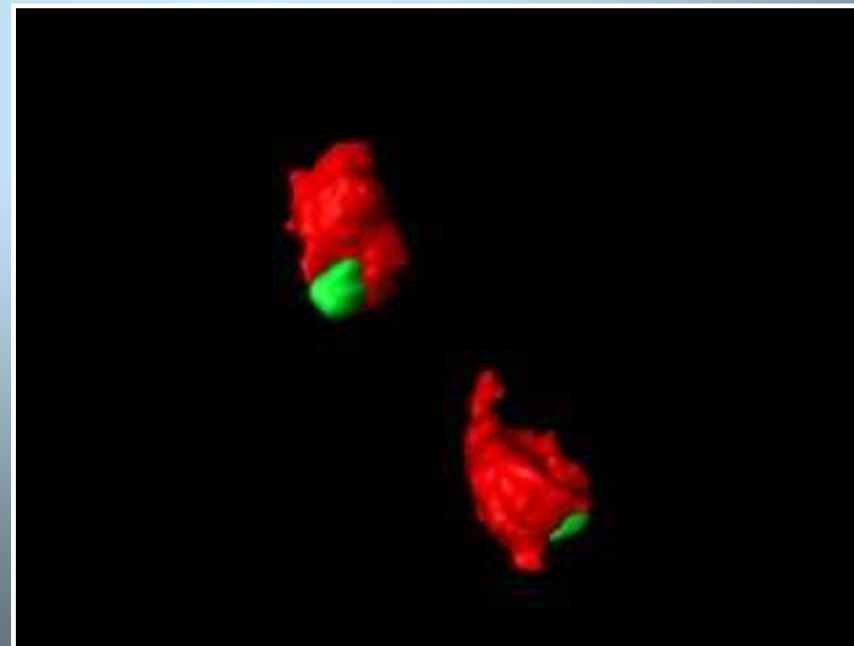
# Single particle tracking
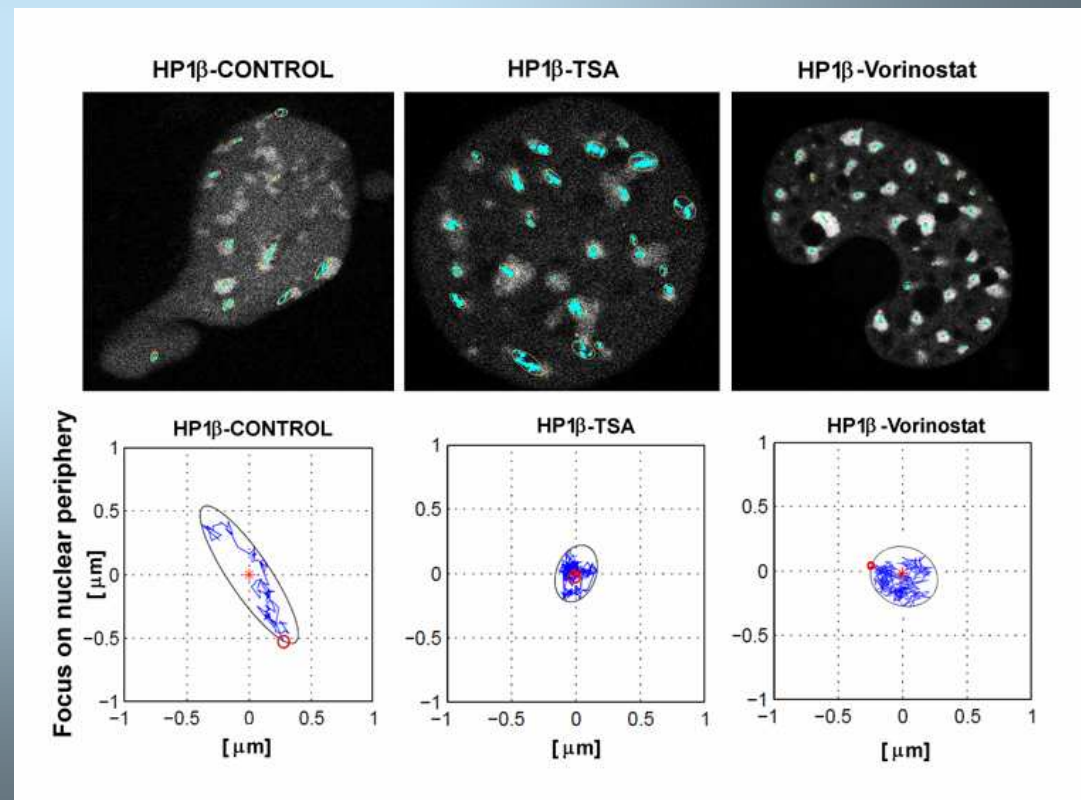
Fluoresence In Situ Hybridization

# 3D-FISH a konfokální mikroskopie



Chr. #1 paint
1q12 probe
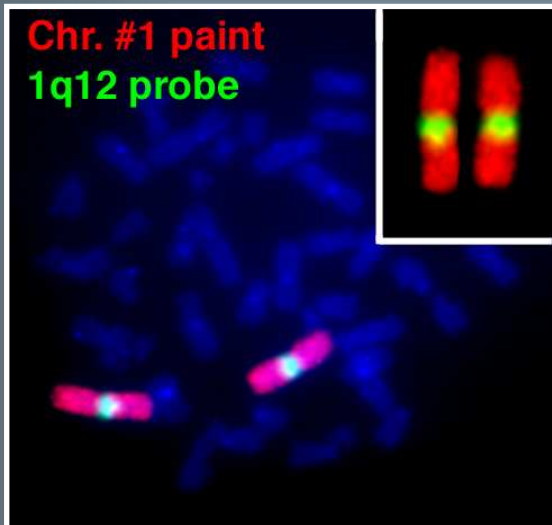
Galerie optických řezů

Maximální obraz
Všech řezů
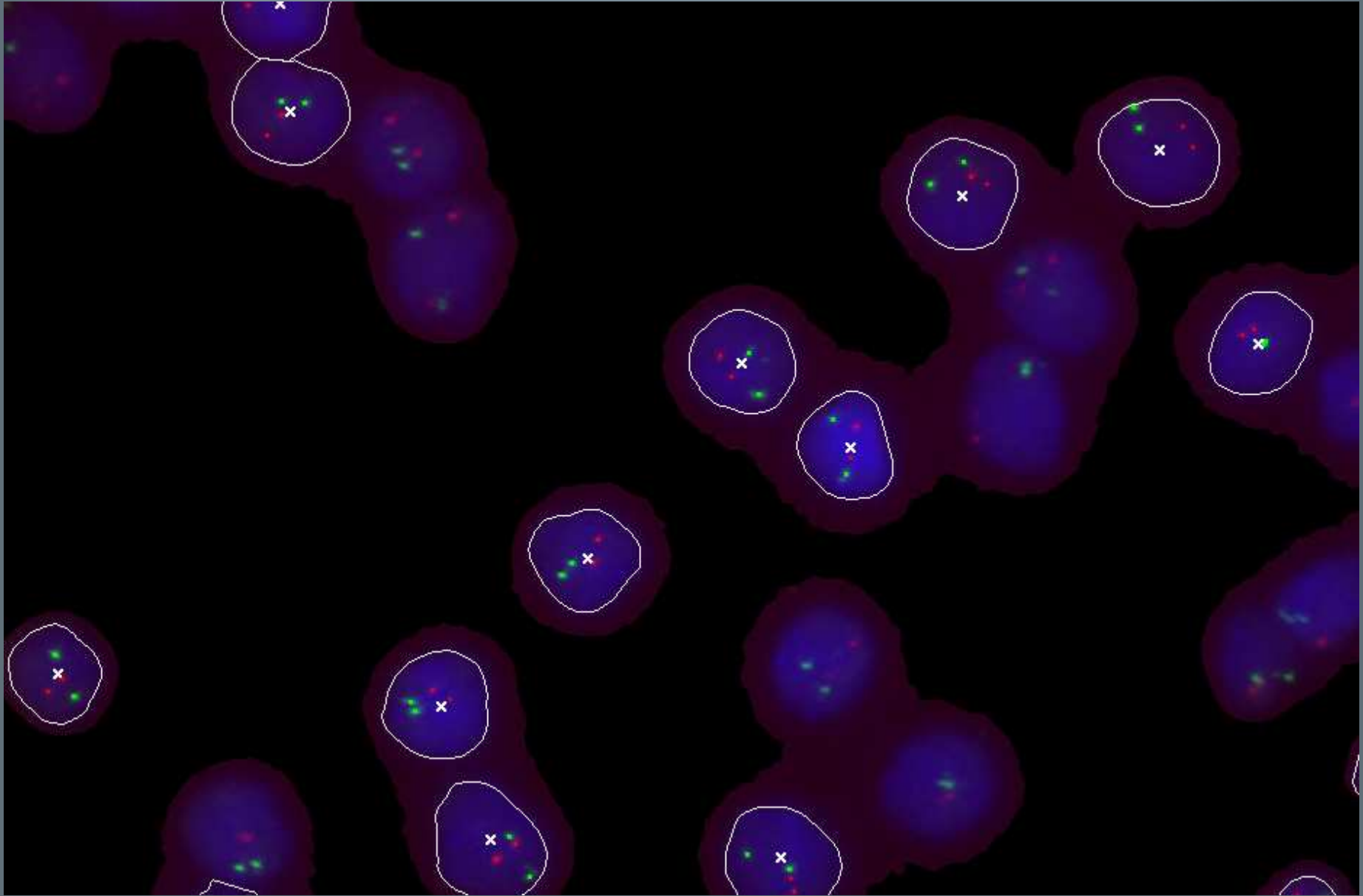
3D reconstrukce CT
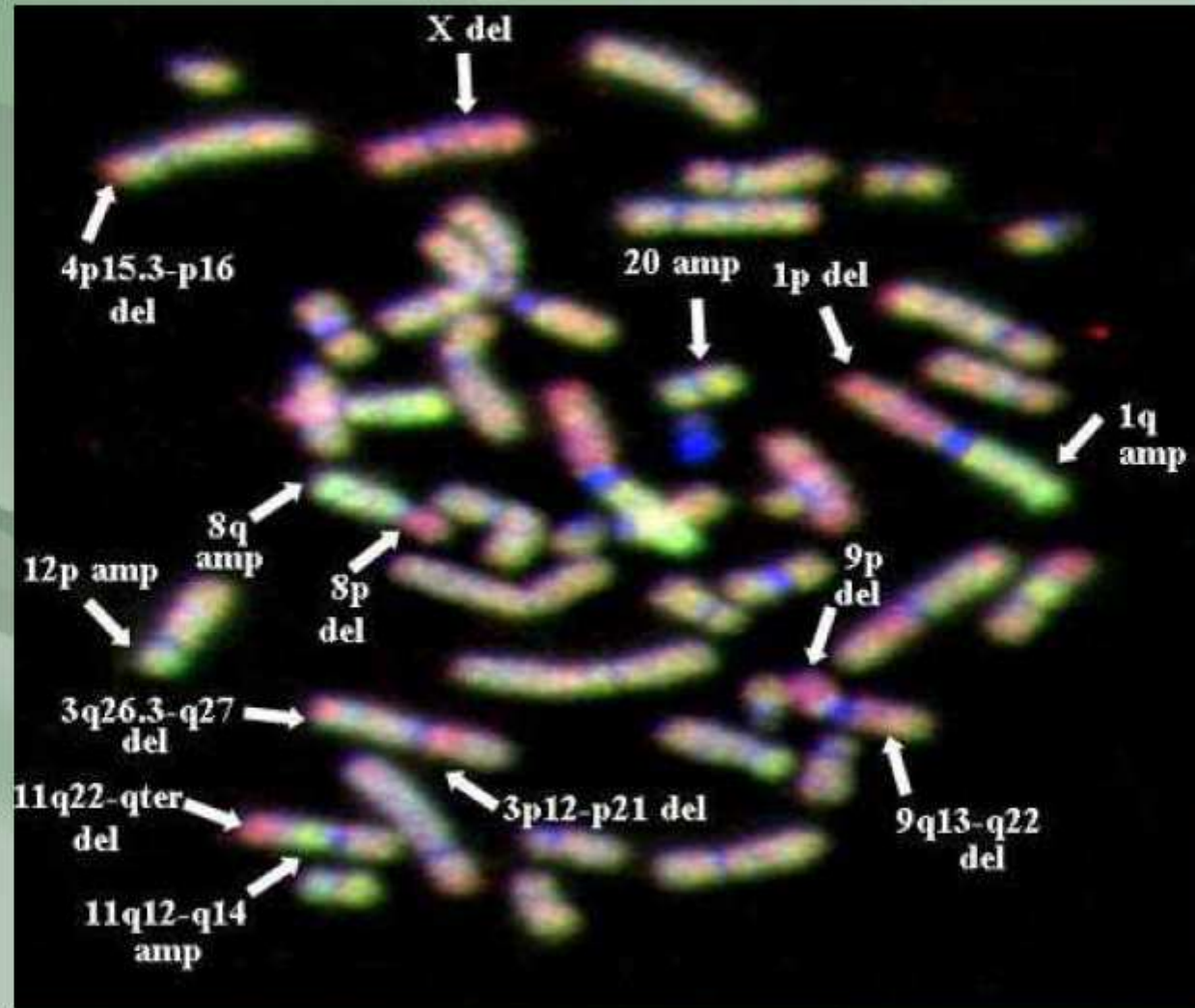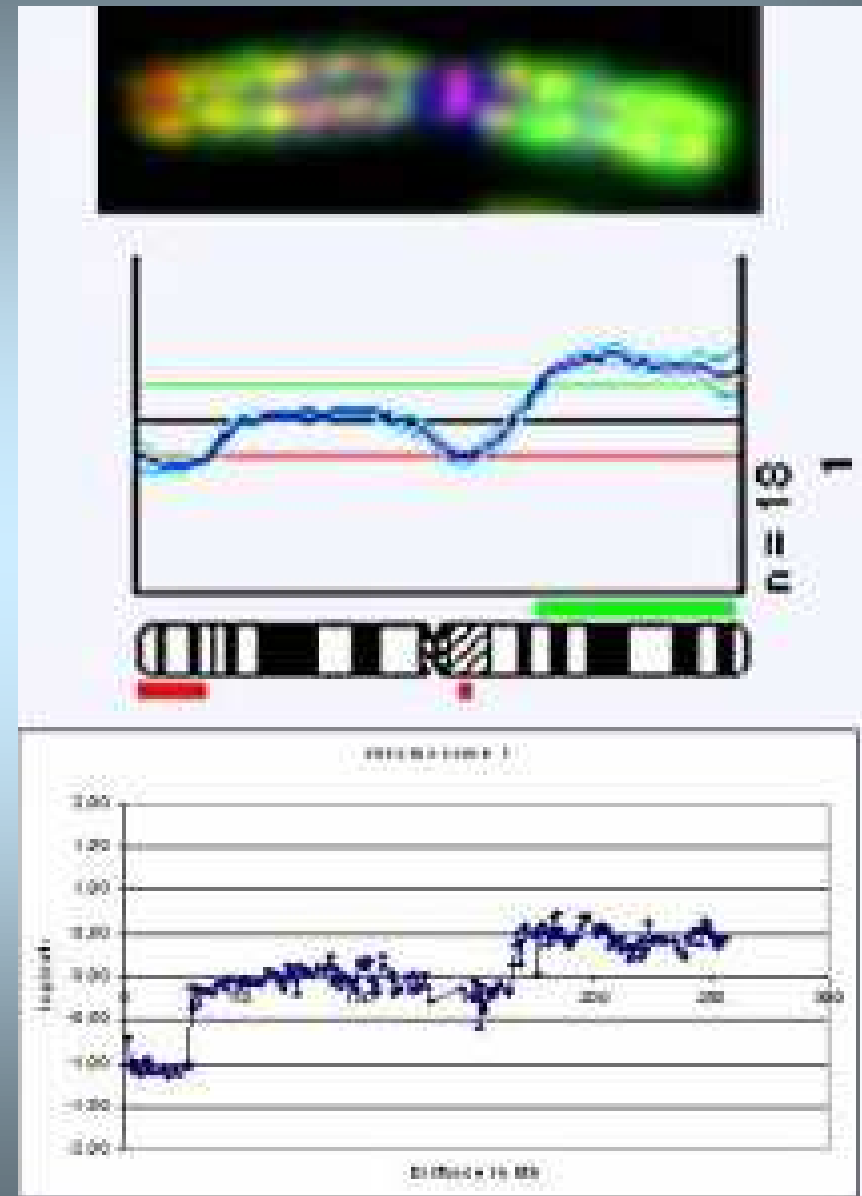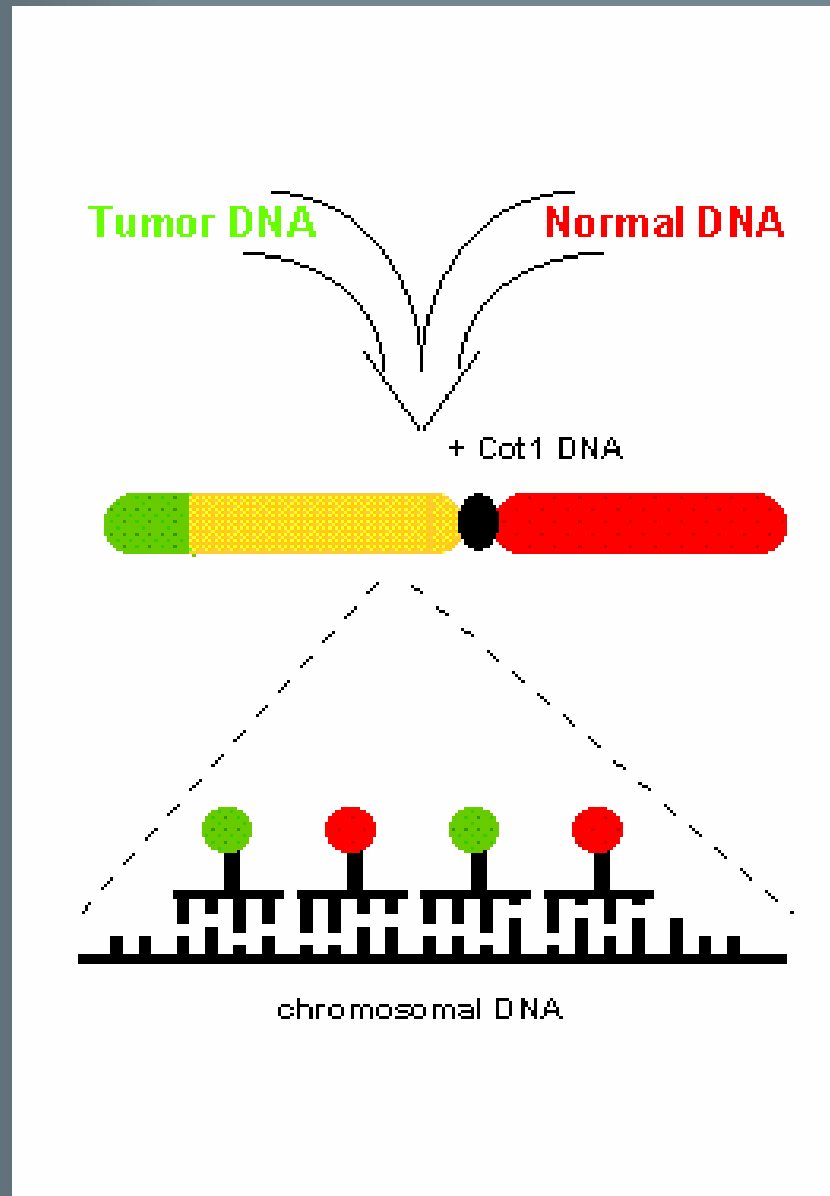
Weierich et al., (2003) in press
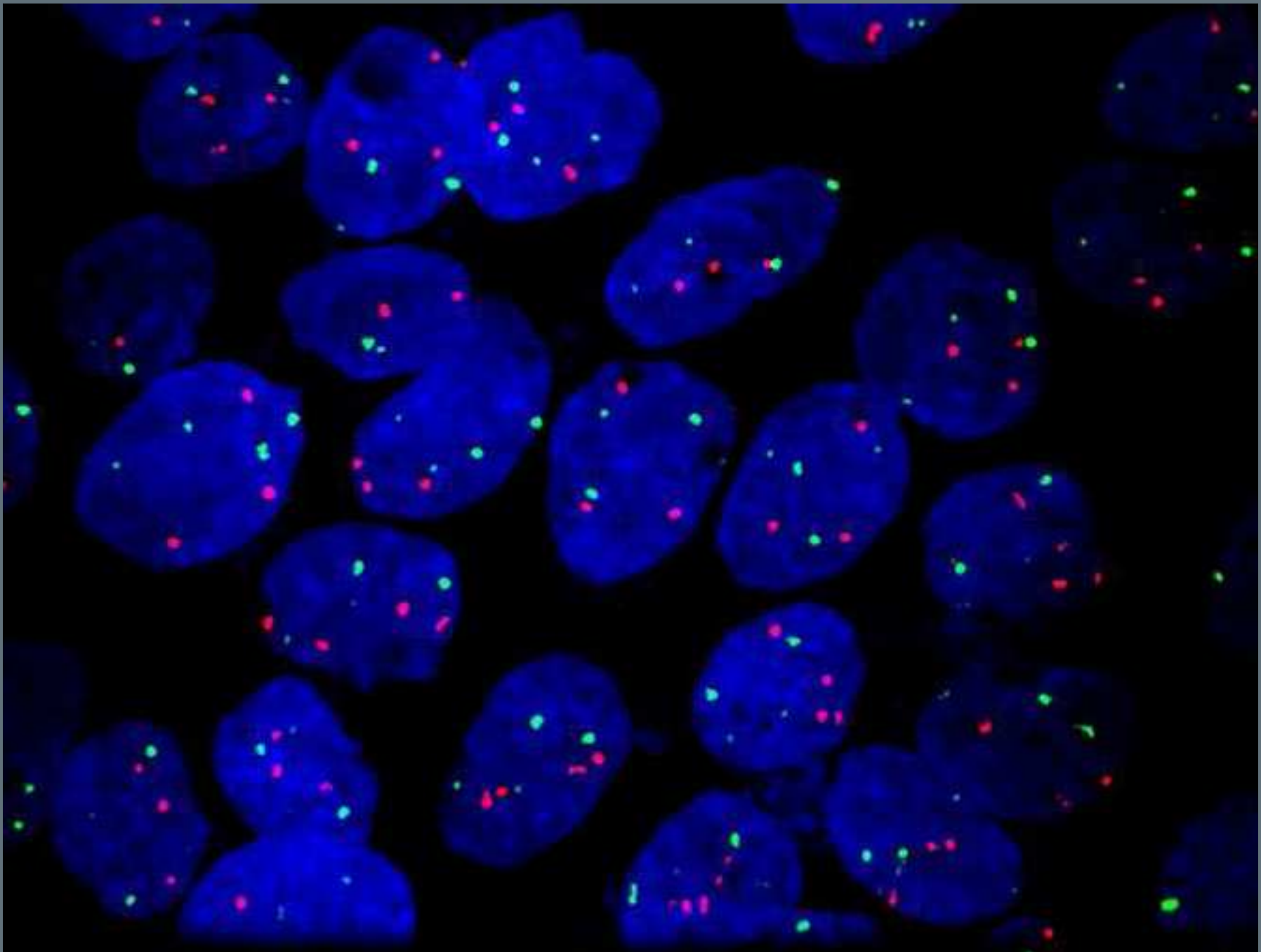
# Image analysis and studies on nuclear radial distribution of selected genomic regions
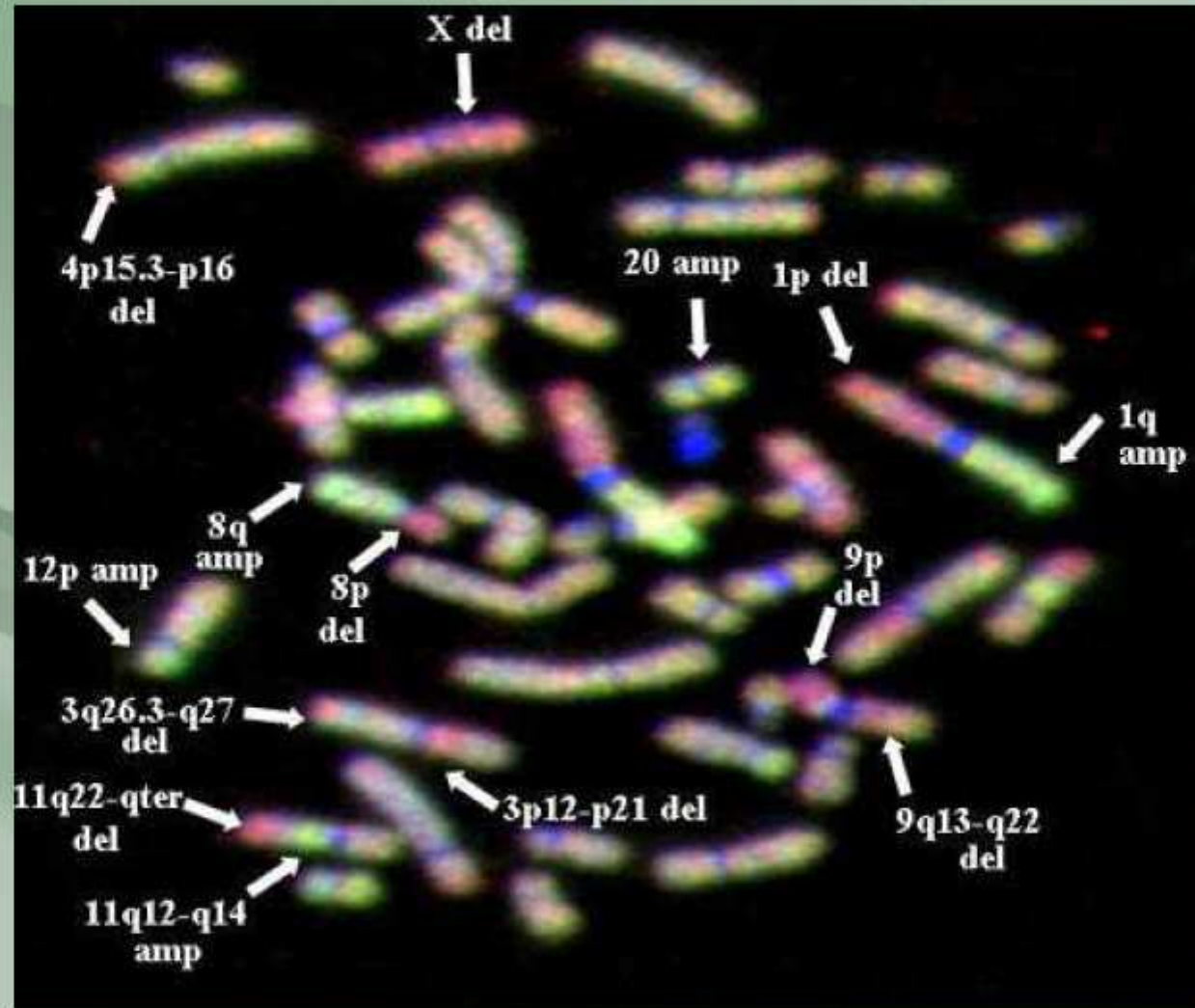
# Comparative genome hybridization

# CGH on metaphase spreads

# Comparative genome hybridization

# Advanced microscopic techniques

**Electron Microscopes are:** scientific instruments that use a beam of highly energetic electrons to examine objects on a very fine scale. This examination can yield the following information:

## Topography
The surface features of an object or "how it looks", its texture; direct relation between these features and materials properties (hardness, reflectivity...etc.)

## Morphology
The shape and size of the particles making up the object; direct relation between these structures and materials properties (ductility, strength, reactivity...etc.)

## Composition
The elements and compounds that the object is composed of and the relative amounts of them; direct relationship between composition and materials properties (melting point, reactivity, hardness...etc.)

## Crystallographic Information
How the atoms are arranged in the object; direct relation between these arrangements and materials properties (conductivity, electrical properties, strength, etc.)
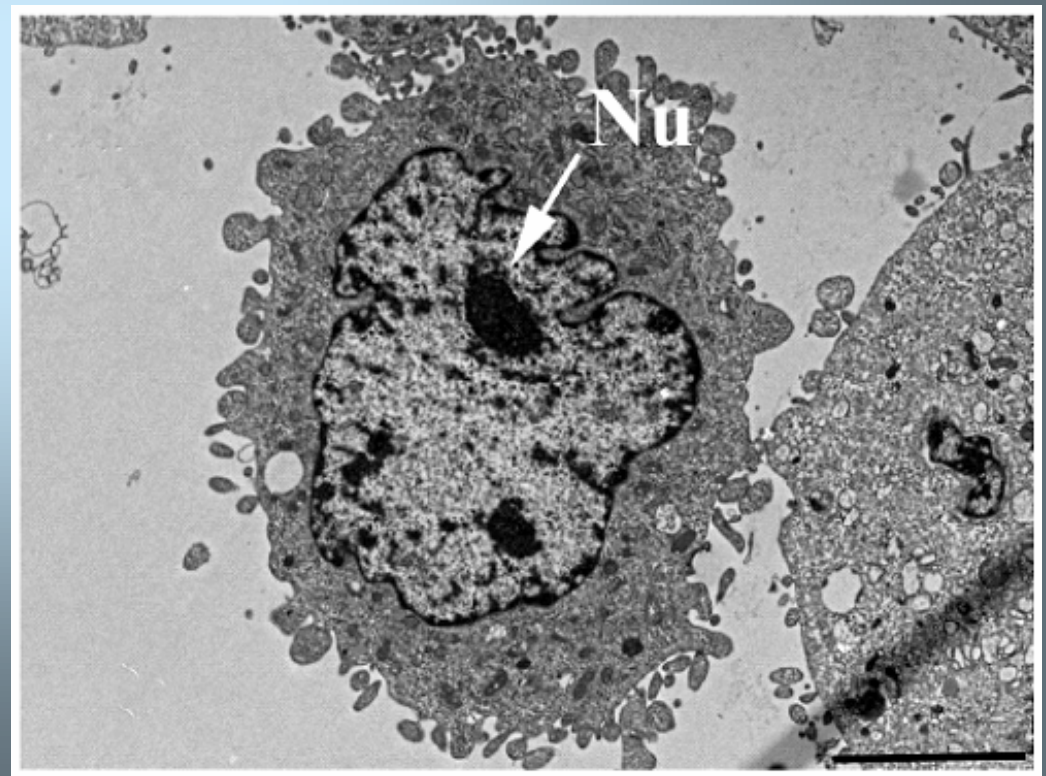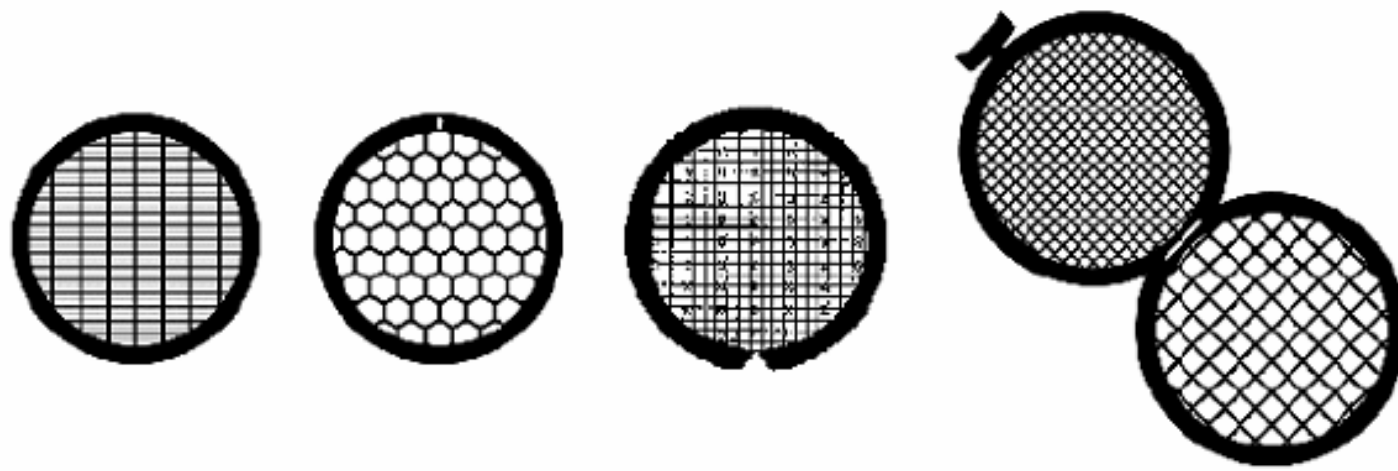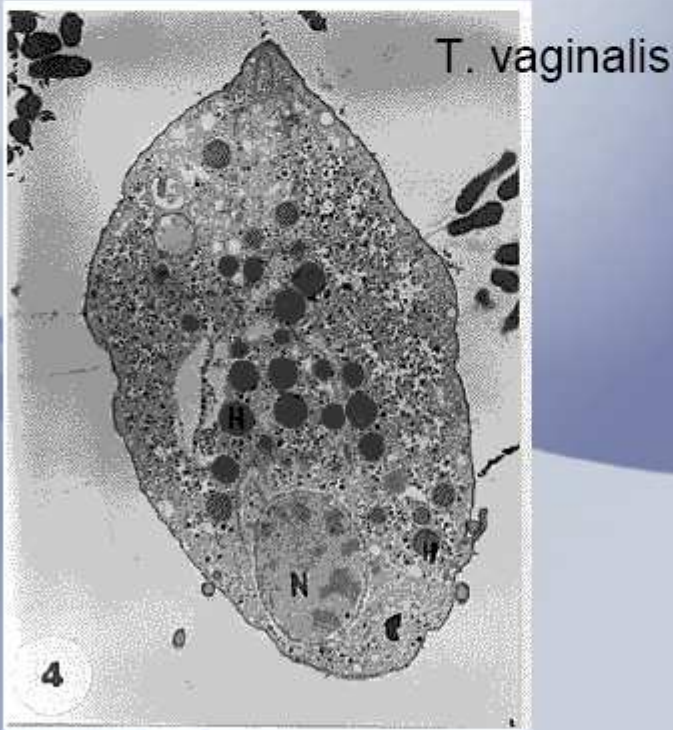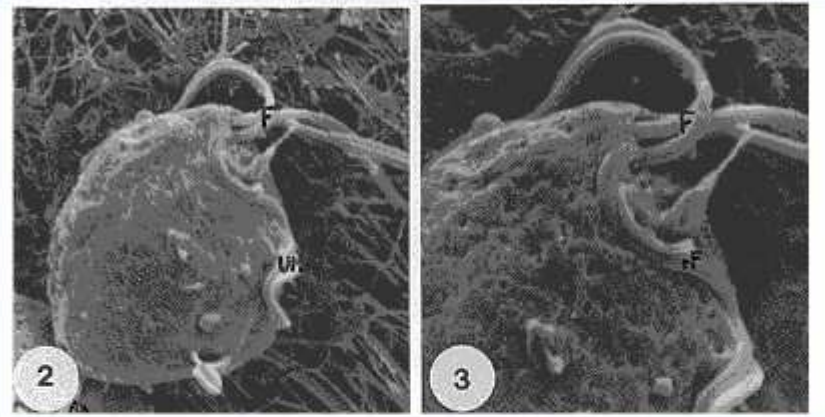
# Transmission Electron Microscopes (TEM)
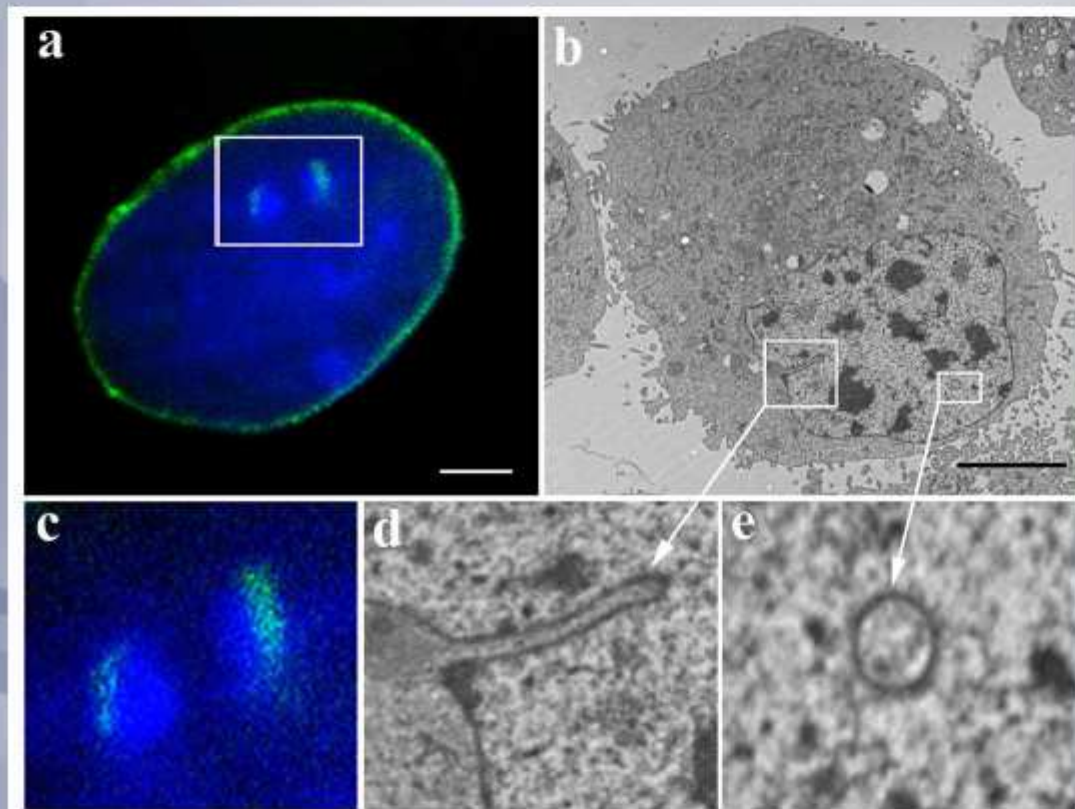


Galiová et al. EJCB (2008)

# Scanning Electron Microscopes



Photo: Gerry Nash
ANTARCTICA



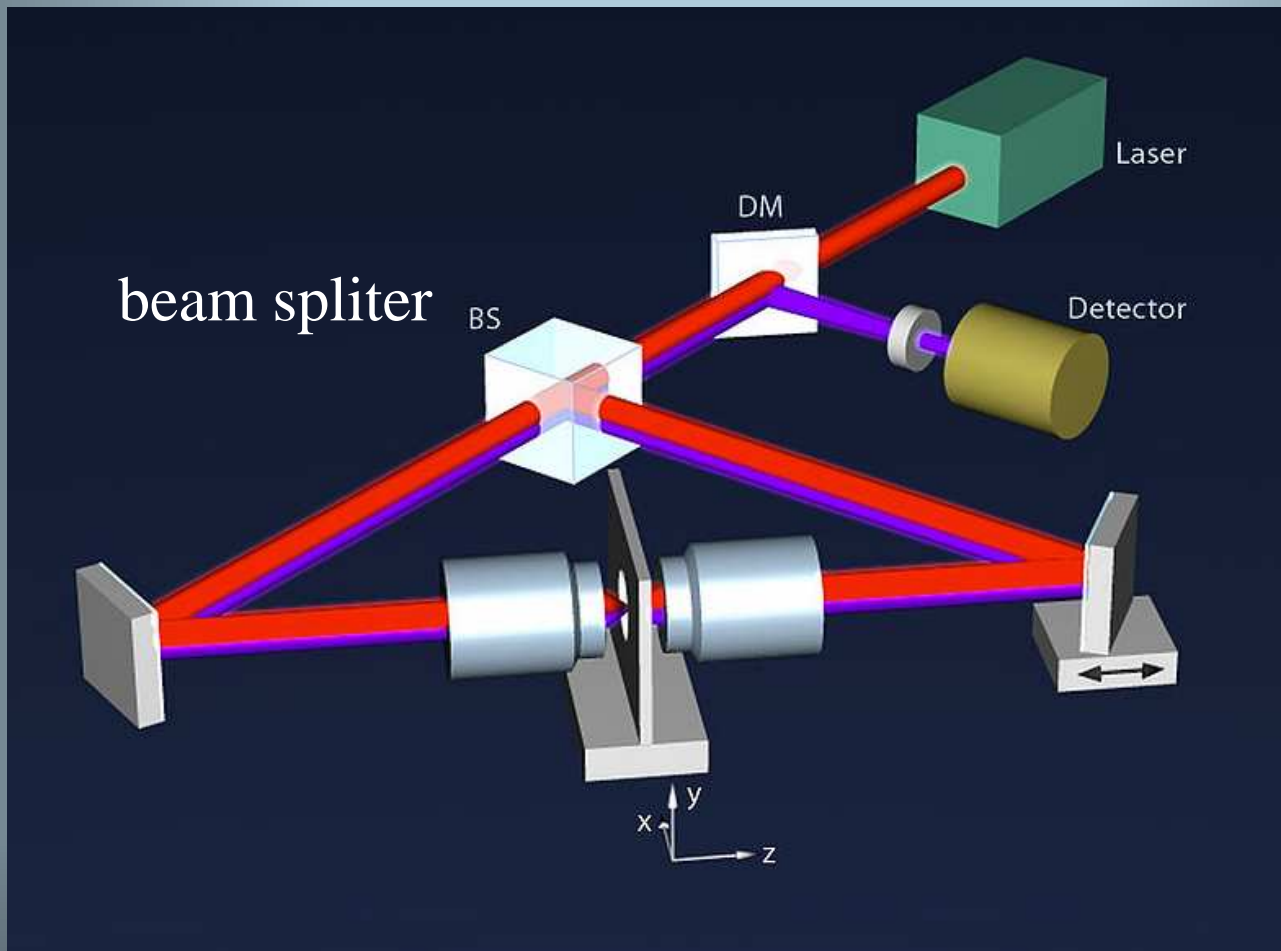T. vaginalis
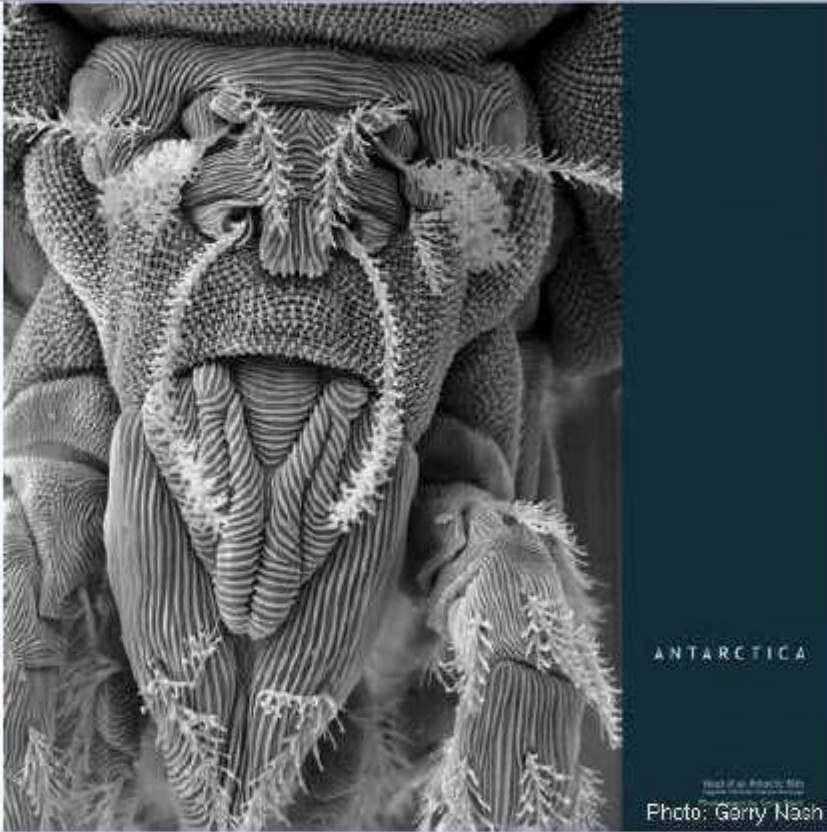
# 4-pi microscopy (CC lab in Heidelberg)
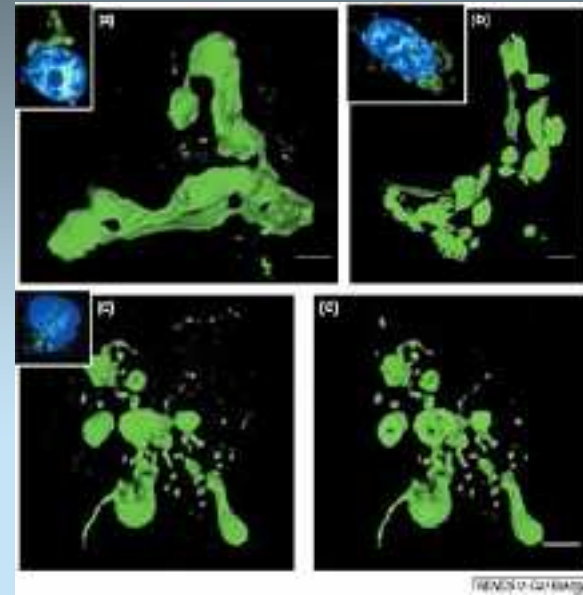
**4pi: improved axial resolution. The typical value of 500-700 nm can be improved to 100-150 nm which corresponds to an almost spherical focal spot with 5-7 times less volume than that of standard confocal microscopy.**

The operation mode of a 4Pi microscope is shown in the figure. The laser light is divided by a beam splitter (BS) and directed by mirrors towards the two opposing objective lenses. At the common focal point superposition of both focused light beams occurs. Excited molecules at this position emit fluorescence light which is collected by both objective lenses, combined by the same beam splitter and deflected by a dichroic mirror (DM) onto a detector. There superposition of both emitted light pathways can take place again.

# 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.
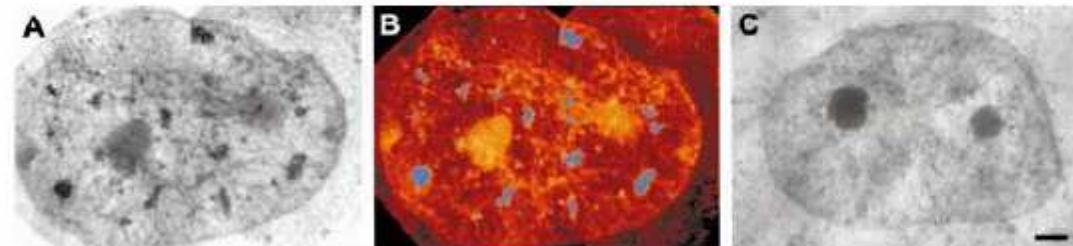


Fig. 4. Nuclei of human mammary epithelial cells (T4) labelled for RNA splicing factor (SRm300). (A) X-ray micrograph of a single nucleus after silver enhancement. This image is a montage compiled from two individual X-ray microscope images. (B) Same nucleus after colour coding to emphasize the label. (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×, 0.034 NA with 20 nm pixel size at 517 eV (λ = 2.4 nm).

(Meyer-Ilse et al., 2000)

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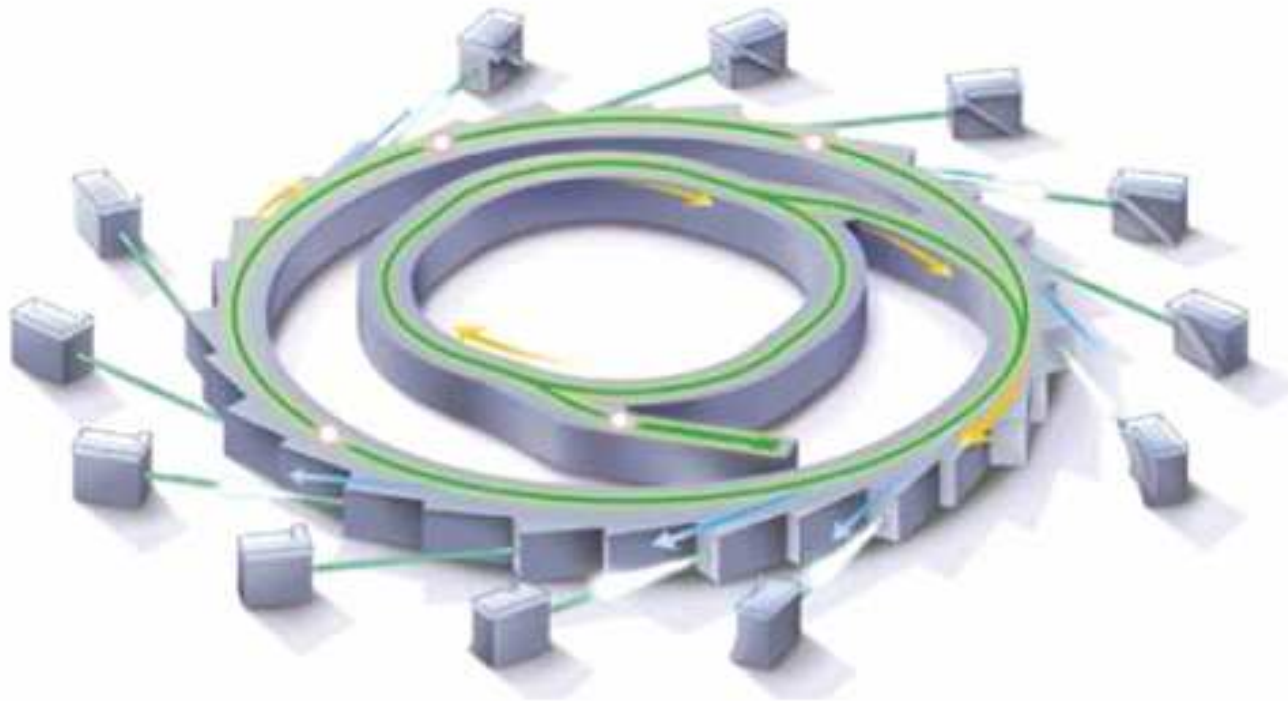
# Subdiffraction Multicolor Imaging of the Nuclear Periphery with 3D Structured Illumination Microscopy

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]*†

**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 μm.
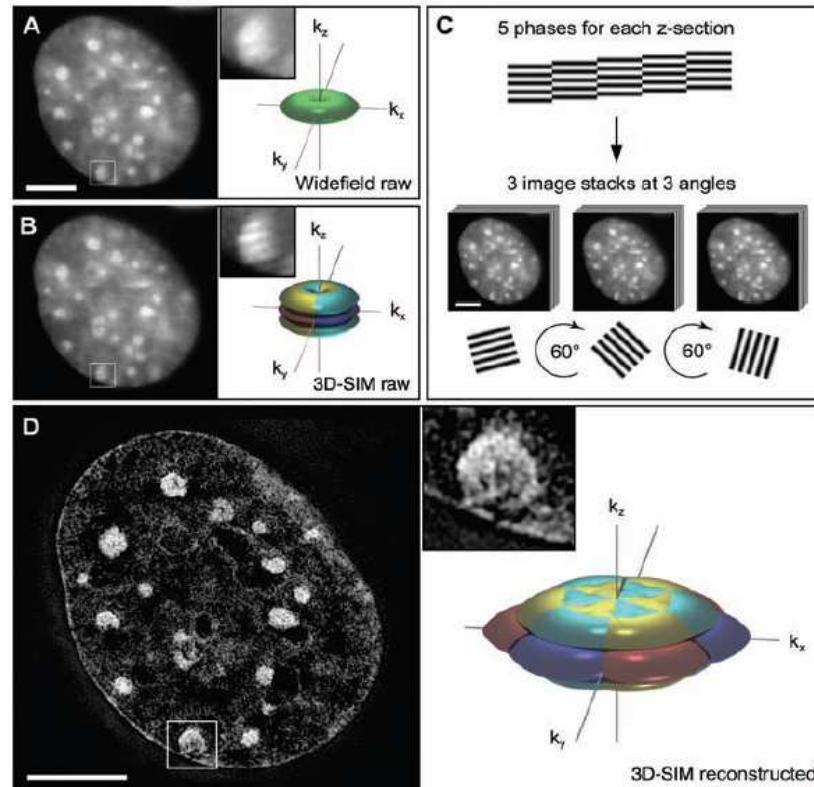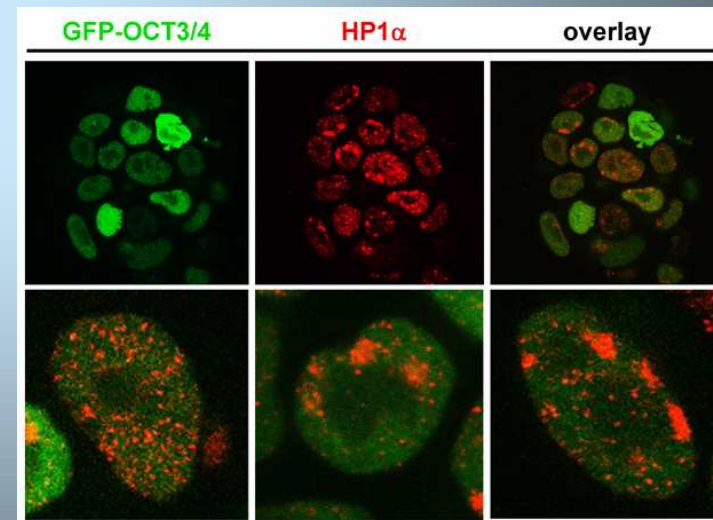
**Stimulated Emission Depletion microscopy, or STED microscopy, is a fluorescence microscopy technique that uses the non-linear de-excitation of fluorescent dyes to overcome the resolution limit imposed by diffraction with standard confocal laser scanning microscopes and conventional far-field optical microscopes.**
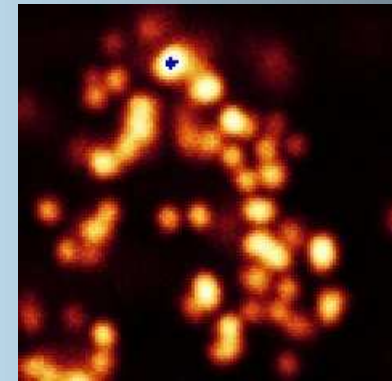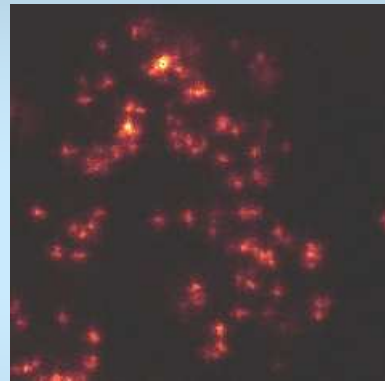
**STED**
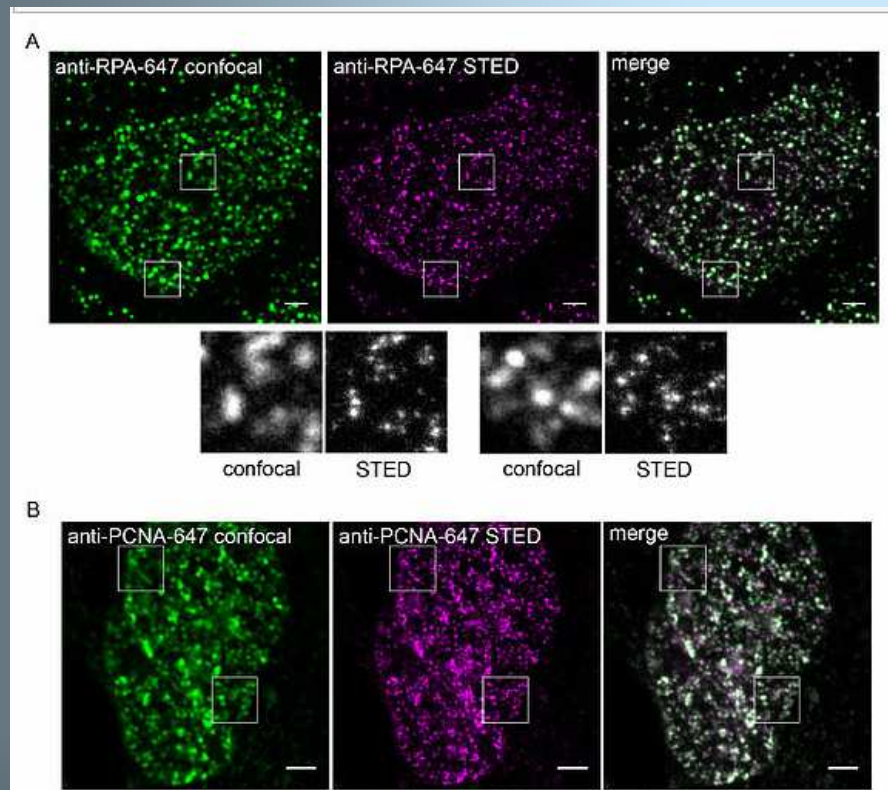
**SP-5 LSCM**

**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"*



$$\Delta X = \frac{\lambda}{2n\sin\alpha\sqrt{1+\frac{I}{I_{sat}}}}$$

neurobiology
membrane biology
membrane rafts
intracellular transport

Willig KI et al. *Nature* 2006

Sieber JJ et al. *Biophy J* 2006

Kittel RJ et al. *Science* 2006

Fitzner D et al. *EMBO J* 2006

Kellner RR et al. *Neurosience* 2007

Lin W et al. *PNAS* 2007

Seebach J *Cardiovas. Res.* 2007

Sieber JJ *Science* 2007

Unique

# Super-resolution microscope systems from Carl Zeiss ELYRA combines PAL-M (Photo-activated localization microscopy) and SR-SIM technology

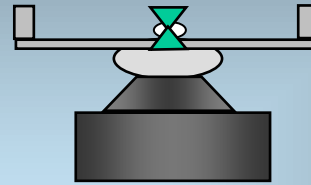# Fluorescence microscopy technique comparison
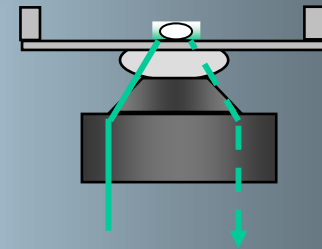


Petri Dish
Oil
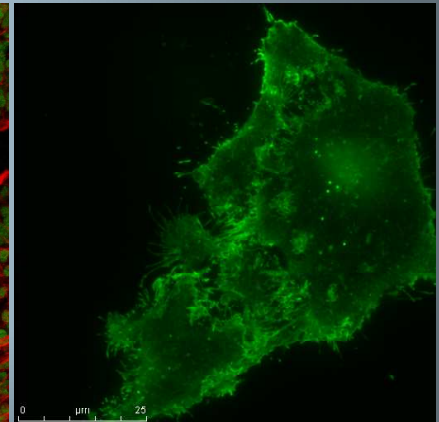Objective
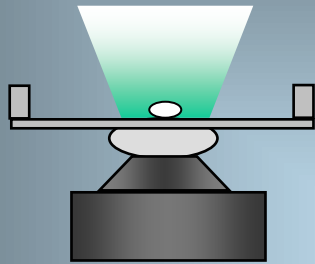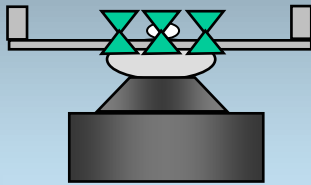
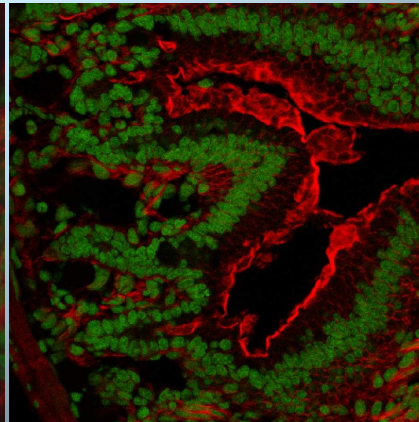**WFM**　　　**SDCM**　　　**CLSM**　　　**TIRF**

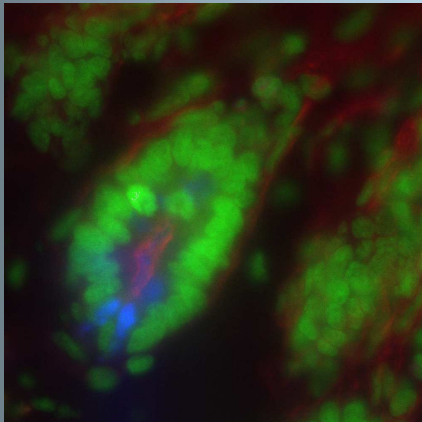**Z resolution measured**

widefield: ~ 1.13 µm　　SD6000: ~ 0.84 µm　　SP5: ~ 0.50 µm　　**TIRF: 0.1 – 0.3 µm**
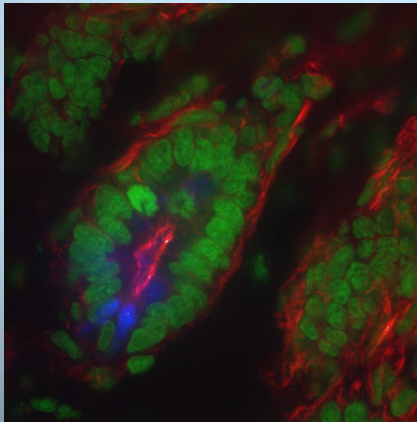
**samples**

thin samples < 5 µm
moderately thick + deconvolution

moderately thick
5 µm – 30 µm

thick > 30 µm

**! does not matter !
Only evanescent waves**

**4PI: 0.1 – 0.12 µm!**

# Confocal Laser Scanning Microscopy – advanced systems

**Leica TCS SP5 – universal system for everything!**



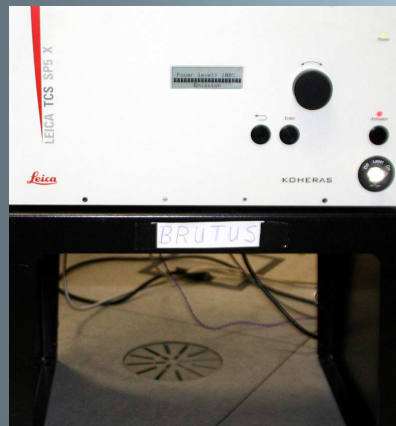**FRAP
FRET AB, SE
Live Data Mode
ROI spectrophotometer
APD
SMD – FCS, FLIM, FCCS
Spectral FLIM
High Content Screening Auto
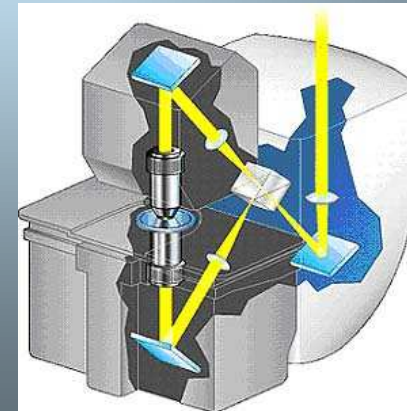2-photon, 3-photon**

**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



## 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 – super-resolution in xy plane

## Separate UNIQUE systems

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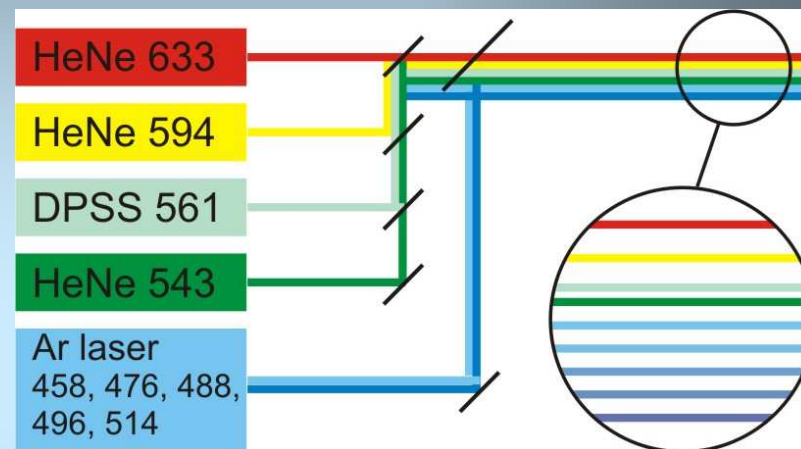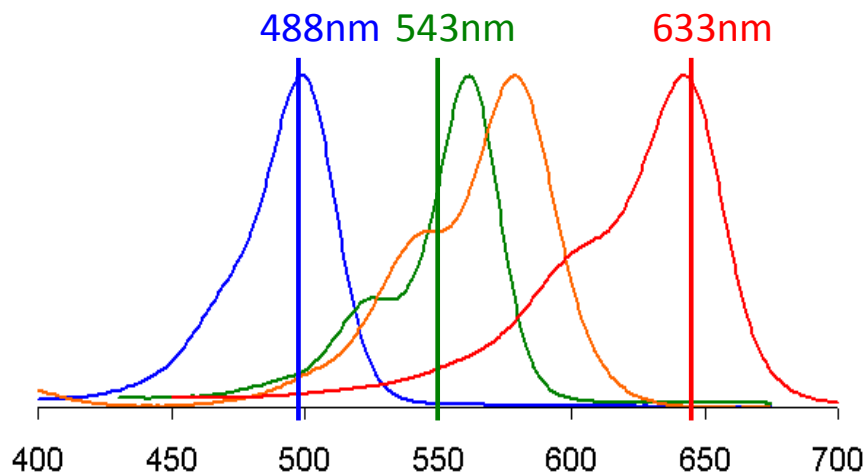- Leica 4PI – high resolution in z axis

Unique

Unique

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



Excitation Spectra

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

488nm  543nm      633nm

HeNe 633
HeNe 594
DPSS 561
HeNe 543
Ar laser
458, 476, 488, 496, 514

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

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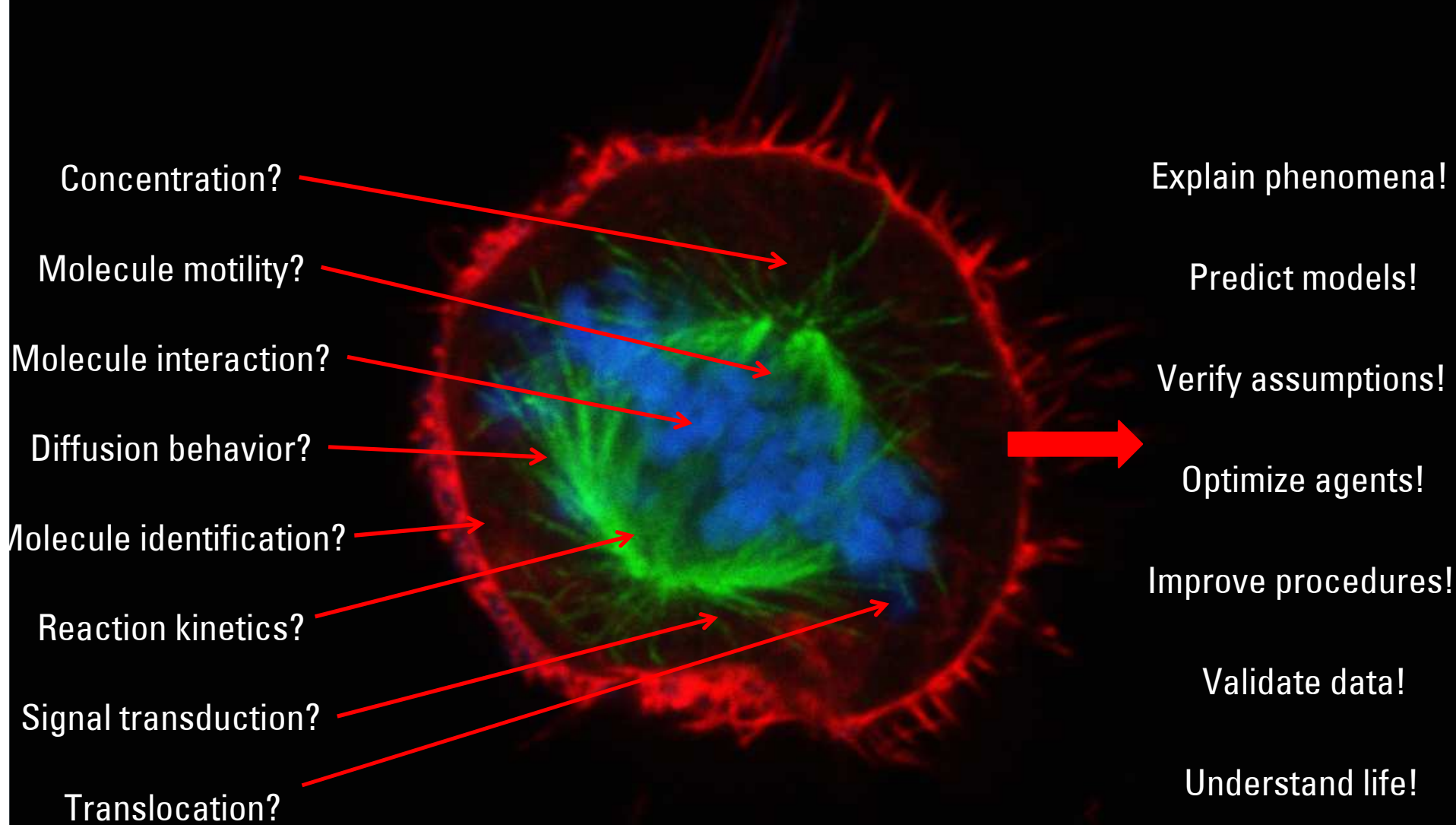- Only several combinations of wavelengths

## White Light Laser – new <u>lambda scan</u>, 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

# Quantify Life! – The Challenge

Concentration?

Molecule motility?

Molecule interaction?

Diffusion behavior?

Molecule identification?

Reaction kinetics?

Signal transduction?

Translocation?

Explain phenomena!

Predict models!

Verify assumptions!

Optimize agents!

Improve procedures!

Validate data!

Understand life!

**Eva Bártová, Gabriela Šustáčková,**
**Lenka Stixová, Soňa Legartová, Darya Orlova, Veronika Foltánková, Pavel**
**Matula, Petra Sehnalová**
**Institute of Biophysics, the Academy of Sciences of the Czech Republic, v.v.i., Brno**