OUTLINE

Group 1:

Jessica BRUNETTI
David COTTET-DUMOULIN
Manon LOCATELLI
Olga RUSIECKA

Group 2:

Axel TOLLANCE
Marina ZAGATTI
Karolina LUCZKOWSKA
Alix GARCIA

Advanced fluorescence imaging techniques for life sciences.

Bioimaging course, April 2019

(4 sessions, 4 hours each). ECTS : 1.5.

Program

Session 1- Introduction to widefield and confocal microscopy **Friday 5 April 2019**	
Time	
13 :00-14 :00 (room A04.27.06)	General presentation of the Bioimaging course Introduction to widefield and confocal microscopy
14 :00- 15 :20 (room C06.1742.a)	**Hands on session 1 (widefield/confocal, group work)** *During the session basics of optics and microscopy will be covered. The goal is to make two types of measurements (Point Spread Function or PSF, lateral and axial resolutions) in widefield and confocal microscopy.*
15 :20-15 :30	Break
15 :30-17 :00 (room C06.1742.a)	**Hands on session 2 (Widefield/ confocal, group work)**
Session 2- F-techniques (FRAP, FRET) **Friday 12 April 2019**	
Time	
13 : 00- 13 :20 (room A04.27.06)	**Fluorescence, fluorochromes and F-techniques**
13 :20-14 :50 (room C06.1742.a)	**Hands on session (group work)** • *FRAP experiments*
14 :50-15 :00	**Break**
15 :00- 17 :00 (room A04.27.06)	**Hands on session (group work)** • *Basics of digital imaging* • *Data handling*

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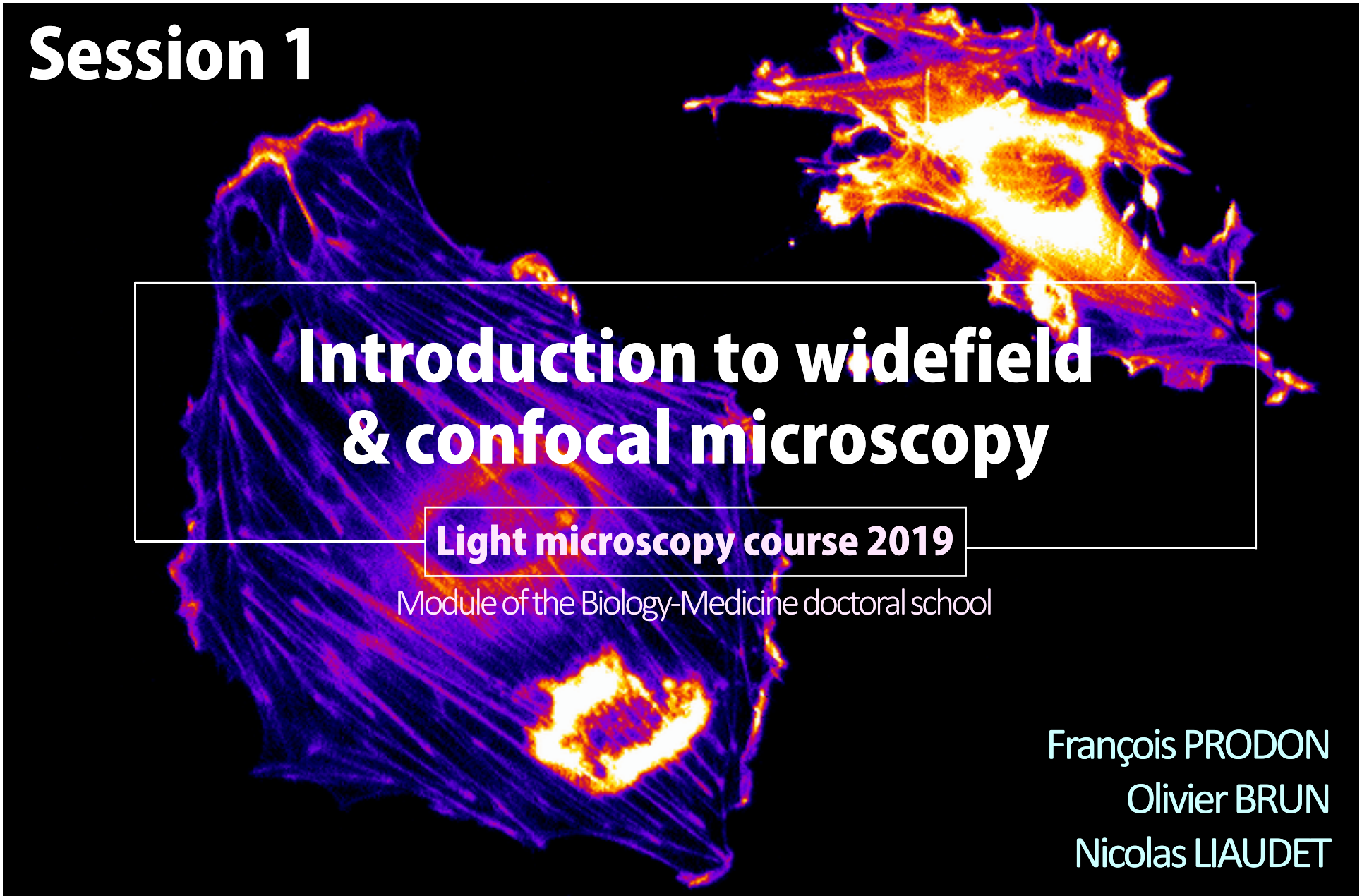
Session 3- Super-Resolution techniques **Tuesday 16 April 2019**	
Time	
13 :00-13 :20 (room A04.29.10)	**General introduction** *STED, SMLM (PALM, STORM), Airyscan.*
13:20- 14:20 (room C06.1742.a)	**Hands on session 1 (group work)** *STED and Airyscan*
14 :20-15 :20 (room C06.1742.a)	**Hands on session 2 (group work)** *STED and Airyscan*
15 :20-15 :30	**Break**
15 :30-17 :00 (room A04.29.10)	**Data Handling acquired in widefield and in confocal:** • *Concept of Deconvolution* • *Use of Huygens sofware*
Session 4- Image manipulation **Friday 26 April 2019**	
Time	
13 :00- 13 :05 (room C06.1542.a)	**Group picture**
13 :05- 14 :05 (room C06.1542.a)	**Tips and tricks for data presentation**
14 :05-14 :20 (room C06.1542.a)	**Open discussion/ remarks**
14 :20-14 :30	**Break**
14 :30-17 :00 (room C06.1542.a)	**Comparison between the images acquired in widefield and confocal (before and after deconvolution) and also in Airy scan and STED:** • *Compilation of these data* • *Determination of the resolving powers of these different types of microscopy and comparison*

Session 1



Introduction to widefield & confocal microscopy

Light microscopy course 2019

Module of the Biology-Medicine doctoral school

François PRODON
Olivier BRUN
Nicolas LIAUDET

FACULTÉ DE MÉDECINE

UNIVERSITÉ DE GENÈVE

2 NP awarded over the past 10 years

2018

Laser physics

2017

Cryo-microscopy

2014

Nobel Prize Chemistry Winners 2014

Stefan Hell | Eric Betzig | William E. Moerner

2014

Blue light emitting diodes

2009

CCD sensor

2008

Eugene Paul Wigner | Maria Goeppert Mayer | J. Hans D. Jensen

Frits Zernike

1953

Richard Zsigmondy

1925

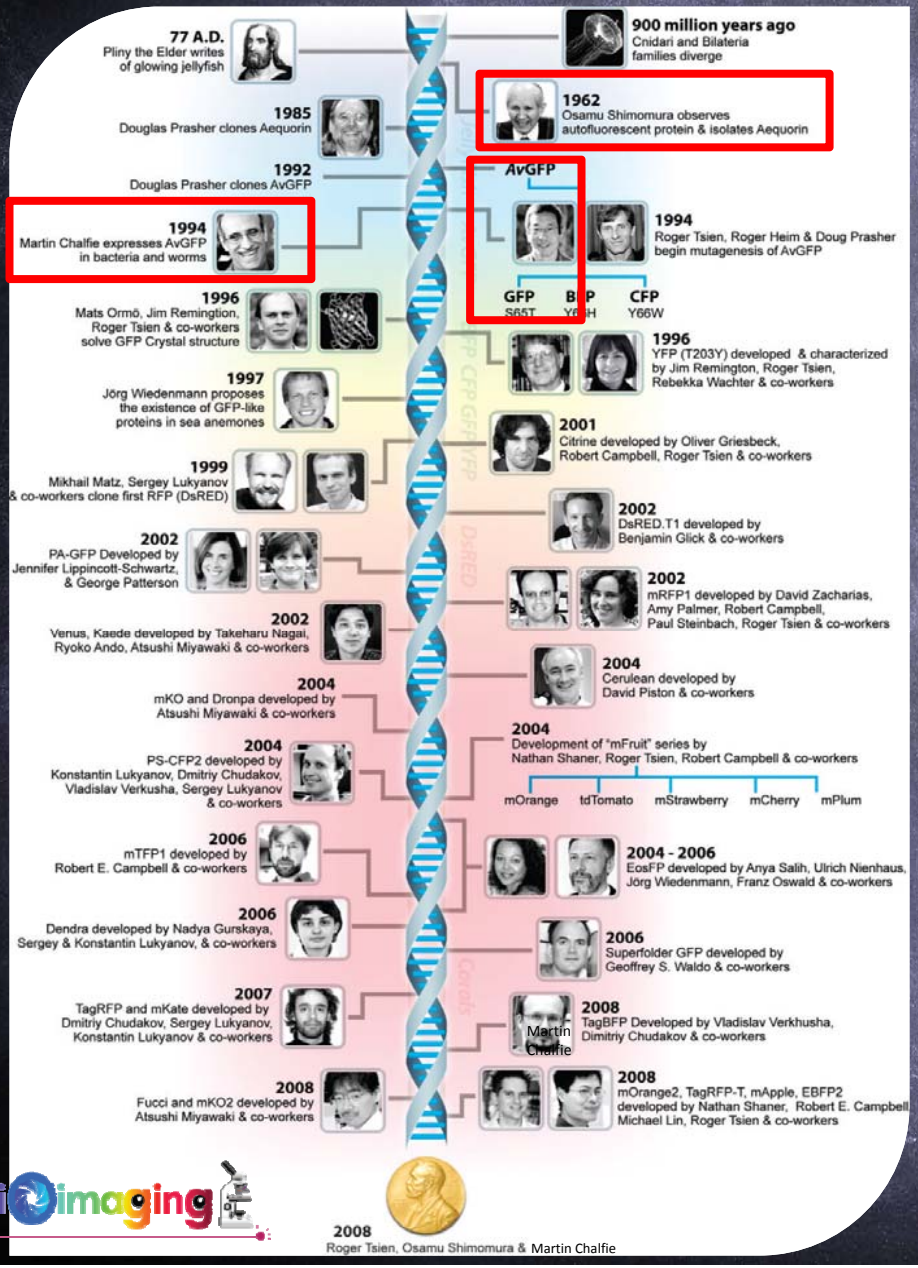
Marwin Minsky: confocal scanning microscope (1957) !!!

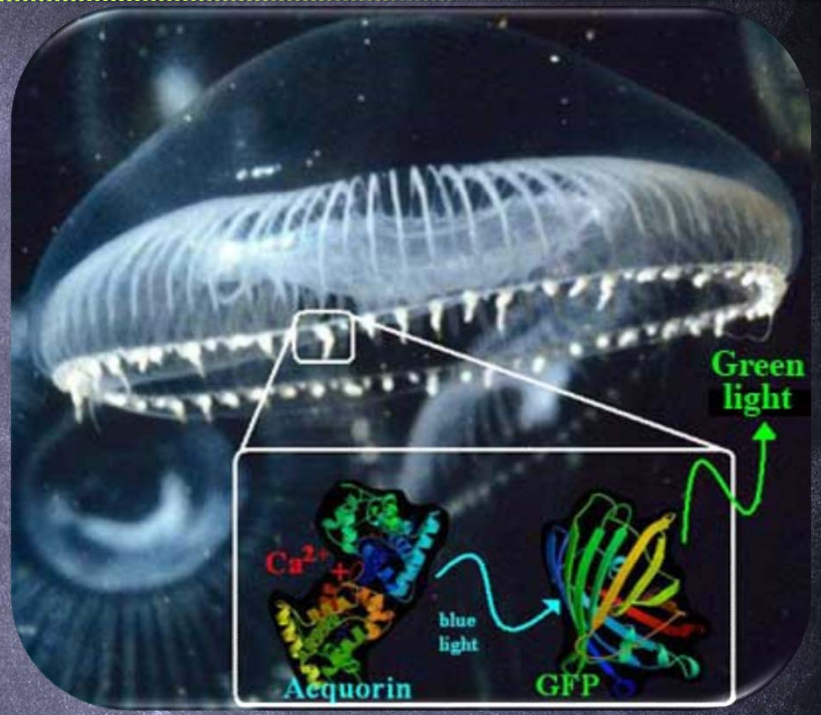
Adapted from:

www.nobelprize.org/educational/physics/microscopes/timeline/index.html

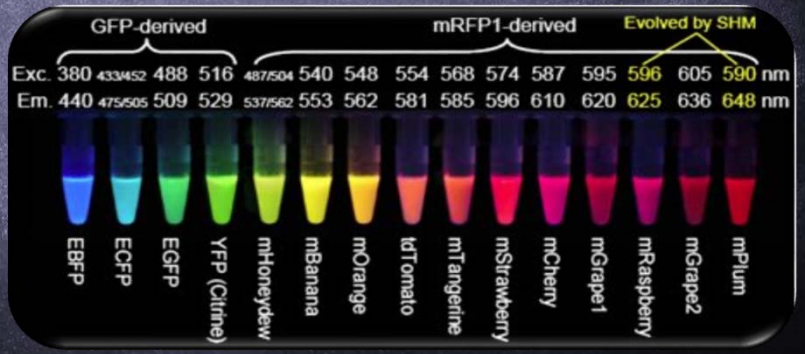
BioImaging

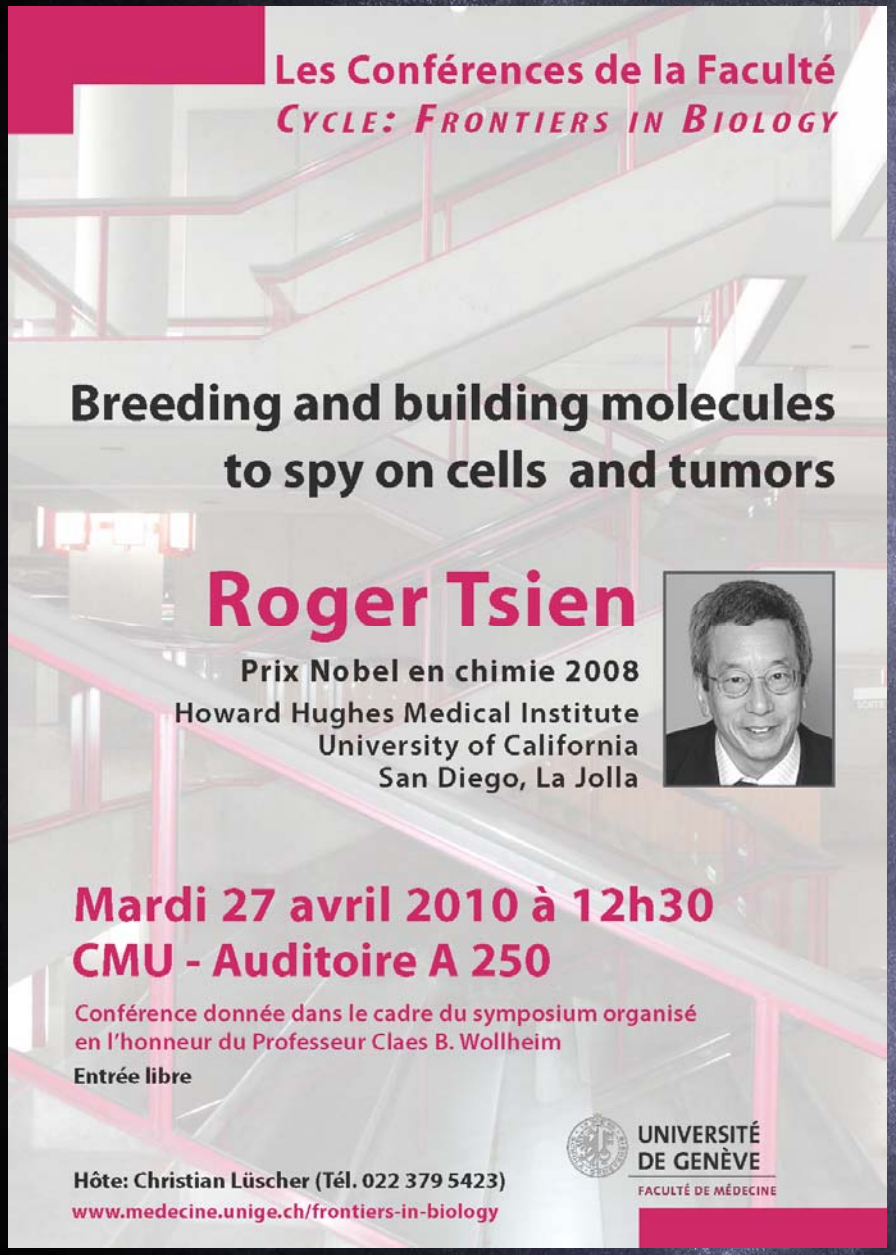
The GREEN revolution: GFP & variants





Day and Davidson (2009) | *Chem. Soc. Rev.* | 38, 2887–2921





Un Prix Nobel crée des molécules espionnes

Quand la chimie vient au secours de la médecine: conférence Frontiers in Biology

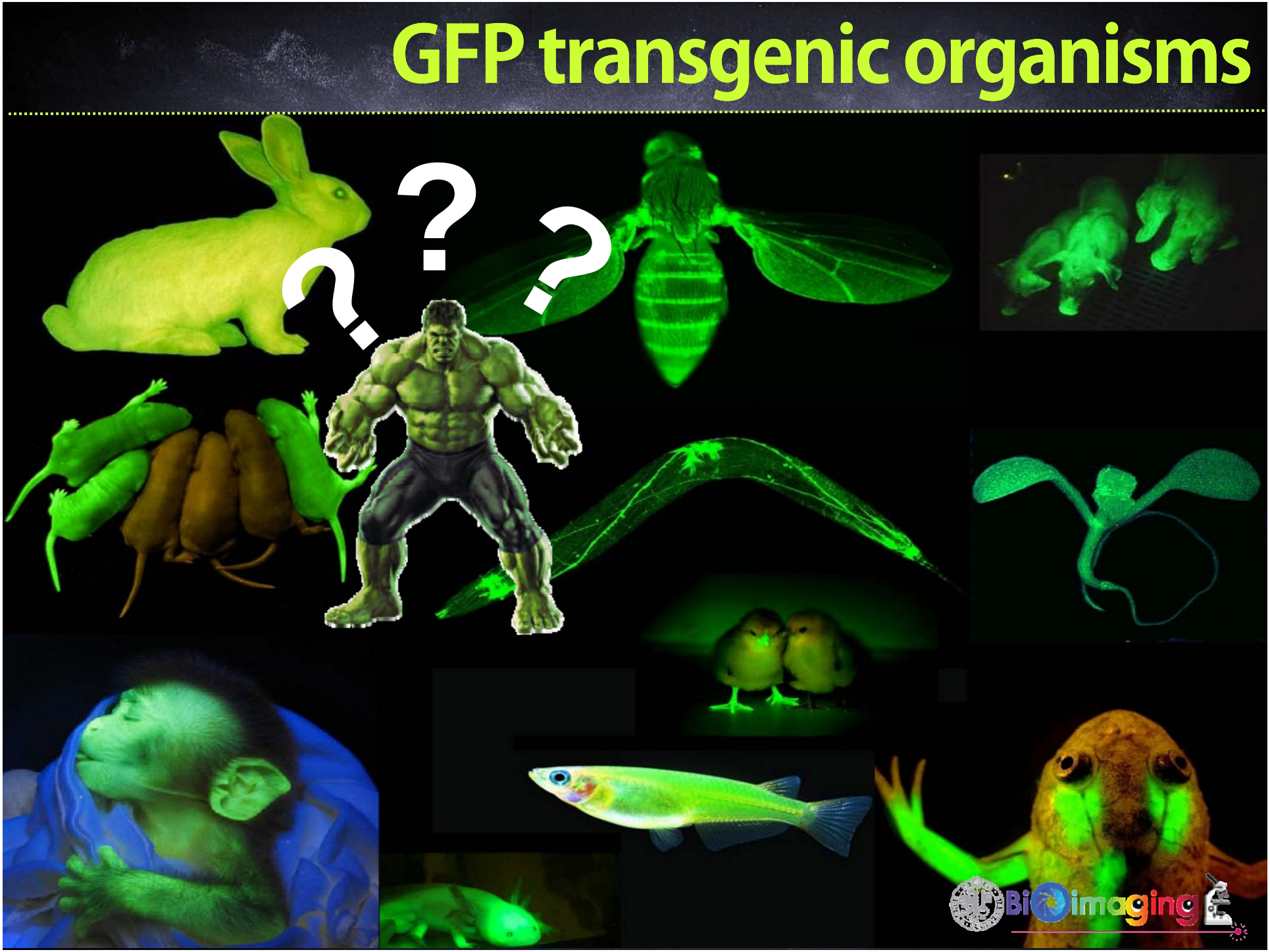
| CONFÉRENCE |
Dans le cadre du symposium organisé en l'honneur du professeur Claes B. Wollheim, le mardi 27 avril au Centre médical universitaire, Frontiers in Biology propose une conférece donnée par le prix Nobel de chimie 2008, le professeur Roger Tsien. Biochimiste et biophysicien américain d'origine chinoise, Roger Tsien, est né à New York en 1952. Il passe son bachelor en sciences (chimie et physique) à Harvard en 1972 avant de traverser l'Atlantique pour rejoindre le Laboratoire de physiologie de l'Université de Cambridge. Il obtient son doctorat au Collège Churchill, à Cambridge en 1977 où il reste encore quatre ans avant de rentrer aux Etats-Unis. Il travaille à l'Université de Berkeley en Californie, puis à l'Université de San Diego où il enseigne actuellement la pharmacologie, la chimie et la biochimie.

PRIX NOBEL
Co-récipiendaire du Prix Nobel de chimie en 2008 avec Osamu Shimomura et Martin Chalfie, Roger Tsien est connu avant tout pour ses travaux sur la protéine fluorescente verte (GPF), protéine découverte initialement dans l'organisme de la méduse *Aequorea victoria* en 1962. Il a contribué à la compréhension du mécanisme de la fluorescence de la GFP et a créé des protéines dérivées capables d'émettre d'autres longueurs d'onde (dans le jaune, le bleu et le cyan) sous excitation. Ces protéines sont devenues des outils essentiels et courants en biologie moléculaire et cellulaire.

| MARDI 27 AVRIL |
Breeding and building molecules to spy on cells and tumors
par Roger Tsien,
Prix Nobel de chimie 2008
à 12h30
CMU, auditoire A250
www.diabete.unige.ch


Roger Tsien, Prix Nobel de chimie 2008. Photo: DR

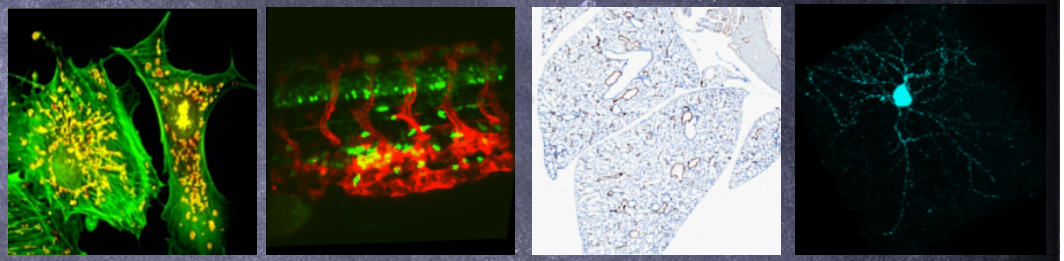
GFP transgenic organisms



Main applications in light microscopy

1- Localization

(co-localization, structure,
large image, 3D, super-resolution…)

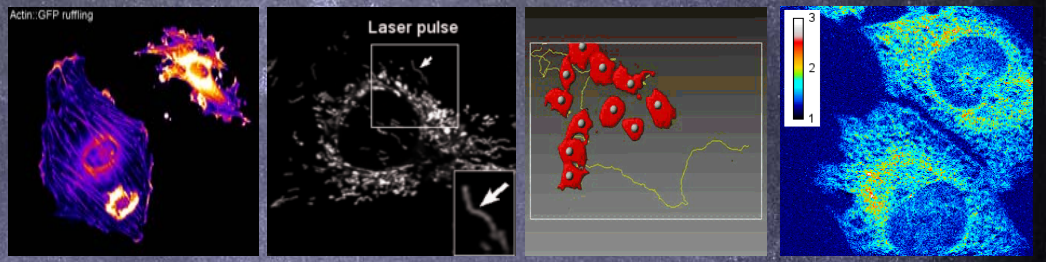


2- Isolation/ ablation

(laser microdissection)

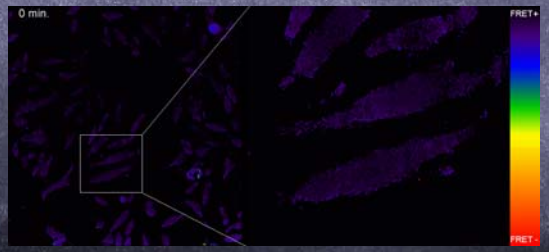
3- Dynamics

(time-lapse, FRAP/ photoactivation,
tracking, Ca2+ imaging/ ratiometric
Imaging…)



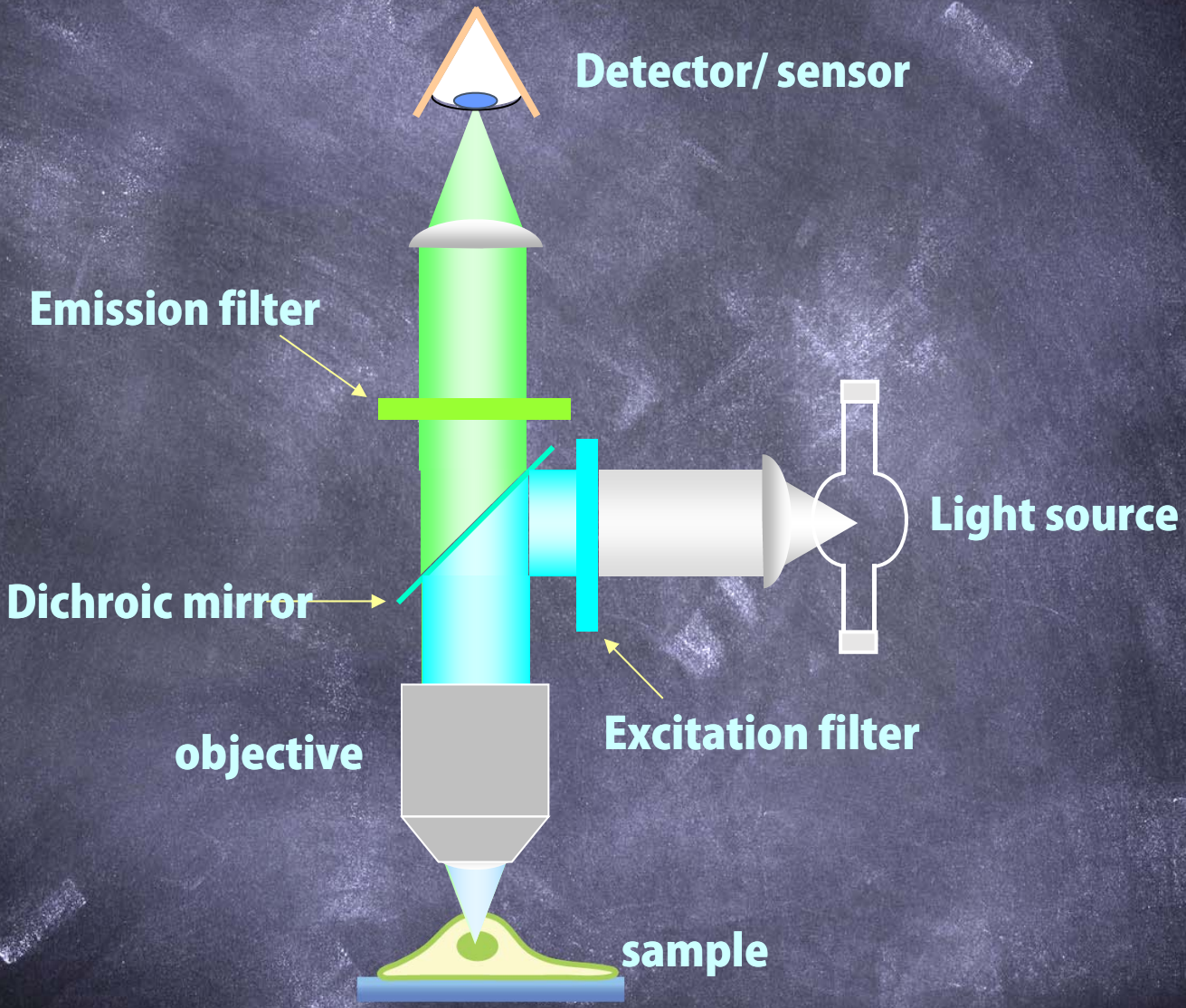
4- Interactions

(FRET, FLIM, FC(CS)…)

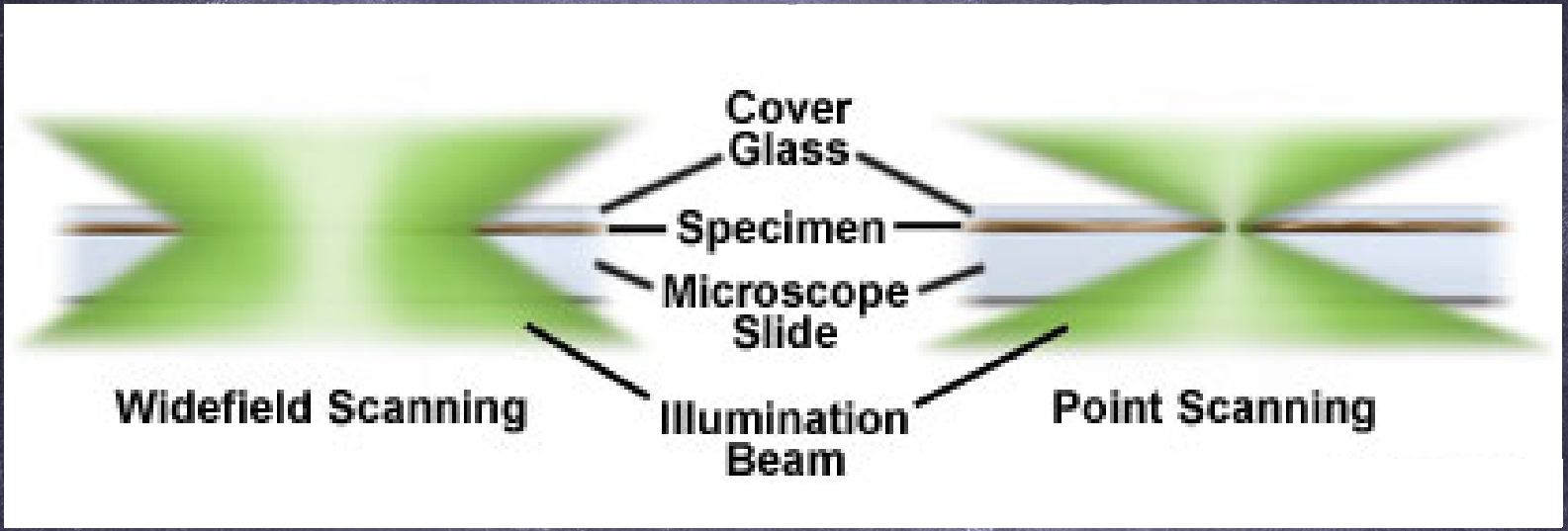


BiOimaging

Anatomy of an epi-fluorescence microscope

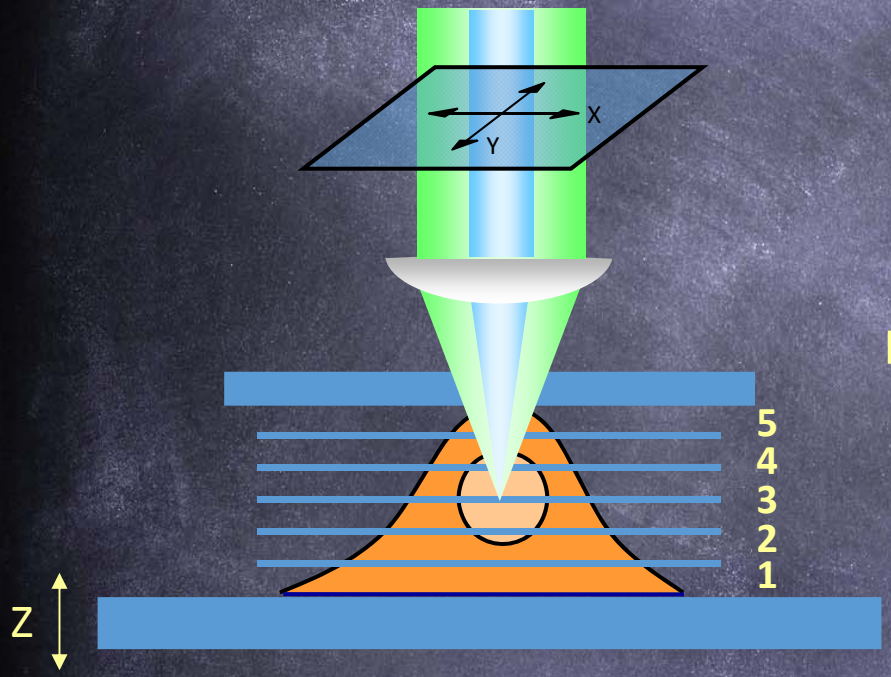


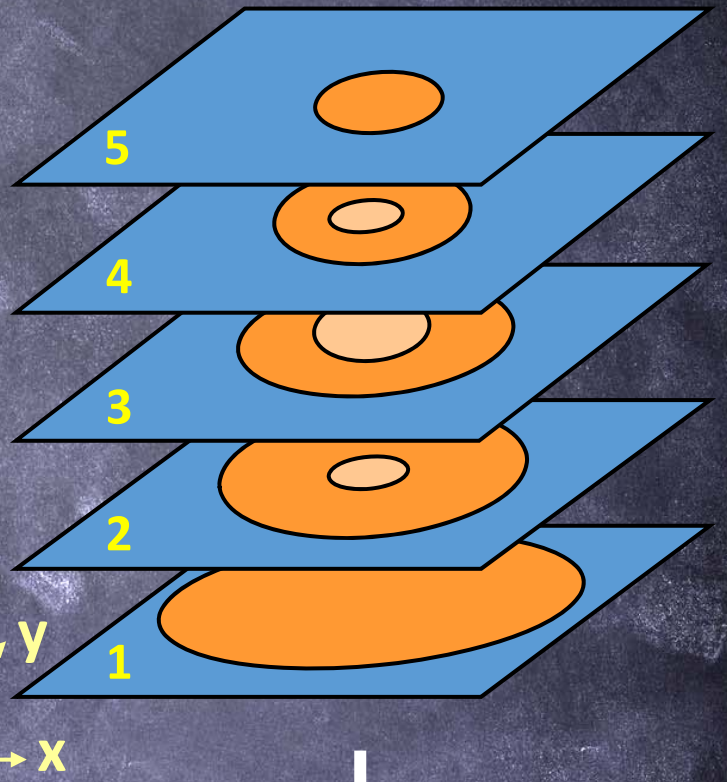
Widefield illumination cone VS point scanning of specimens



- **Widefield microscope: entire depth of the specimen over a wide area is illuminated**
- **Confocal microscope: the sample is scanned with a finely focuse spot of illumination centered in the focal plane.**

Optical sectionning

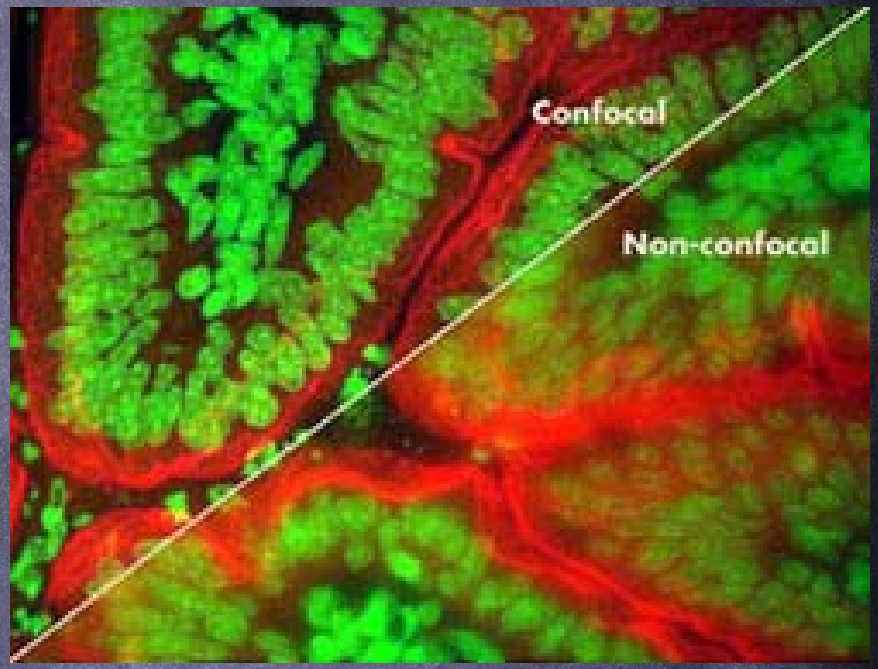




3D reconstruction

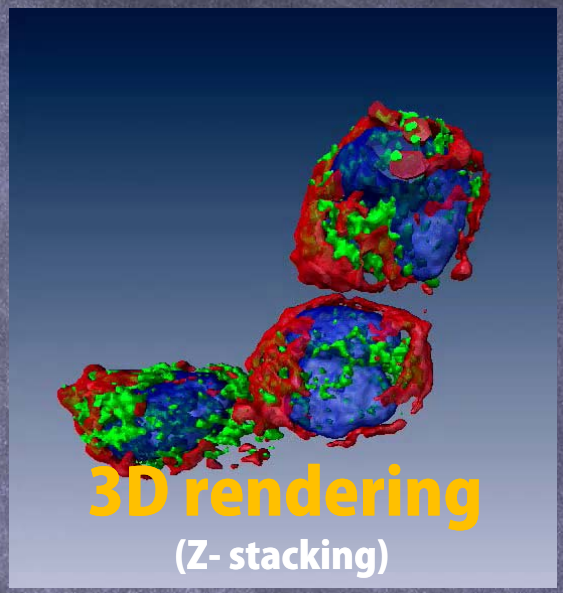
Advantages of confocal microscope

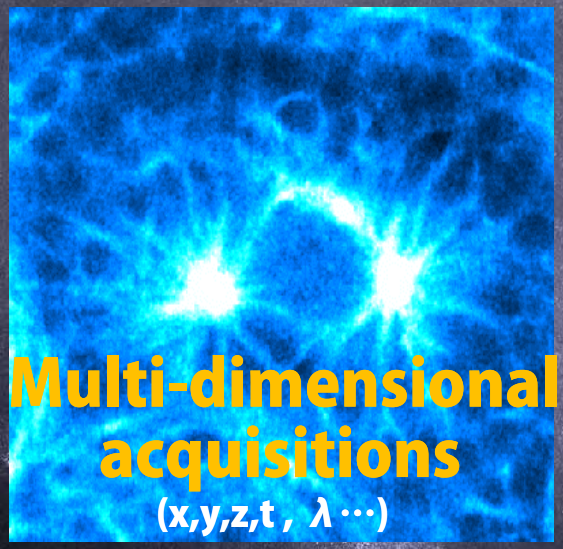
- Ability to control **depth of field**
- **Elimination or reduction of background** information away from the focal plane
- Capability to collect **serial optical sections** from thick specimens

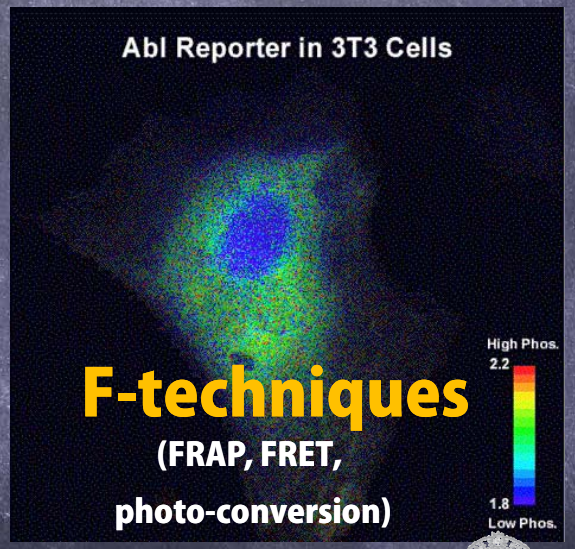


A section of mouse intestine imaged with both confocal and non-confocal microscopy

Main applications in CLSM







BioImaging

Epiaxial confocal microscope for fluorescence

① **Different laser lines** as sources of excitation

② **Galvanometric mirrors** to XY scan the field of view

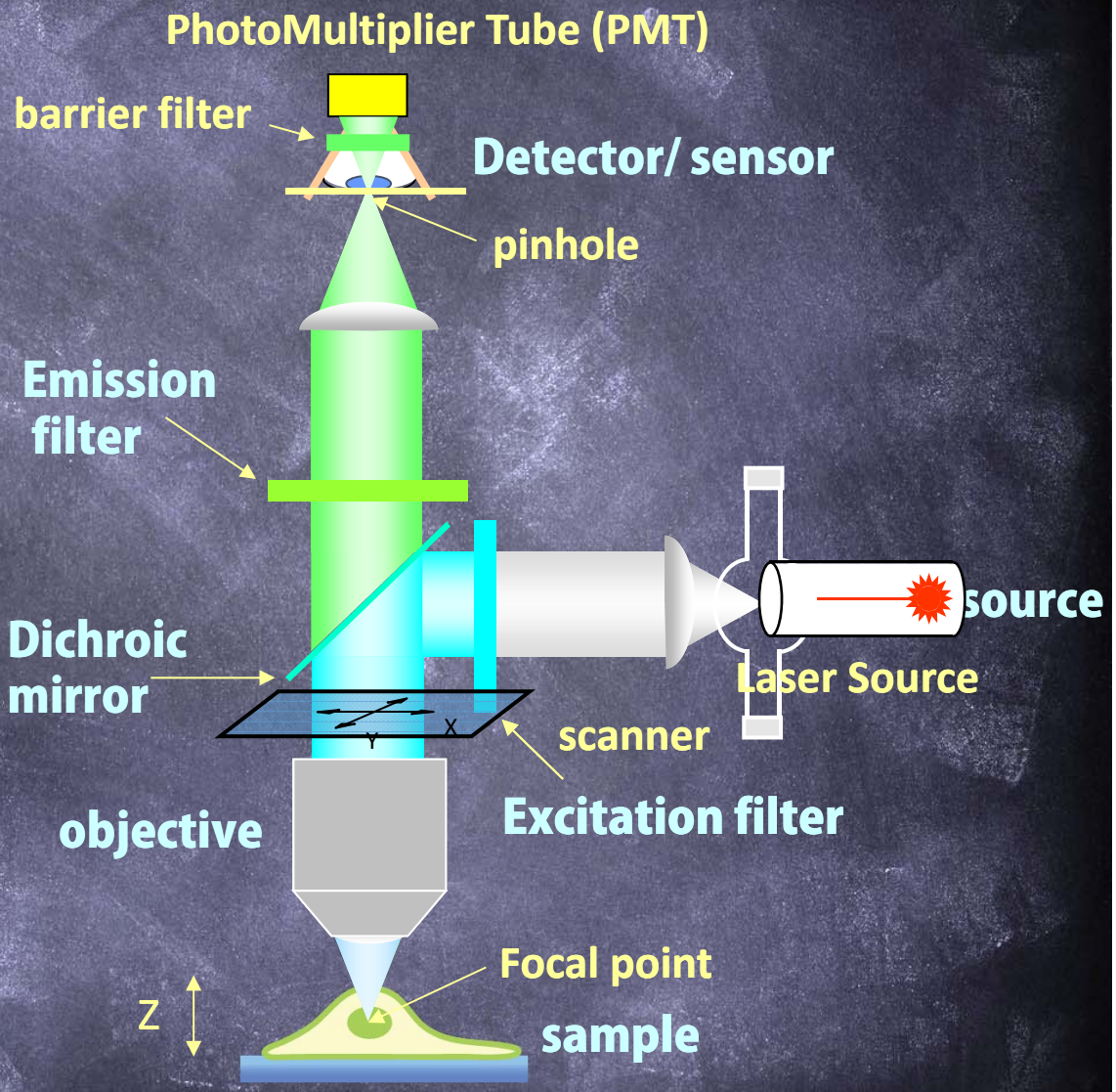
③ **Acousto-optically activated crystals** are used to separate the excitation & emission signals

④ **Same objective** for illumination & detection

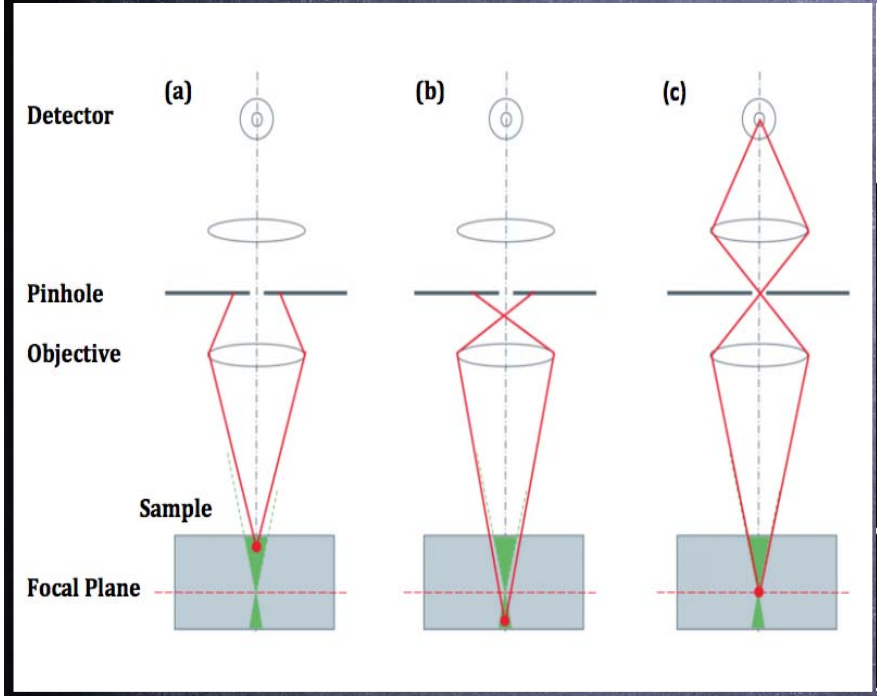
⑤ **Stepping motor** in the Z direction to make optical slices in the sample

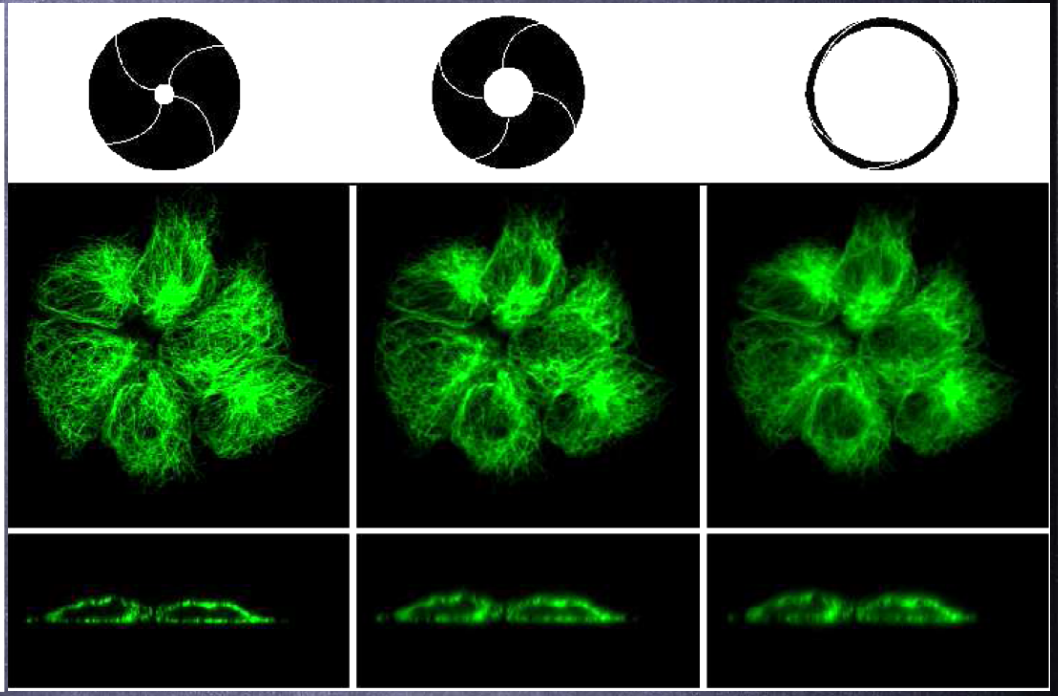
⑥ **Pinhole** to reject photons coming from the planes out of the focal plane

⑦ **PMT** behind the **pinhole** to make photon counting for each focal point



Pinhole and optical sectionning

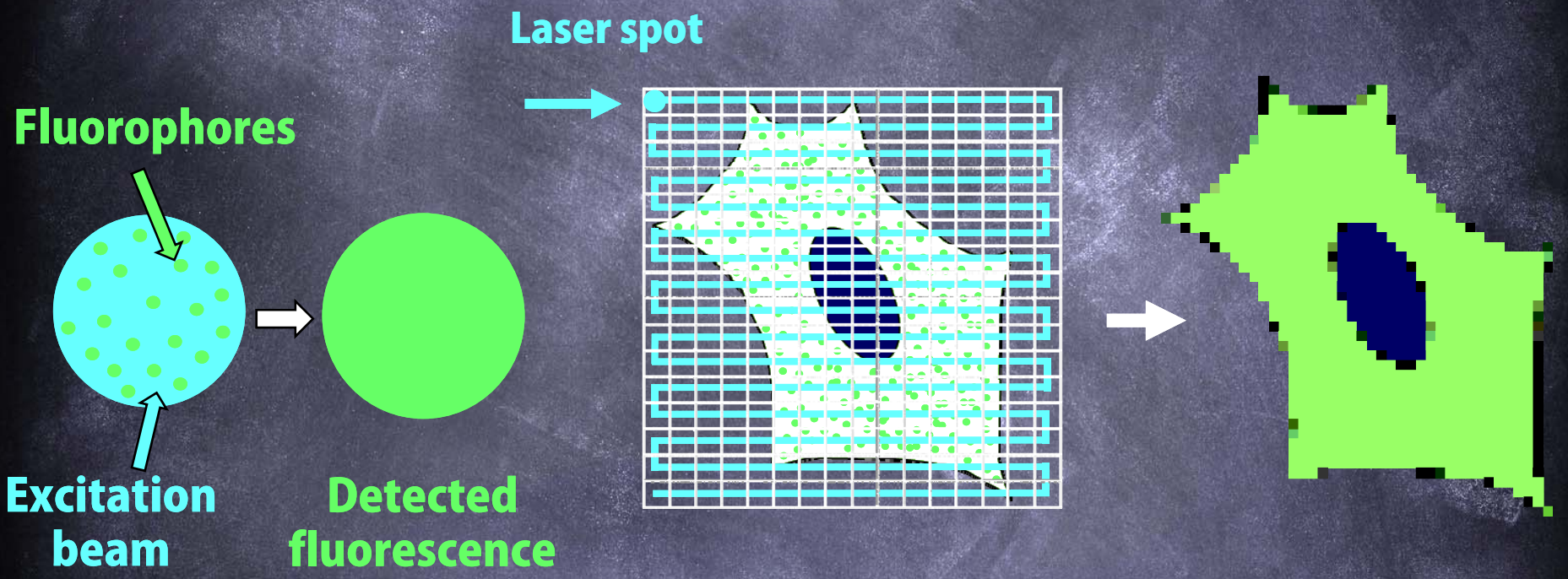




Role: rejection of photons from out-of-focus-light → The pinhole directly influences the thickness of optical slices.

CLSM: one single beam scanning

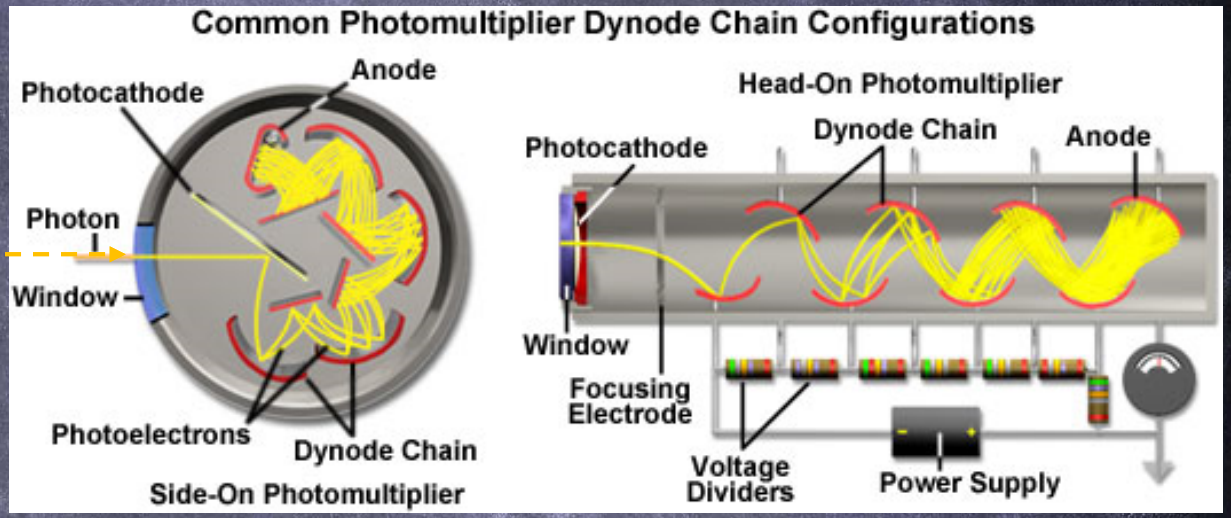
Excitation must be focused to a diffraction limited spot.
Laser= perfectly collimated and high power



Create image by scanning laser point-by-point over sample and recording intensity at each spot

PhotoMultiplier Tubes (PMT)

analog-to-digital converter



Gain varies with the voltage across the dynodes and the total number of dynodes : with typically 9 dynodes, gain of 4×10^6 can be achieved

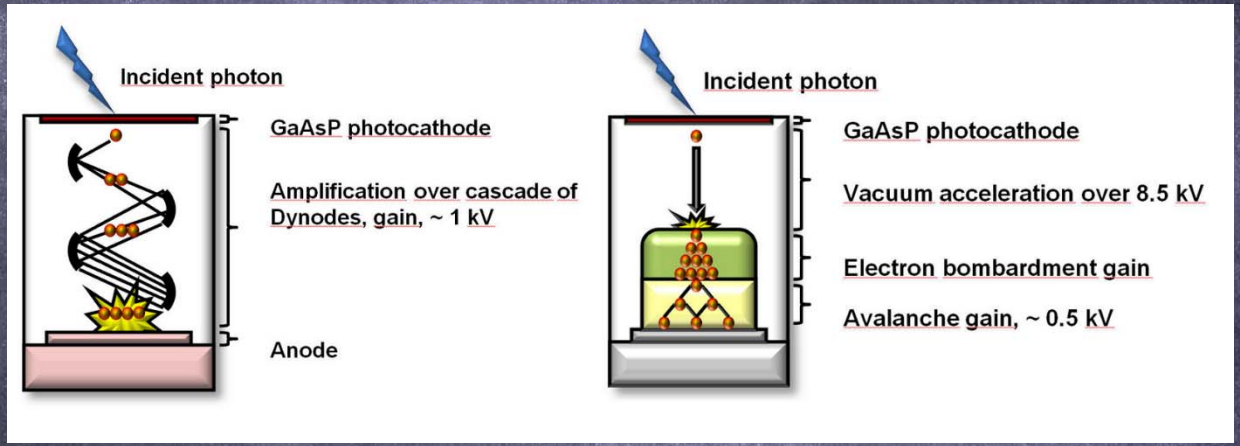
http://www.hi.helsinki.fi/amu/AMU%20Cf_tut/cf_tut_part1-7.htm

New generation of confocal detectors

PMT

HyD

(Hybrid Detector)



GaAsp: Gallium arsenide phosphide

- Next generation of detectors are significantly more sensitive.
- Particularly useful with photon limited samples & high speed imaging (ex. Live cell confocal with a resonant scanner)

Confocal Laser Scanning Microscopy

Pros:

(+) Very thin optical sectioning
(+) Multi-dimensional acquisitions, (+) Multi-labelings
(+) F-techniques

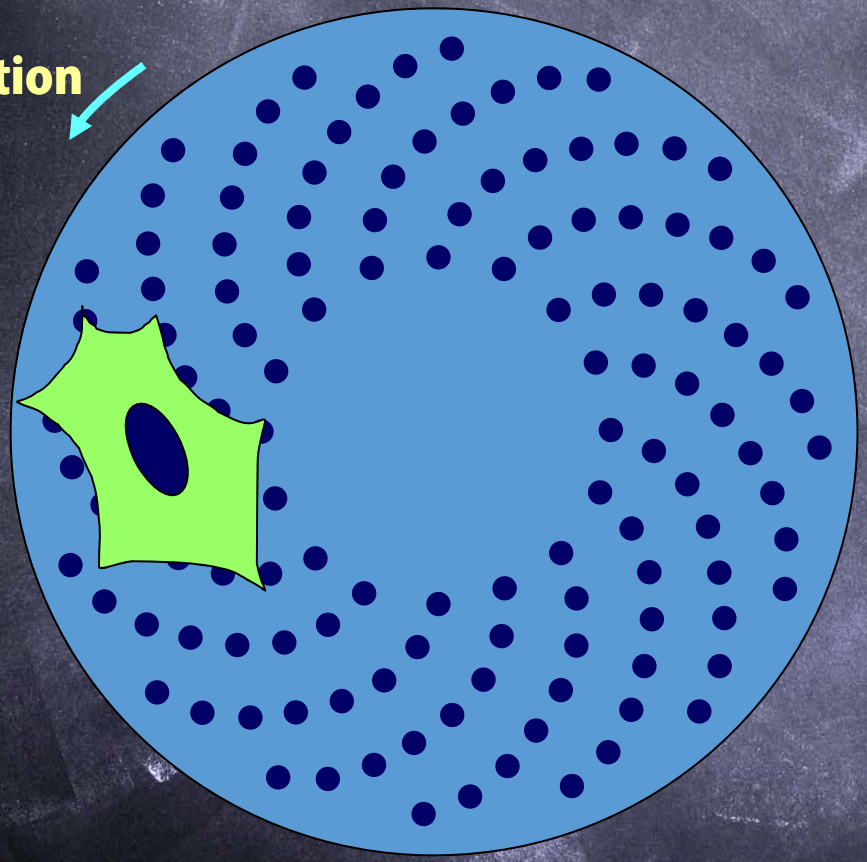
Cons:

(-) Small pinhole: large fluo. rejection, requires a strong signal of fluorescence
(-) Strong photobleaching, photo-cytotoxicity
(-) Slow acquisition
(-) Limited number of lasers (ex.)...
Now there is a solution: White laser (but expensive)

BiOimaging

Other beam scanning techniques

Multiple beam scanning : the Nipkow disk

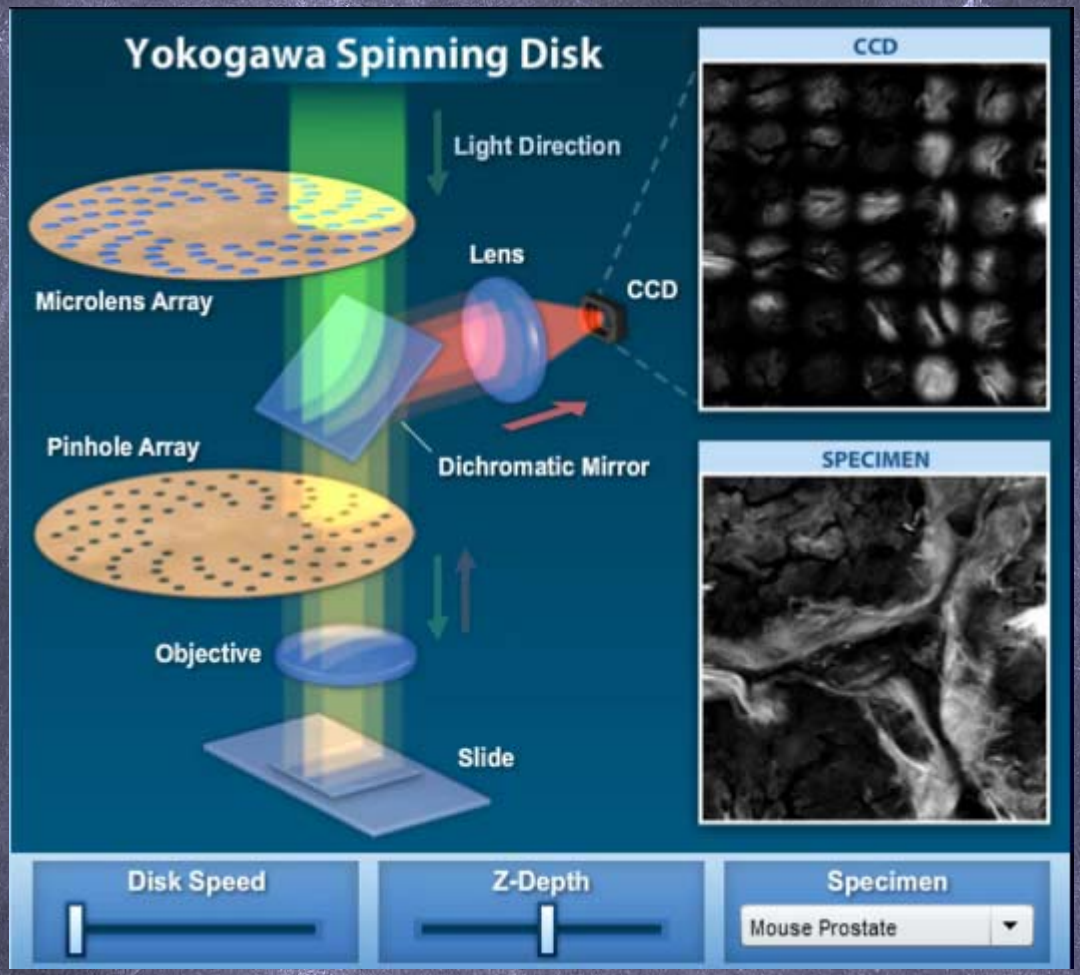


One way to increase the scanning speed is to increase the number of scanning spots.

The spinning disk with pinholes was introduced into a microscope by Mojmir Petran in 1968.

→ only strong signals could be imaged

Scanning with the Nipkow disk



The spinning disk fills the spaces between the holes to create a real-time confocal image

Scanning with the Nipkow disk

Pros:

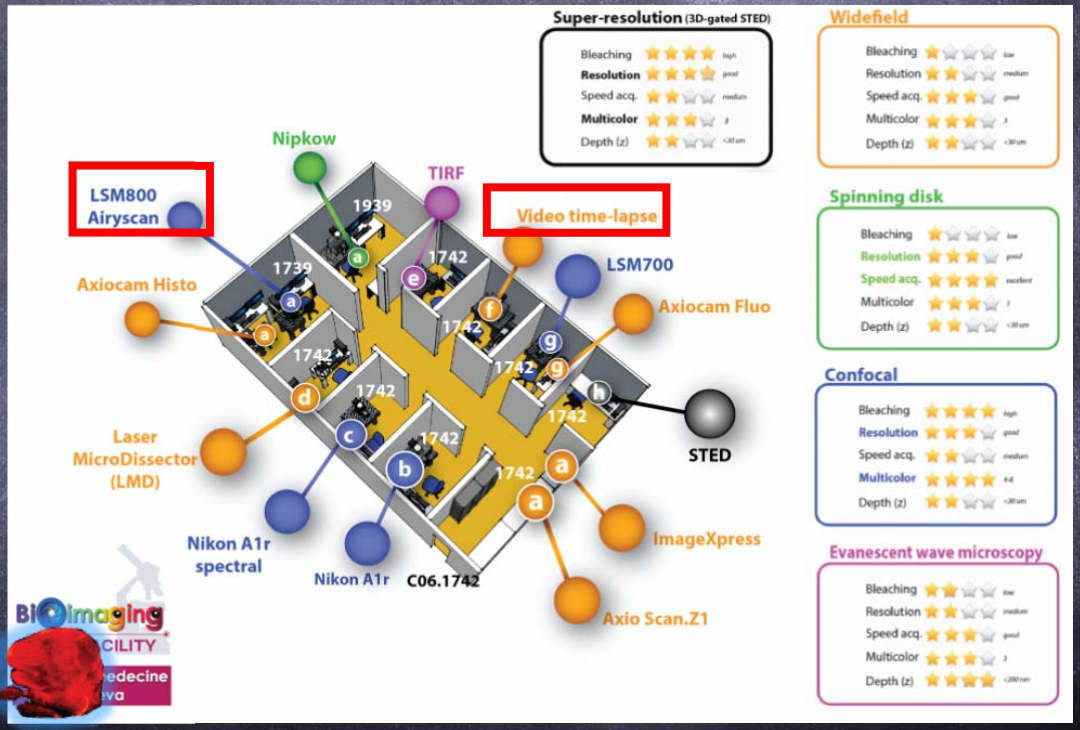
(+) Fast-live cell imaging (high frame rates)
(+) Increased illumination time per pixel
(+) Low photobleaching/ photodamage

Cons:

(-) Fixed pinholes'size (→ fixed optical section.)
(-) No optical zoom

Hands-on session

	14:00-15:20	15:20-15:30	15:30-17:00
Group 1 (Jessica, David, Manon, Olga)	**Video time-lapse** (Olivier)	*Break*	**LSM800** (François)
Group 2 (Axel, Marina, Karolina, Alix)	**LSM800** (François)	*Break*	**Video time-lapse** (Olivier)



Deconvolution (definition)

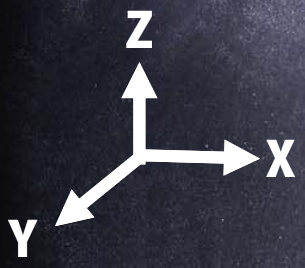
- A mathematical processing technique for improving the contrast and resolution of digital images captured in the microscope.
- Many different devolution algorithms available.
  (Richardson-Lucy,...)

(Images courtesy of A. Ferrand, University of Dundee, UK.)

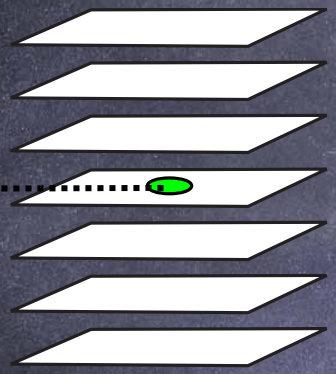
Principle of deconvolution

In a perfect world...

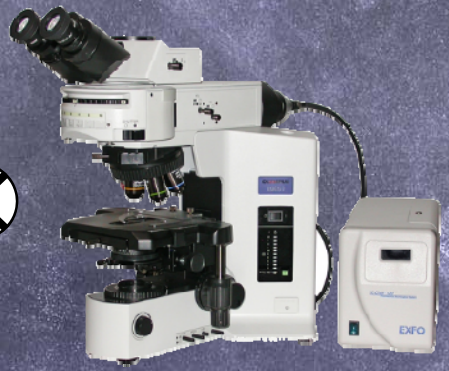
...in real life !

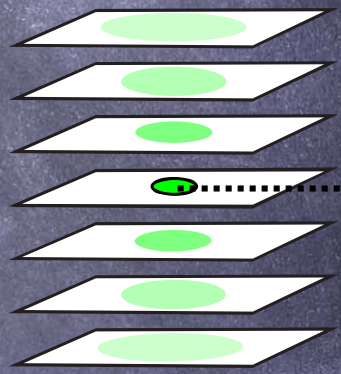


Sub-resolution fluorescent bead



Z-stack

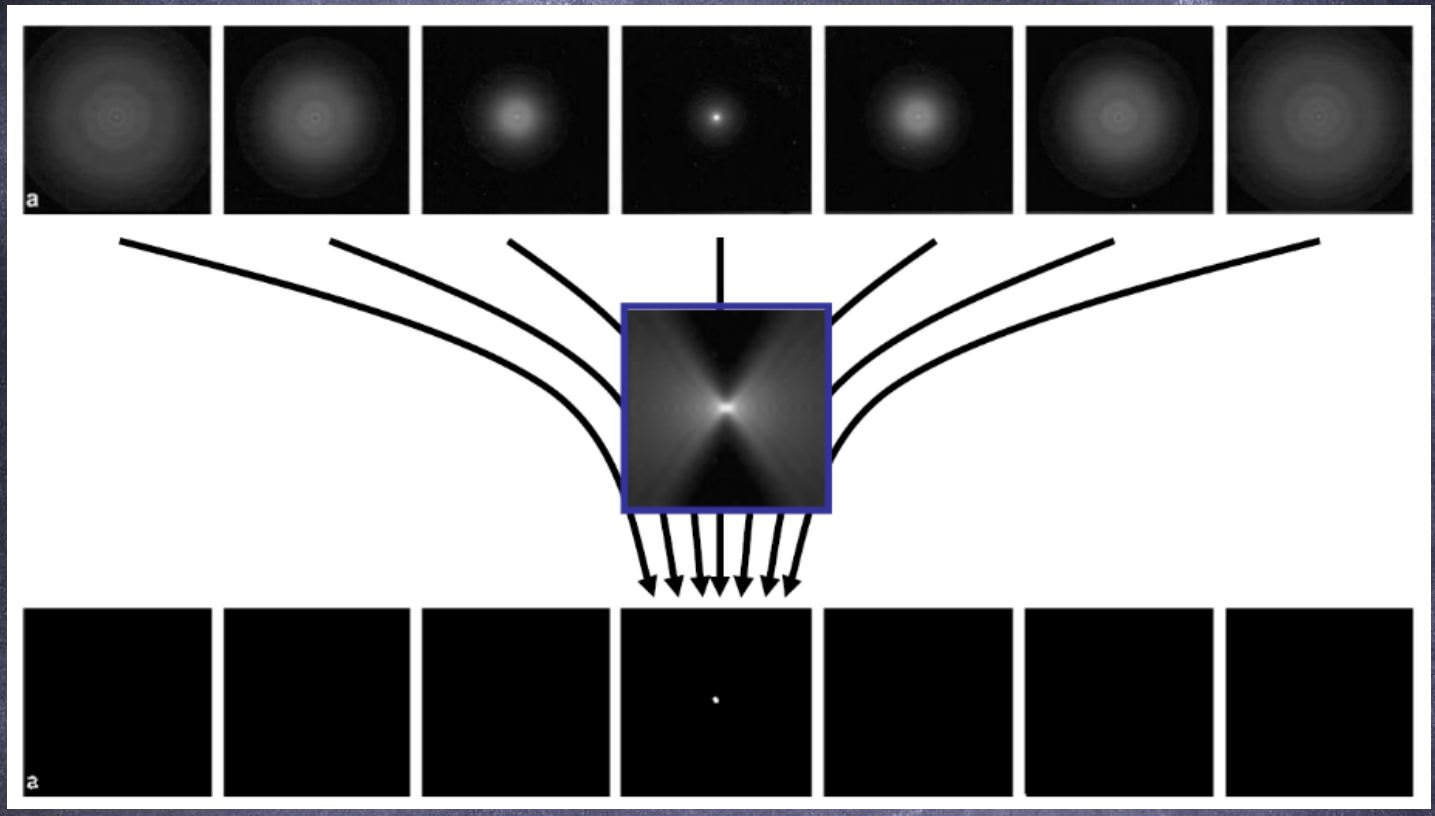




Z-stack

Sub-resolution fluorescent bead

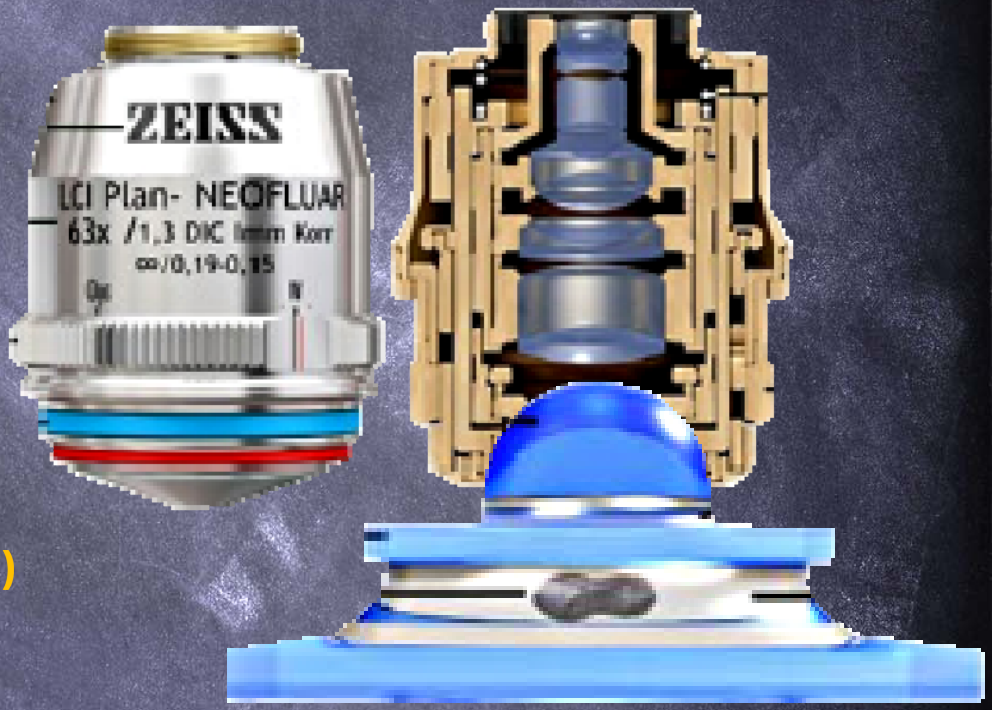
Measuring the PSF



Out-of-focus light is essential for deconvolution !!

➡ Don't crop PSF and image it in X, Y, Z, intensity (saturation)

Measuring the PSF



Needed information:

- Imaging mode (widefield, confocal, …)
- Magnification
- Numerical aperture
- Pixel dimensions (X,Y,Z)
- Refractive index immersion oil
- Refractive index mounting medium
- Thickness coverslip (water objectives)
- Emission wavelength
- Distance from coverslip

Rules for PSF and sample

❶ Clean sample, high-quality coverslips

❷ Z-spacing
→ See online Nyquist calculator

❸ Choice of objective
Water: least problems with refractive index mismatch
Oil: least affected by uneven coverslip thickness

❹ Immersion oil

❺ Avoid/ correct imaging aberrations
Uneven illumination/ camera sensitivity, sample movement, unstable light)

❻ Don't crop image and out-of-focus light in X, Y, Z, intensity