Microscopy Nanocourse 2015

PHYME department

February 3rd & 5th

UNIVERSITÉ DE GENÈVE
FACULTÉ DE MÉDECINE

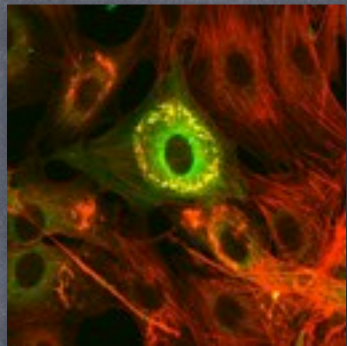
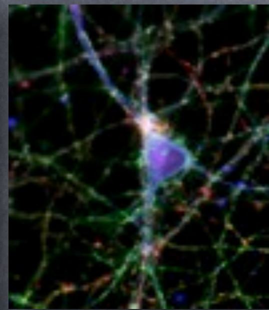
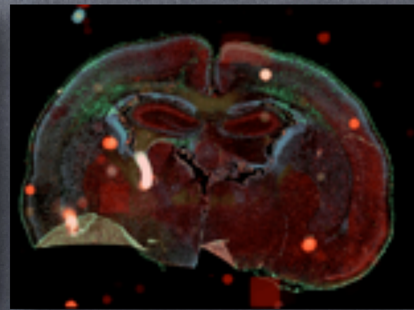
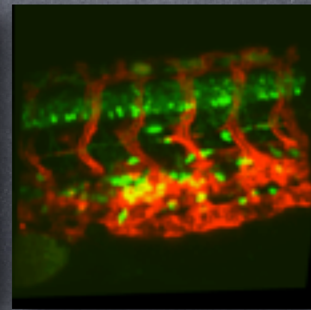
François PRODON
Olivier BRUN
Sergei STARTCHIK

Bioimaging core facility

Goal of the microscopy in *Cell Biology*

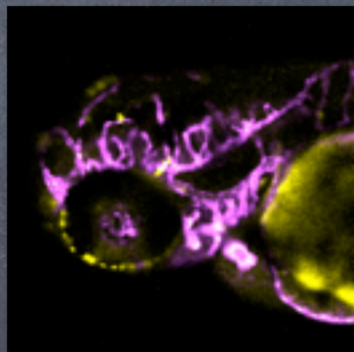
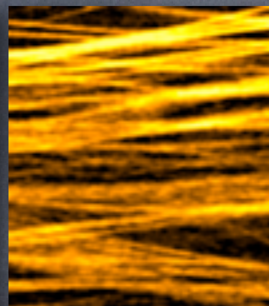
- **Localization**

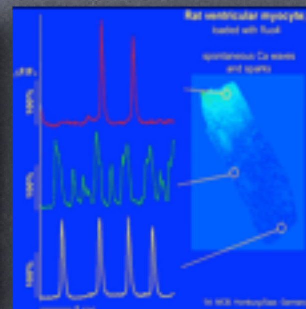
  (Co-localization, structure, large image, 3D,...)

- **Dynamics**

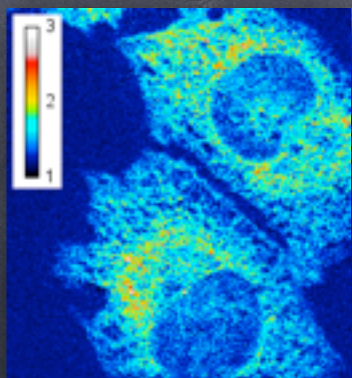
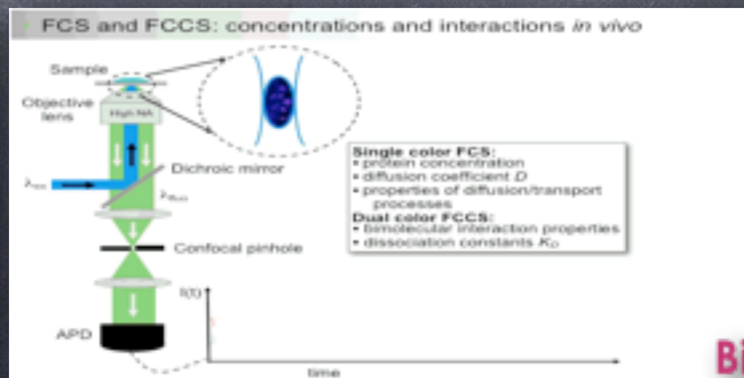
  (time-lapse, FRAP, tracking, Ca2+, ...)



- **Interactions**

  (FRET, FCCS, FLIM, ...)

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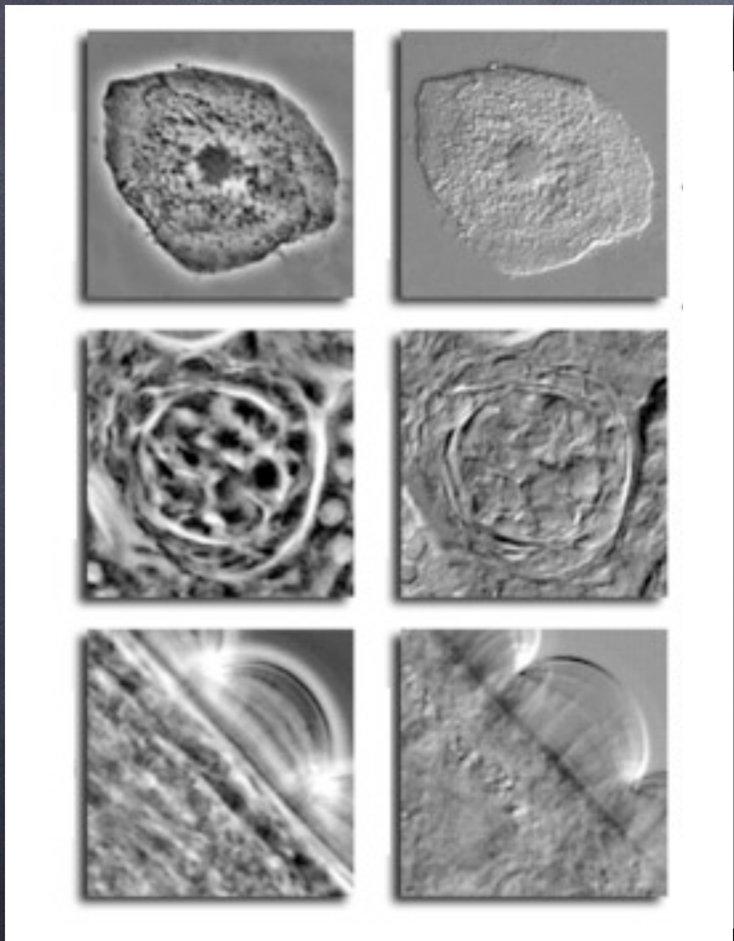
Transmitted light

Brightfield

Phase contrast DIC optics



Darkfield

(C. Sardet, Plancton, Ed. Ulmer)

http://www.olympusmicro.com/primer/techniques/dic/dicphasecomparison.html

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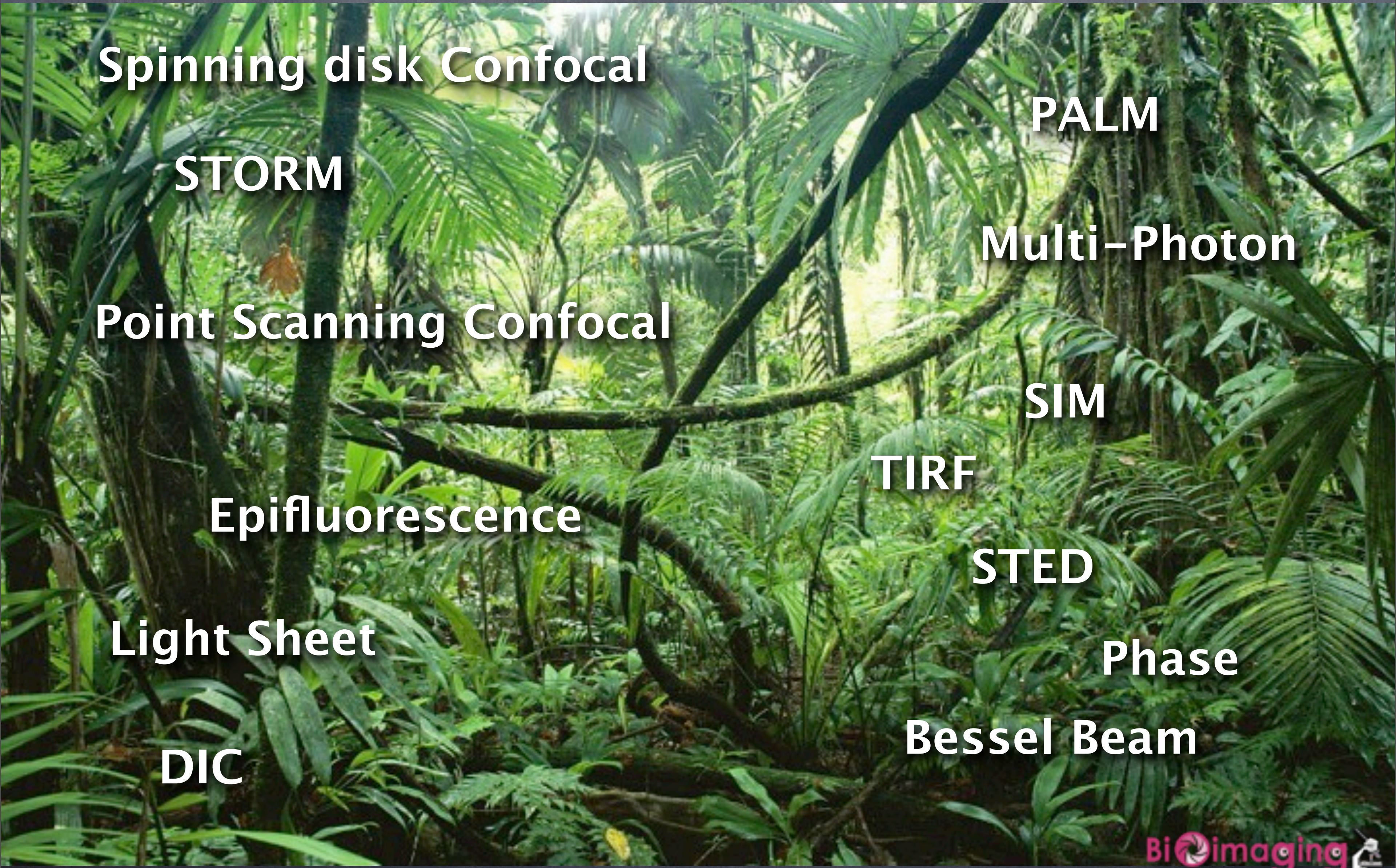
Choosing the right microscopy technique



Adapted from: iBiology (Ron Vale, Univ. of California, San Francisco)

Bioimaging core facility

Choosing the right microscopy technique

Adapted from: iBiology (Ron Vale, Univ. of California, San Francisco)

Did you know?

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

Bioimaging core facility

Did you know?

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

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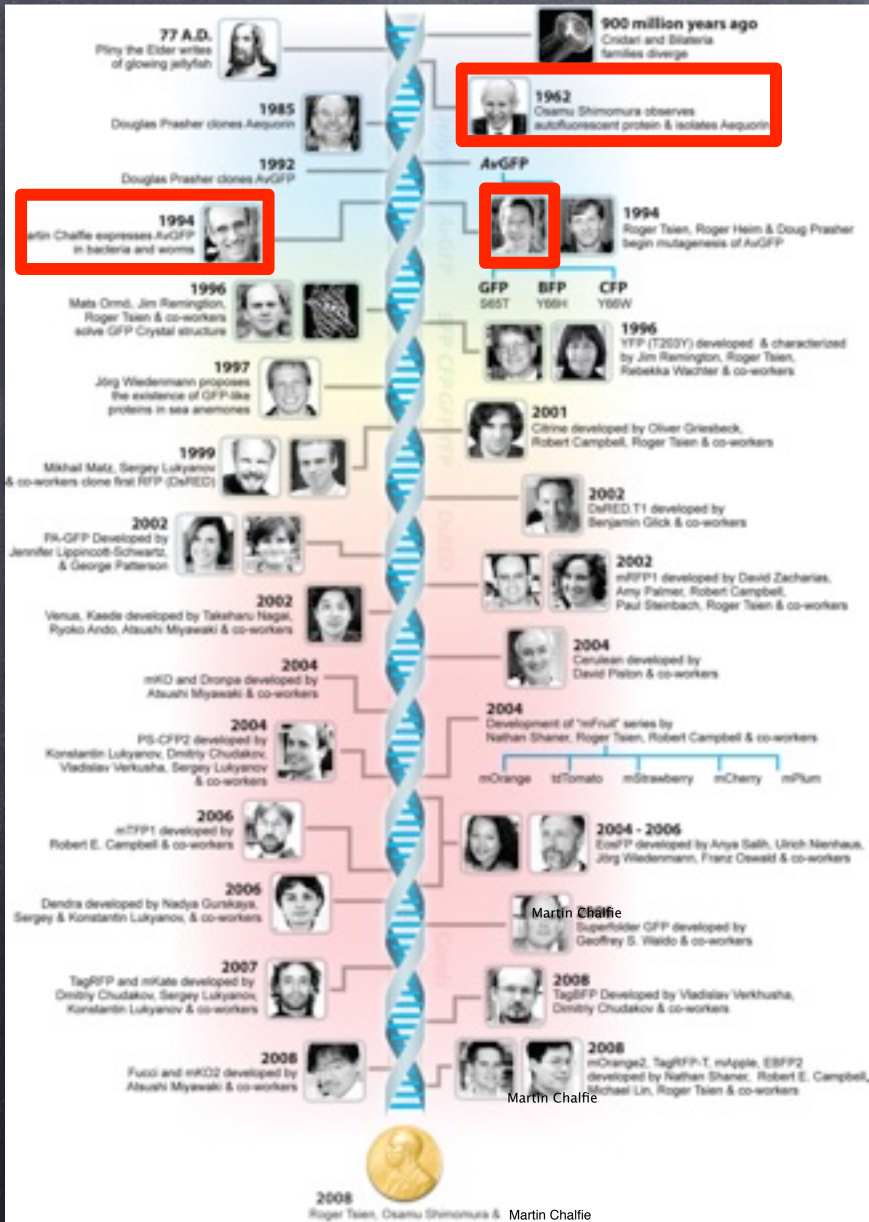
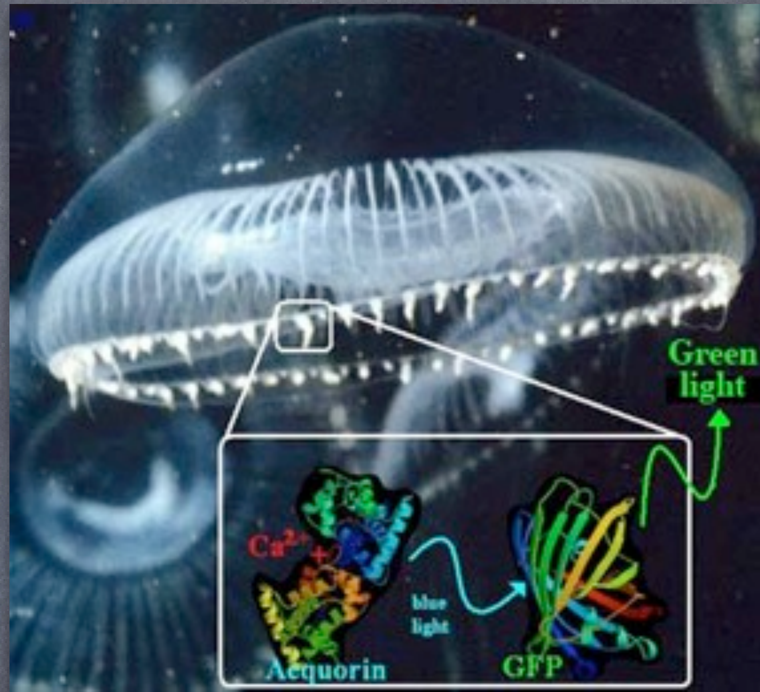
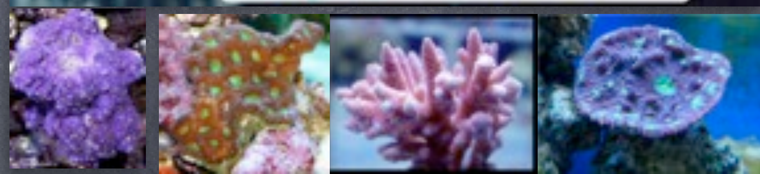
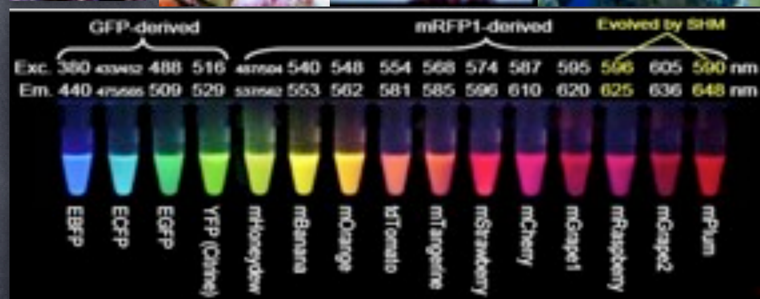
The GREEN revolution: GFP & variants









Day and Davidson (2009).
Chem. Soc. Rev., 38, 2887-2921

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Fluorescent dyes & proteins

Fluorescence:

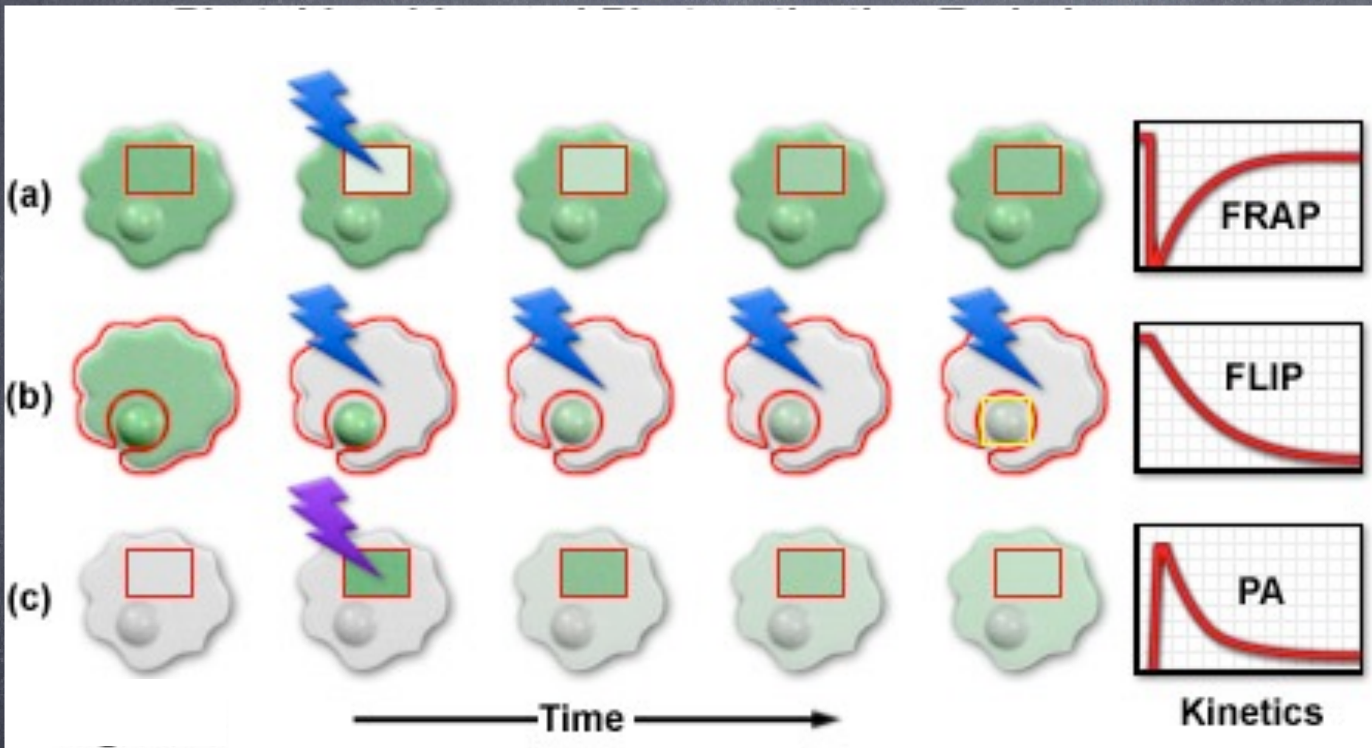
- Maximum excitation and emission wavelength
- Extinction coefficient
- Quantum yield
- Lifetime
- Stock shift

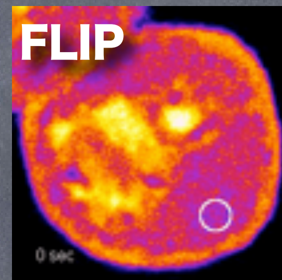
Different families of fluorophores:

- Fluorescent proteins
- Non-protein organic fluorophores
- Reactive and conjugated dyes

Photobleaching & Photoactivation techniques

(tools for measuring protein dynamics)





(FRAP)....... Fluorescence Recovery After Photobleaching
(FLIP)......... Fluorescence Loss in Photobleaching
(PA) .... PhotoActivation

Adapted from: http://www.olympusmicro.com/primer/techniques/confocal/applications/opticalhighlighters.html

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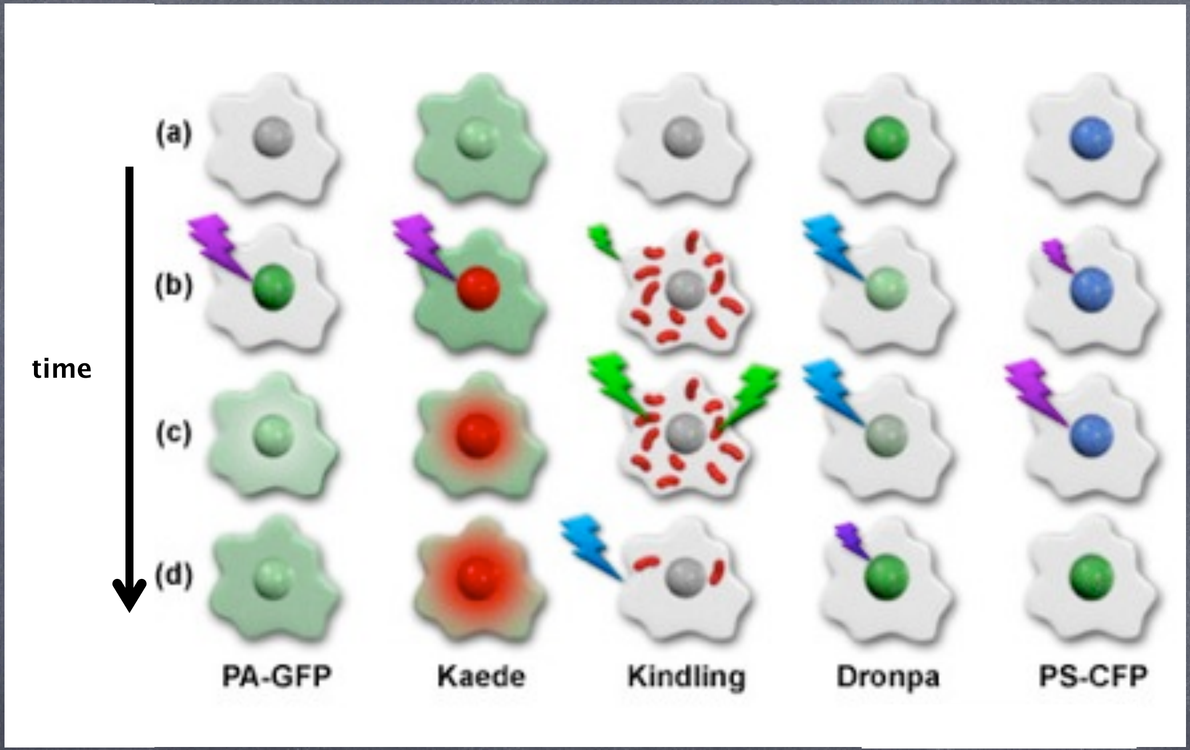
Photoconversion reactions in Optical Highlighters

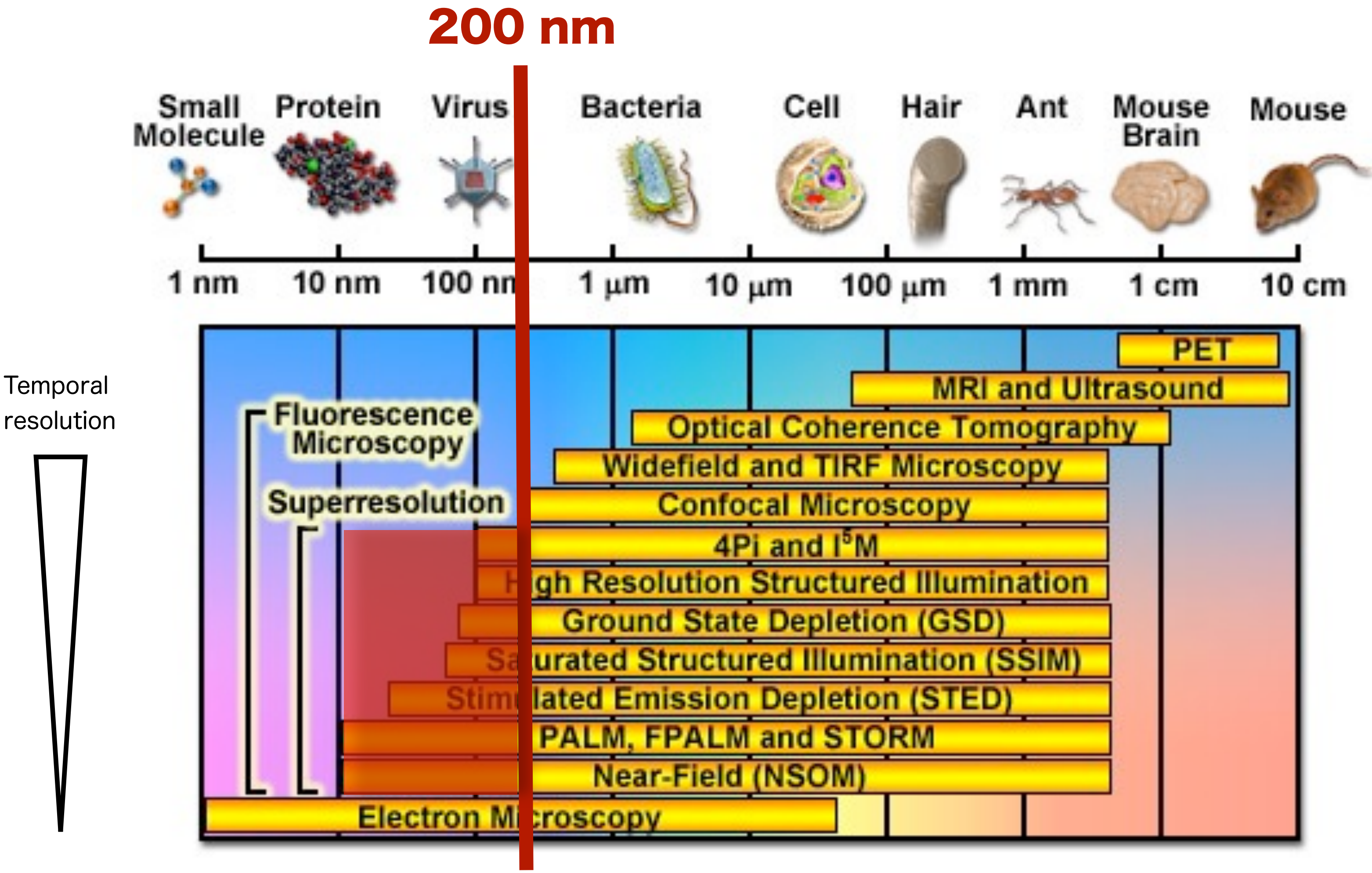


Palette of PA-FPs:

- -1)..... Photoactivated (PA-GFP, PA-mCherry1)
- -2)..... Photoconverted (Kaede, Kindling, mEos2, Dendra2, ···)
- -3)..... Photoswitching (Dronpa, ···)

Adapted from: http://www.olympusmicro.com/primer/techniques/confocal/applications/opticalhighlighters.html

Spatial and temporal resolution of biological imaging techniques



(from Zeiss at: http://zeiss-ampus.magnet.fsu.edu/articles/superresolution/introduction.html)

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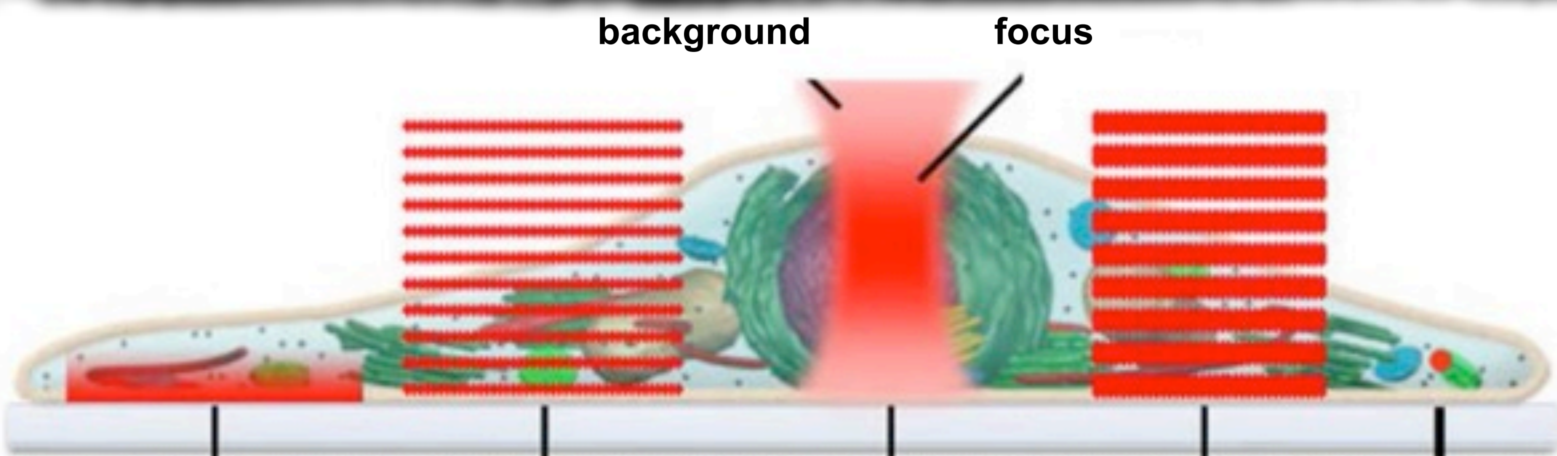
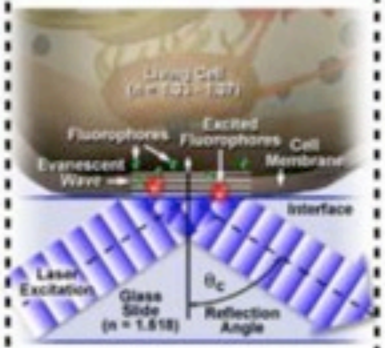
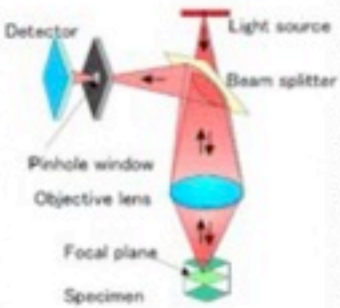
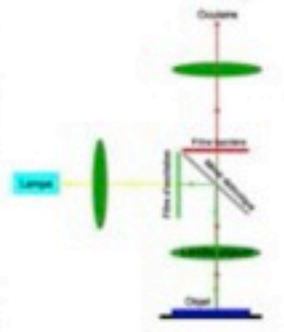
What type of technology is available?

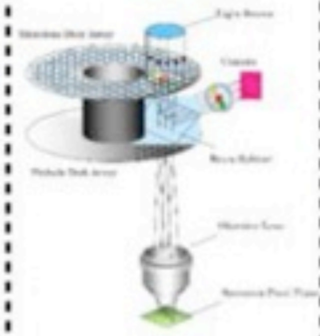


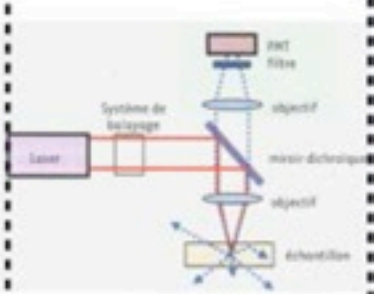
Evanescent Wave Microscopy (TIRFM*)	**Confocal Laser Scanning Microscopy (CLSM)**	**Fluorescence Widefield Microscopy**	**Nipkow Spinning Disk Confocal Microscopy**	**Two-Photon Microscopy**











(*TIRFM :Total Internal Reflection Fluorescence Microscopy)

+ Laser Dissection Microscope
+ automated systems (Mirax/ slide scanner, ImageXpress/plate reader,….)

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Comparison of spatial resolutions

	R(X,Y) **Lateral** Resolution	R(X,Z) **Axial** Resolution
WIDEFIELD	**0.62λ/NA** 195 nm 273 nm	**2λn/NA2** 683 nm 954 nm
CONFOCAL Microscopy	**0.4λ/NA** 126 nm 176 nm	**1.4λn/NA2** 478 nm 668 nm
TWO-PHOTON Microscopy	**0.7λ/NA** 221 nm 308 nm	**2.3λn/NA2** 786 nm 1097 nm

400 nm < λ < 700 nm

Examples (63x/1.4 objective, n= 1.516) :

1- Alexa Fluor 350: Ex./Em.= (346/ 442)
2- Alexa Fluor 594: Ex./Em.= (590/ 617)

(always based on the EMISSION wavelength !)

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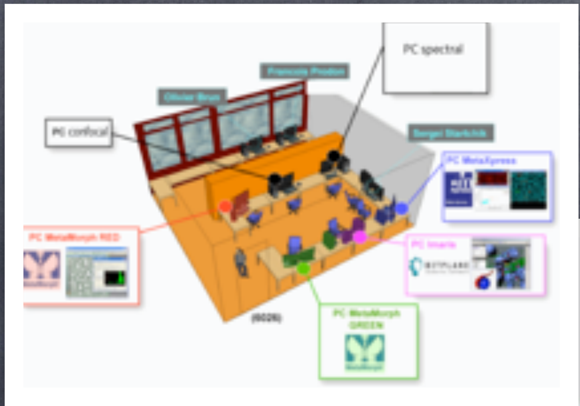
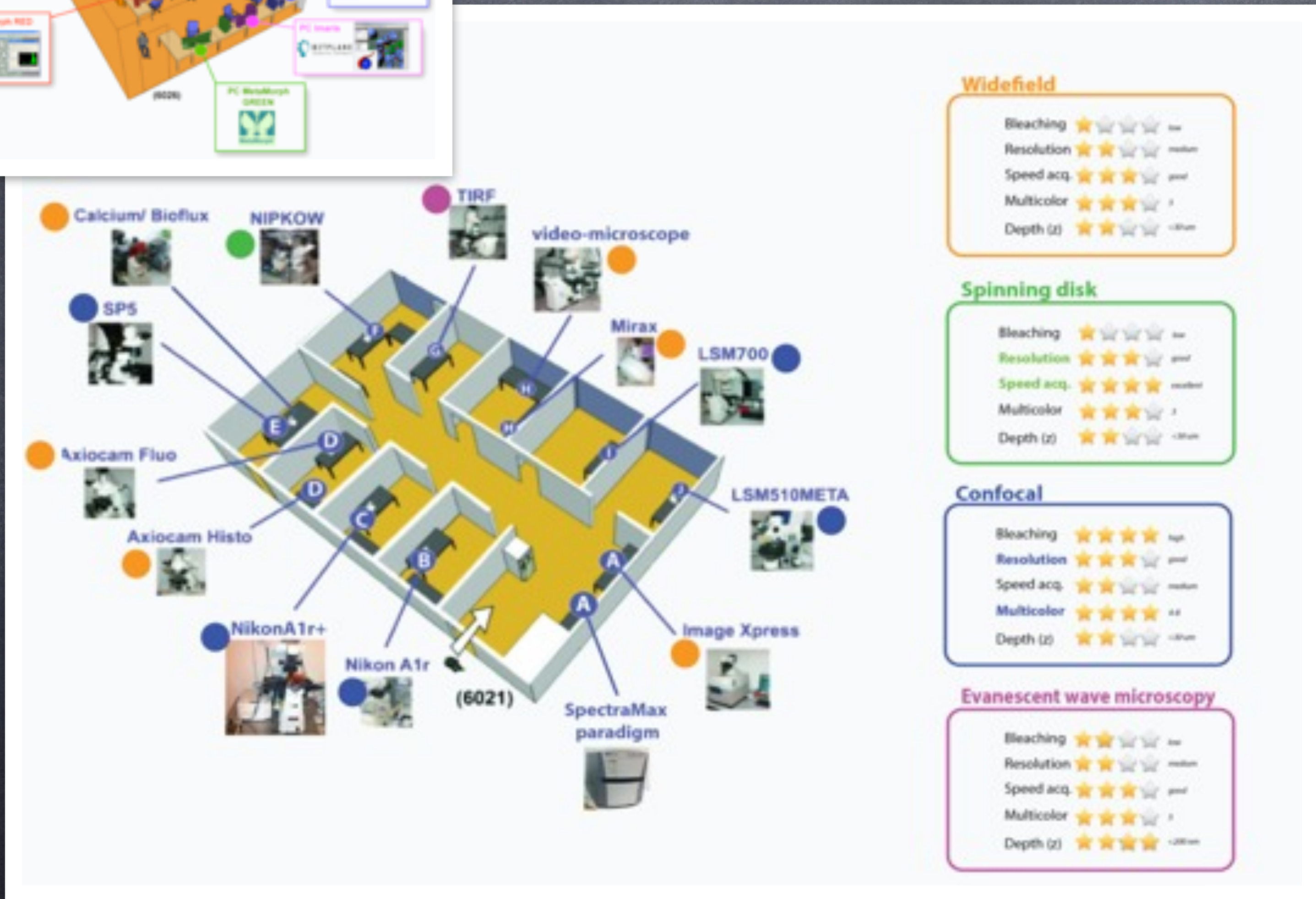
What type of machine is available?





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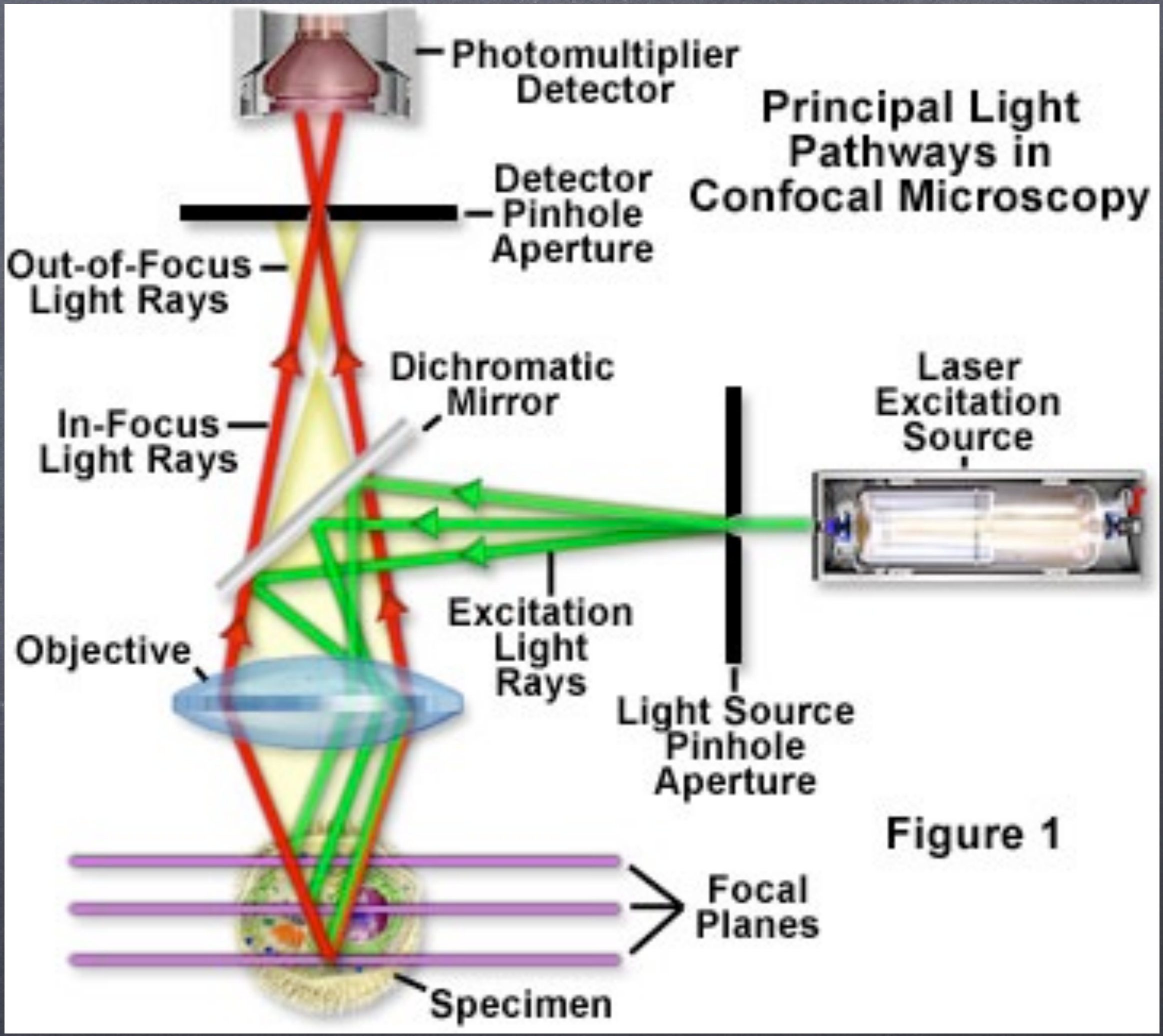
Point scanning confocal microscope



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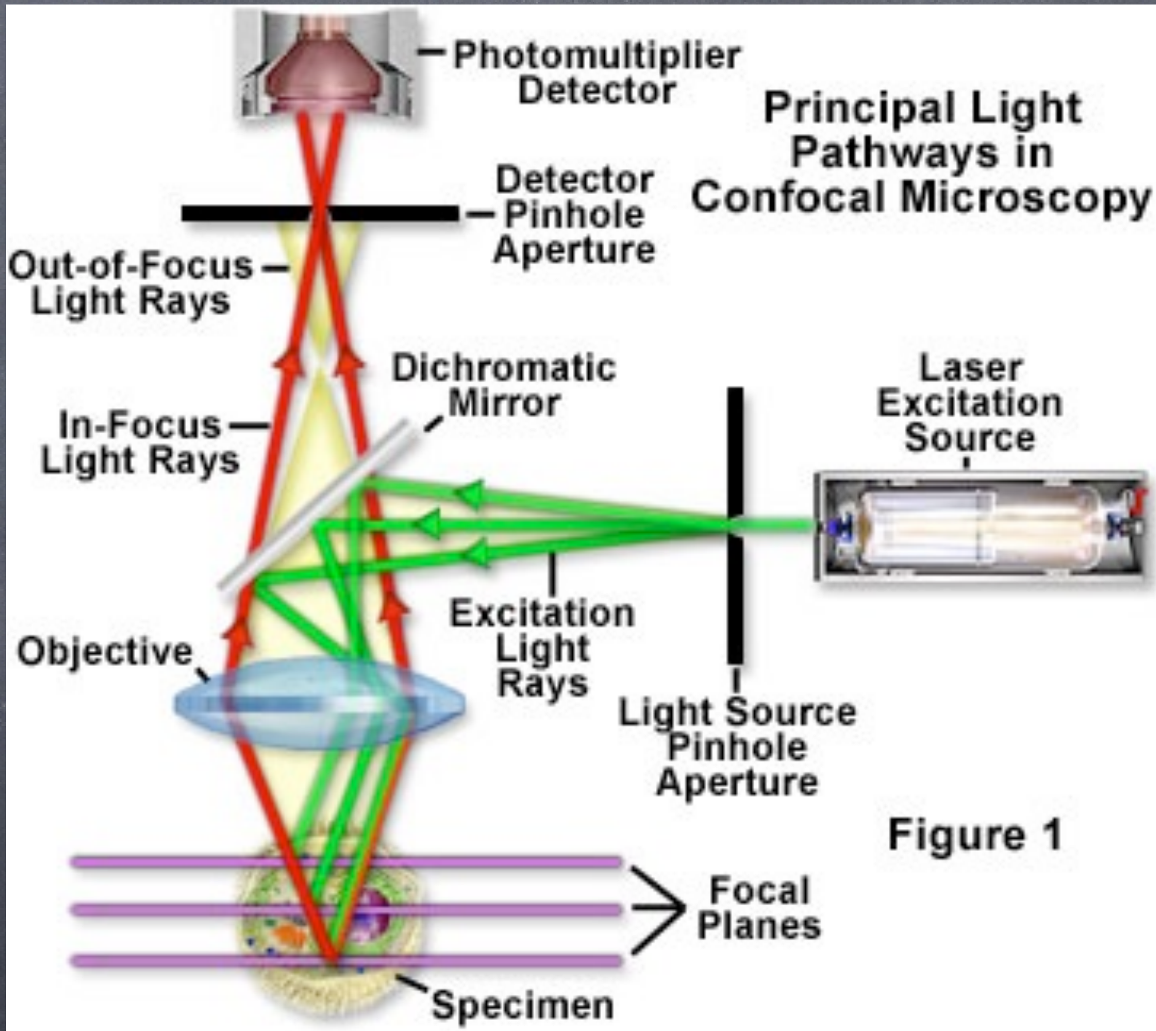
Point scanning confocal microscope

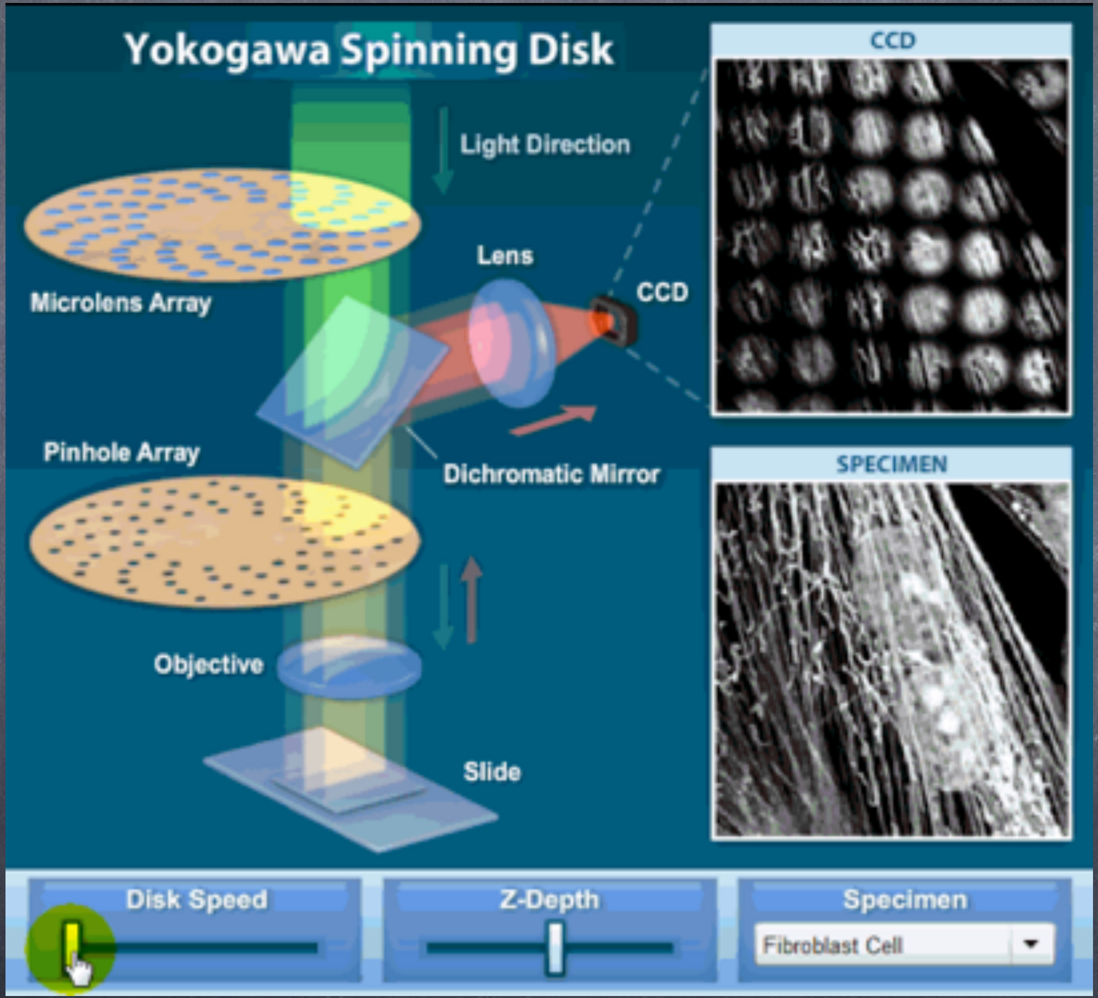
Confocal microscope drawbacks:

- Scans excitation spot point-by-point to build up image
- Slow (~1 sec to acquire an image)
- Low light efficiency (due to use of PMT as detector)
- Solution: use multiple pinholes and a camera



A solution: spinning disk confocal (Nipkow)

- -1) Image with many pinholes at once, so fast
- -2) Use CCD as detector, so high light efficiency



http://zeiss-campus.magnet.fsu.edu/tutorials/spinningdisk/yokogawa/indexflash.html

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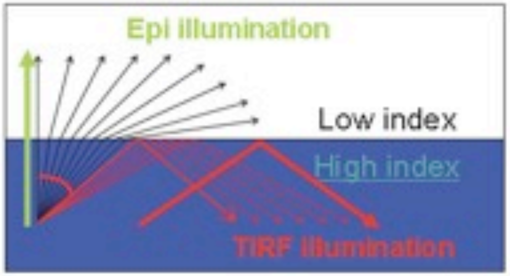
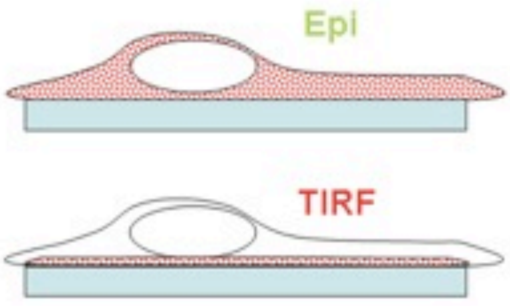
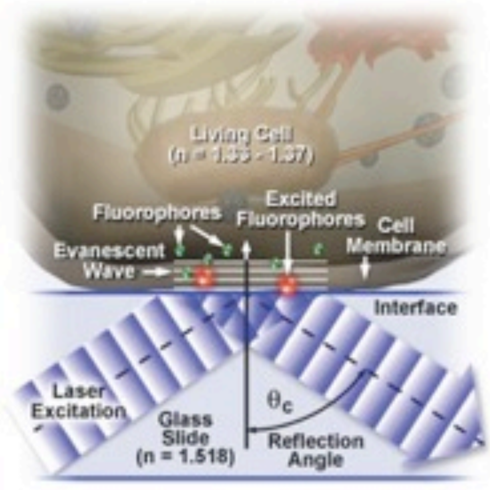
How does TIRF microscopy work?

(Total Internal Reflection Fluorescence Microscopy)







In TIRF, only those fluorophore molecules that are near the cover glass surface are excited

When the incident angle is greater than the critical value, the laser light undergoes **total internal reflection** from the glass-water interface.

http://www.olympusmicro.com/primer/techniques/fluorescence/tirf/tirfhome.html

Some key TIRF formulas

But electric and magnetic fields **cannot** be discontinuous at a boundary, therefore:

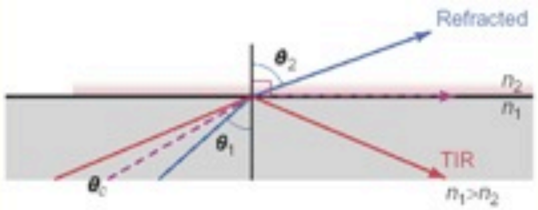


Fig. I. TRENDS in Cell Biology

Snell's law:

$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

θ = angle of incidence, n = refractive index

Critical angle:

At the critical angle, θ_c, $\theta_2 = 90°$; $\sin 90° = 1$;

$$n_1 \sin\theta_c = n_2$$

$$\theta_c = \sin^{-1}(n_2/n_1)$$

If $n_1 = 1.515$ and $n_2 = 1.36$, $\theta_c = \sin^{-1}\left(\frac{1.36}{1.515}\right) = 63.85°$

Evanescent field:

$$I_z = I_0 \exp^{-z/d_p}$$

$$d_p = \frac{\lambda}{4\pi\sqrt{n_1^2 \sin^2\theta_1 - n_2^2}}$$

I = intensity, z = distance, λ = wavelength, d_p = penetration depth

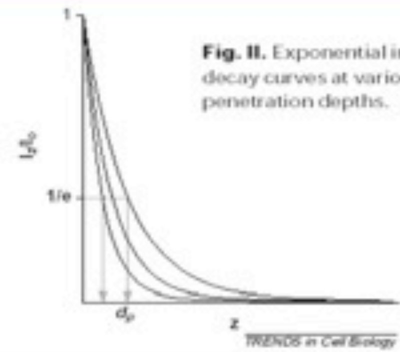
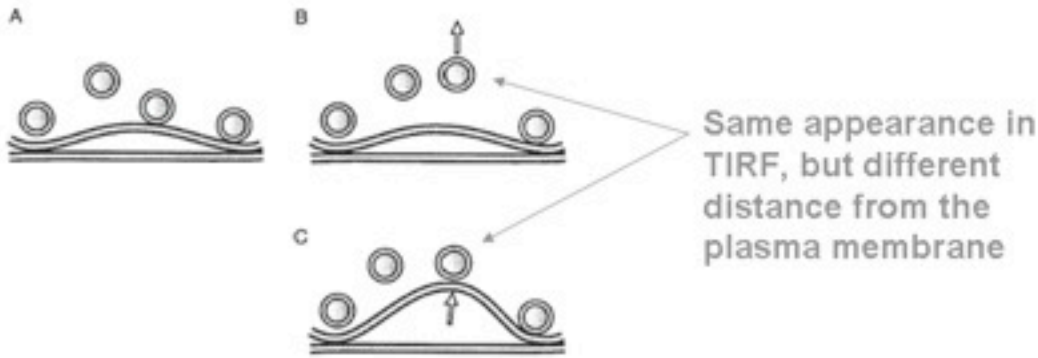


Fig. II. Exponential intensity decay curves at various penetration depths.

Toomre and Manstein (2001) Trends in Cell Biol 11:298

Ambiguities in TIRF data interpretation



Vesicles of equal brightness are **not necessarily** located at equal distances from the plasma membrane, because:

1. The plane of reference for evanescent-wave distance measurements is the **glass/water interface** – at which the light is reflected – rather than the plasma membrane.

2. The value of the penetration depth of the evanescent wave, determined in aqueous solution, is modified by the **higher-refractive index medium of the cytoplasm**.

Oheim et al (1999) Eur Biophys J 28: 91–101

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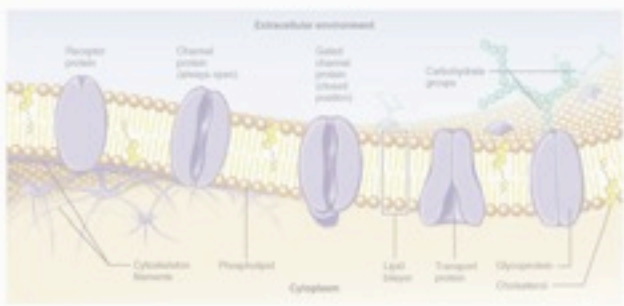
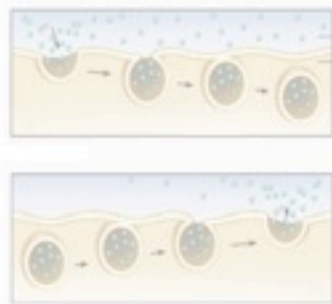
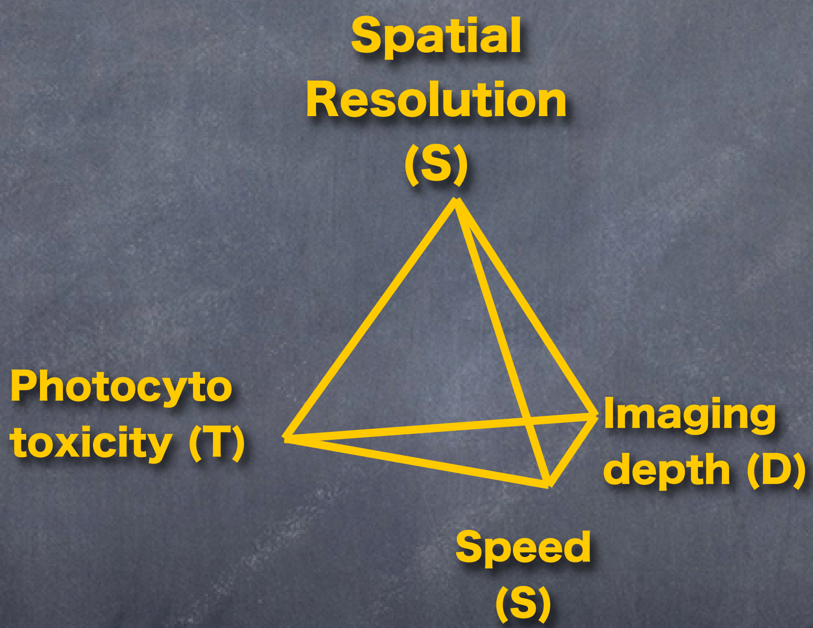
Summary I-III: features of TIRF imaging

- TIRF microscopy is a powerful tool for studying membrane-associated signaling with **supra-optical resolution** (optical sections thinner than in confocal microscopy);
- **Single molecules** can be visualized and tracked with time-lapse TIRF, and their **conformational changes** as well as **ion flux** can be detected with TIRF-FRAP;
- TIRF imaging is largely **limited to the basal plasma membrane**, although some groups have reported first studies in which apical membrane was also visualized;
- Ambiguities in TIRF image interpretation may arise from complex membrane topology, and **light scattering on organelles** may compromise the TIR effect.



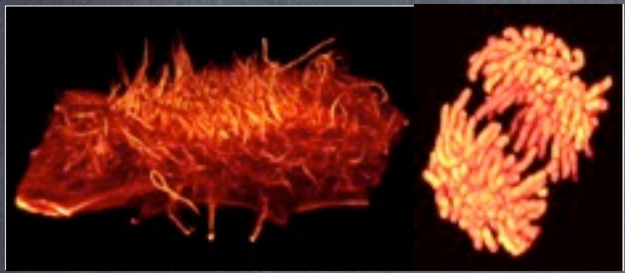


Performance Metrics of new imaging technologies (E. Betzig)



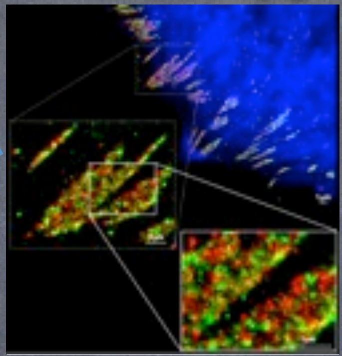
Performance Metrics of new imaging technologies (E. Betzig)

2D structured illumination

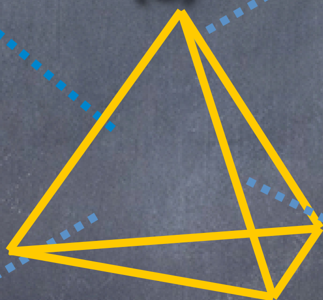


R: 60-100 nm (xy)
S: 1-10 fps
T: 20-200 frames
D: 0.05- 3.0 um

Superresolution localization microscopy

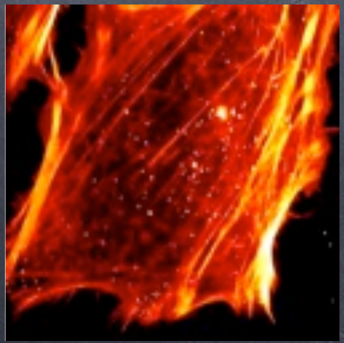


R: 30*30*50nm (xyz)
S: 0.1-5.0 fps
T: N.A. (fixed)
D: 5-50 um

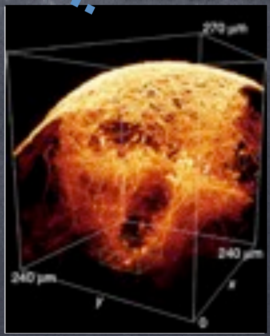


Lattice light sheet microscopy

R: 150-240*240*280-370nm (xyz)
S: 50-1000 planes/sec
T: 50- inf. volumes
D: 20-100 um



Two-photon/ confocal adaptive microscopy



R: 300*300*1000nm (xyz)
S: ~ 1 fps
T: N.A. (fixed)
D: ~300-600 um

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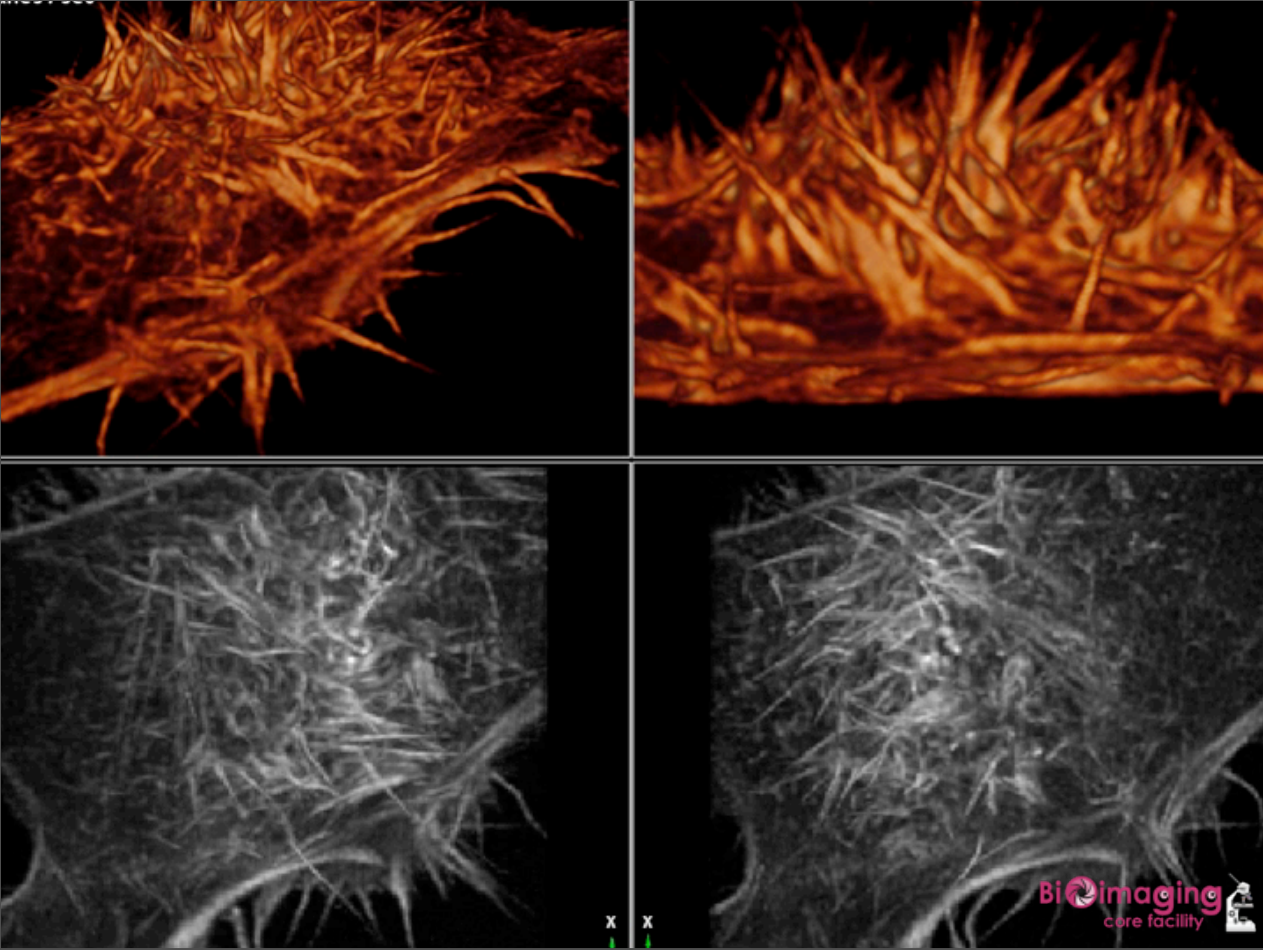
x
x
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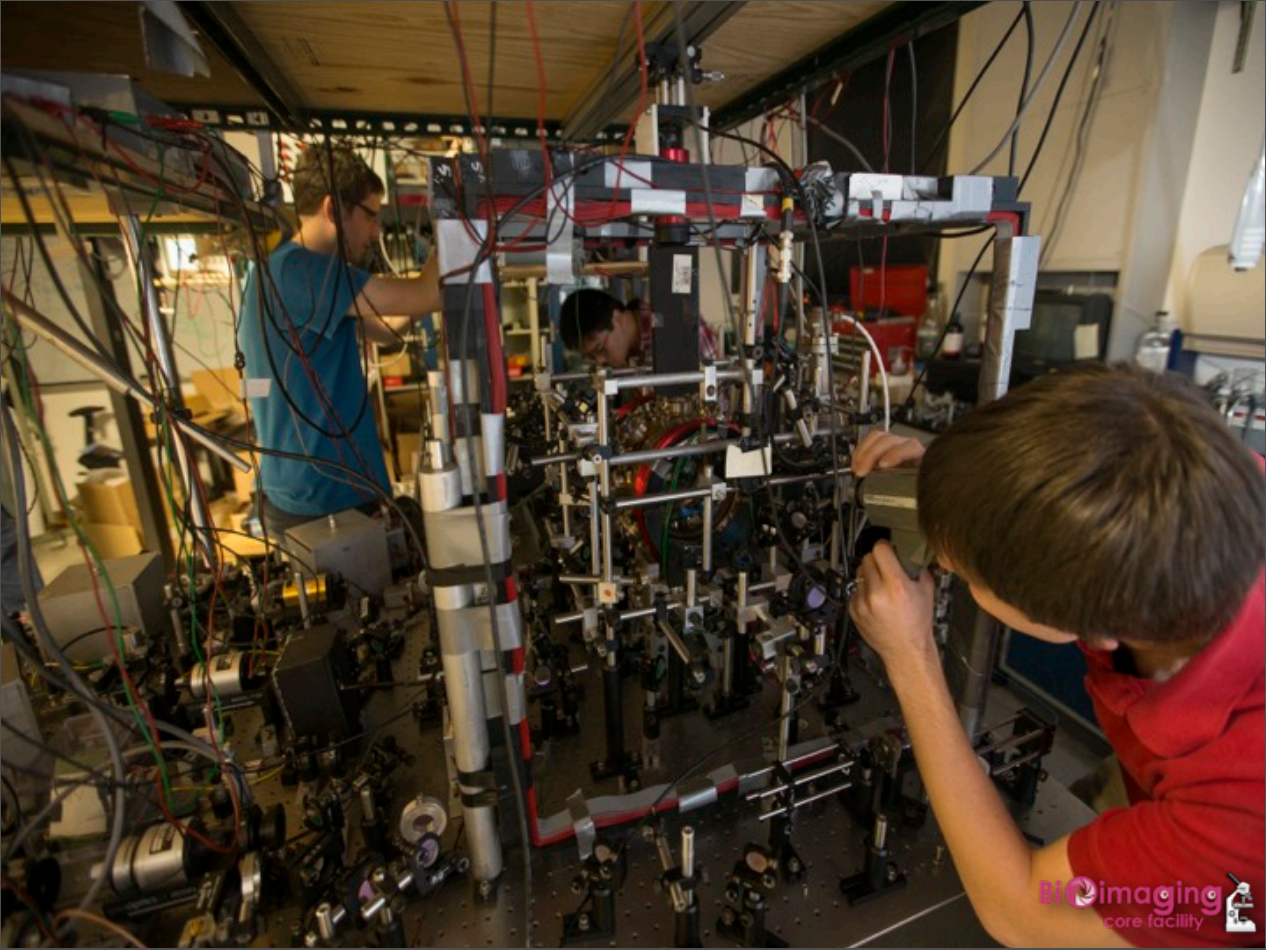
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EDUCATION

http://www.ibiology.org/ibioseminars.html

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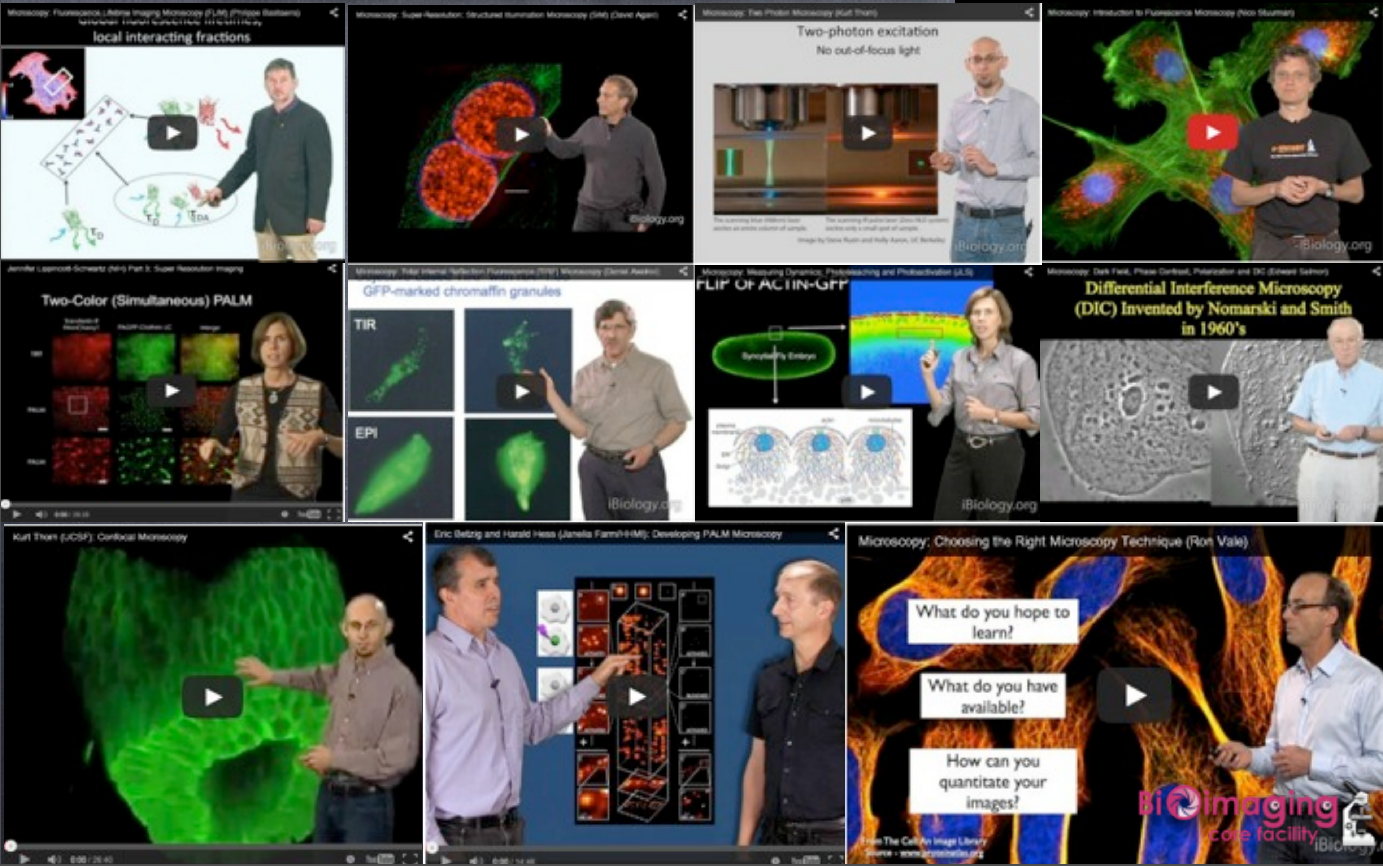

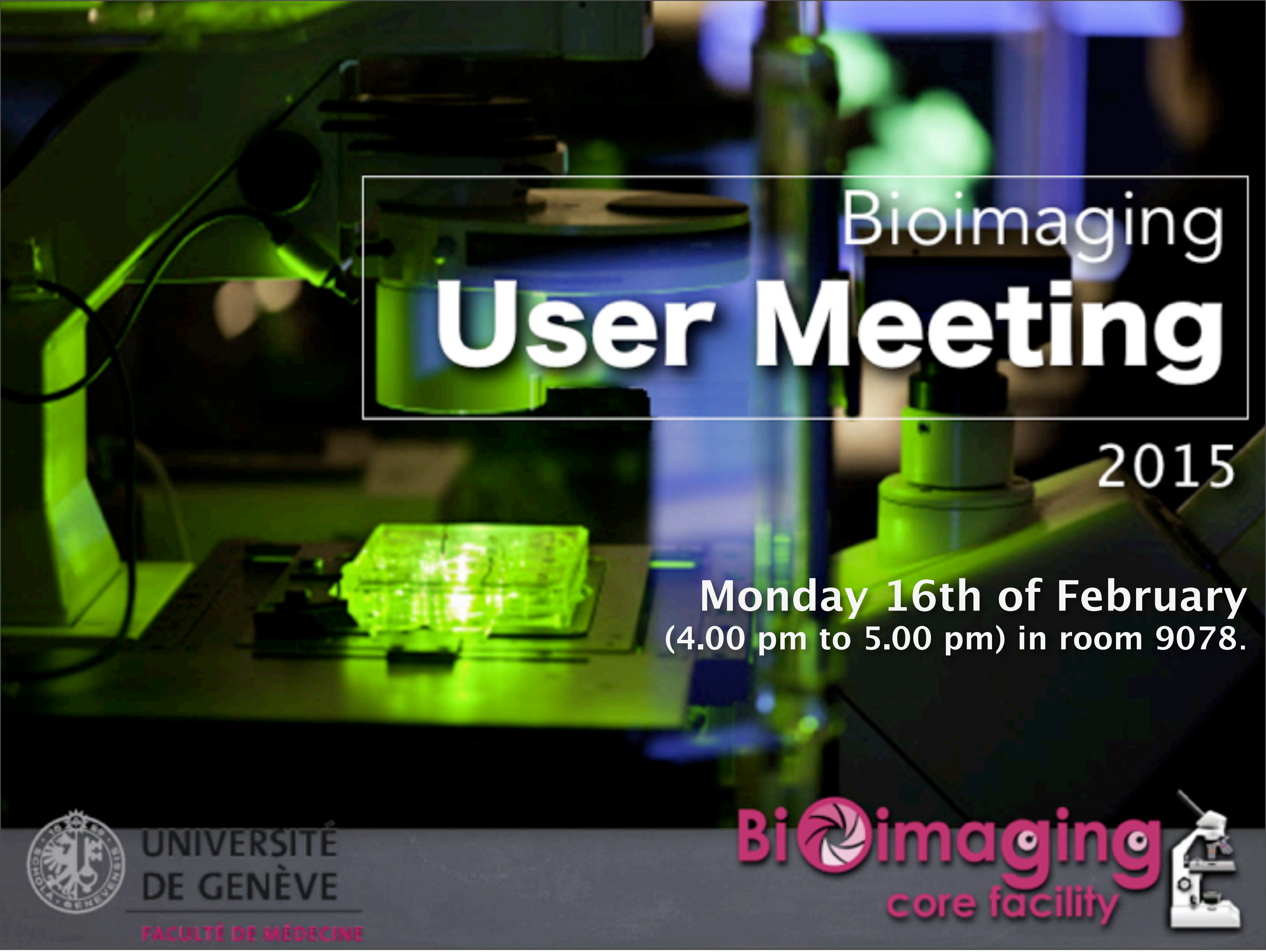
Bioimaging
User Meeting
2015
Monday 16th of February
(4.00 pm to 5.00 pm) in room 9078.
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Organization of four workshops

	Group 1	Group 2	Group 3	Group 4
Spinning disk confocal (Olivier)	13.30-14.15	14.15-15.00	16.00-16.45	15.15-16.00
Point scanning confocal (Byung Ho, François)	14.15-14.45	13.30-14.15	15.15-16.00	16.00-16.45
TIRF (Patricia, Daniele)	15.15-16.00	16.00-16.45	13.30-14.15	14.15-15.00
Image processing (Sergei)	16.00-16.45	15.15-16.00	14.15-15.00	13.30-14.15

15.00-15.15 coffee break

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