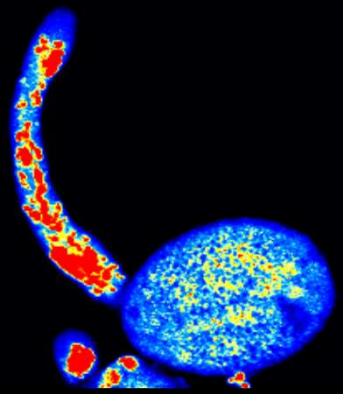
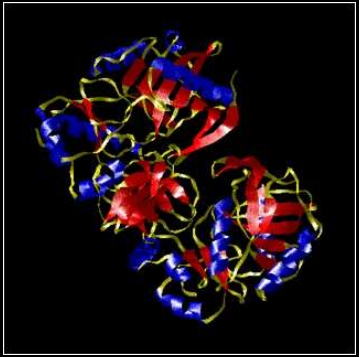
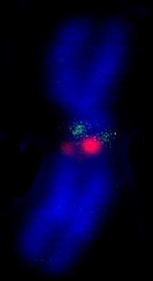
Methods for mapping and quantification of intracellular 2nd messengers



Rui Malhó
r.malho@fc.ul.pt
http://webpages.fc.ul.pt/~rmmalho





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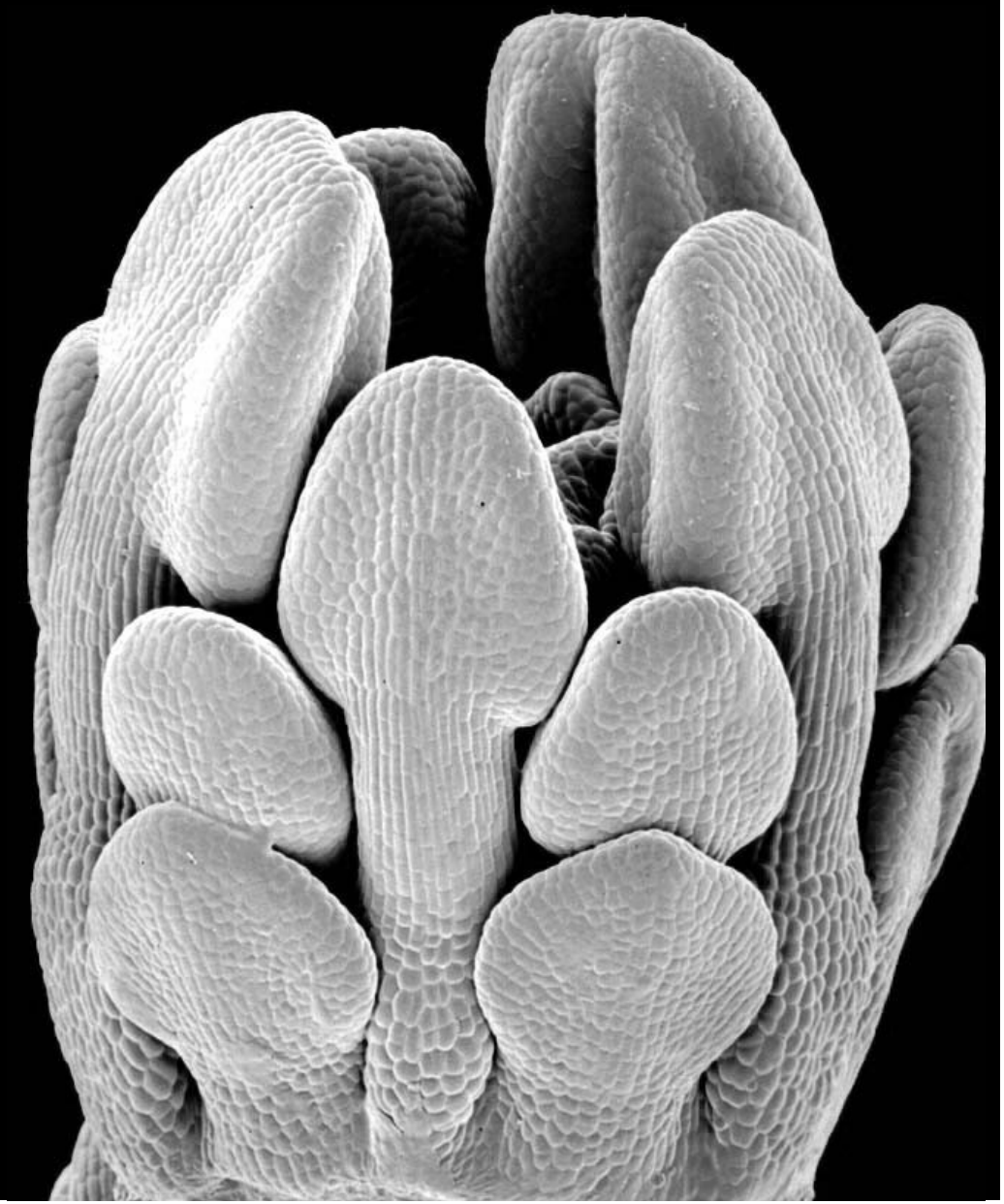
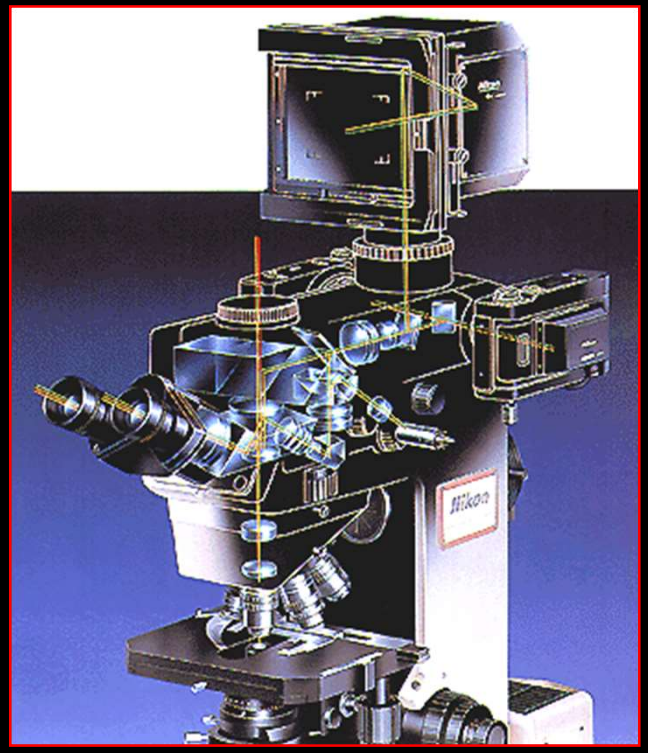
Microscopy Techniques:

- Optical Microscopy
  - bright field
  - dark field
  - phase contrast
  - DIC
- Fluorescence Microscopy
  - Video/Digital
  - Confocal
  - Conventional
- Electron Microscopy
  - Transmission
  - Scanning
- Atomic Force Microscopy
- Micro-electrodes
  - Vibrating Probe
  - Patch-clamp



Essentials for Imaging

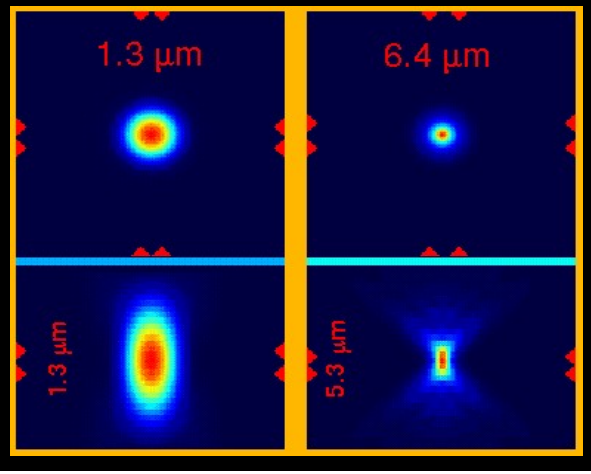


System architecture

Optics

Quality of image &
Signal captured

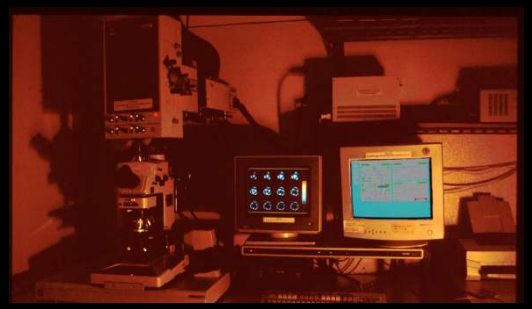
Detectors

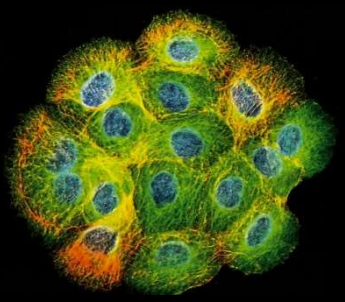


System
resolution

Image acquisition devices

- "Analogic": Eyesight, film and video signal *vs*:
- CCD & CMOS digital cameras (spatial detectors)
  - All pixels are acquired simultaneously
  - Used with conventional light microscopes (wide-field)
  - Low noise esp. if electronics cooled ("cooled CCDs")
  - To detect colors "pixel-size" filters are added
  - CCDs are more sensitive and noise-free (& expensive!)
  - CMOS are faster
  - emCCDs are more sensitive and have virtually "zero" noise
- PMT (photo multiplier tube)+A-D-Converter (point detection)
  - Point detector (registers fluctuations in light intensity...when synchronized with scanning generates 2D images...like a TV!)
  - Used in Laser Scanning Confocal Microscopes
  - Less efficient and more noisy! Requires powerfull light exposure

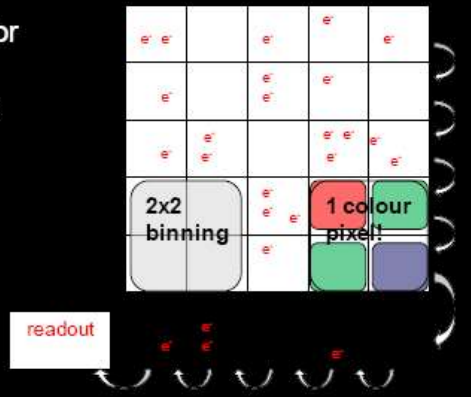


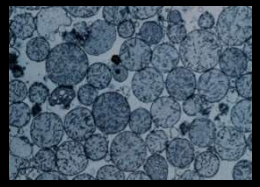


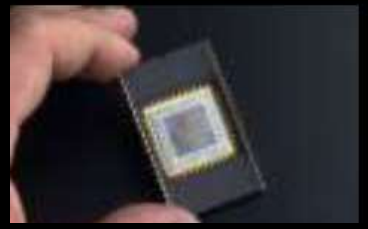
CCDs & CMOs cameras

High sensitive (scientific grade!) cameras don't see colors, i.e, they don't "see" energy, just convert photons into current which is accumulated in a ""bucket" (pixel!) and then passed on to readout.

Important parameters for image acquisition are exposure time and area of detection (binning)







Digital cool CCD Cameras 8-16 bit

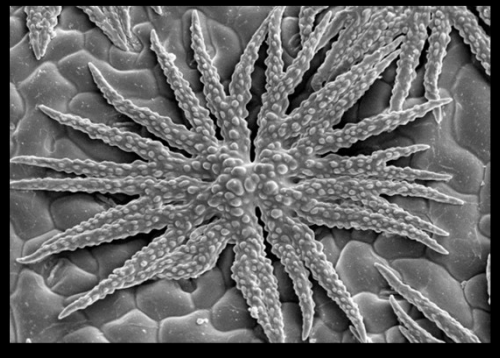
Confocal system

laser, spinning disc, *multi-photon*

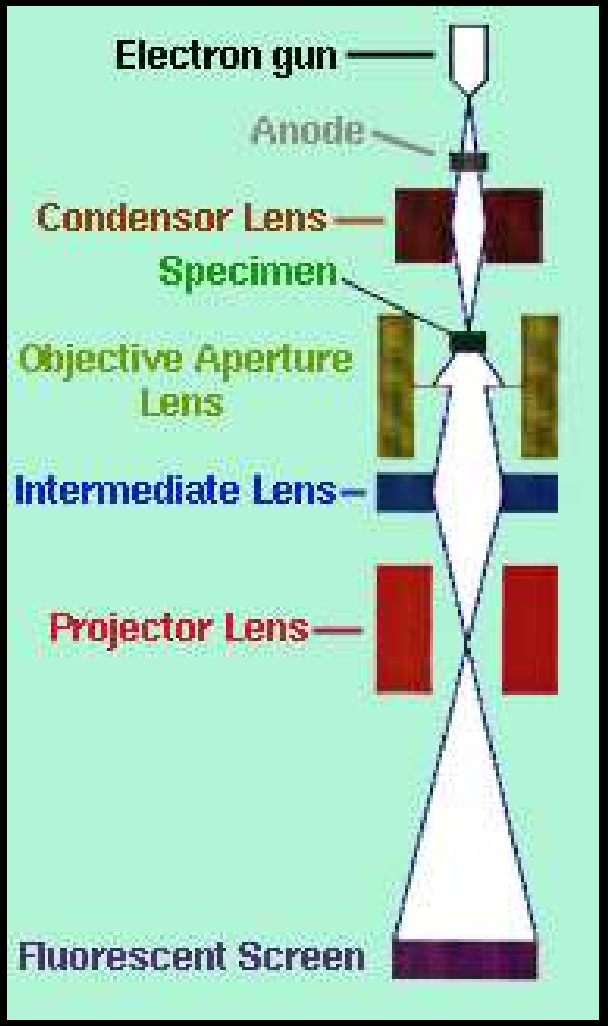
35 mm cameras

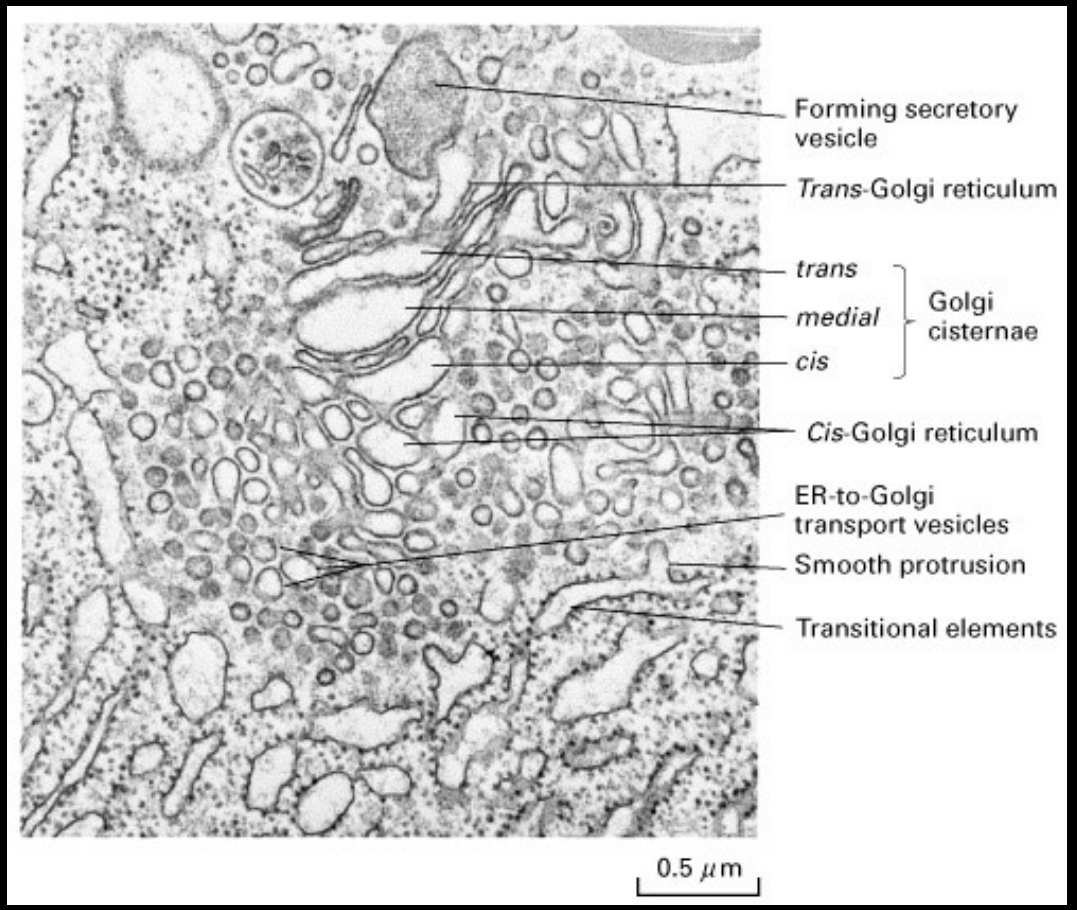
Luminometers

Photomultipliers

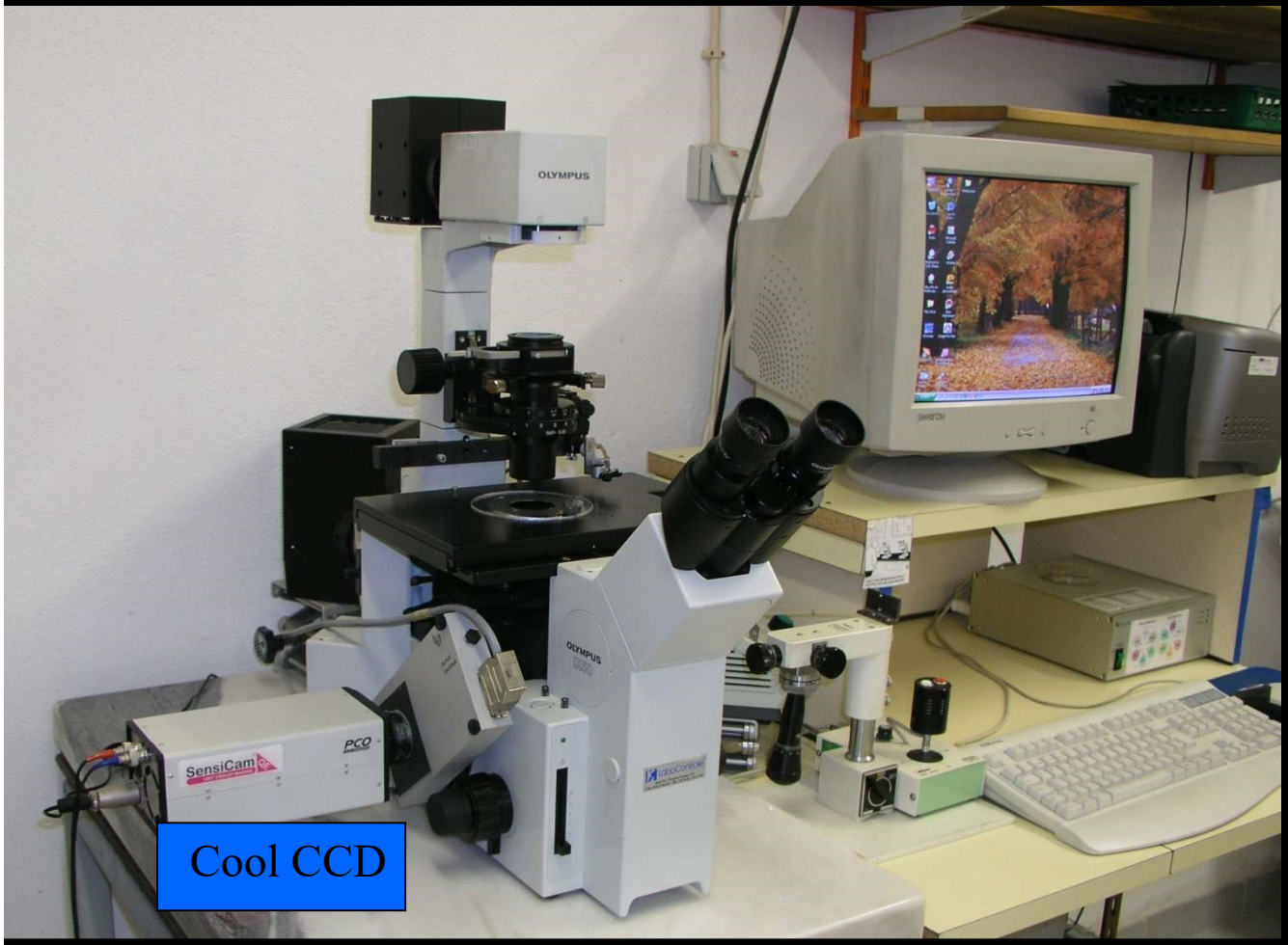


Electron Microscopy – High resolution, no dynamics





Wide-field system



- Fast (ms rate)
- Sensitive
- Ideal for SW dyes
- Coupled to deconvolution

- No Z-sectioning / time-course

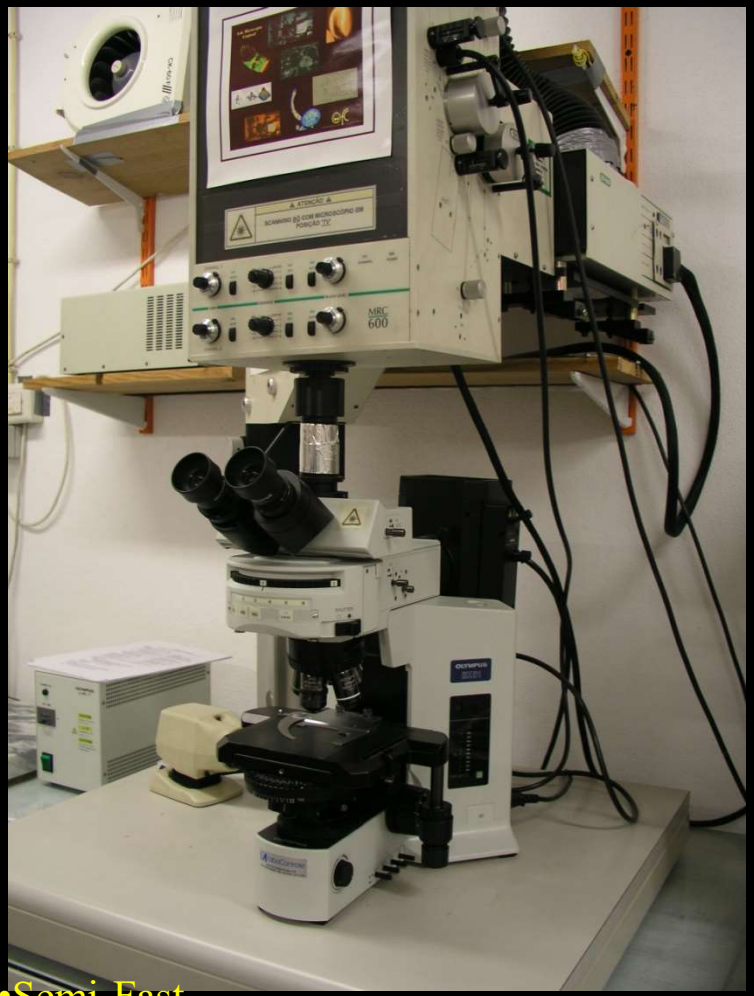
- Co-localization with temporal delay (ok in fixed or immobilized material)

- Imaging of ANY wavelenght

- Mostly manual
- Cheap
- User-friendly

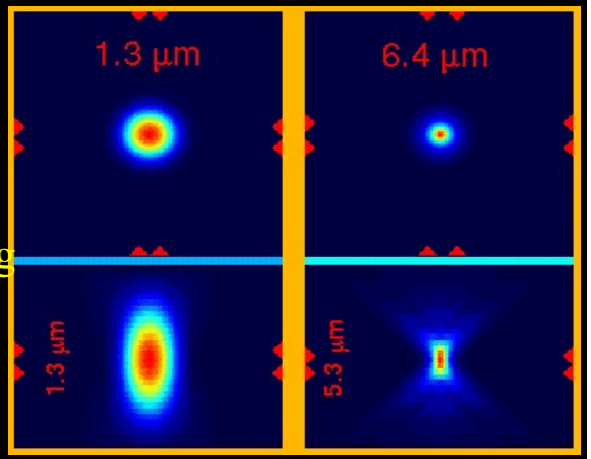
Inverted configuration:
- Caution with immersion obj
- Mouting in coverslides

Confocal system



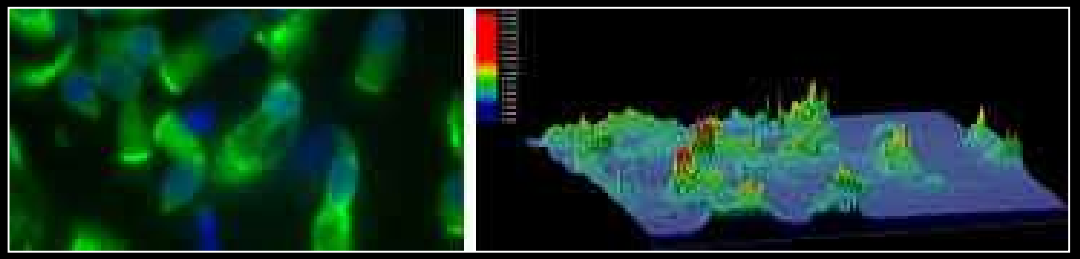
•Semi-automatic
•Expensive
•User-friendly

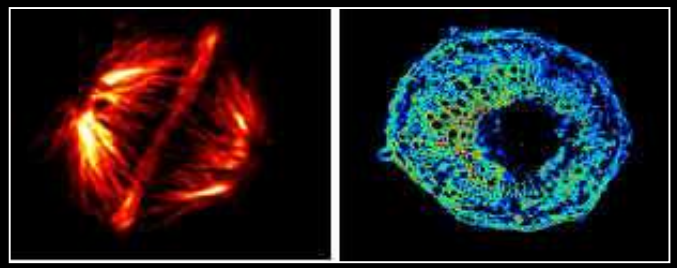
Upright configuration:
- Good for immersion obj
- Problem with “deep” imaging



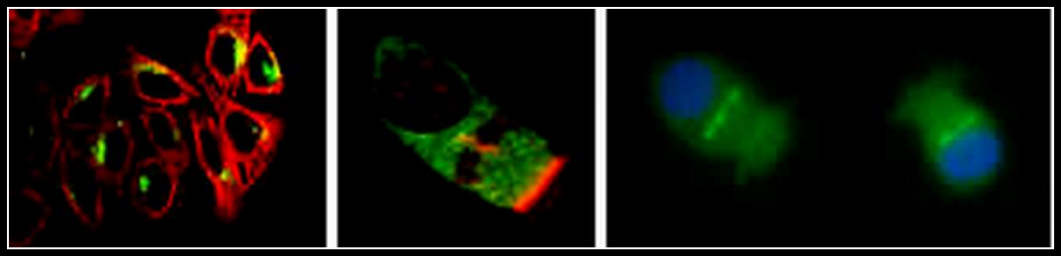
•Semi-Fast
•Sensitive
•Ideal for two dyes - Co-localization with NO temporal delay (optimum for live material)
•Not Coupled to deconvolution
•Z-sectioning /time course
•Imaging of SPECIFIC wavelenghts

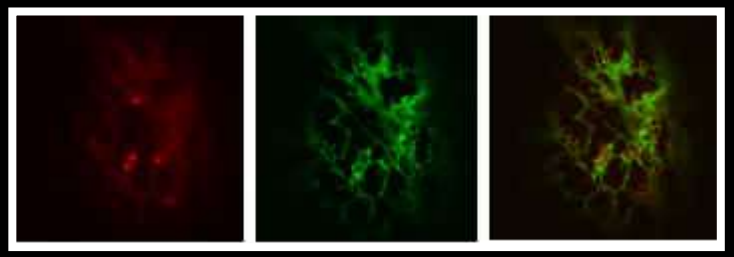
Imaging techniques: Confocal and video fluorescence microscopy

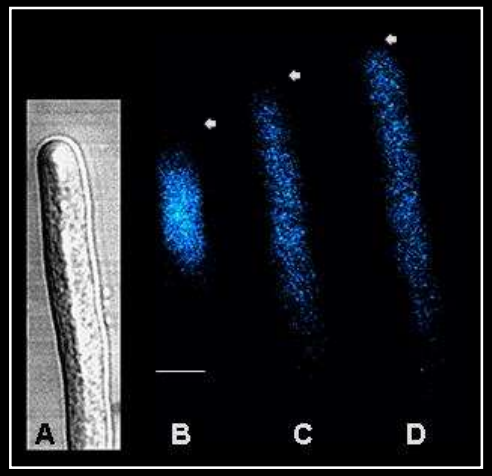




1. ***Cellular labelling with specific fluorescent probes / molecular constructs***
2. ***Temporal and Spatial Mapping of ions and molecules***
3. ***Three-dimensional reconstruction of cellular optical sections***
4. ***Analysis of Digitized Images*** *(quantification, volume determination, statistical analysis)*
5. ***Microinjection of fluorescent probes, antibodies and nucleic acids***
6. **Non-invasive manipulation of intracellular concentration of signalling molecules (w/ caged-probes)**



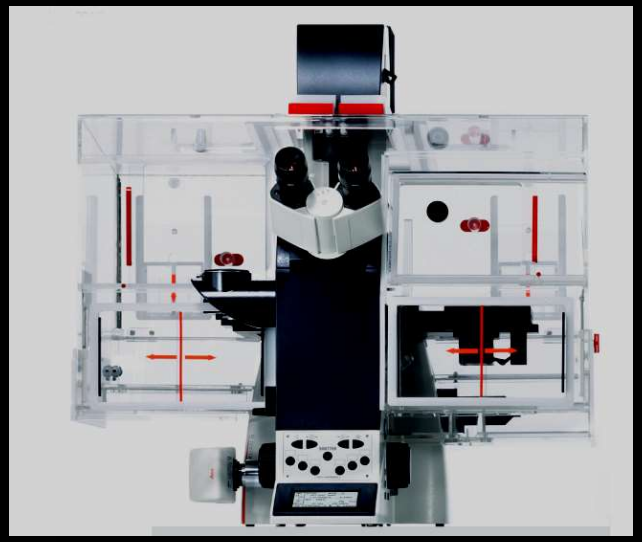




Phototoxicity

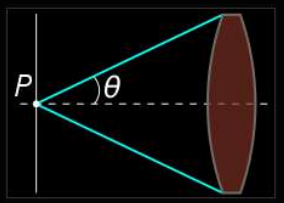
Phototoxicity is the absolute limiting factor in live cell imaging

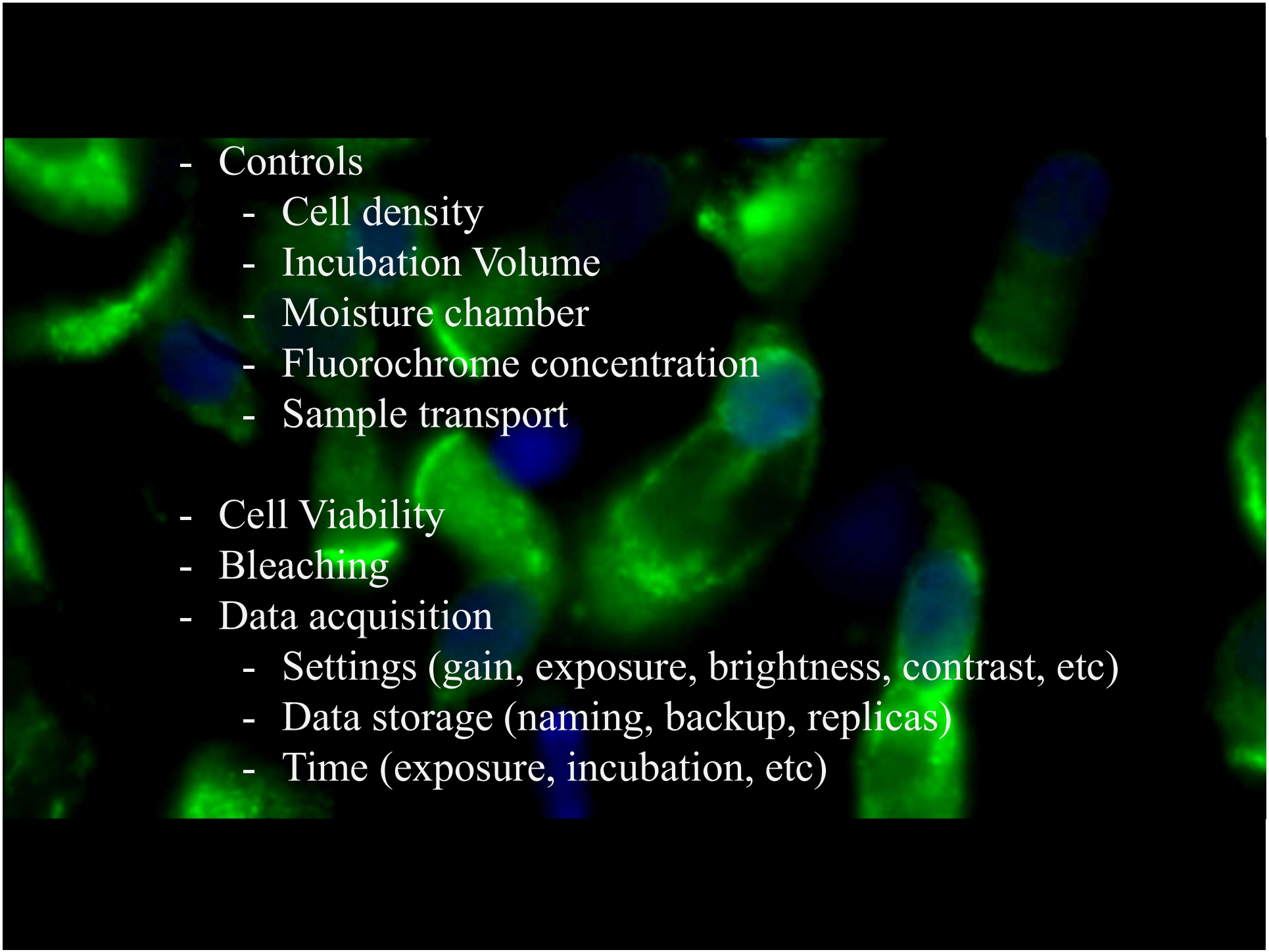
- Excitation of fluorescent molecules in the presence of oxygen leads to fluorochrome bleaching and free radical generation.
- Free radicals kill cells.
- The interaction of light with cells > heat
- Heat kills cells.

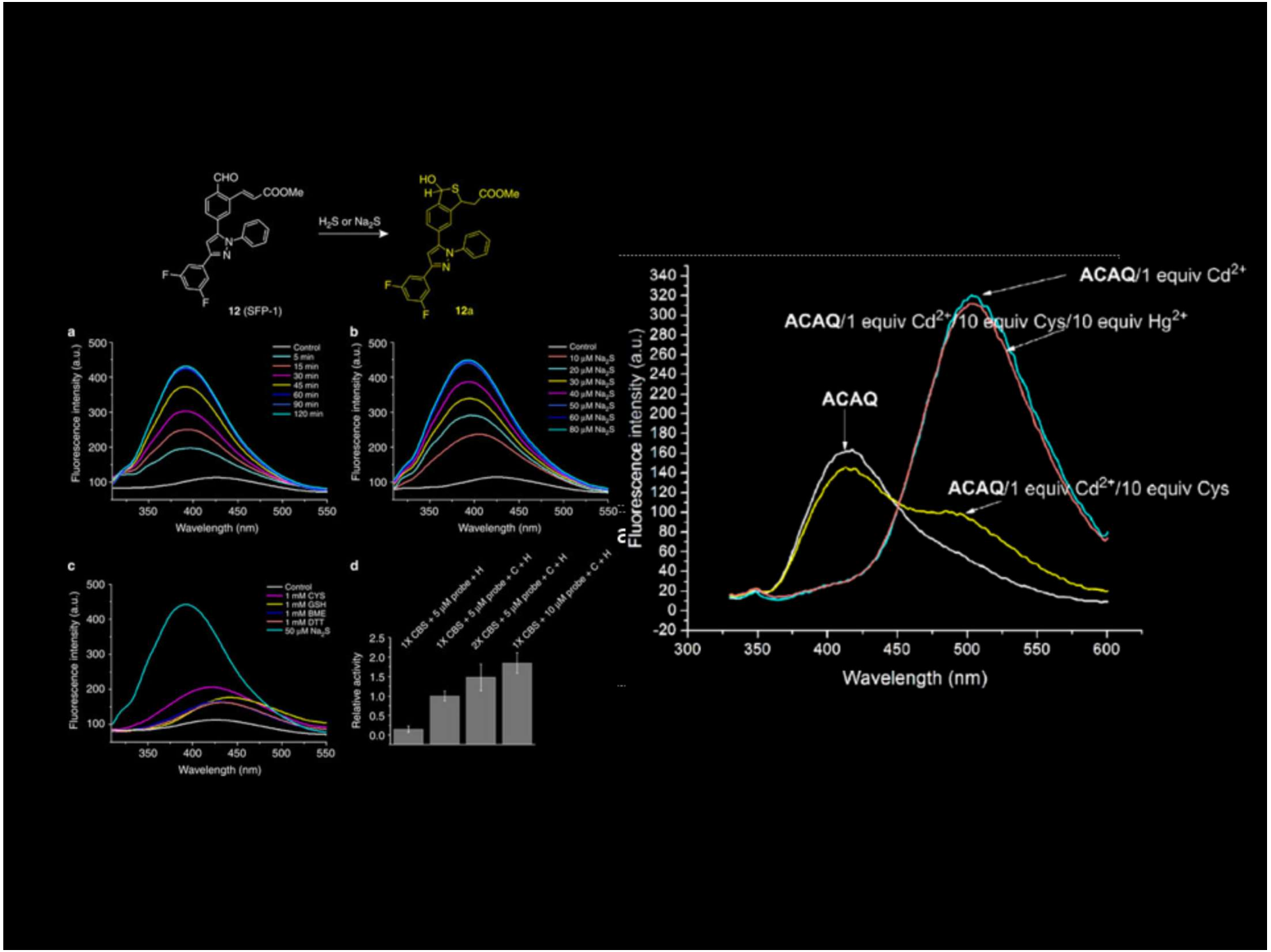


Limiting Phototoxicity

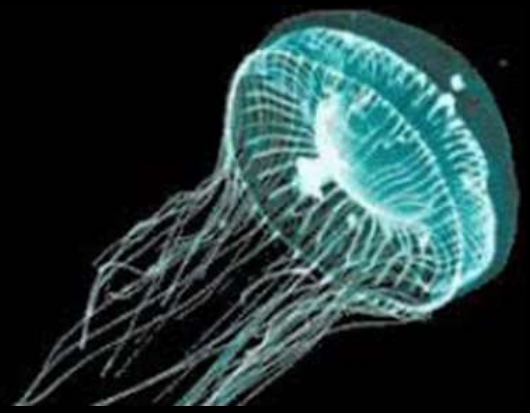
- Maximize light collection.
  - Use HIGH NA
  - Immersion optics
    - This type of optic (for example 60Xplan-apo) has the best NA (1.4) and best light collection.
    - However, immersion optics generally lead to thermal problems - a big issue.
    - Thus we make optical choices dependant on how long the experiment goes on for, how frequently we need to take images.

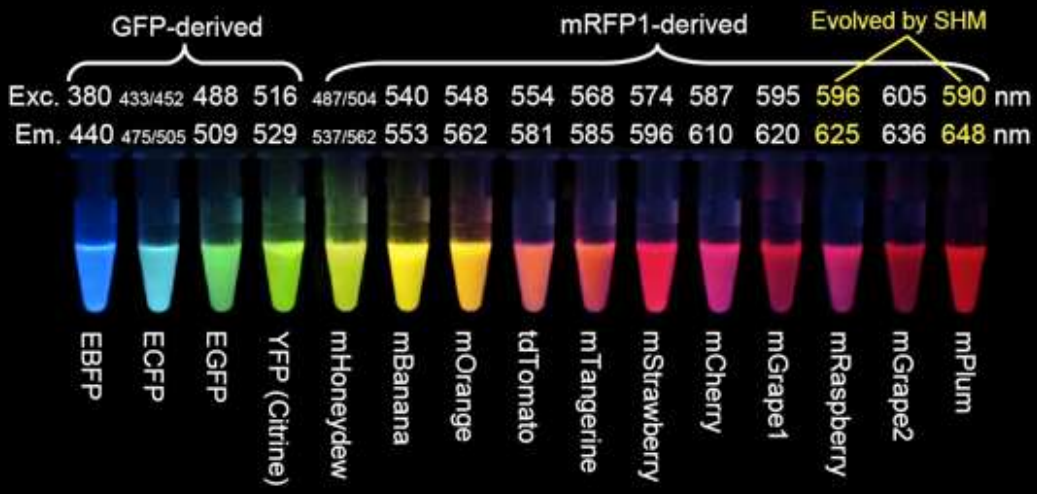


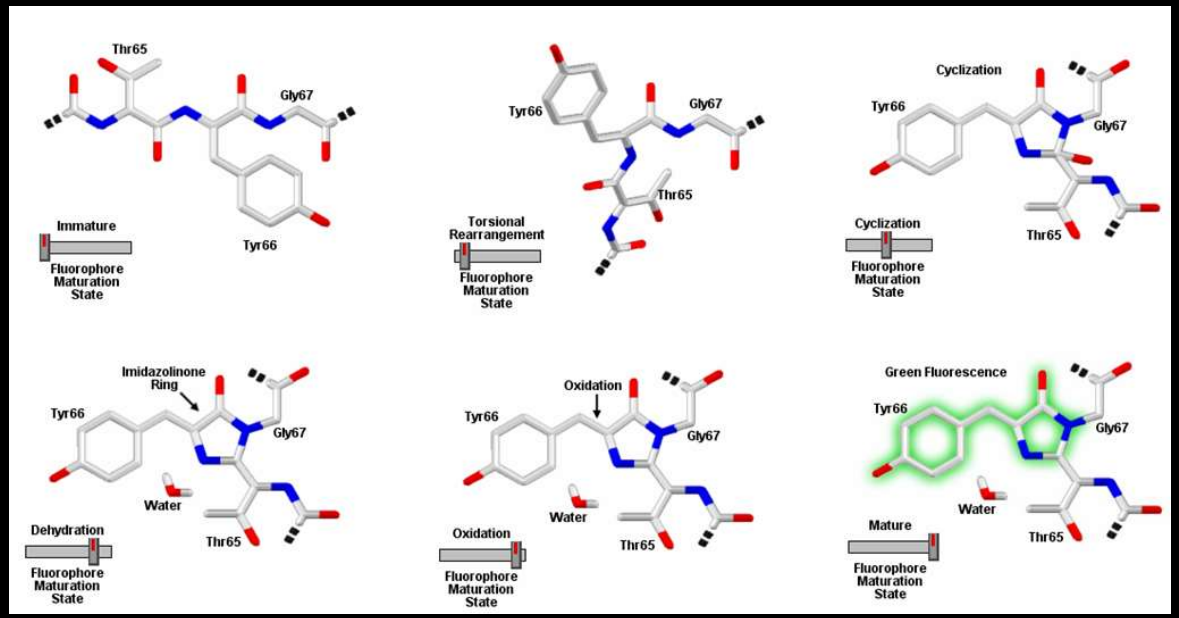

- Controls
  - Cell density
  - Incubation Volume
  - Moisture chamber
  - Fluorochrome concentration
  - Sample transport
- Cell Viability
- Bleaching
- Data acquisition
  - Settings (gain, exposure, brightness, contrast, etc)
  - Data storage (naming, backup, replicas)
  - Time (exposure, incubation, etc)

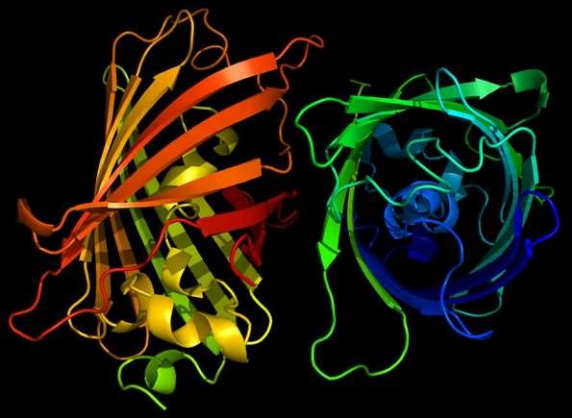

CHO
COOMe
H_2S or Na_2S
HO
H
S
COOMe
12 (SFP-1)
12a
a
Fluorescence intensity (a.u.)
Wavelength (nm)
Control
5 min
15 min
30 min
45 min
60 min
90 min
120 min
b
Control
10 μM Na_2S
20 μM Na_2S
30 μM Na_2S
40 μM Na_2S
50 μM Na_2S
60 μM Na_2S
80 μM Na_2S
c
Control
1 mM CYS
1 mM GSH
1 mM BME
1 mM DTT
50 μM Na_2S
d
Relative activity
1X CBS + 5 μM probe + H
1X CBS + 5 μM probe + C + H
2X CBS + 5 μM probe + C + H
1X CBS + 10 μM probe + C + H
ACAQ/1 equiv Cd^{2+}
ACAQ/1 equiv Cd^{2+}/10 equiv Cys/10 equiv Hg^{2+}
ACAQ
ACAQ/1 equiv Cd^{2+}/10 equiv Cys
Fluorescence intensity (a.u.)
Wavelength (nm)

Imaging techniques: The GFP breakthrough



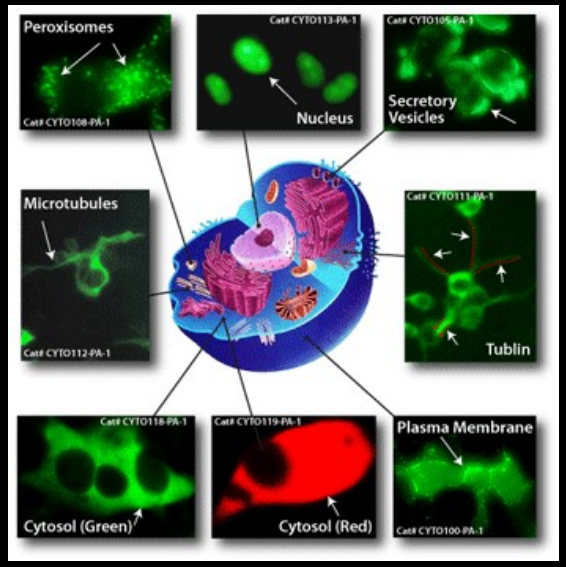


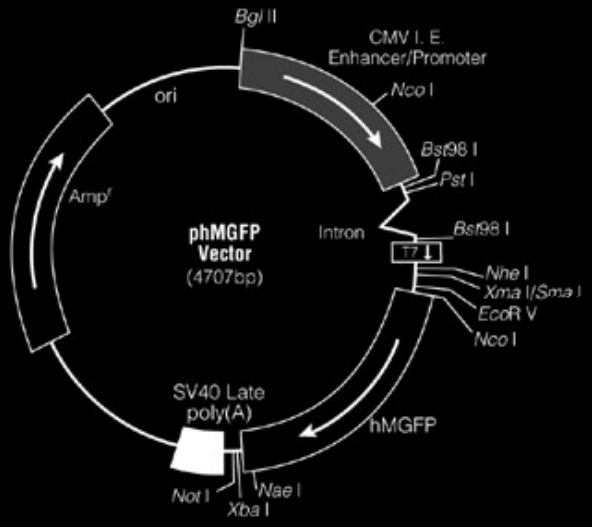


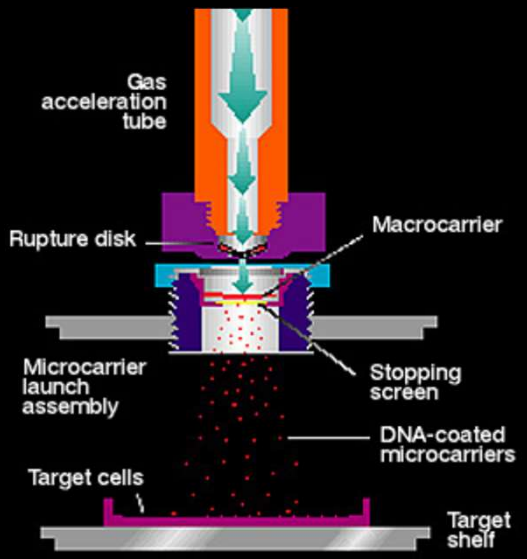


GFP can be inserted by genetic transformation

- Obtention of stable/transient mutants (cells, tissues, organisms)
- 1 gene transcription = 1 GFP molecule
- Quantifications (theoretically) possible
- Construction of fusion proteins ("tag")







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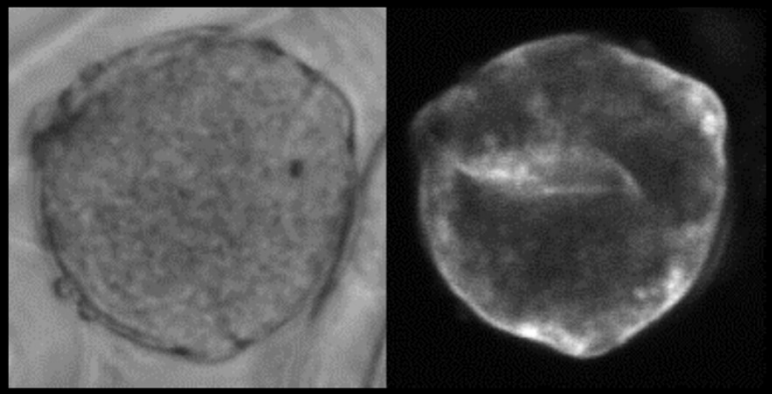
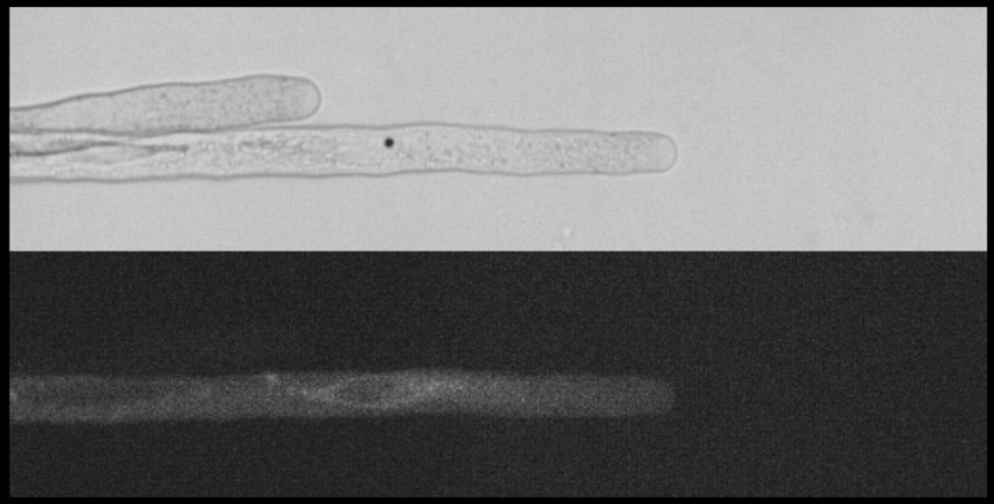
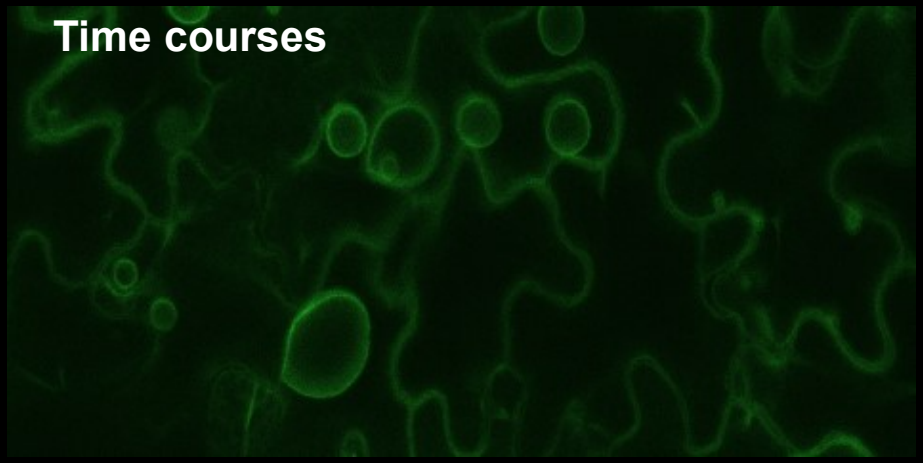
NVsmGFPKDCV (766 a.a)

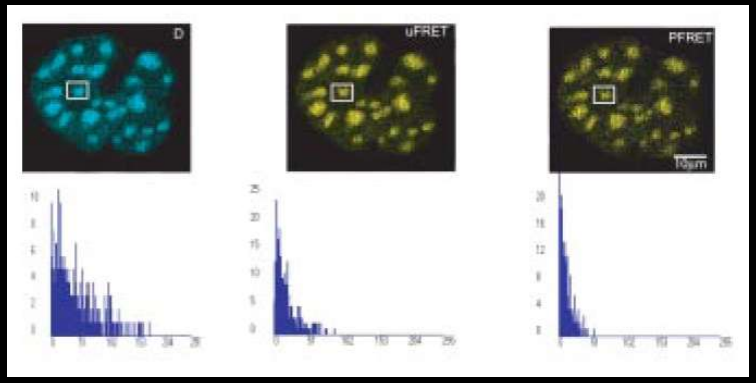


NVsmGFPKDK82MCV (766 a.a)

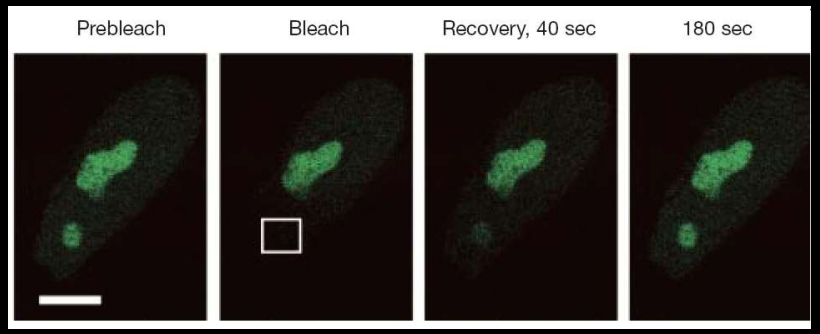


Imaging techniques: The GFP applications

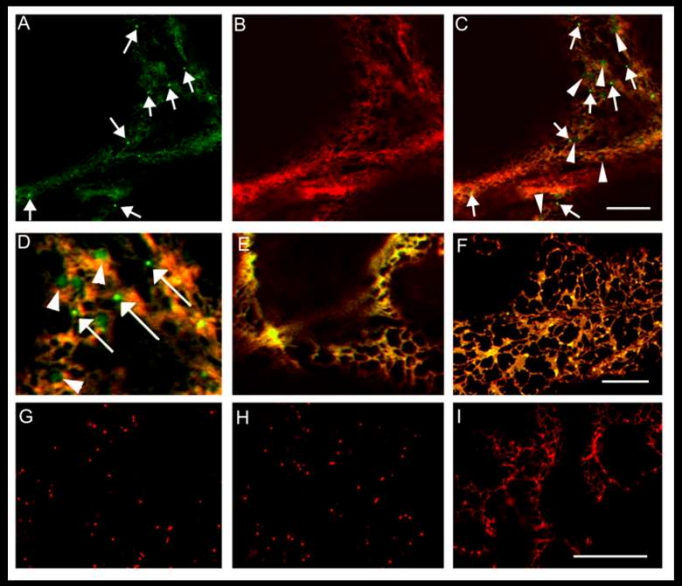




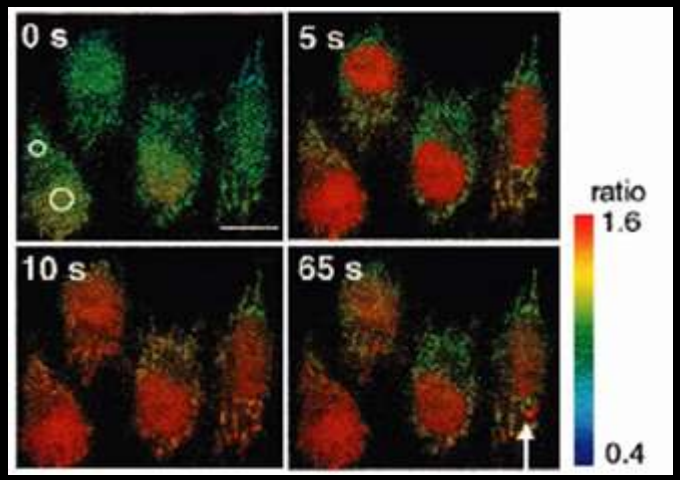
FRET



FRAP

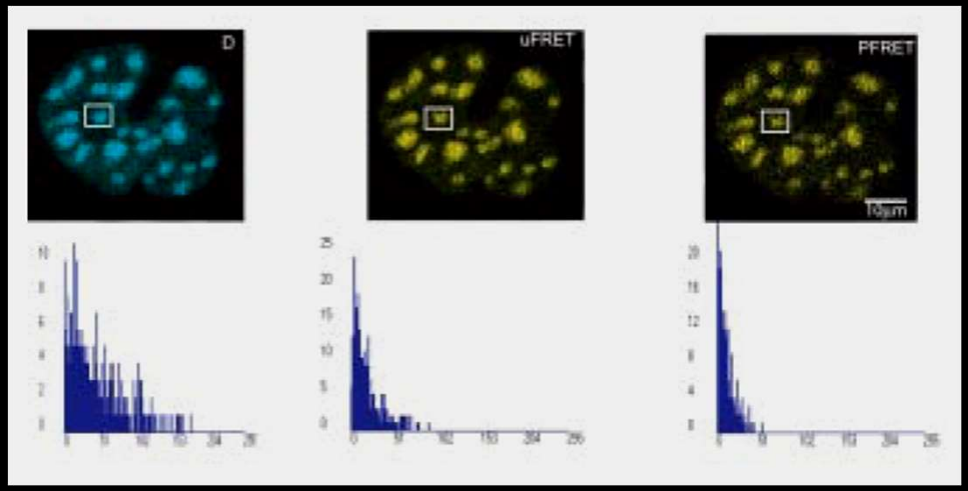


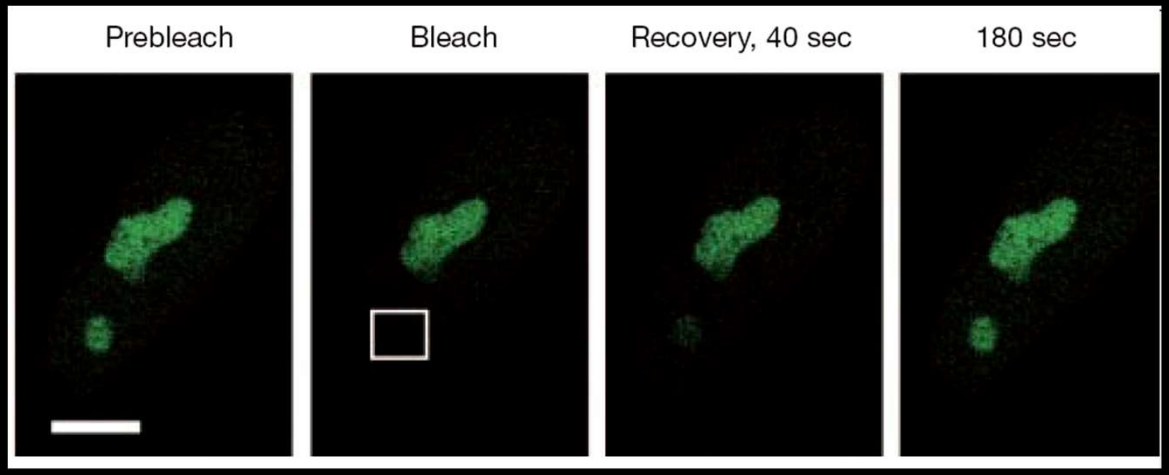
Co-localization

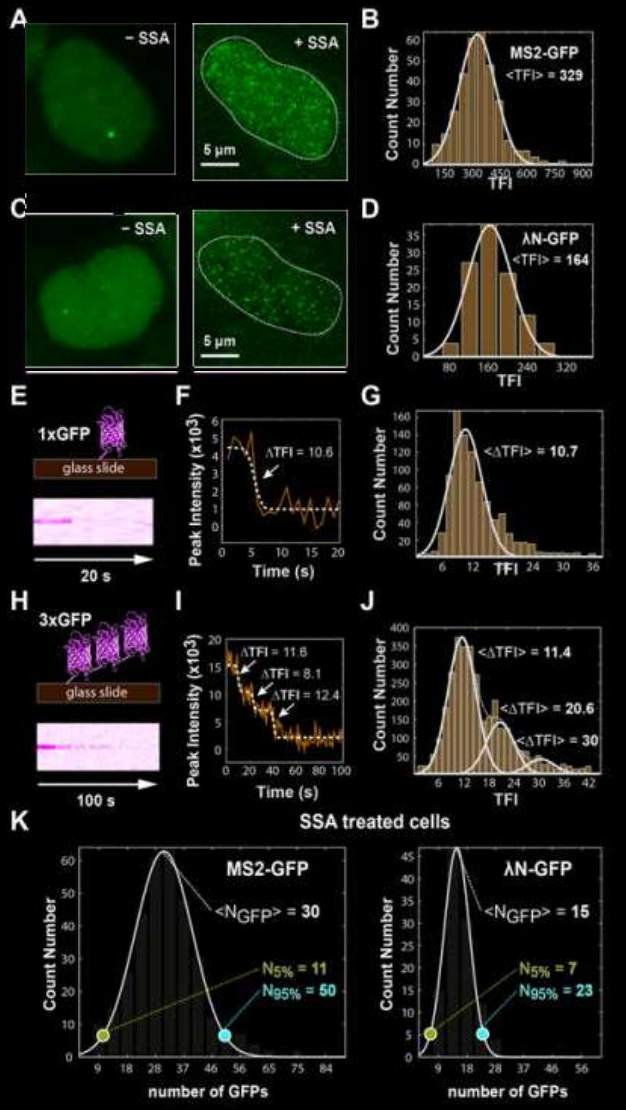


Ion dynamics

FRET / FRAP / FLIM / FLIM-FRET







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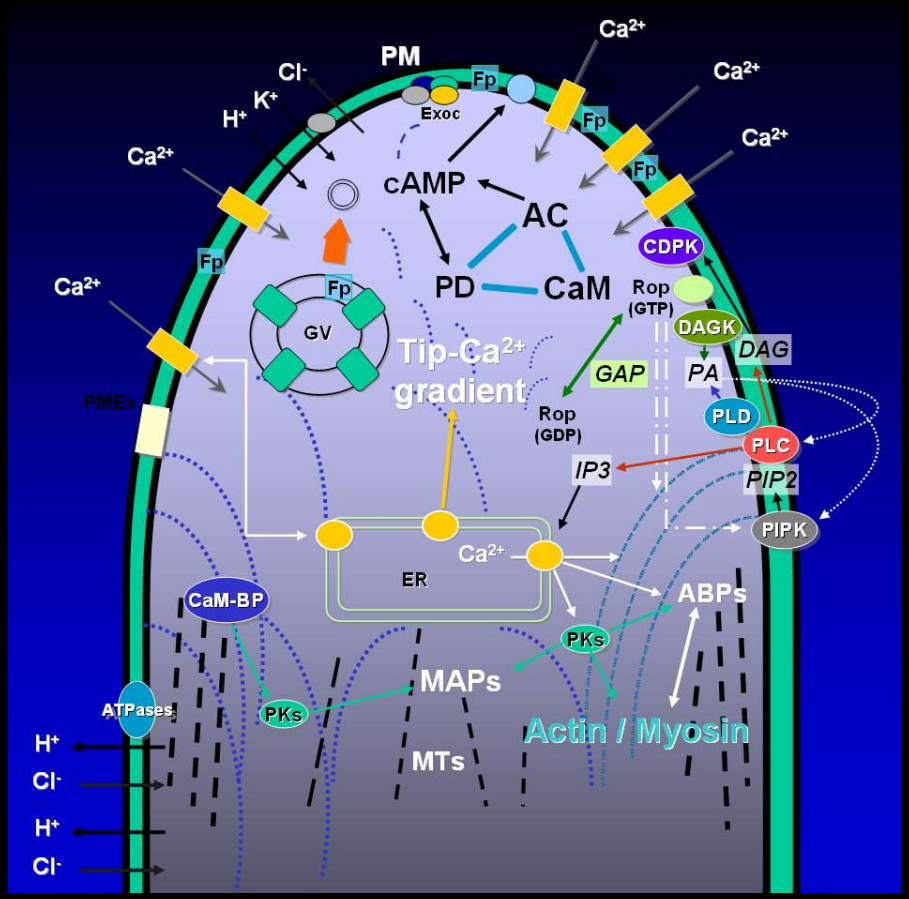
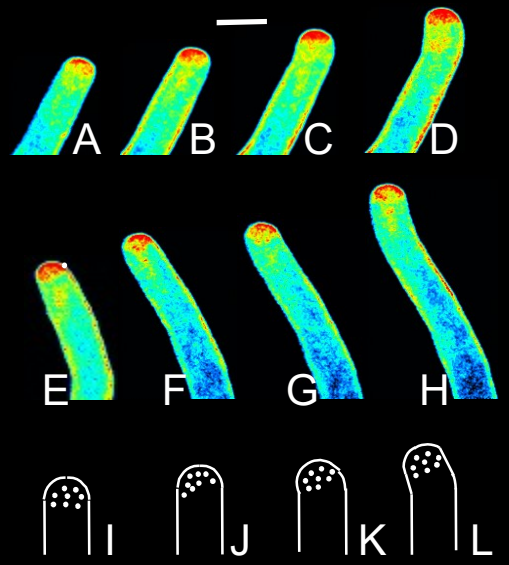
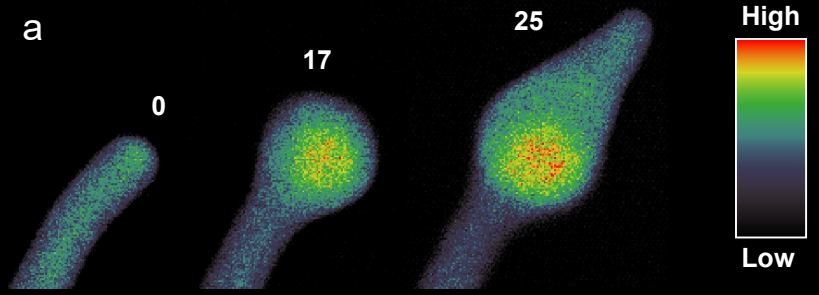
Correlative analysis of apical secretion and $[Ca^{2+}]_C$ in pollen tube growth and reorientation

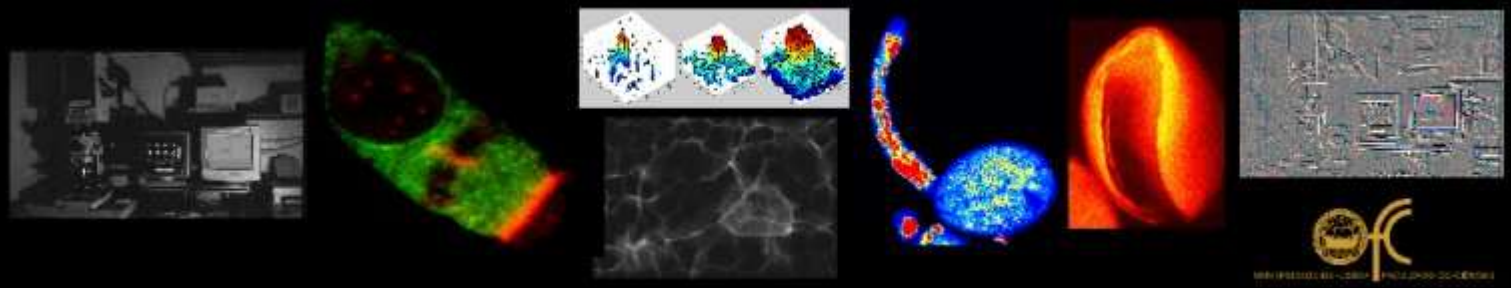




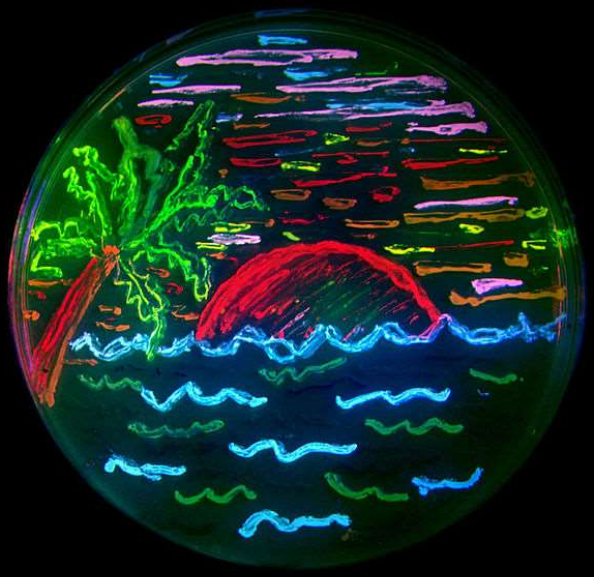
FM 1-43 under CLSM imaging

Ca2+ imaging





Our results are as good as the techniques used to obtain them



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