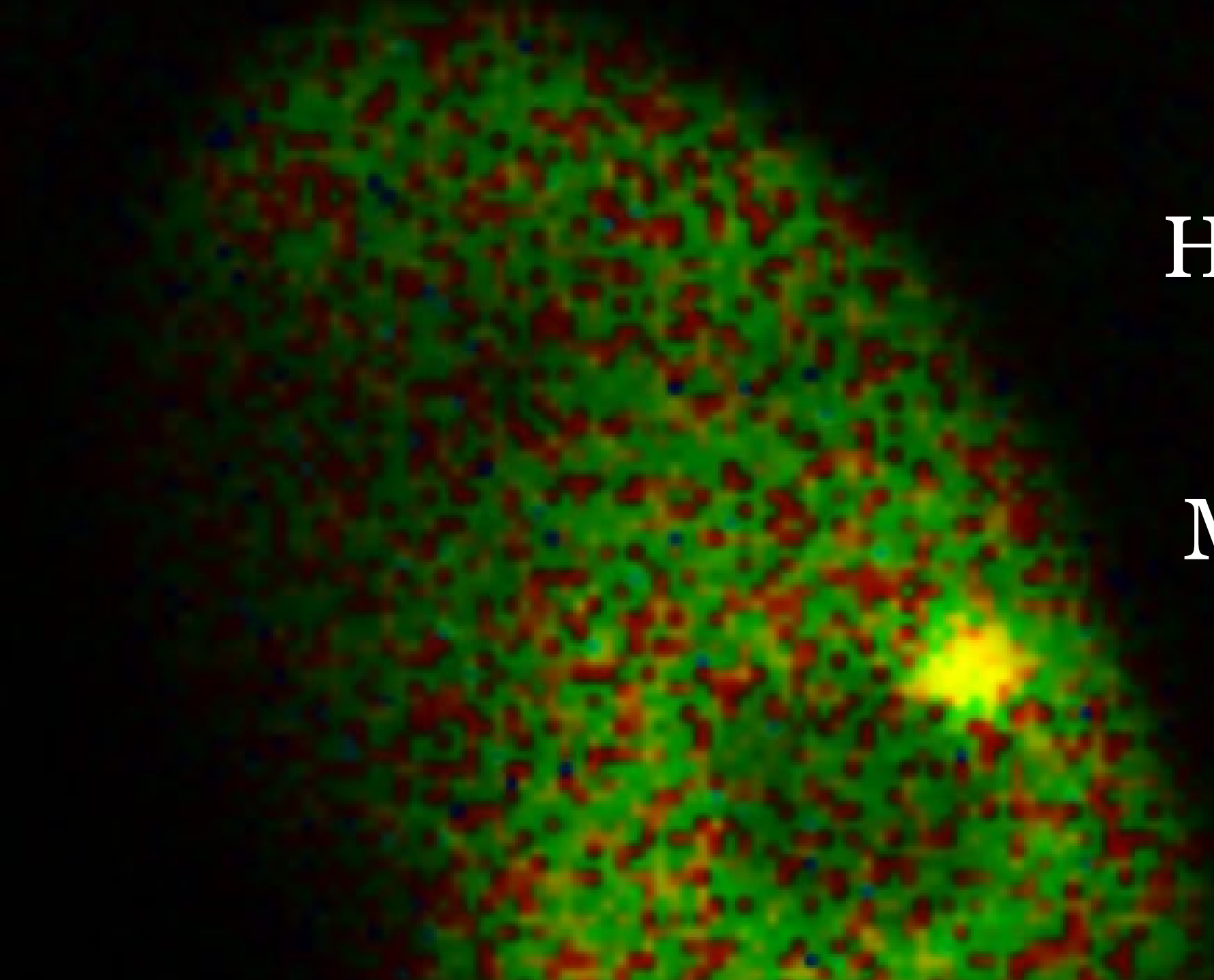


How Do You Do It?

—

Microscopy Edition

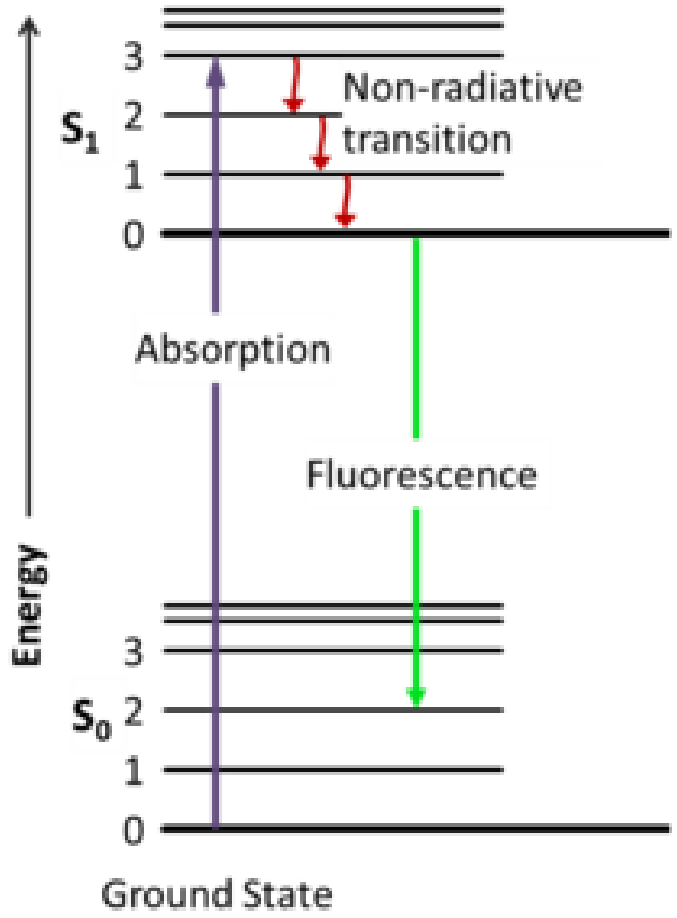
Alberto Danieli
Tanja Kaufmann

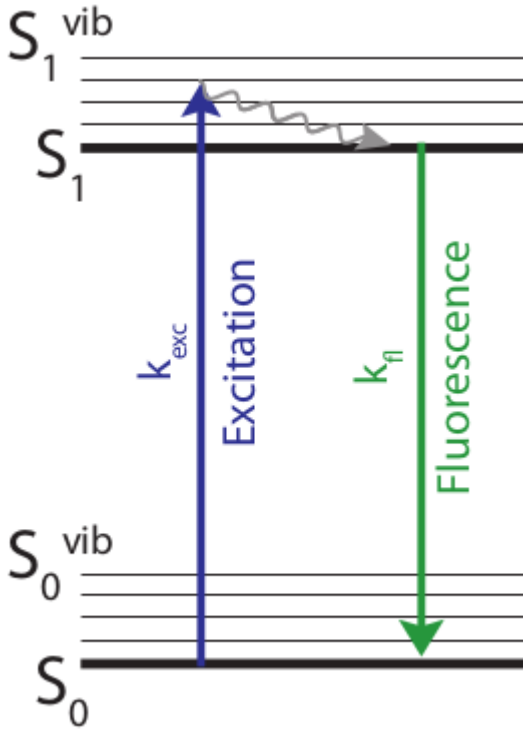
25.03.2019

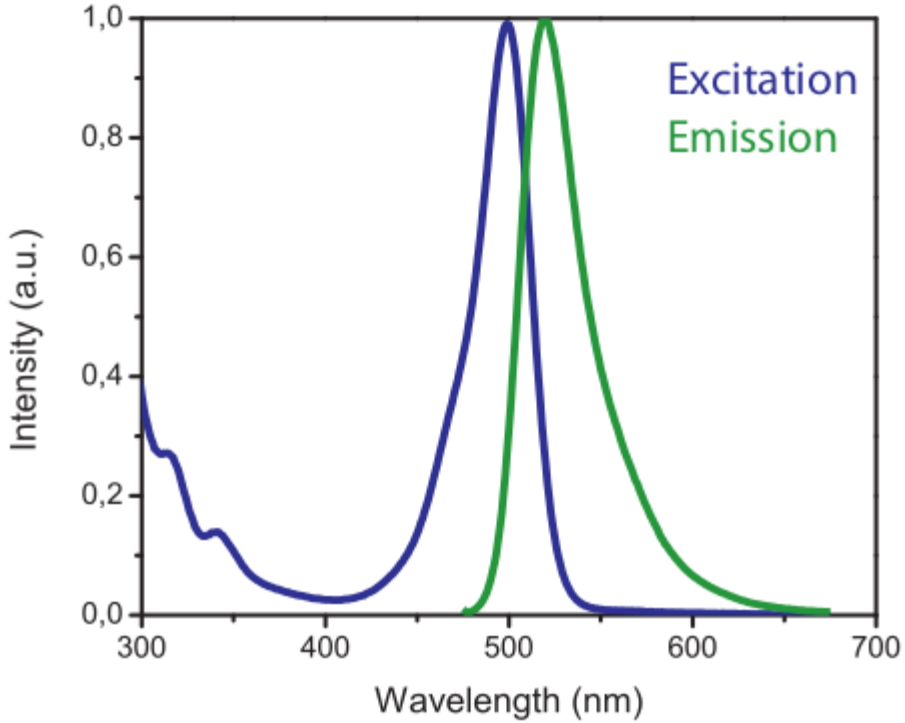
On the menu

- Part 1: Fluorescence microscopy
  - Widefield vs. Confocal microscopy
  - Spinning Disc Microscope and fun things to do with it
  - FLIM (Fluorescence Lifetime Imaging)
  - FRAP (Fluorescence Recovery After Photobleaching
- Part 2: Correlative Lightand Electron Microscopy (CLEM)

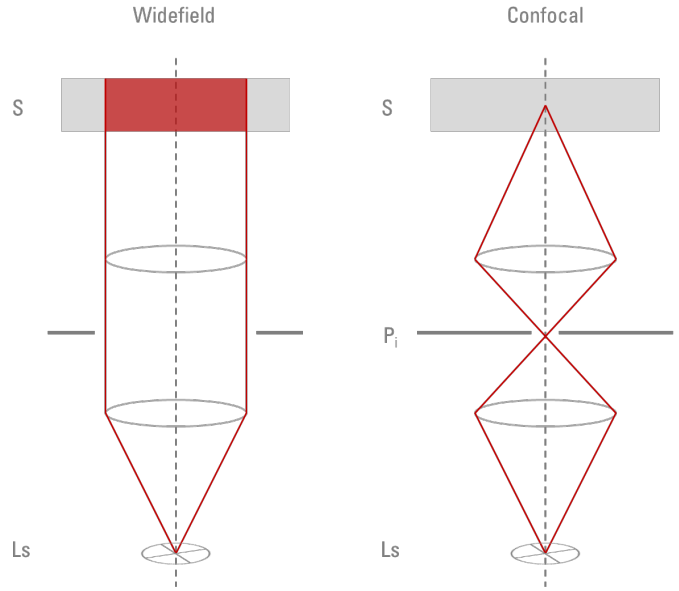
Fluorescence



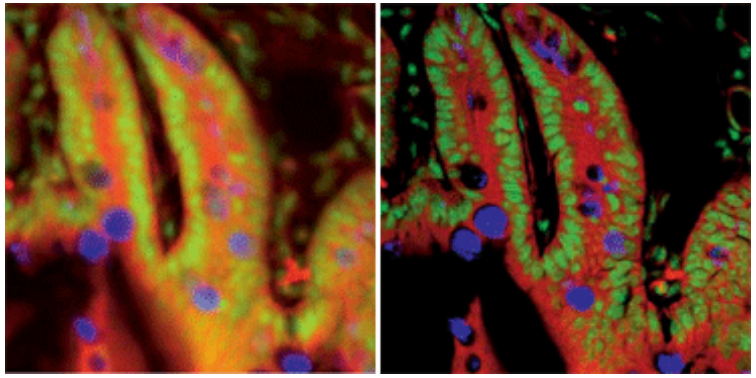


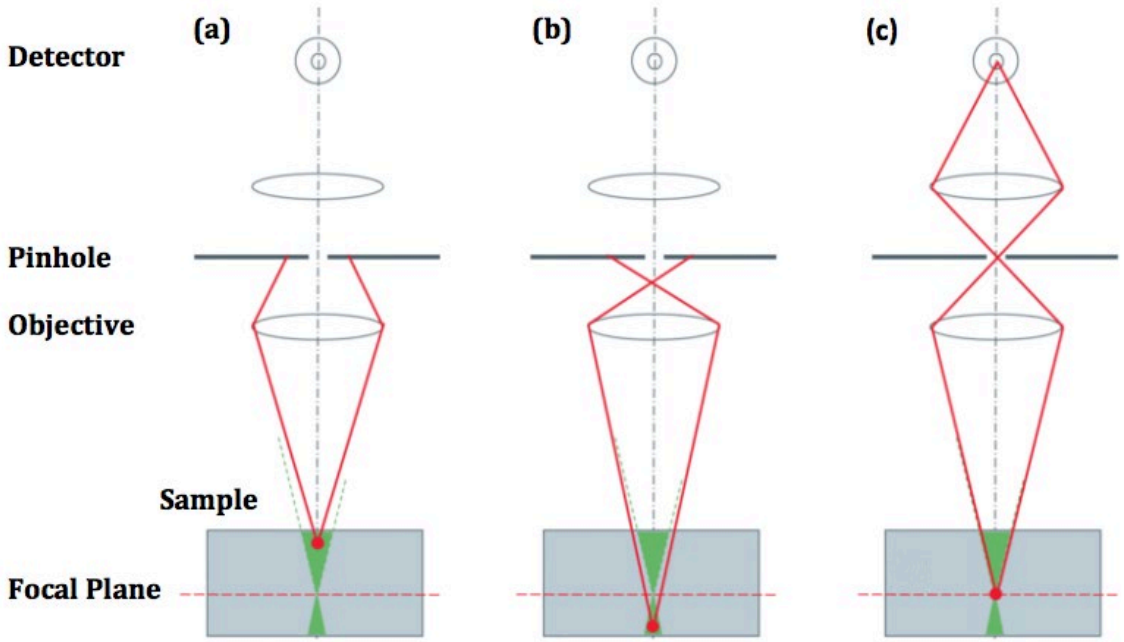


Widefield vs Confocal. What‘s the difference?



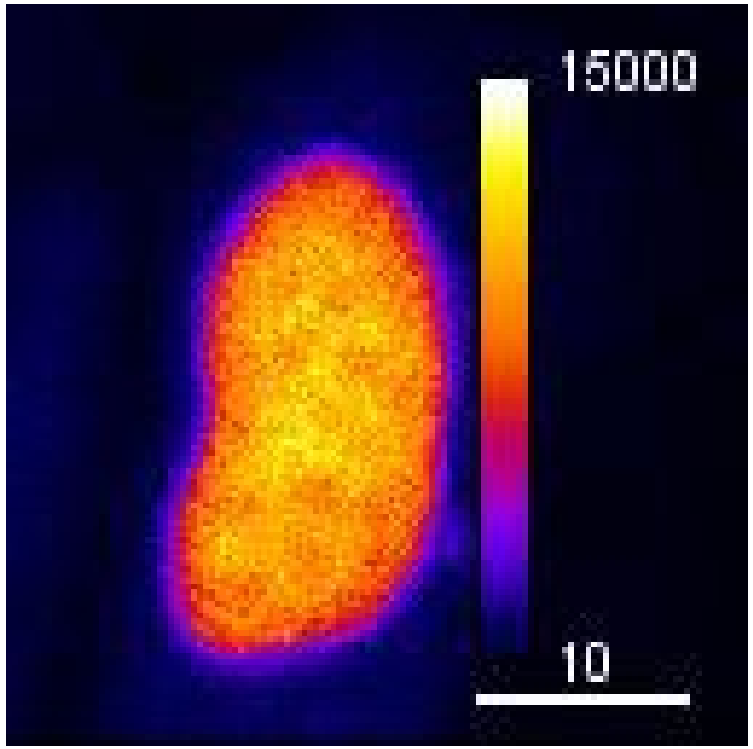
leica-microsystems.com





photometrics.com

Common miscoception: Confocal microcopes are slow



0.264 µm pixel size, 1 second intervals

Confocal is fast, Spinning disc is faster!

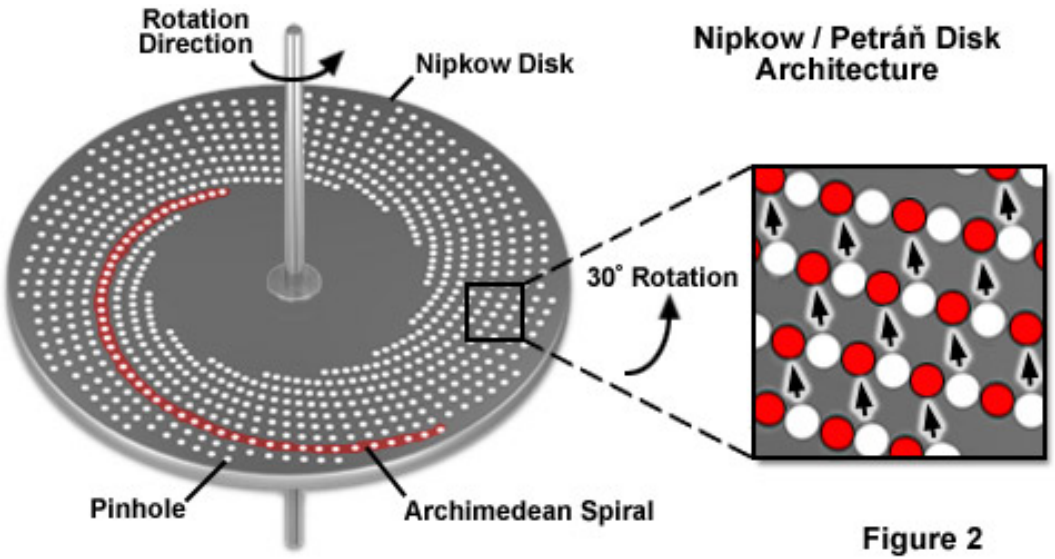
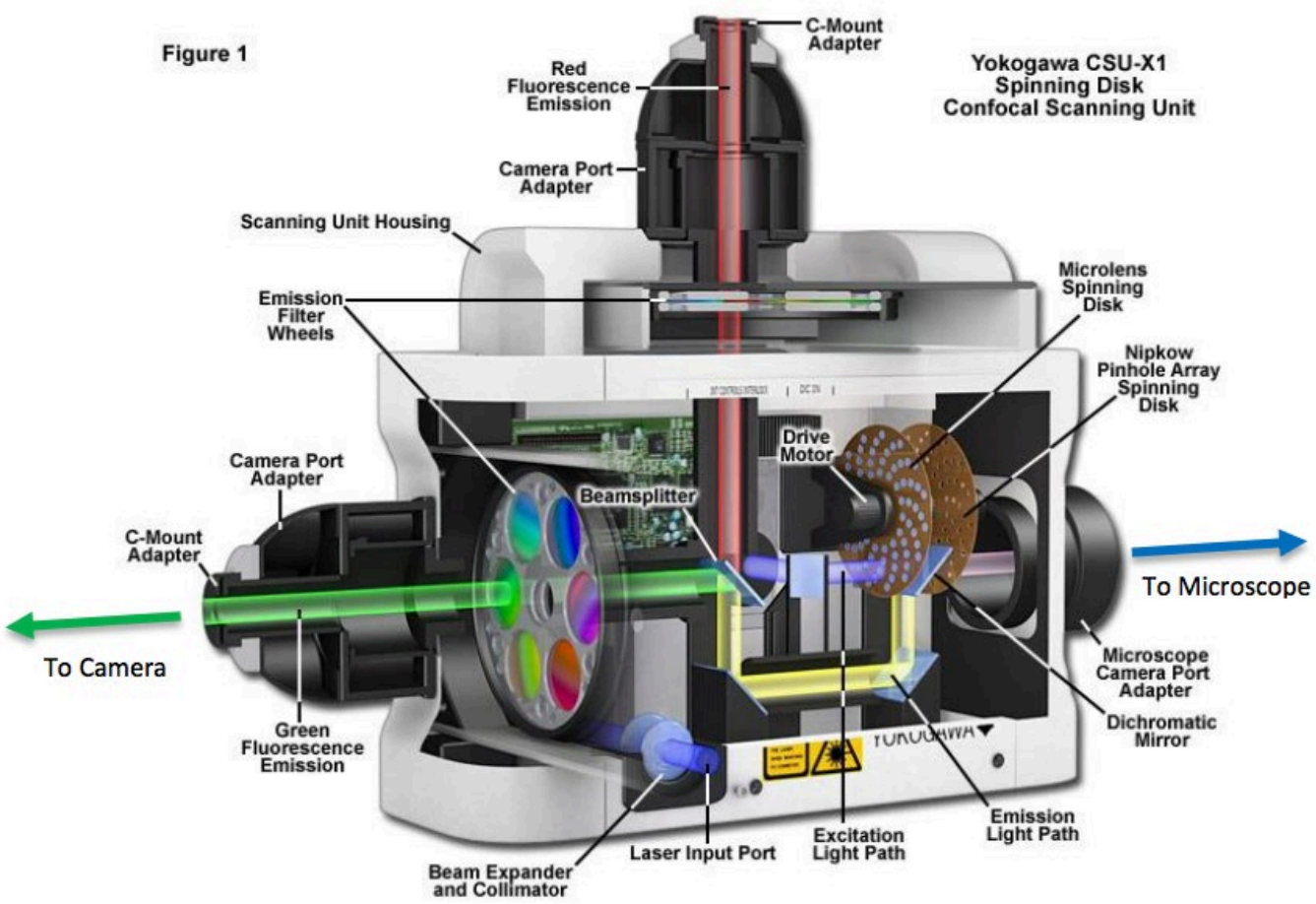
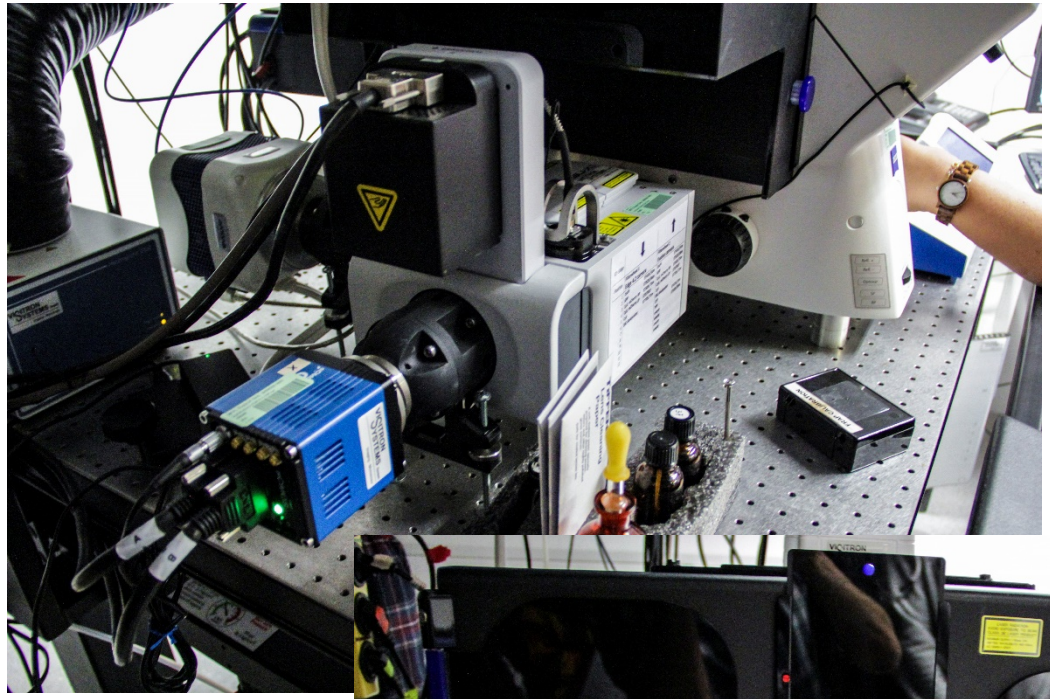
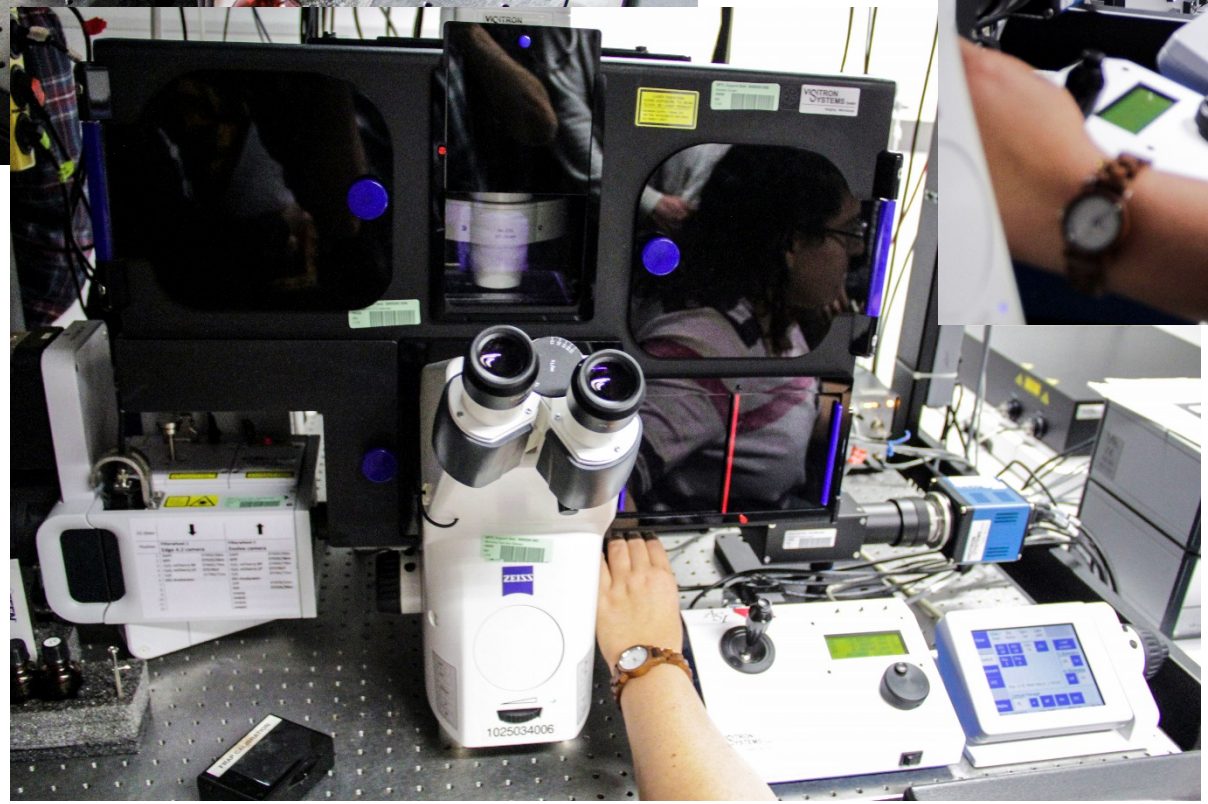


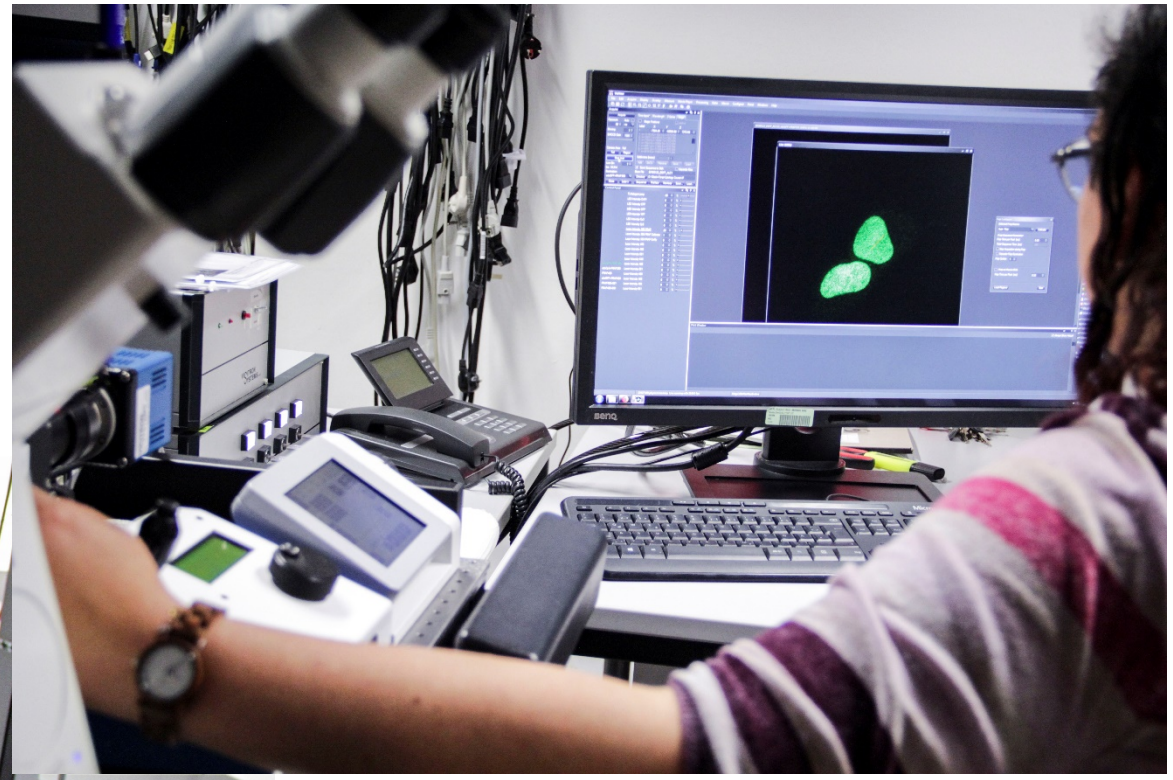
Figure 2



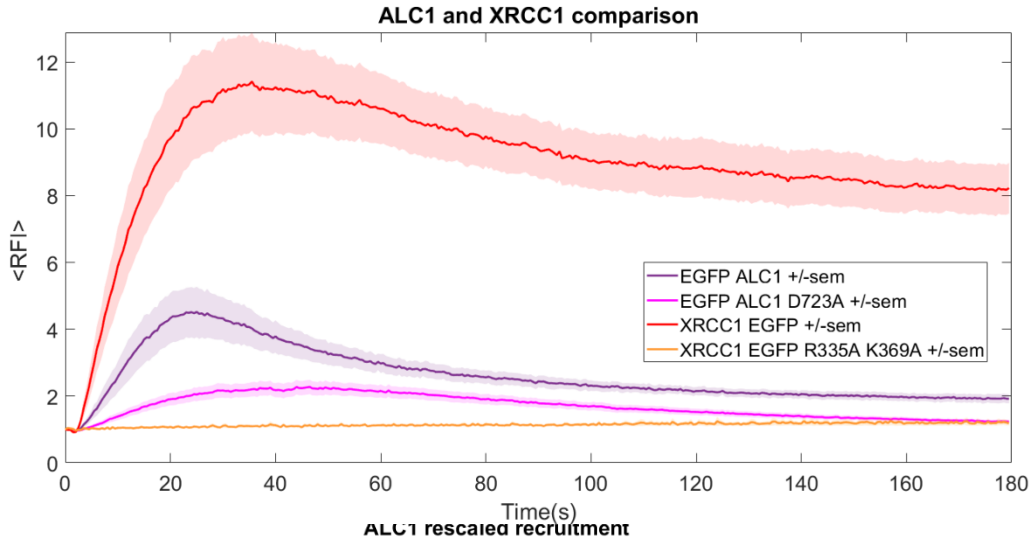
Zeiss-campus

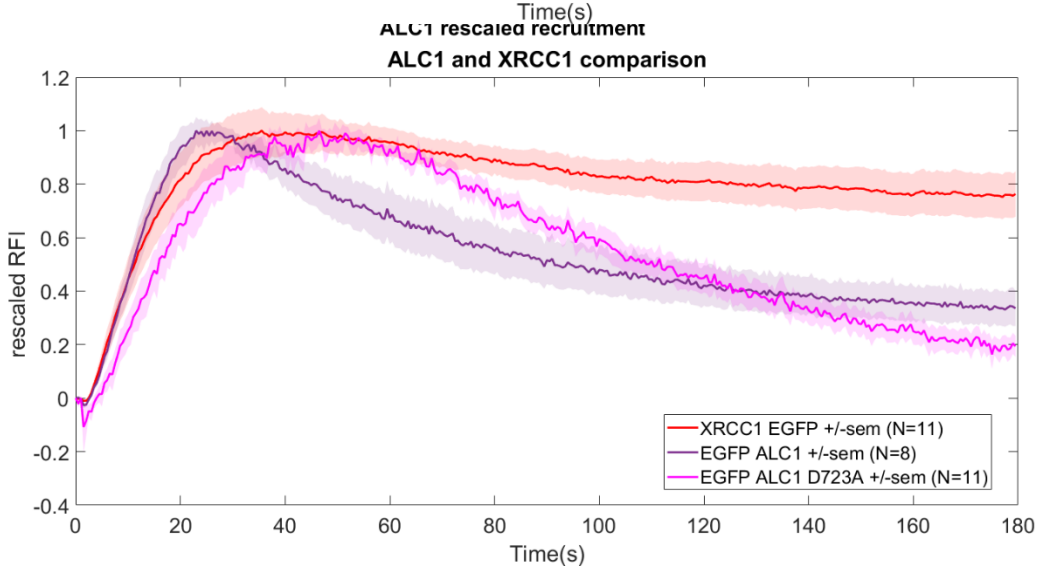


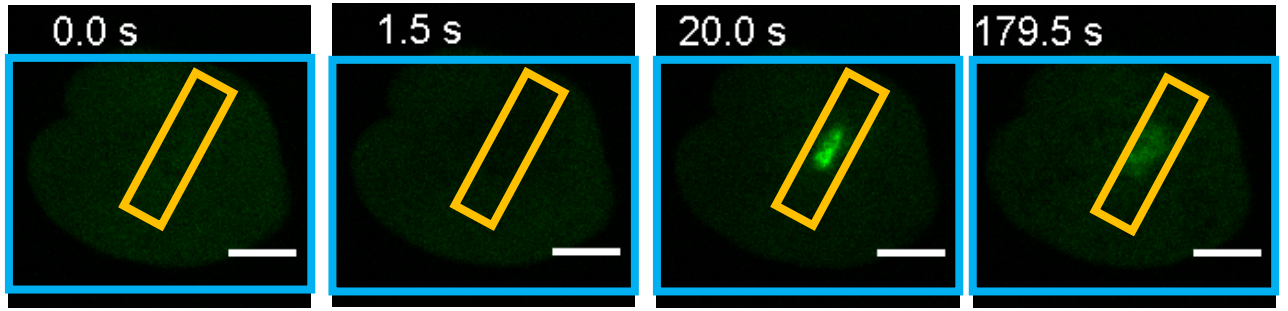

VOLTRON
VOLTRON SYSTEMS
ZEISS
1025034006

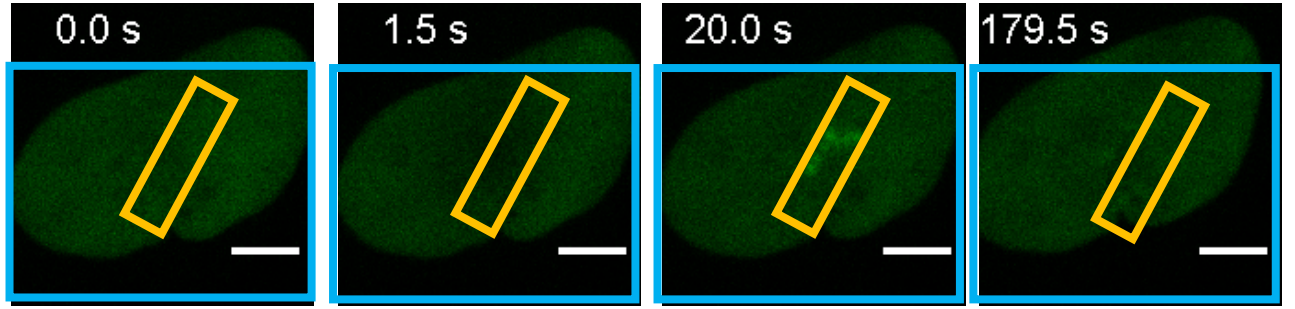

BenQ

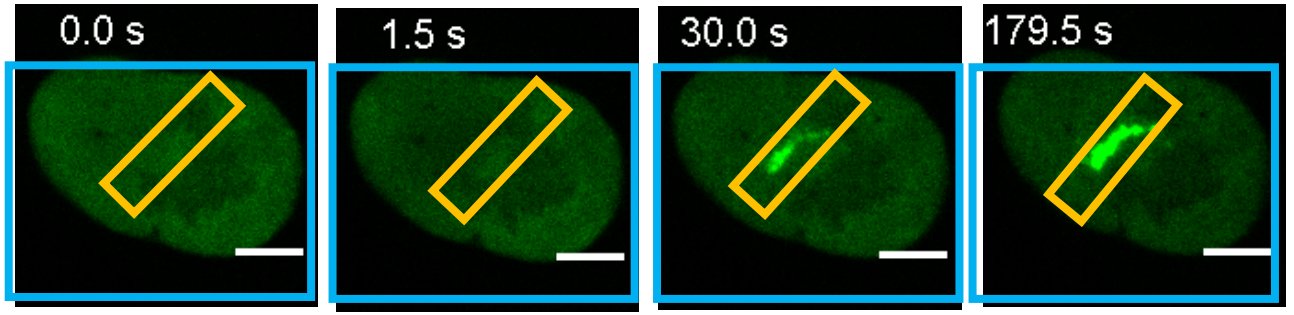
Case study 1: the recruitment of fluorescently tagged proteins to UV-laser induced sites of DSBs



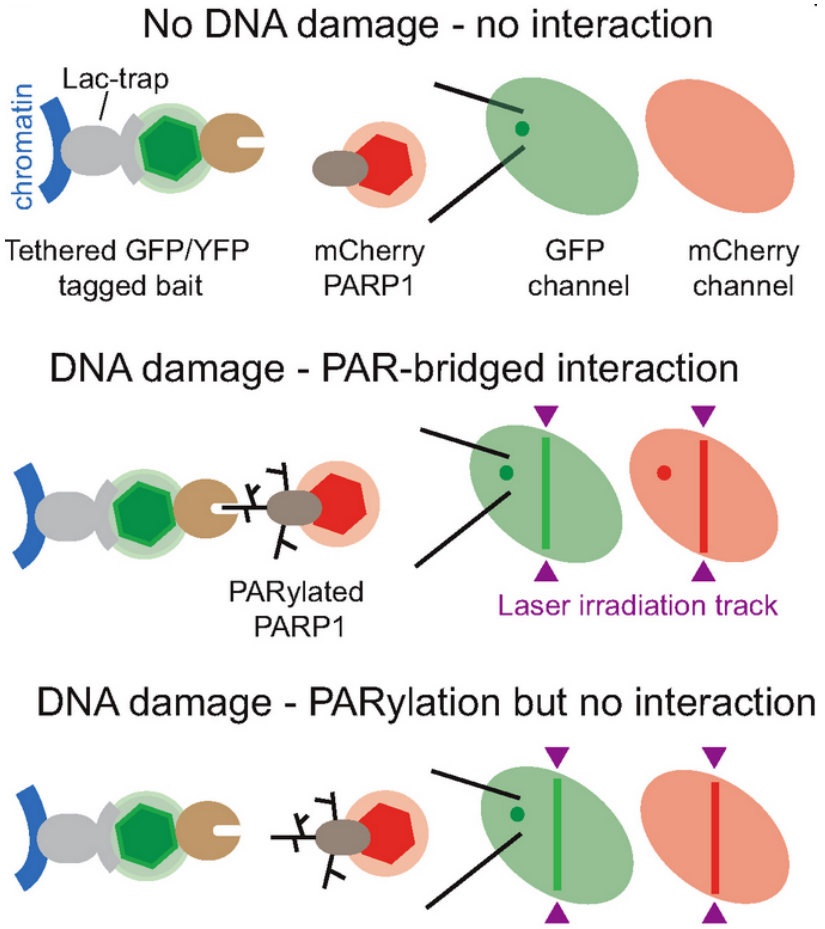


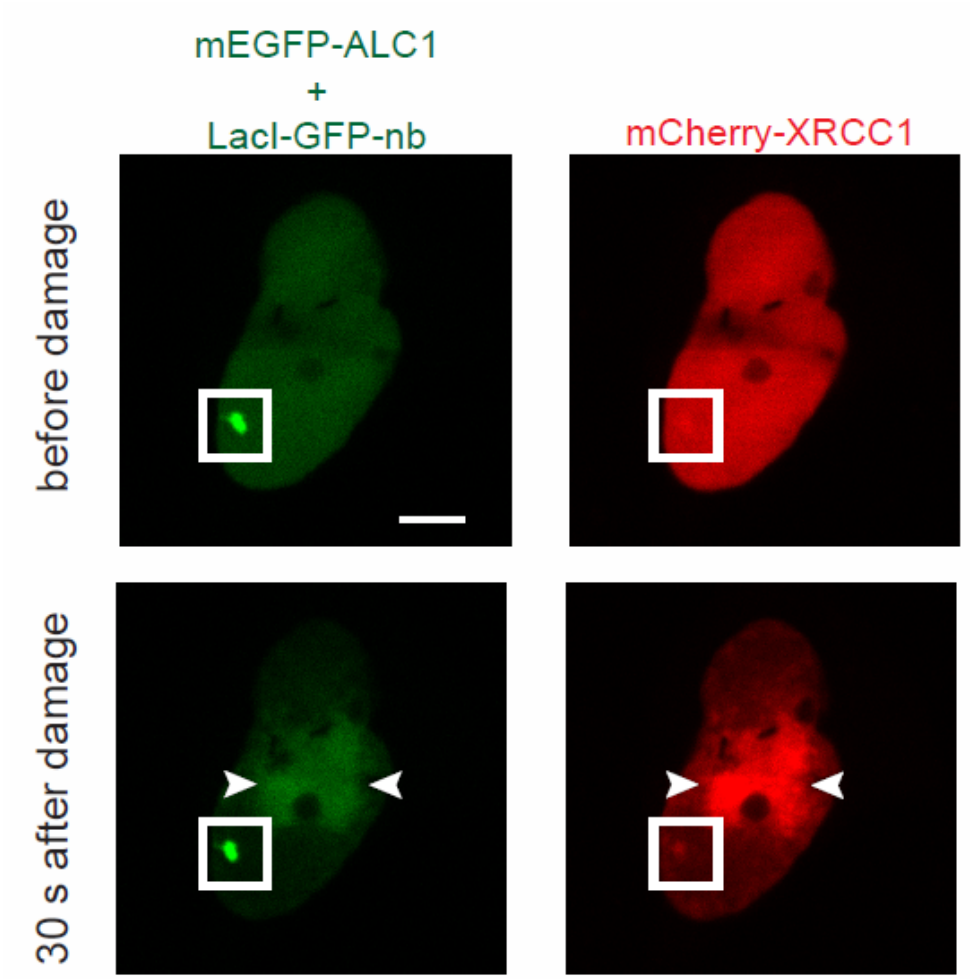




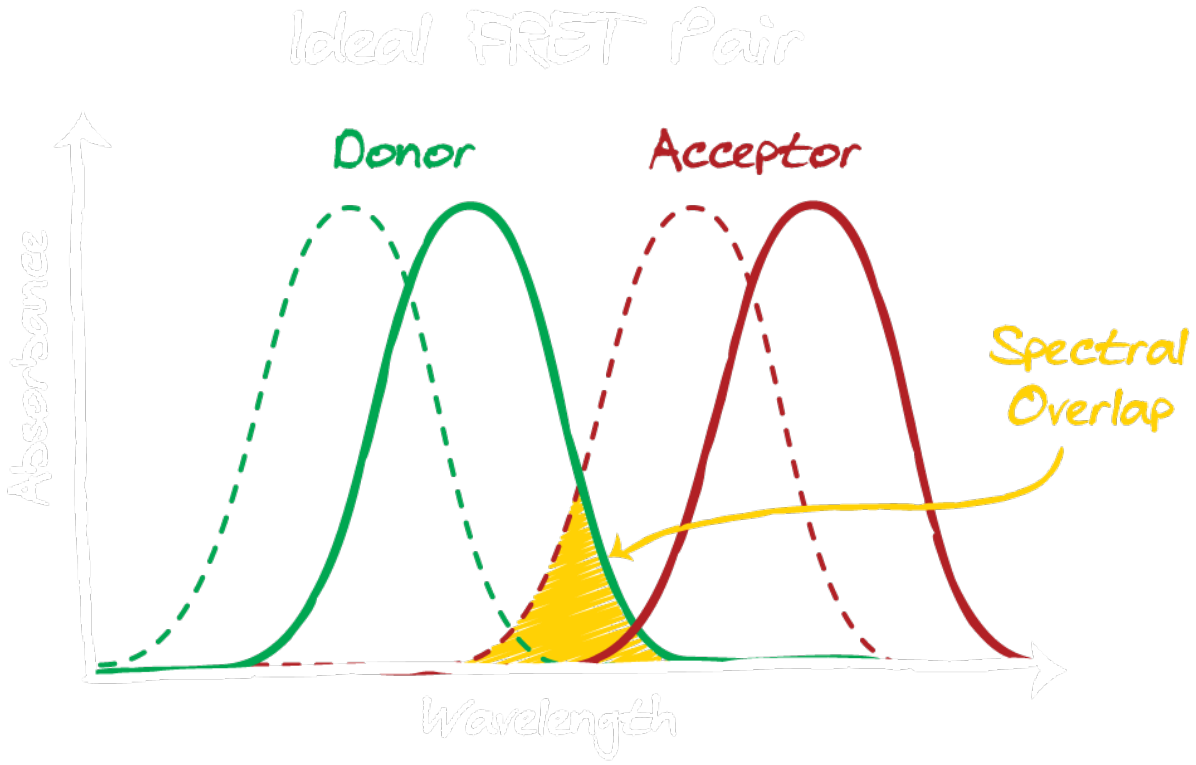


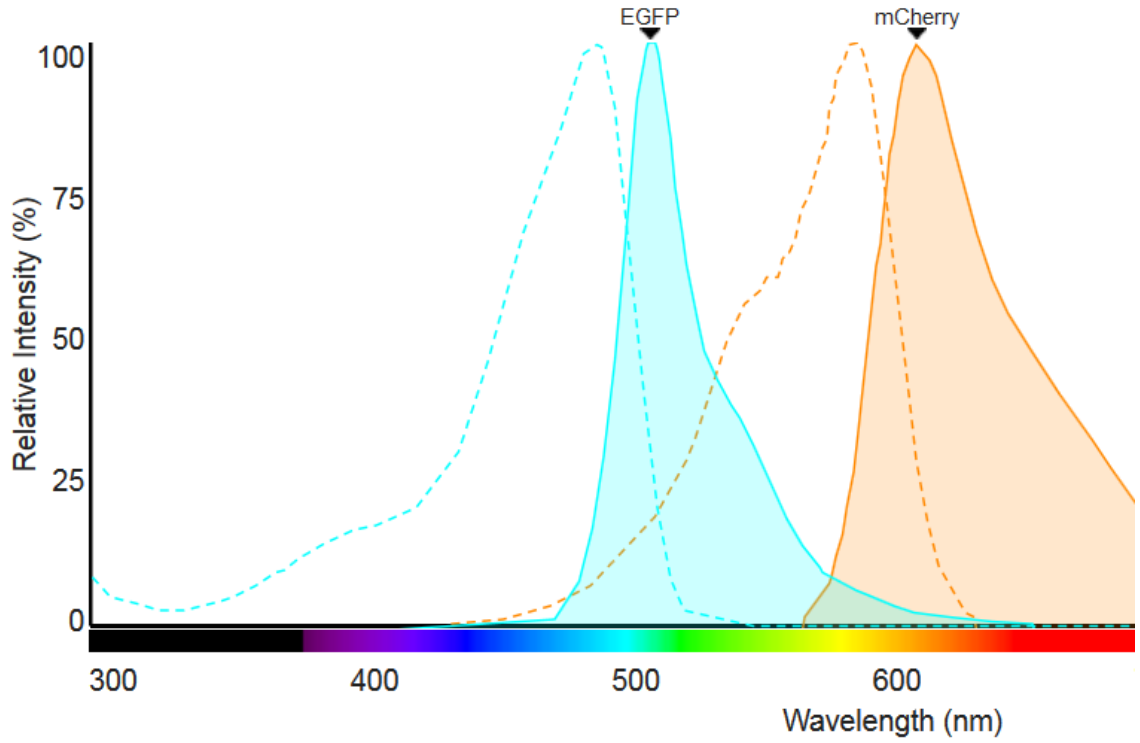
Case study 2: Interaction of fluorescently tagged proteins in response to DNA damage





Is there a more direct way to analyse interaction of proteins (at DNA damage sites, in live cells!)? Yes!

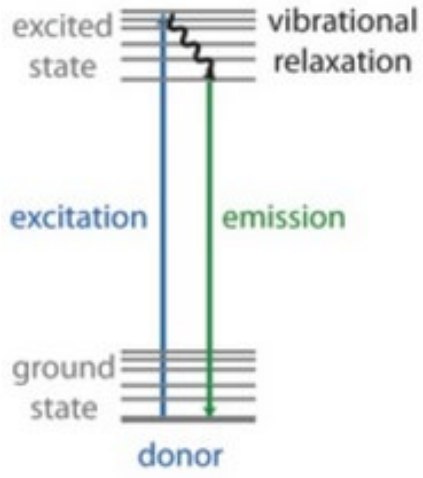




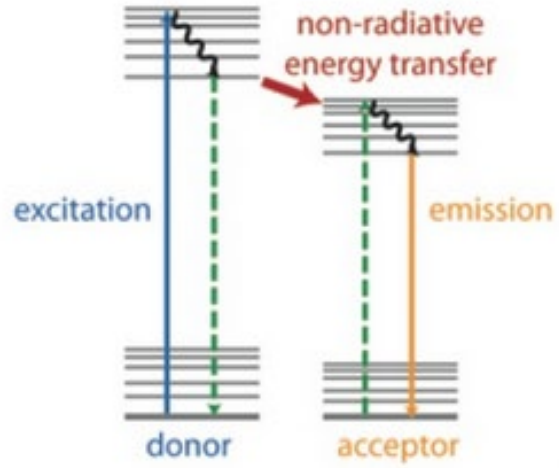
Fluorescence lifetime imaging (FLIM)

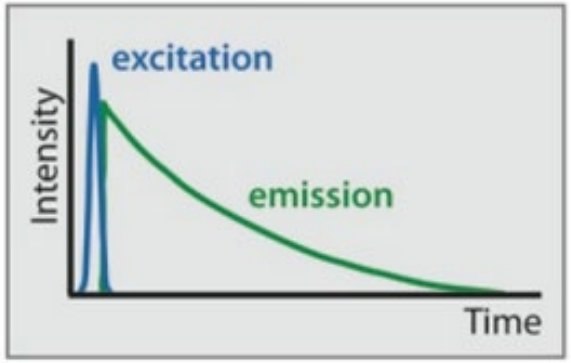
Actually, what is the lifetime?

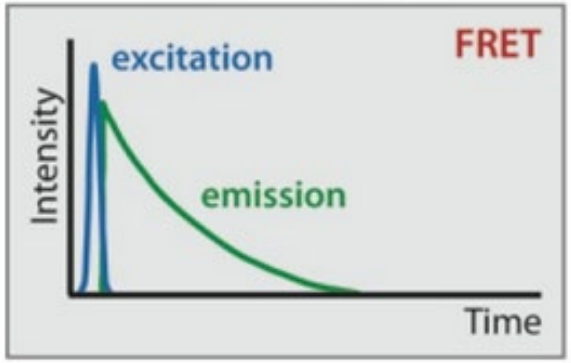
Donor (EGFP) only



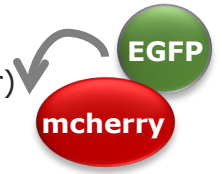
Donor (EGFP) + acceptor (mcherry)



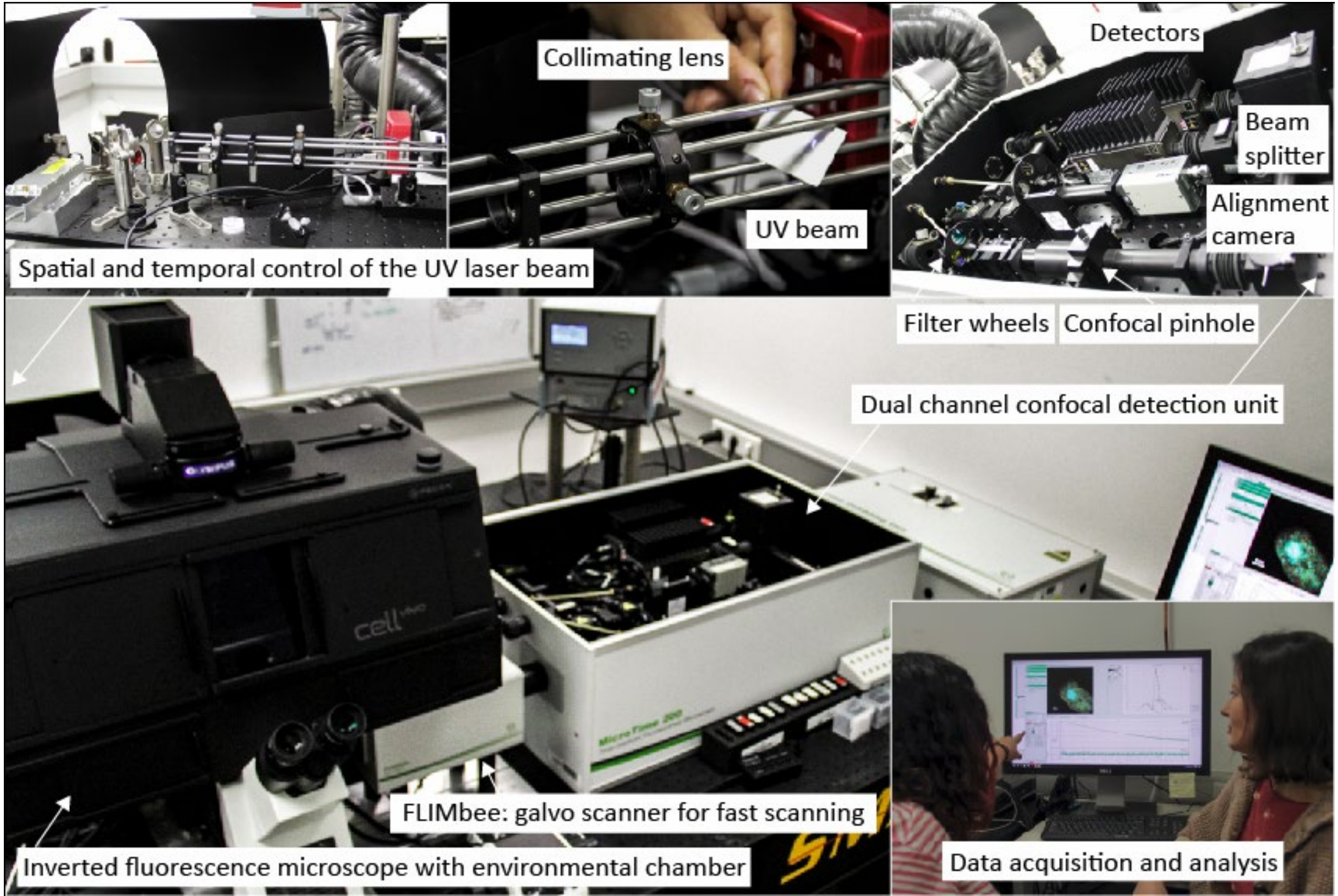


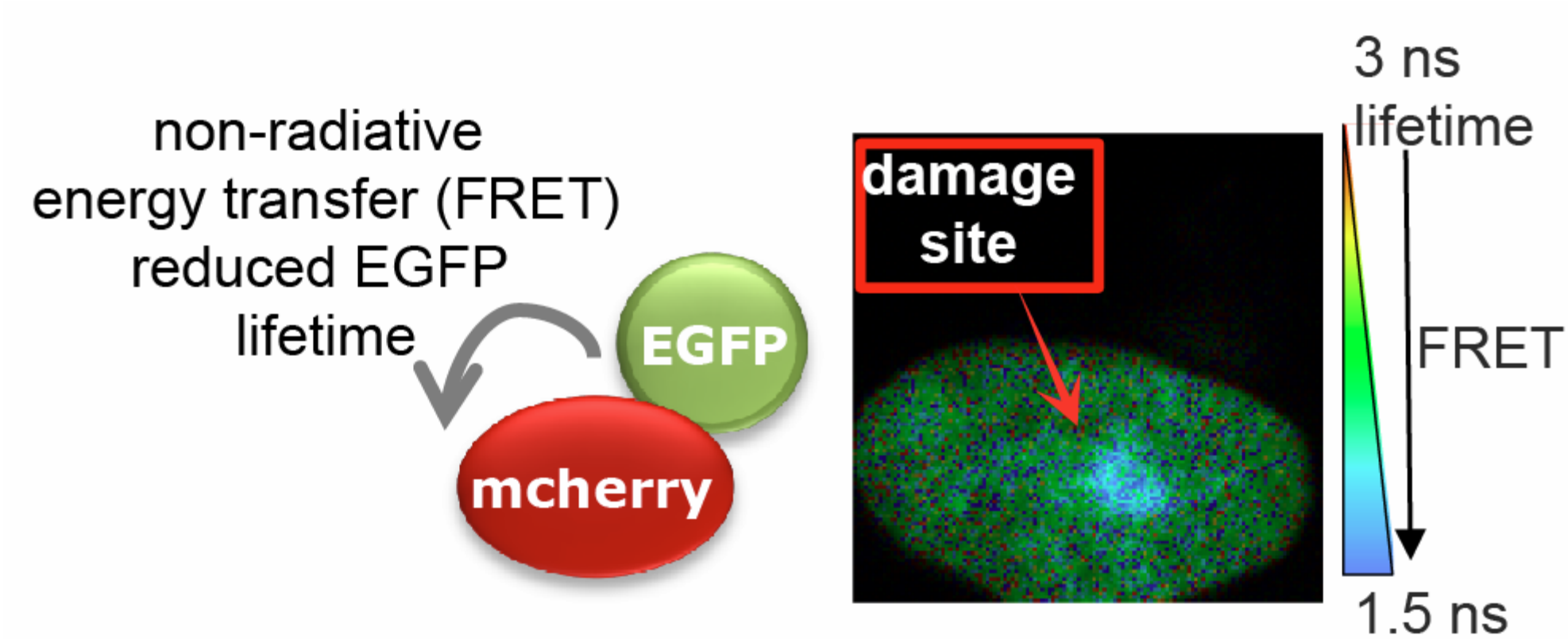


non-radiative Energy transfer **FRET:** Förster resonance energy transfer)

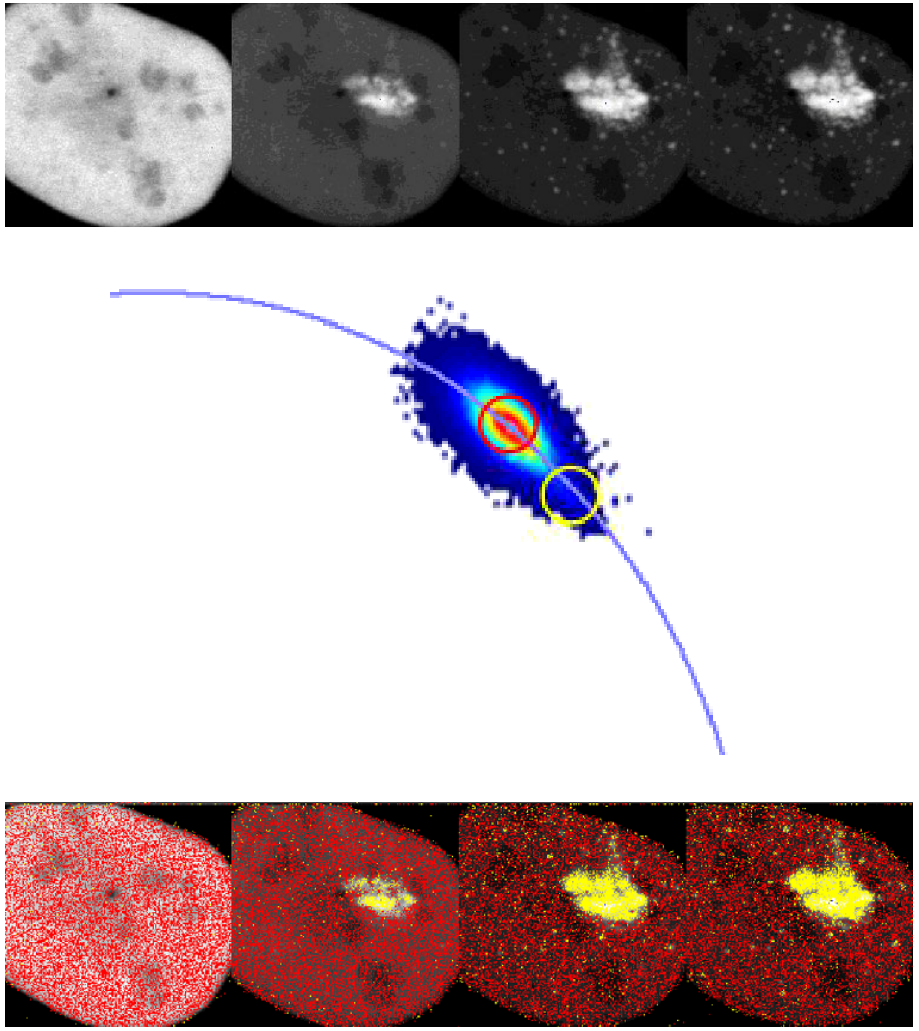


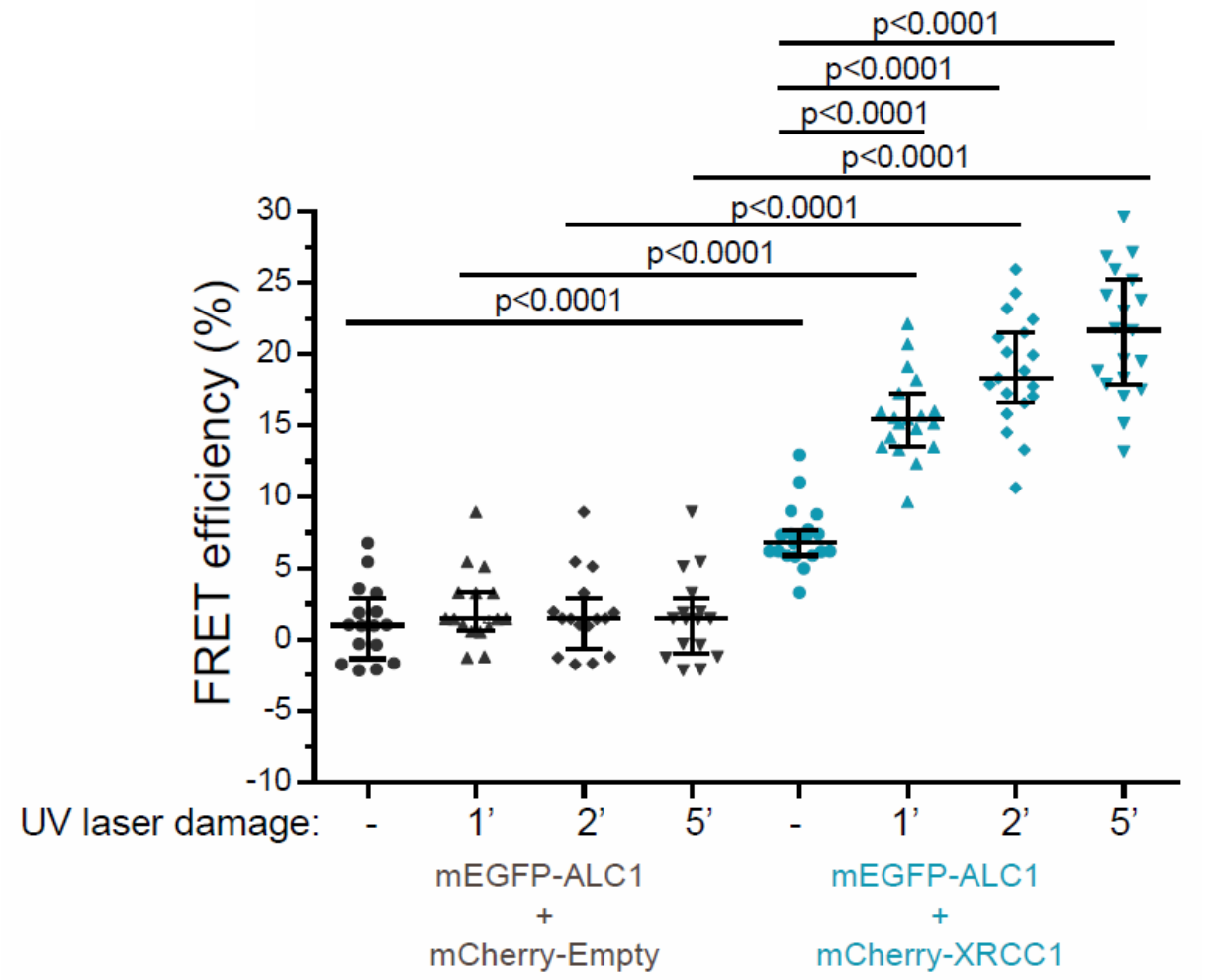
→ **Decreased EGFP intensity** (EGFP quenched by mcherry)
→ **shortened EGFP lifetime**


Collimating lens
UV beam
Detectors
Beam splitter
Alignment camera
Spatial and temporal control of the UV laser beam
Filter wheels
Confocal pinhole
Dual channel confocal detection unit
FLIMbee: galvo scanner for fast scanning
Inverted fluorescence microscope with environmental chamber
Data acquisition and analysis


non-radiative
energy transfer (FRET)
reduced EGFP
lifetime
EGFP
mcherry
damage
site
3 ns
lifetime
FRET
1.5 ns

Case study 3: interaction of ALC1 and XRCC1

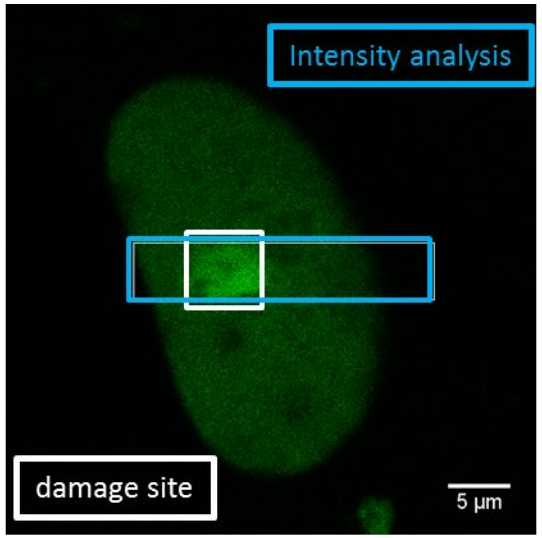


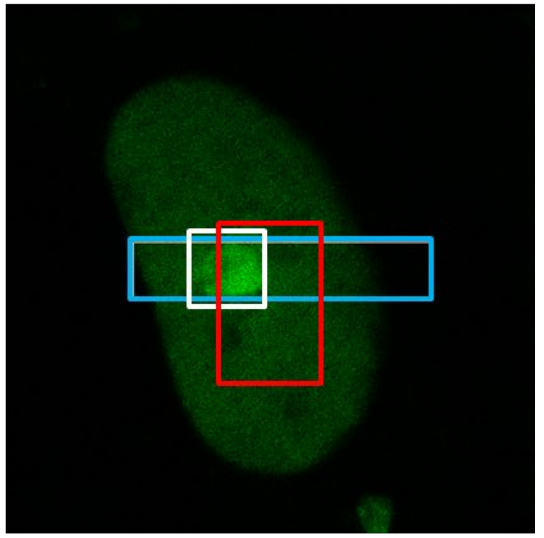


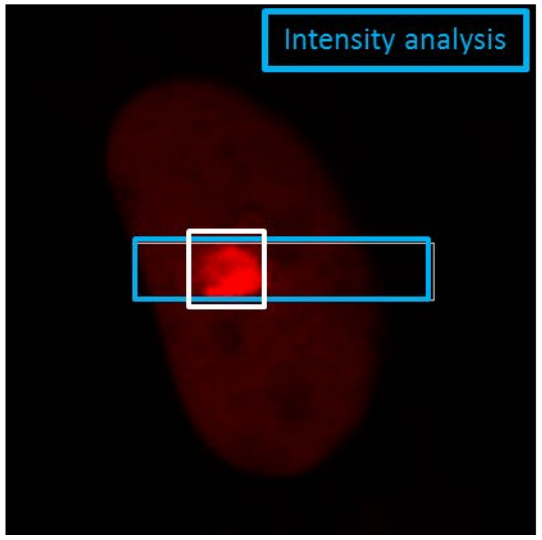
Analysing FRET by acceptor photobleaching

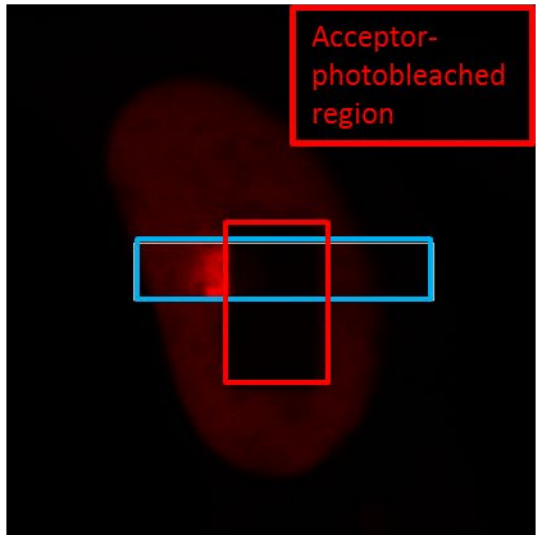
non-radiative Energy transfer (FRET)

→**EGFP quenched by mcherry**
→bleach mcherry
→**intensity of EGFP increases**

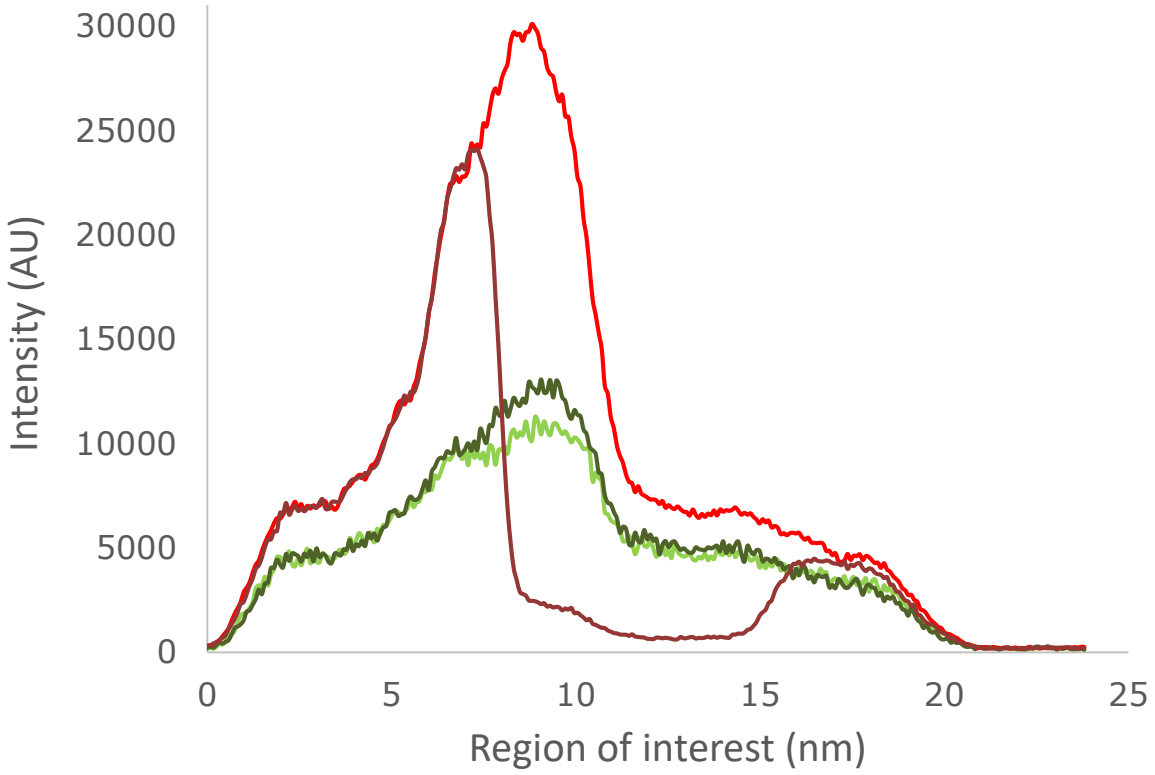


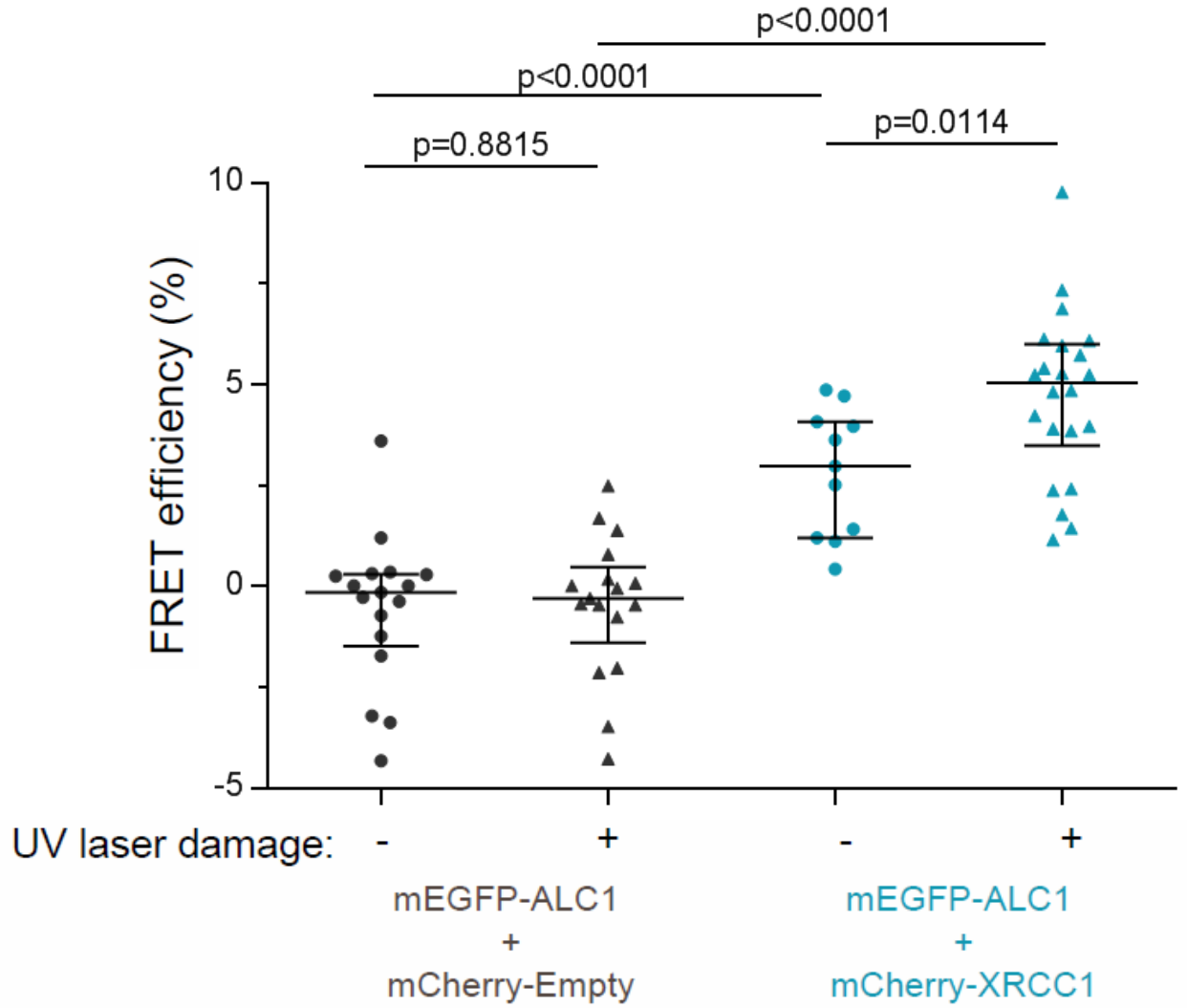




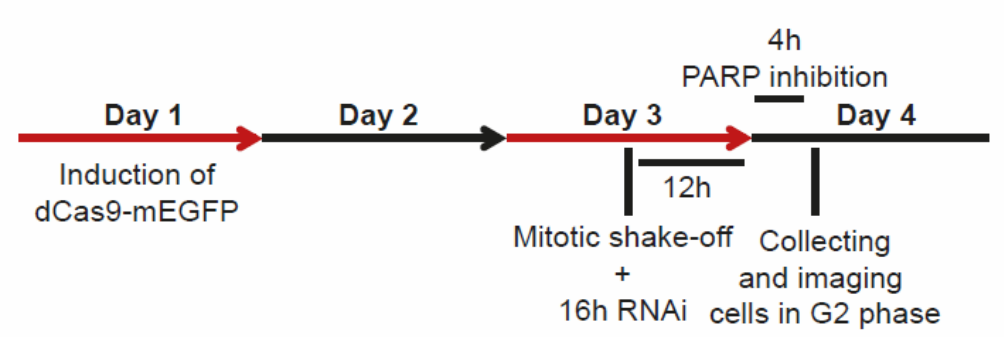


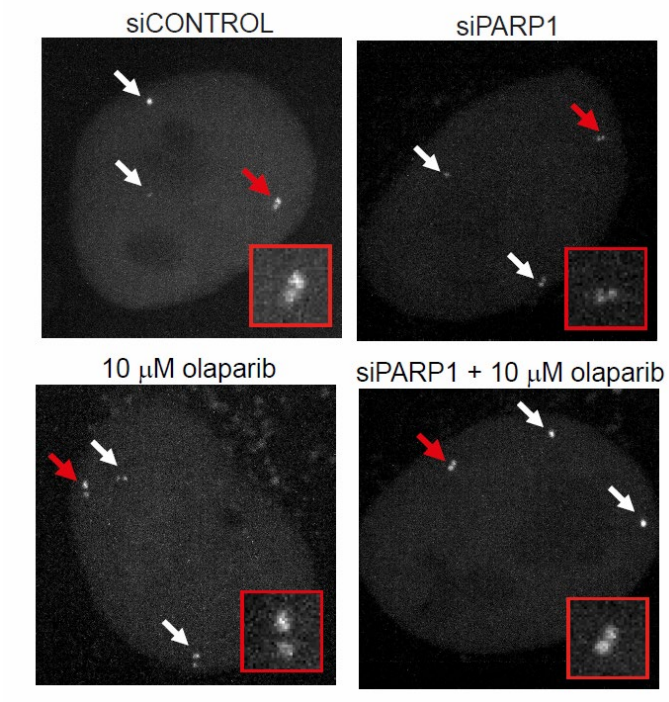
Analysing FRET by acceptor photobleaching

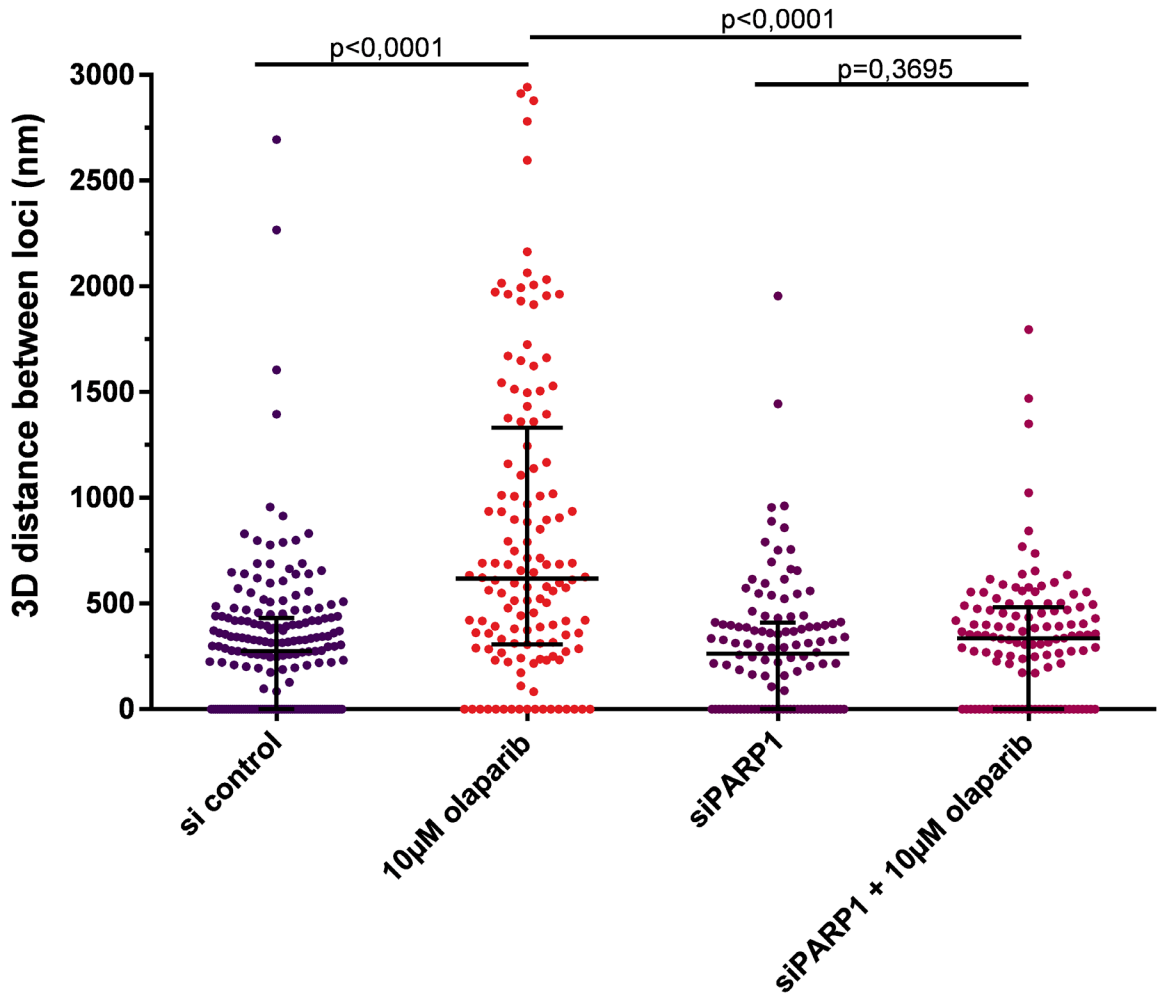




Case study: the effect of drugs on sister chromatid cohesion measured in live cells

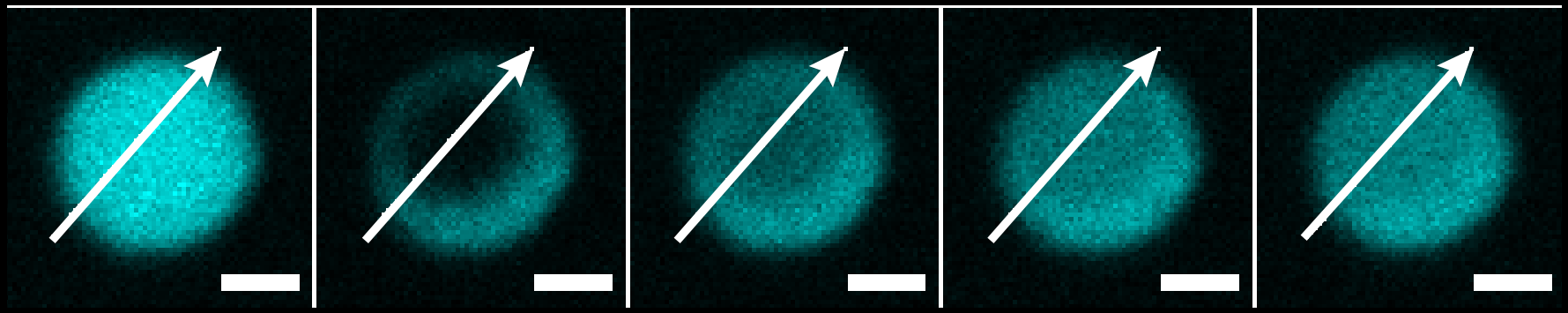






Fluorescence Recovery After Photobleaching (FRAP)

Fluorescence recovery after photobleaching is used to characterize the mobility of cellular molecules a region of interest within the cytoplasm or cellular structures within the cell can be monitored.



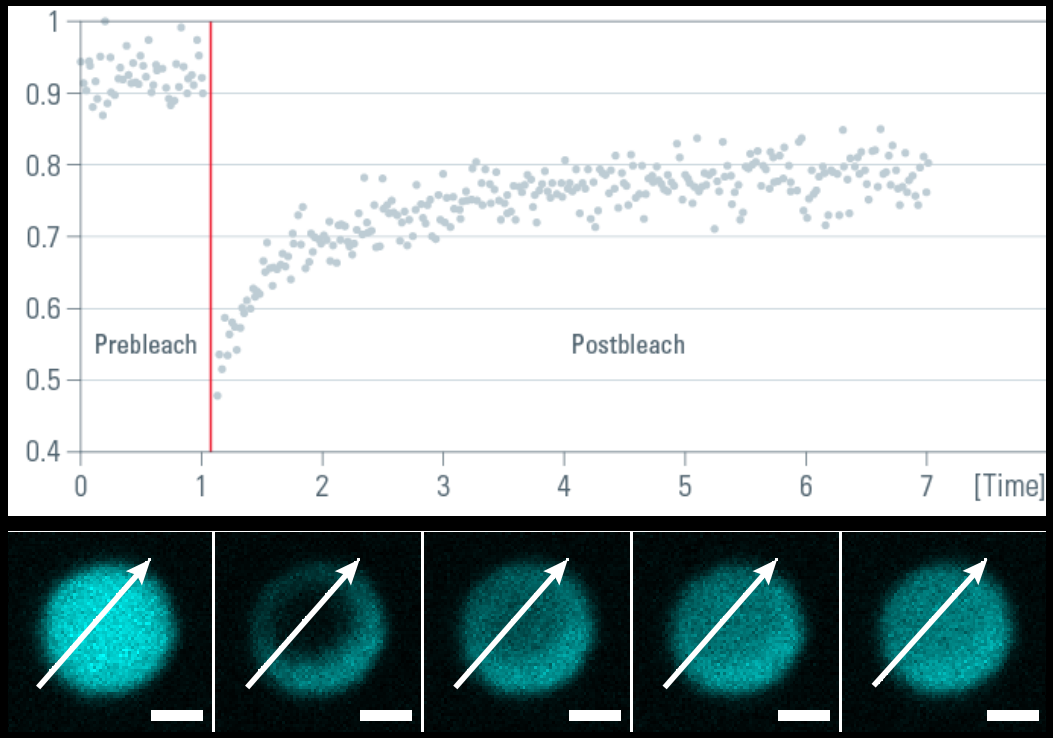
Photobleaching experiments can be conducted with confocal laser scanning microscopes where the laser is used at high intensity for bleaching and low intensity for image recording. It employs irradiation of a fluorophore in a living sample with a short laser to erase fluorescence followed by time-resolved image recording of the sample

Zaffagnini *et al*. 2018

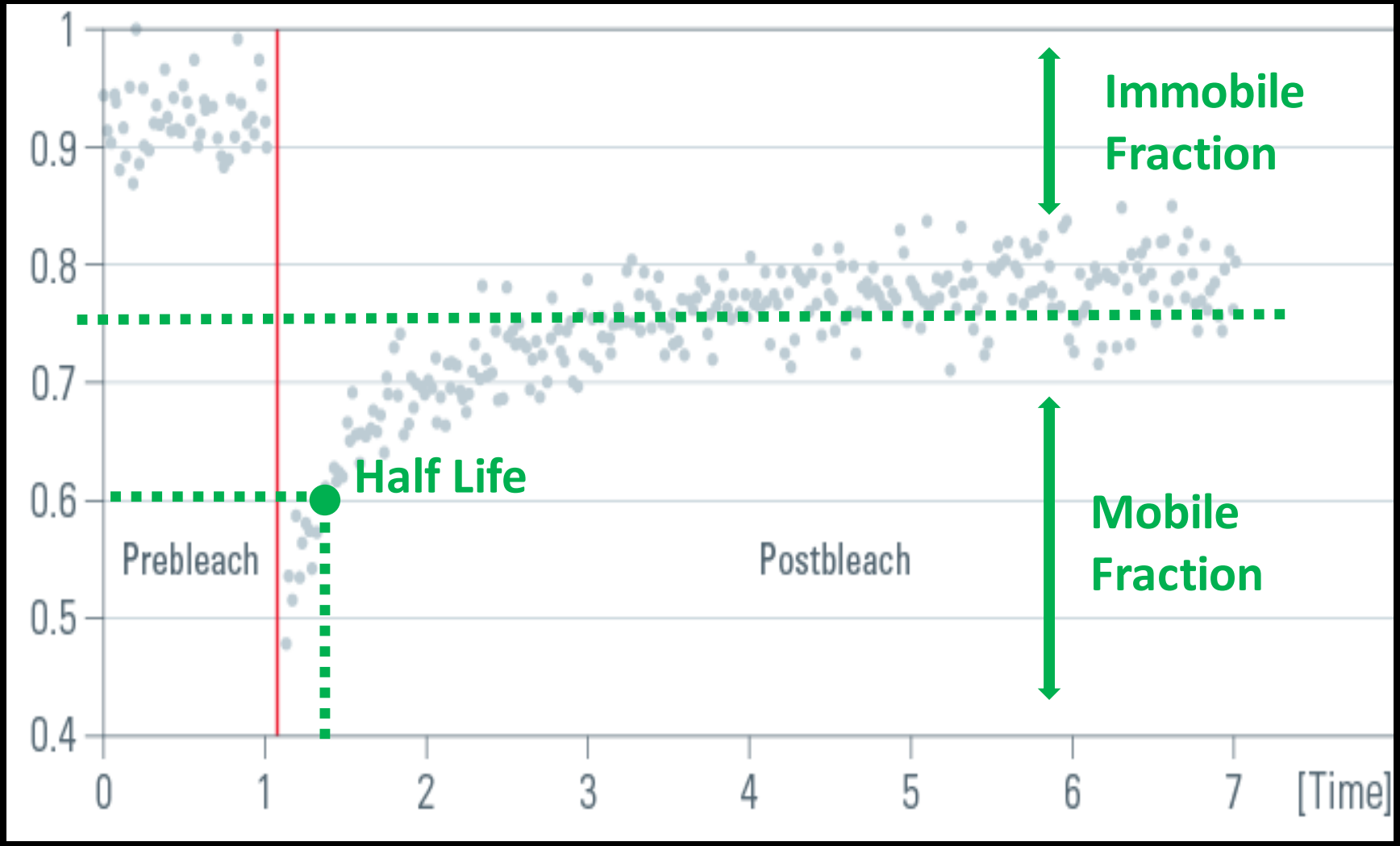
Fluorescence Recovery After Photobleaching (FRAP)

Three different image sequences:

1. **prebleach sequence with low laser power setting to have a reference value;**
2. **bleach sequence with high laser power inside the ROI;**
3. **postbleach setting with low laser power setting to examine the fluorescence recovery**



Fluorescence Recovery After Photobleaching (FRAP)



Fluorescence Recovery After Photobleaching (FRAP)

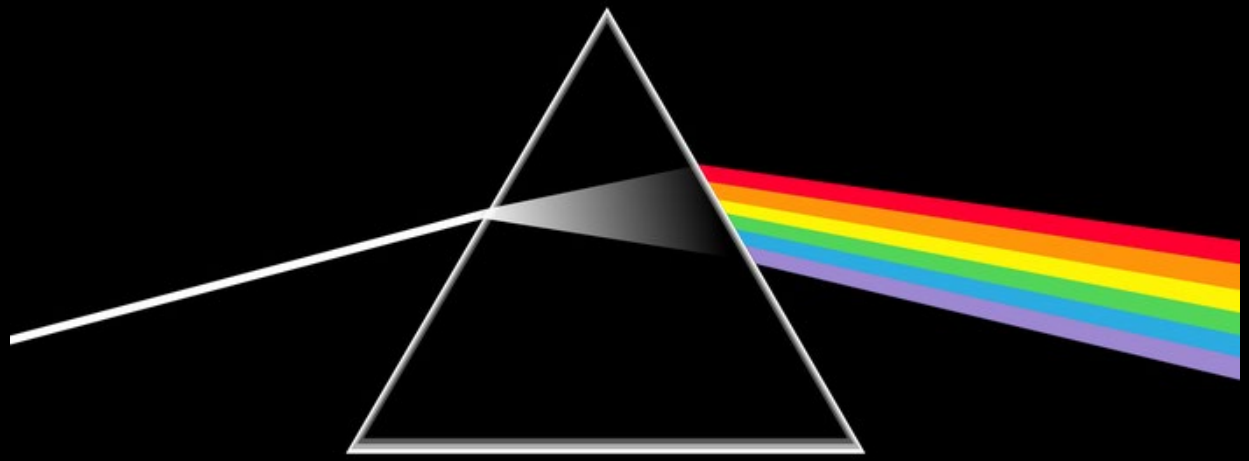
CAVEAT AND PITFALL

The main advantage of FRAP is its straightforward and simple strategy, however when it comes to a more quantitative evaluation of the FRAP, many subtle problems arise.

driving force of the recovery;
stochastic diffusion, directed motion or binding events;
anomalous diffusion of diffusion on membranes

Fluorescence Correlation Spectroscopy (FCS)

Correlative Light and Electron Microscopy (CLEM)

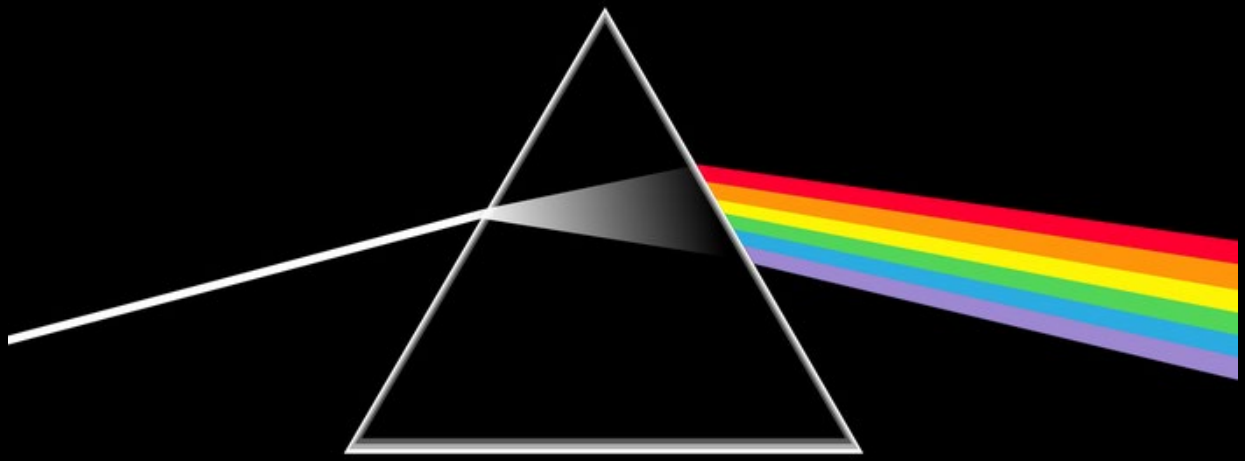


IS TWO BETTER THAN ONE

Correlative Light and Electron Microscopy (CLEM)

Light microscopy permits the observation of dynamic events in living cells, but the spatial resolution is limited to 200-300 nm.

Conversely, EM provides higher spatial resolution but affords only static images



Correlative Light and Electron Microscopy combine the advantages of classical light and electron microscopy

Correlative Light and Electron Microscopy (CLEM)

Markers for correlative microscopy have recently been divided in 3 categories

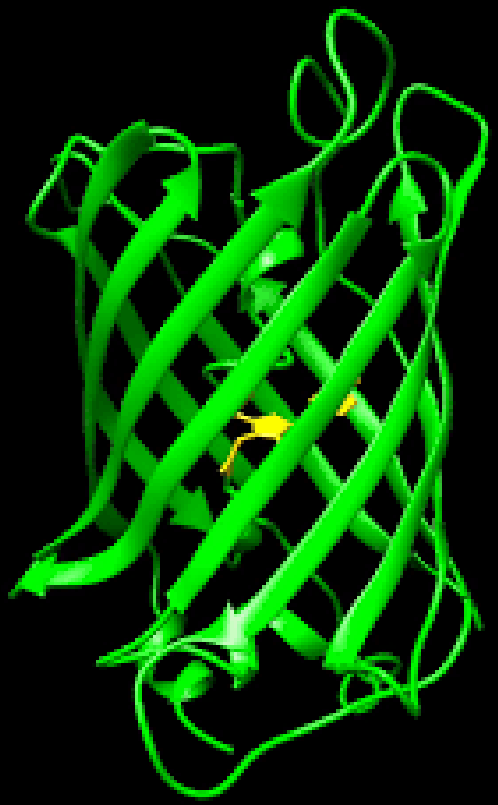
1. fluorescence photooxidation;

2. enzyme-based methods;

3. particle-based methods.

Sosinsky *et al*. (2007

Correlative Light and Electron Microscopy (CLEM)



NEED O_2

NEED DAB

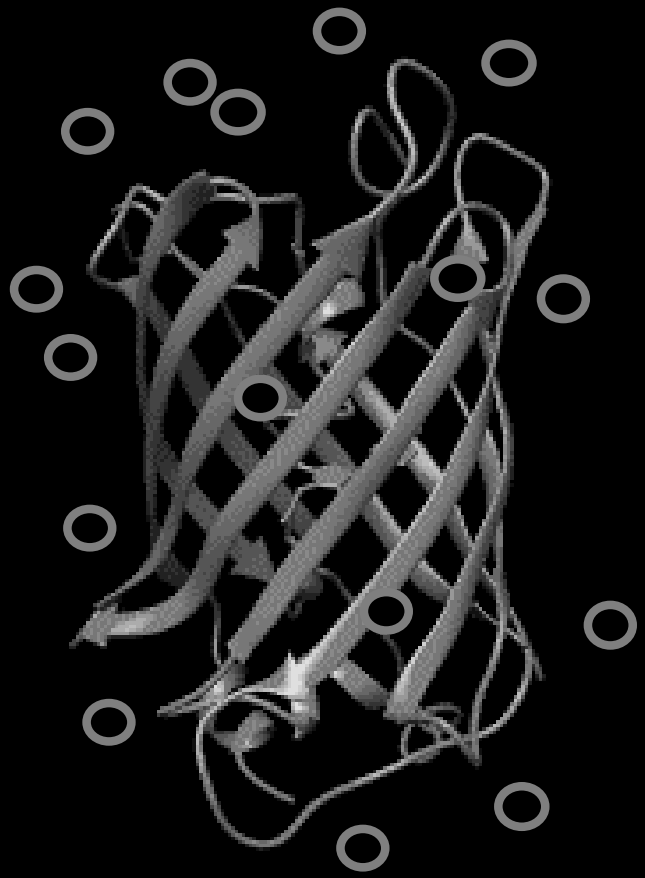
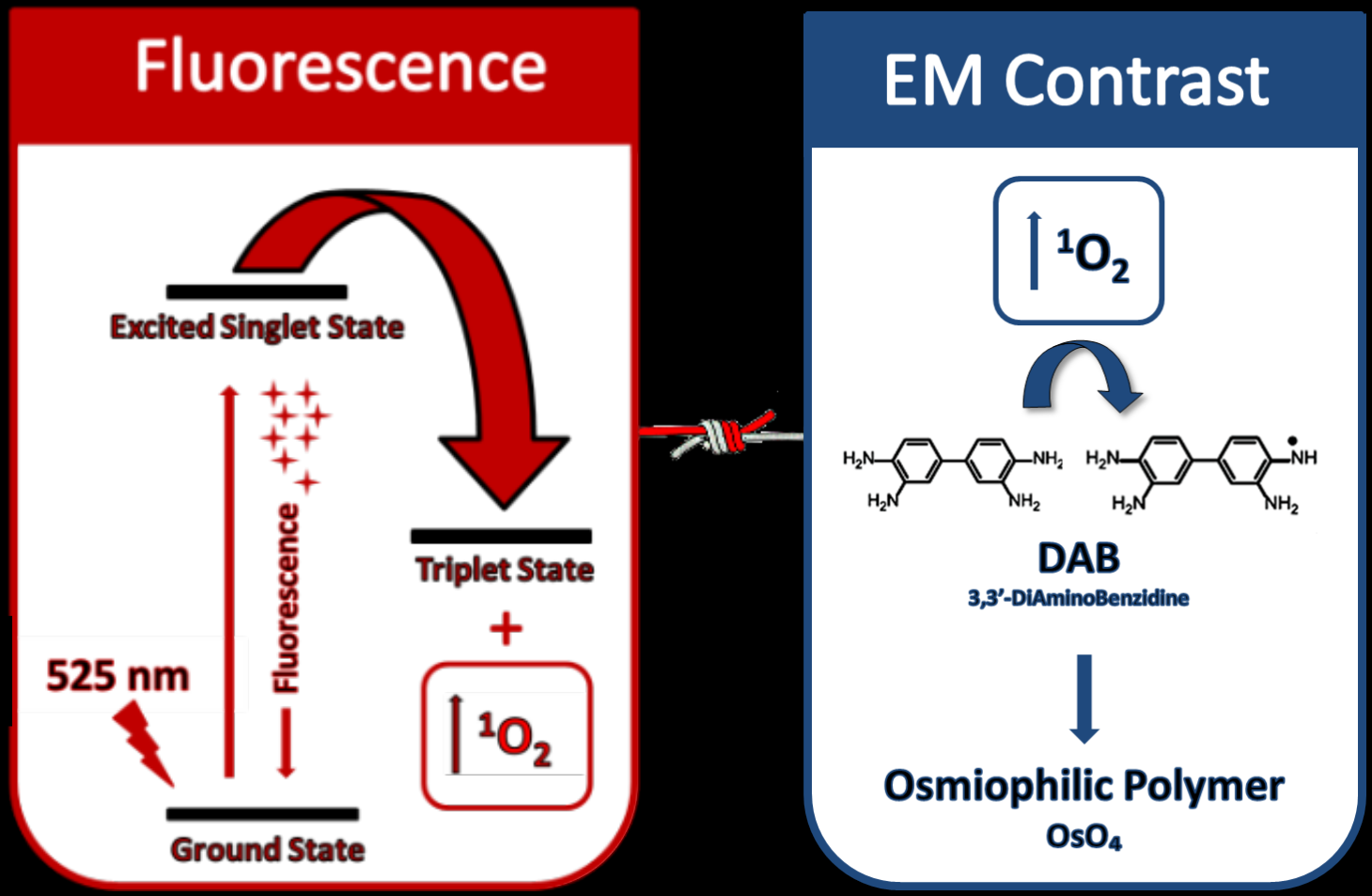


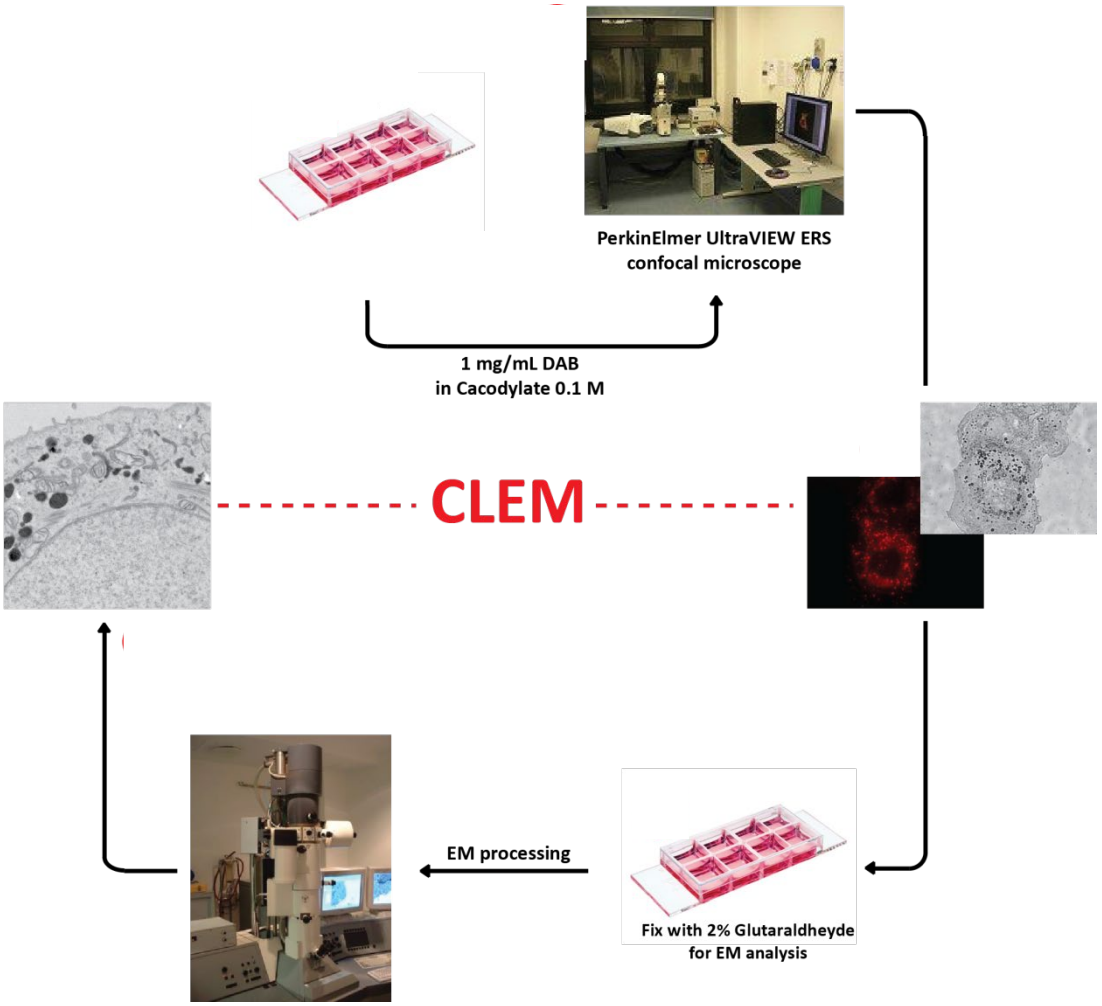
PHOTO-OXIDATION

Correlative Light and Electron Microscopy (CLEM)



NEED O_2

Correlative Light and Electron Microscopy



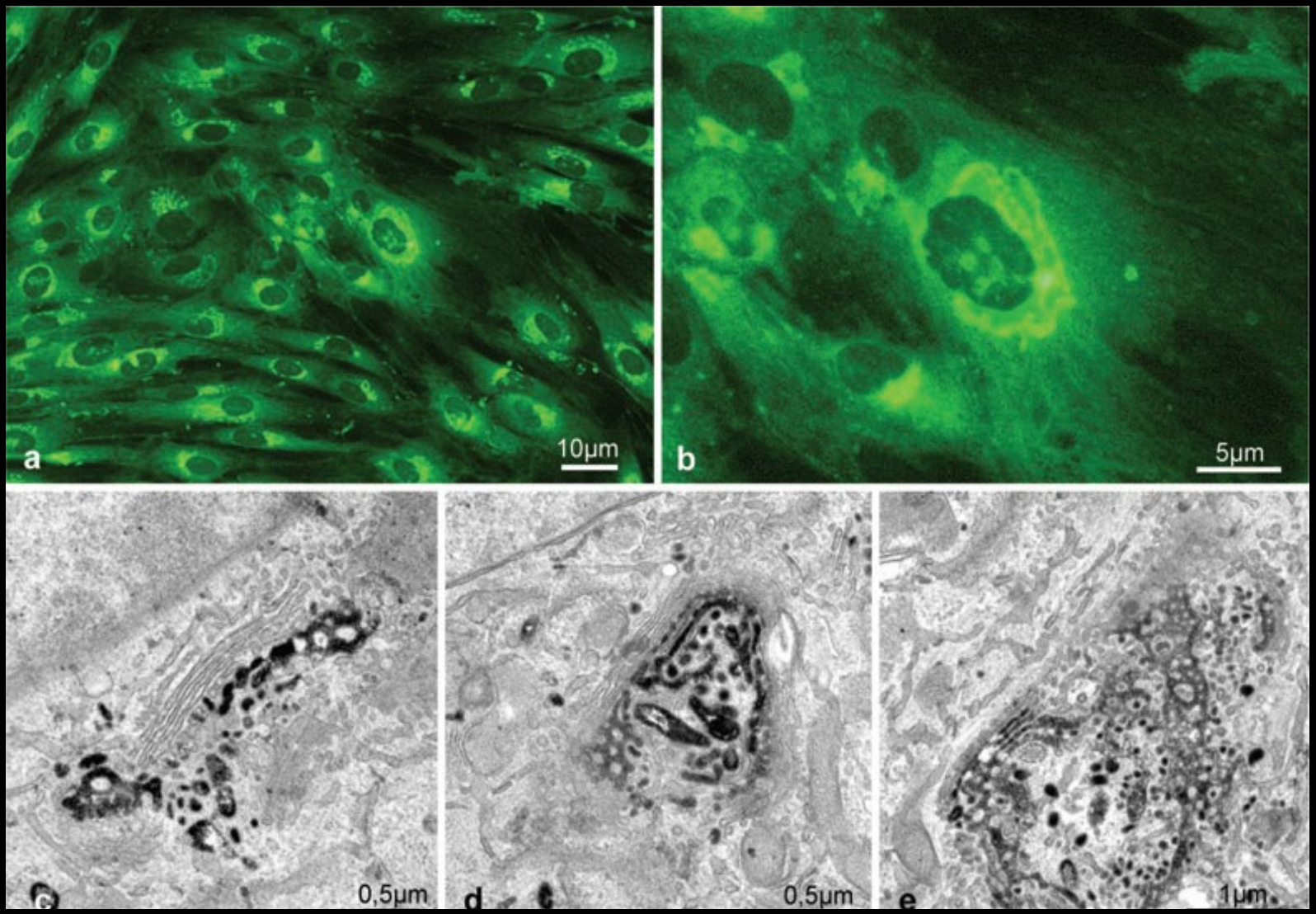
lack of permeabilization facilitate the quality of fine structural preservation.

stable fixation contributes to higher resolution of the reaction, however it might cause background fluorescence

DAB solutions freshly prepared containing 0.5–2 mg/mL in saline buffer

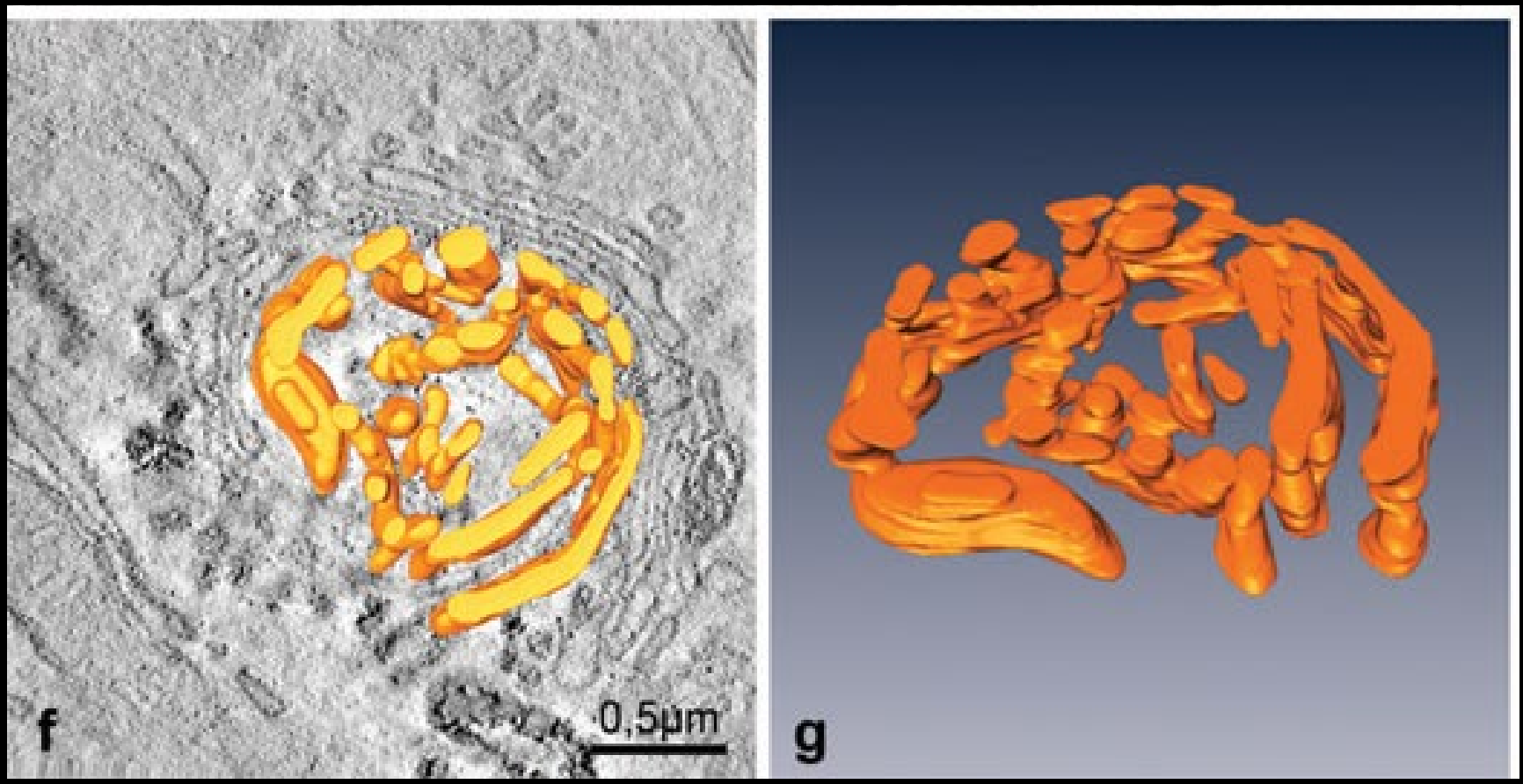
higher magnification objectives and/or enriching the medium with oxygen

Correlative Light and Electron Microscopy (CLEM)



C. Meisslitzer-Ruppitsch *et al.* 2019

Correlative Light and Electron Microscopy (CLEM)



C. Meisslitzer-Ruppitsch *et al*. 2019


THANK FOR YOUR ATTENTION