Superresolution two-photon fluorescence microscopy using photoswitchable fluorescent proteins

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jointly organized by the Institute of Nanobiology and Structural Biology of GCRC,
Academy of Sciences of the Czech Republic,
the University of Szeged, Hungary,
Jagiellonian University, Krakow, Poland,
and Comenius University in Bratislava, Slovakia,
and supported from
International Visegrad Fund

The principal goal

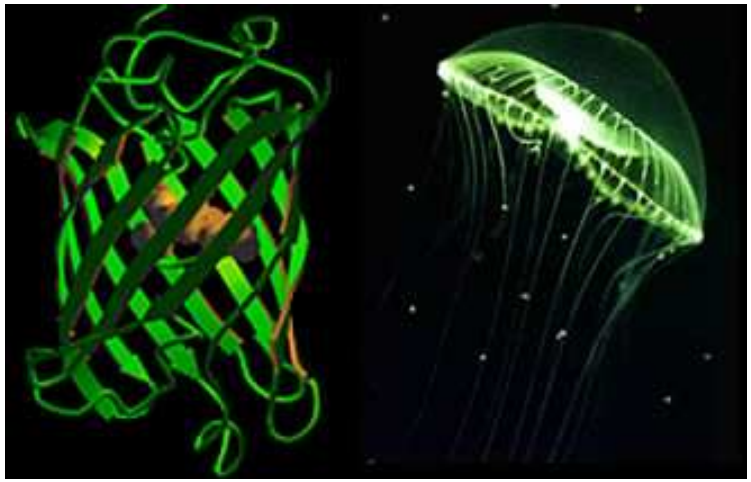
- Combine the method of two-photon polarization microscopy with microscopy techniques allow breaking the diffraction limit on resolution of optical microscopes.

Aim of the project

- Create a membrane-localized photoswitchable fluorescent protein suitable for combining two-photon polarization microscopy with STED superresolution microscopy.

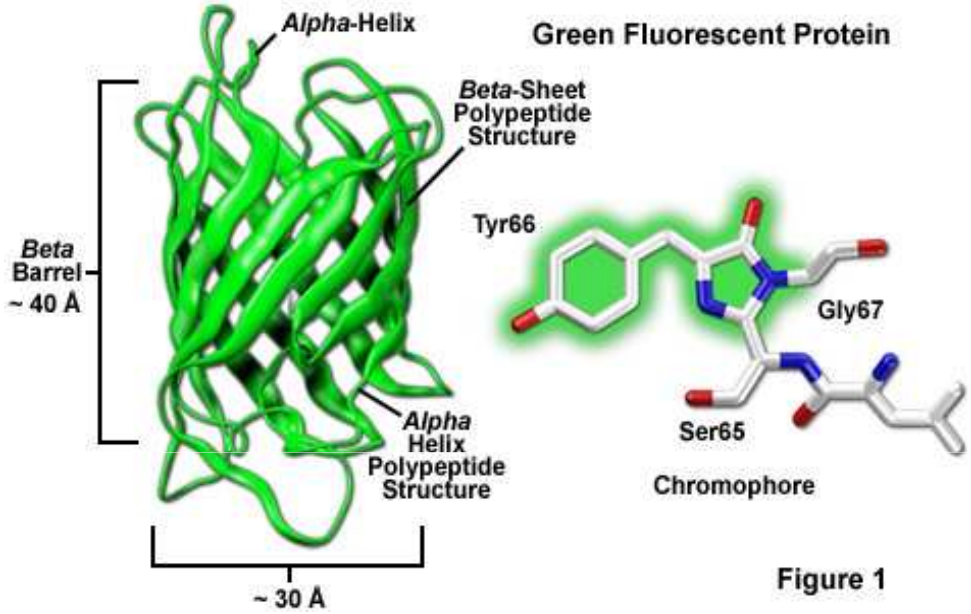
What is the GFP?

- Green Fluorescent Protein
- 238 amino acids
- Fluorescence
- Aequorea victoria



http://www.bscb.org/?url=newsletter/spring2009/news1, http://elprogreso.galiciae.com/nova/18130.html?lang=es

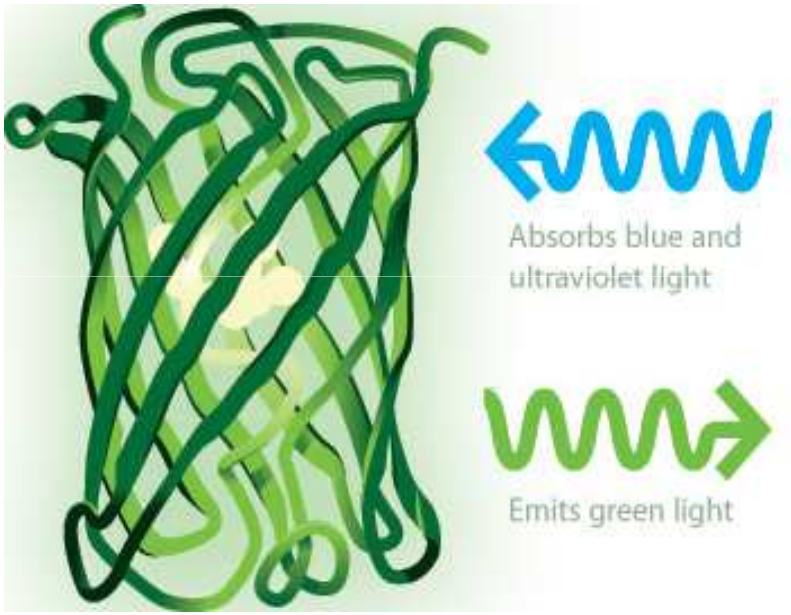
Structure of the GFP



- Chromophore/fluorophore
- Planar structure

http://zeiss-campus.magnet.fsu.edu/articles/probes/fpintroduction.html

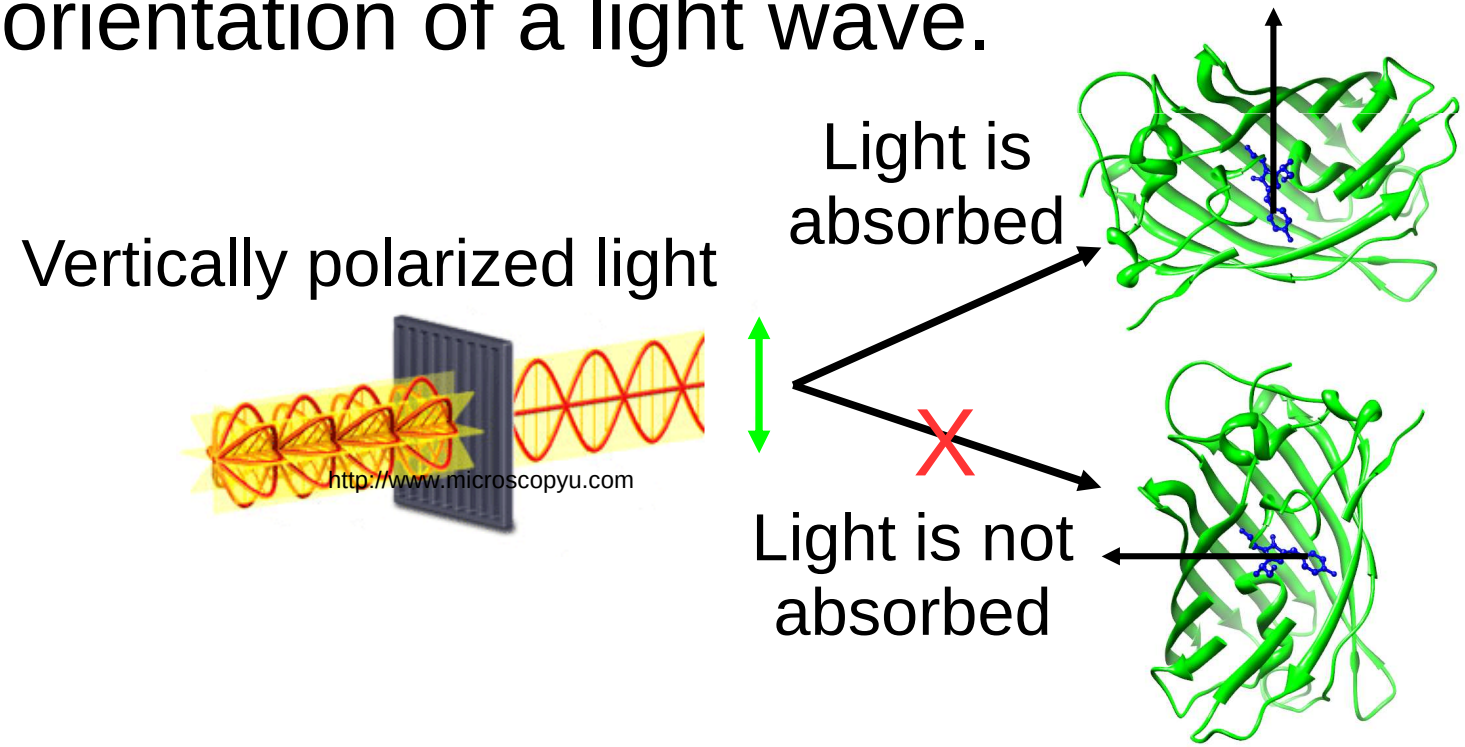
How can we use fluorescent proteins for studies of membrane proteins?



http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2008/illpres.html

Fluorescent proteins are sensitive to excitation light polarization

- *Light polarization* is the electric field vector orientation of a light wave.



Two-photon polarization microscopy (2PPM)

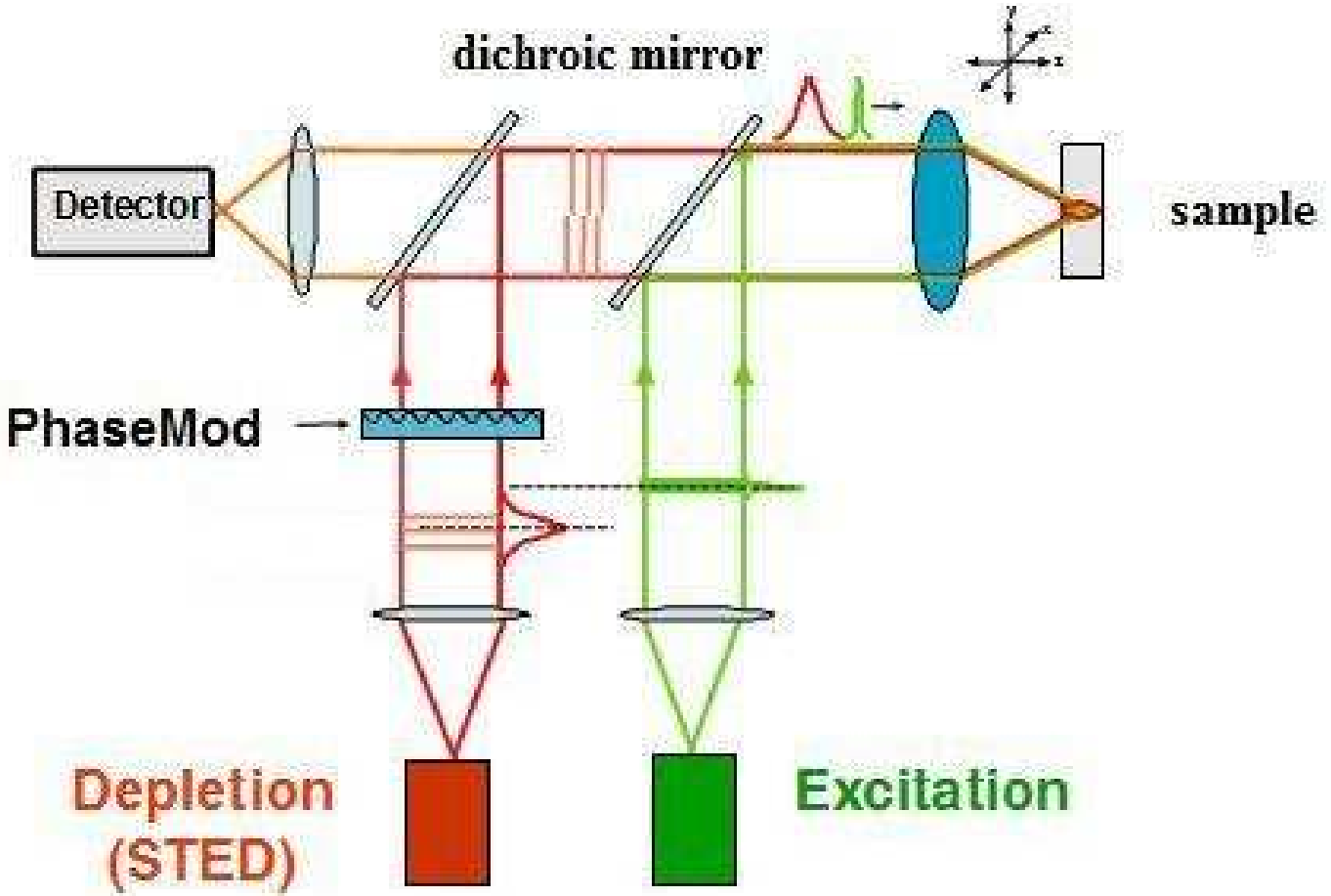
- A method of live cell microscopy which utilizes an optical property of fluorescent proteins called linear dichroism for determination of protein orientation with respect to the cell membrane.

- - live cell microscopy,
- - detects linear dichroism of fluorescent proteins,
- - works for membrane proteins (requires membrane),
- - shows protein orientation with respect to the membrane.

STED and RESOLFT (stimulated emission depletion)

- STED technique utilizes two beams: excitation and doughnut-shaped depletion beam, to excite fluorescence in an area smaller than diffraction limit.
- RESOLFT technique uses photoswitchable fluorescent proteins in order to obtain superresolution.

STED setup


dichroic mirror
Detector
sample
PhaseMod
Depletion
(STED)
Excitation

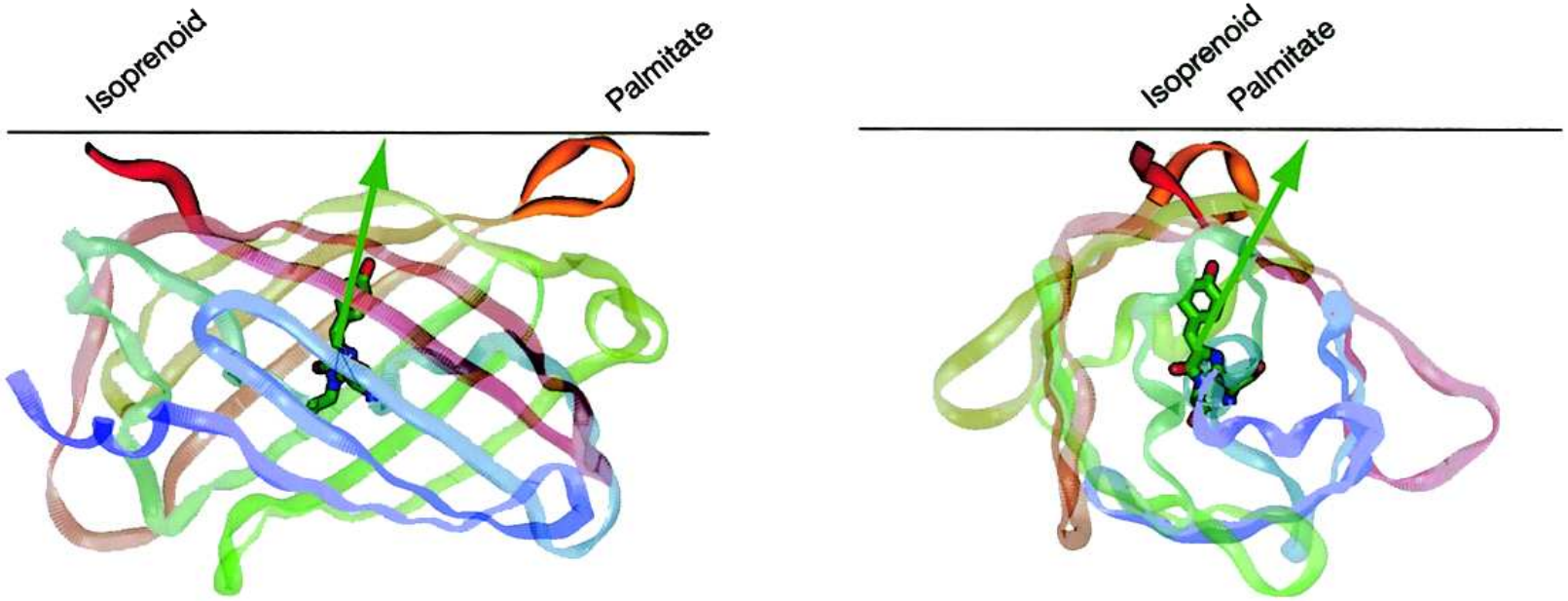
What is required for studies of the membranes?

- Need a protein which is:
- In the membrane or
- Is attached to the membane.

- Original GFP- cytoplasmic localized.
- dleGFP.

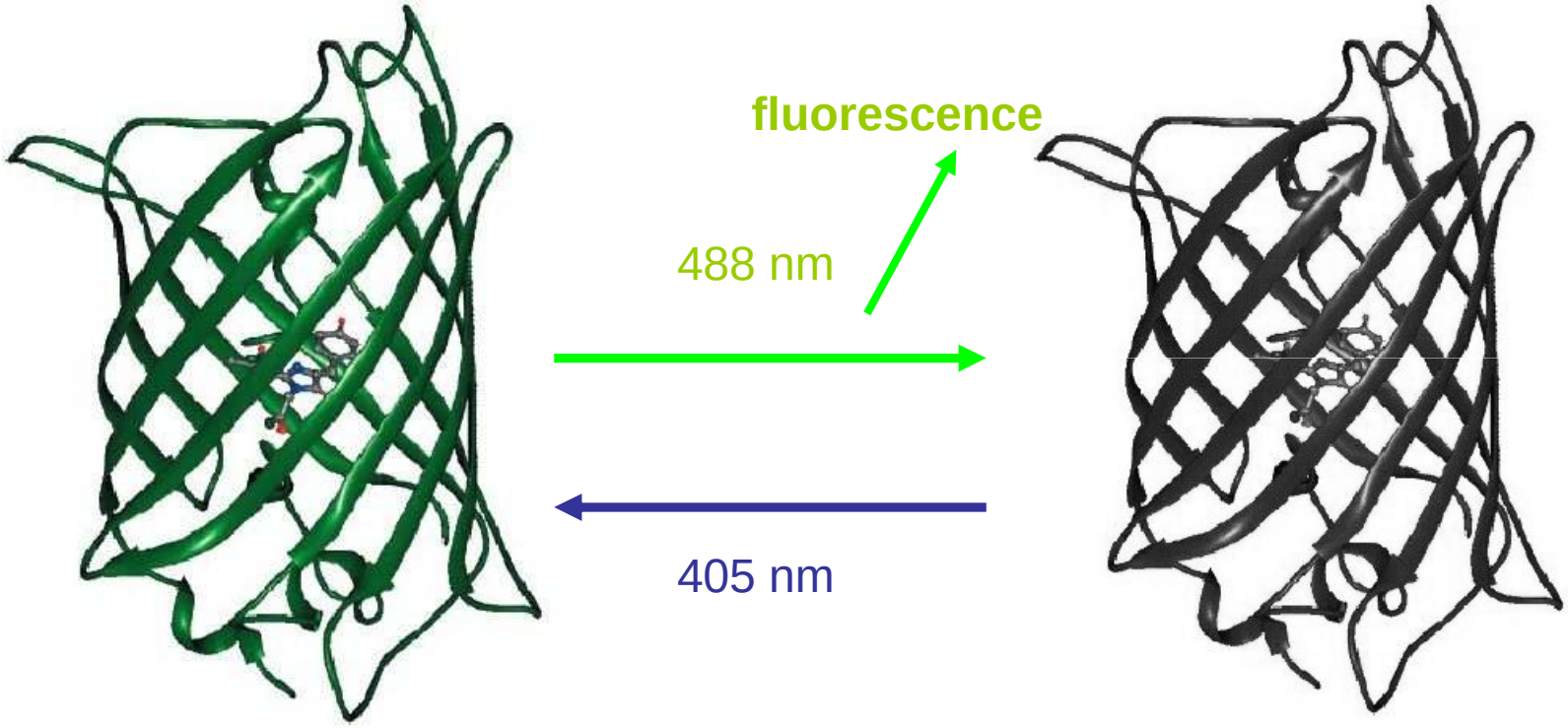
dleGFP

- Doubly lipidated.
- Model construct for observation of eGFP linear dichroism in live cells.



(Roorda et al J. Neurophysiol. 2004)

Photoswitchable GFP



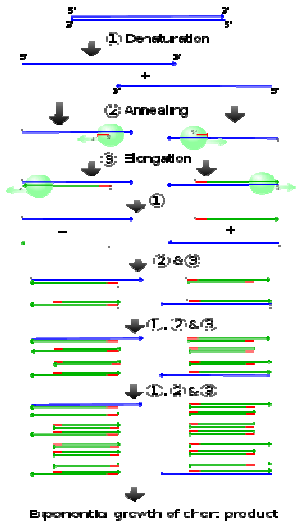
http://www.science.uva.nl/research/molphot/research/sm/peterSMS.html

Experimental steps

- 1. Creating the mutant construct.
- 2. Testing the fluorescence properties.
- 3. Measuring linear dichroism of the mutant.

1.Creating the mutant construct

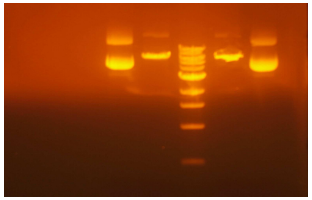
PCR

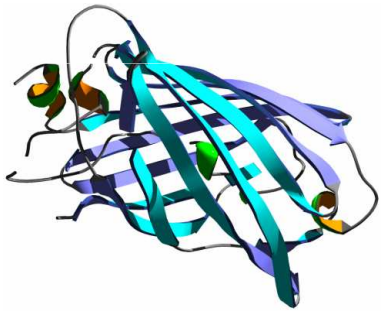


Get mutation in the DNA

Linear DNA

Electrophoresis- cut one band

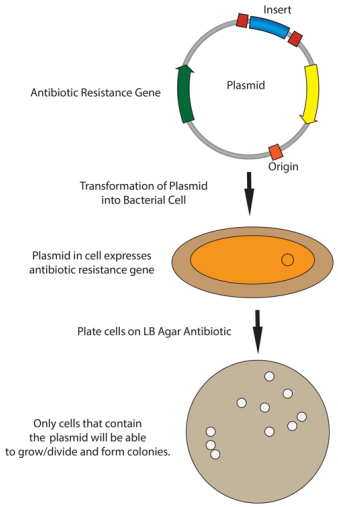




Phosphorylation and ligation

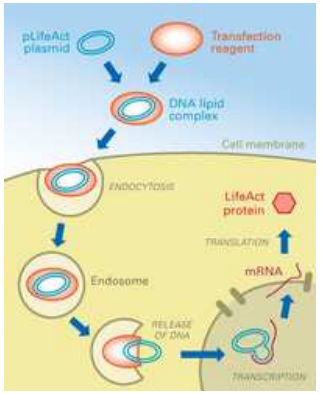
Make DNA circular

transformation



Grow DNA in bacteria in LB

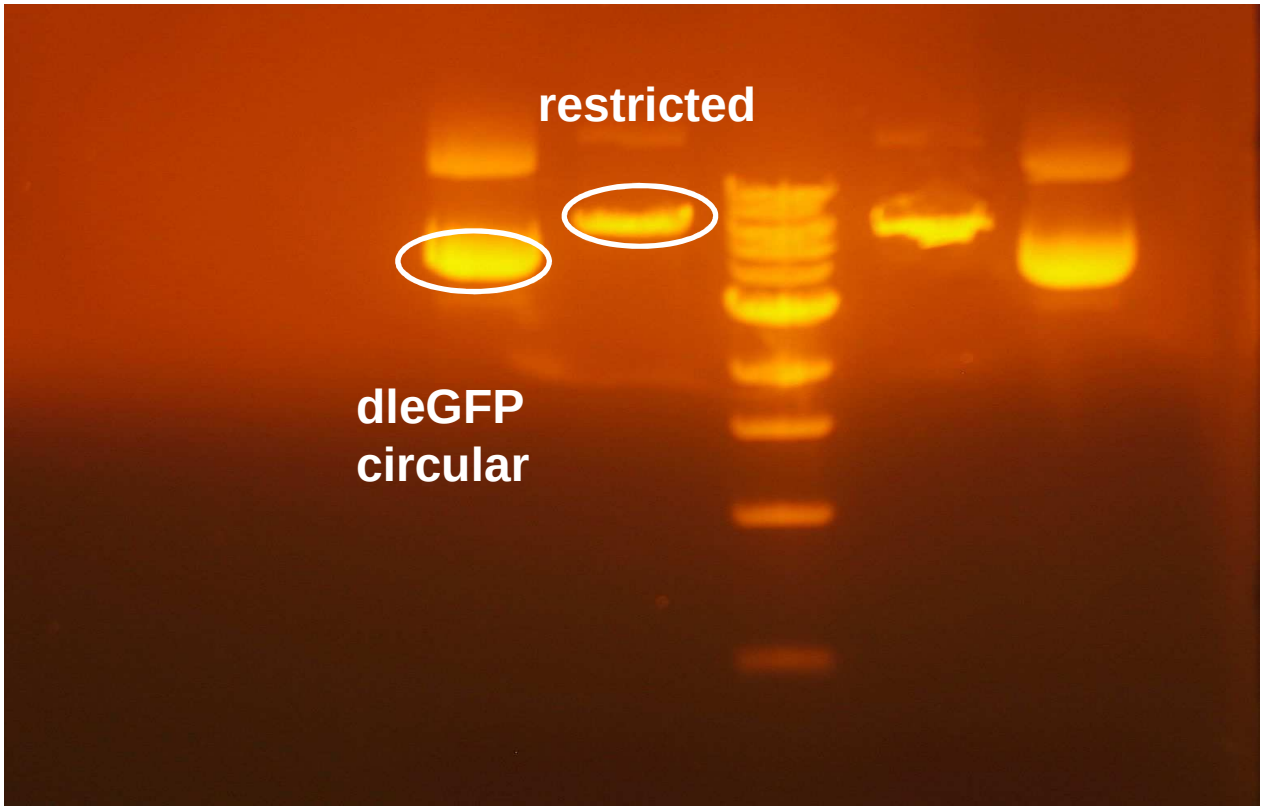
Miniprep -elution the DNA from bacteria

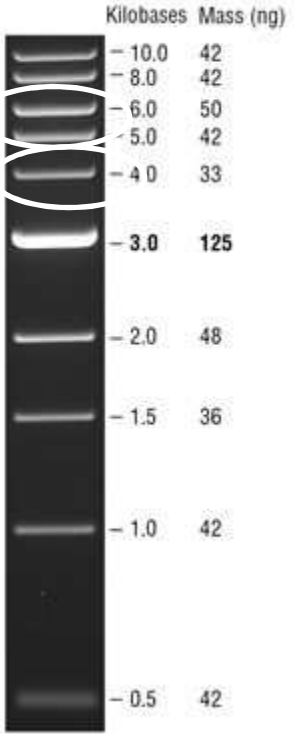


Transfection HEK 293 cells

Microscopy of mutated dleGFP

Original template and restriction with BaeI





Mutation of dleGFP

- Photoswitchable protein.
- EGFP[T65A, Q69L, V163S, A206K]
- PCR (Phusion Hot Start)

(**Grotjohann, 2012)**

PCR with primers A206K,T65A and Q69L

- A206K.

5'CTTATGGTGTTCTATGCTTTTCAA3(F),

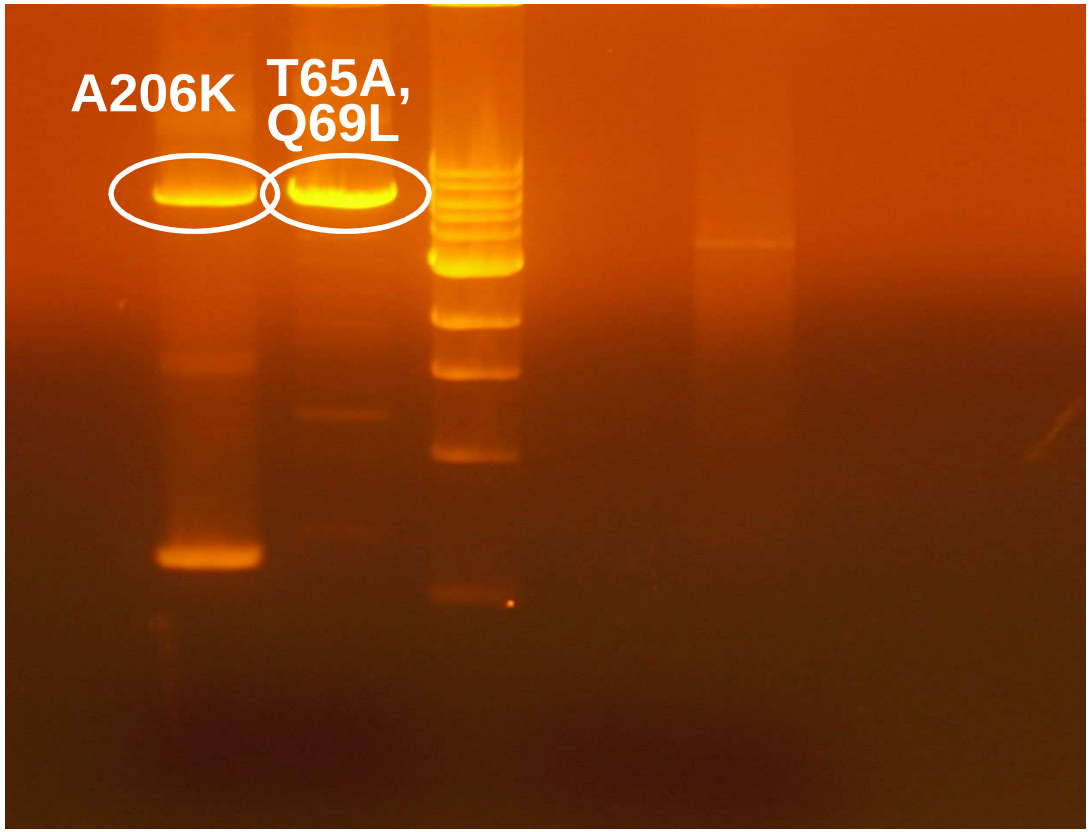
5'CTAAAGTAGTGACAAGTGTTGGC 3(R),

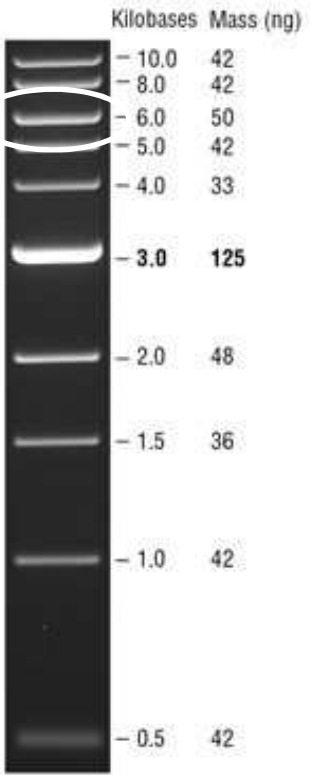
- T65A, Q69L.

5'CTTATGGTGTTCTATGCTTTTCAA3(F),

5'CTAAAGTAGTGACAAGTGTTGGC3,(R).

Mutation A206 K, T65A and Q69L



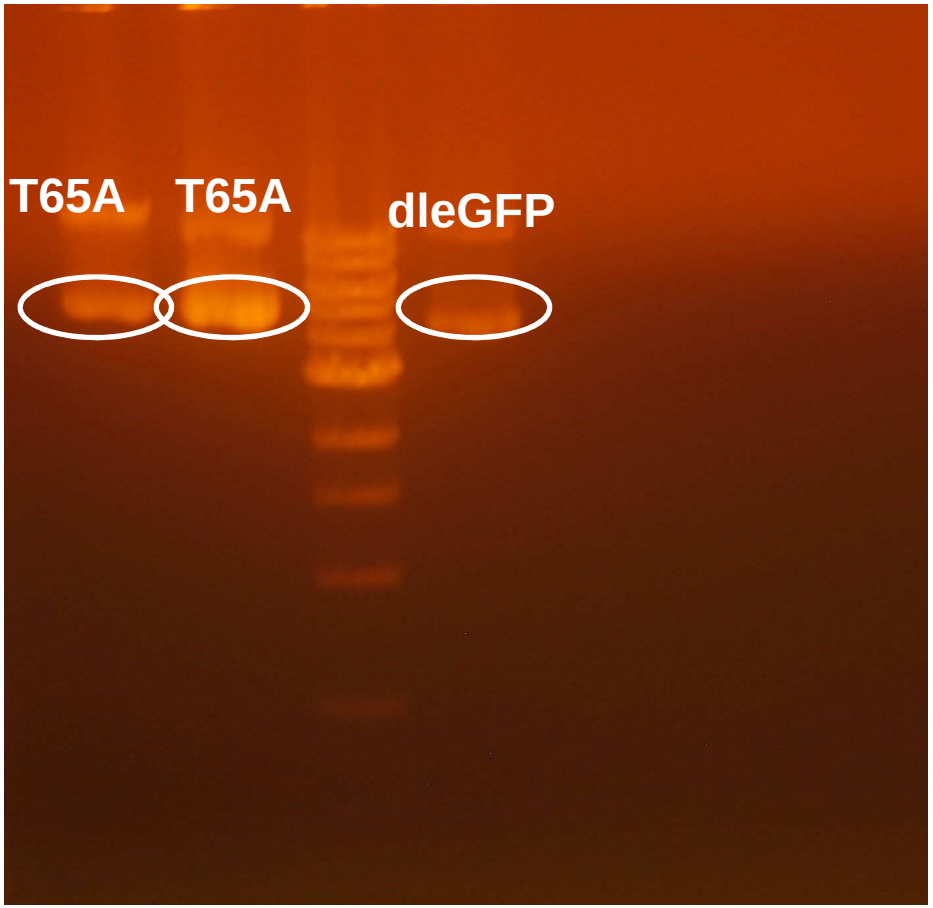


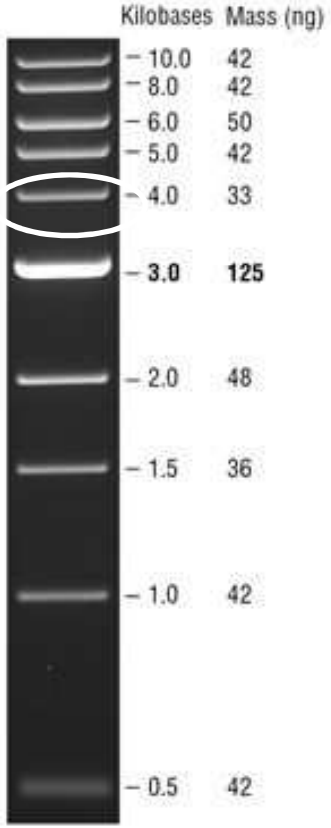
Gel extraction

- Cut one band from gel,
- Extraction from gel (extraction kit),

http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/DNA-Cleanup/QIAquick-Gel-Extraction-Kit

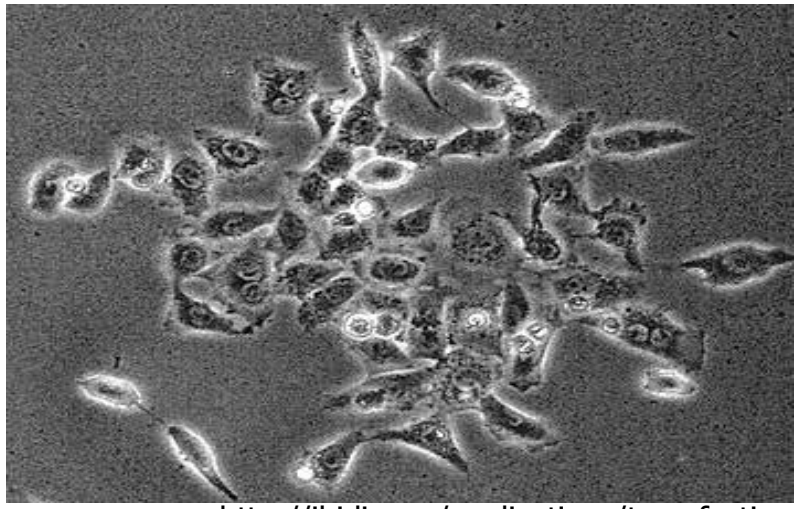
Electrophoresis after miniprep

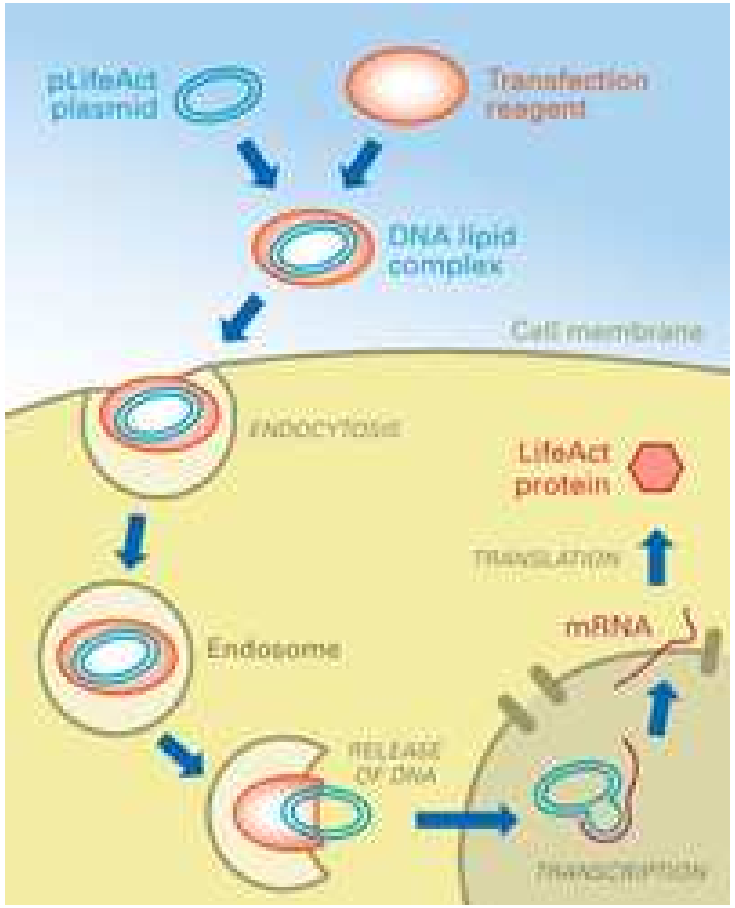




Transfection in mammalian cells

plasmid-> liposome->
endocytosis->core->
transcription->
translation->protein

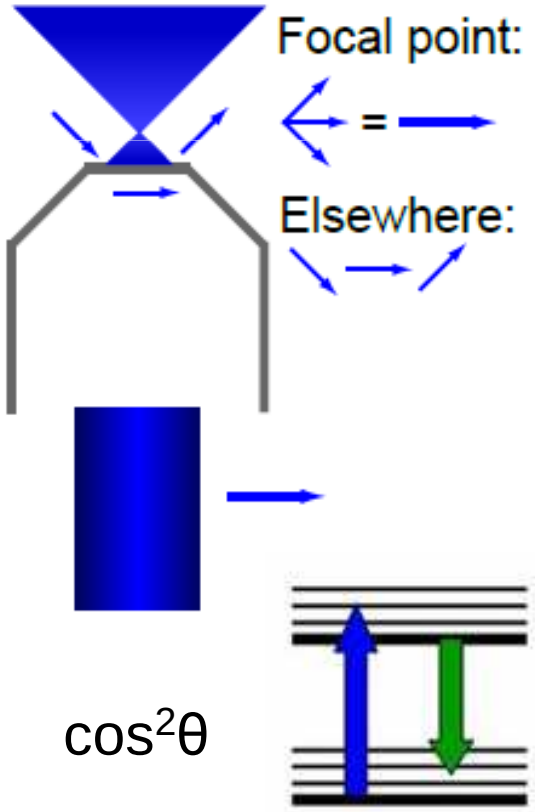




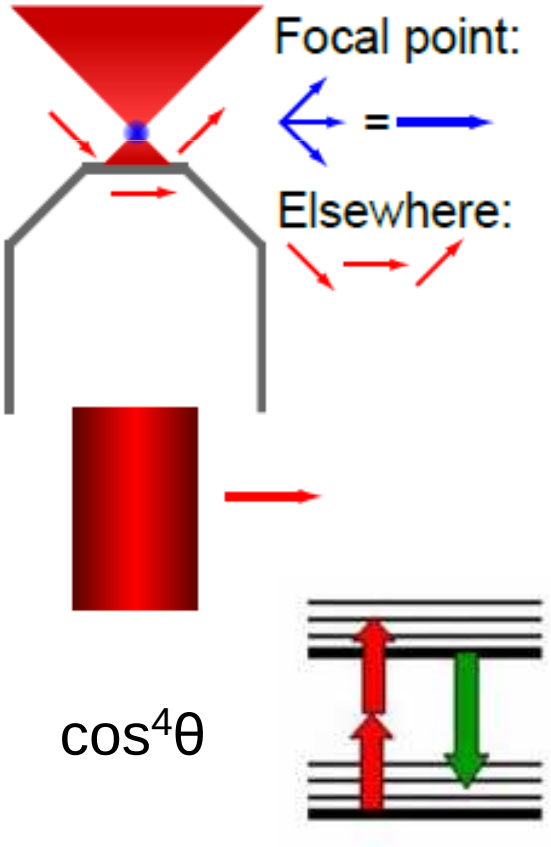
http://ibidi.com/applications/transfection-transduction-and-proteofection/transfection/,
http://en.wikipedia.org/wiki/File:HEK_293_cells_grown_in_tissue_culture_medium.jpg

Comparison of one-photon and two-photon microscopy

One-photon excitation



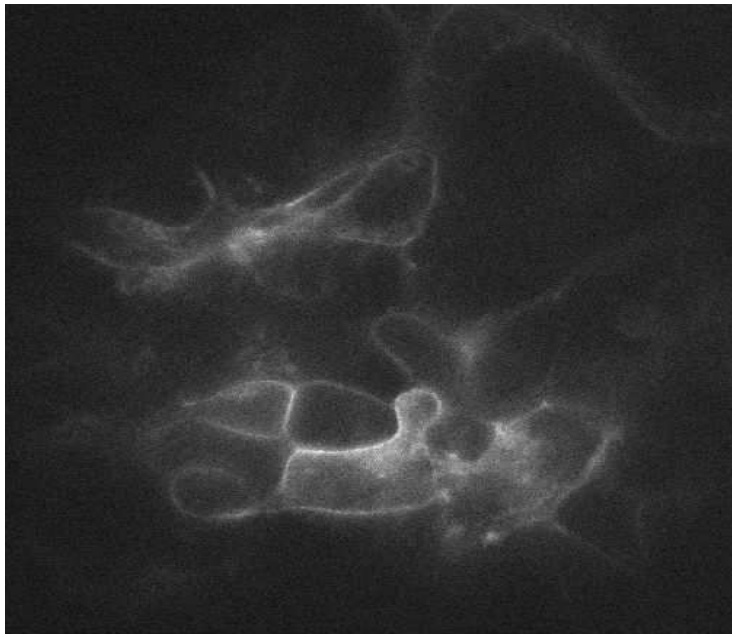
Two-photon excitation



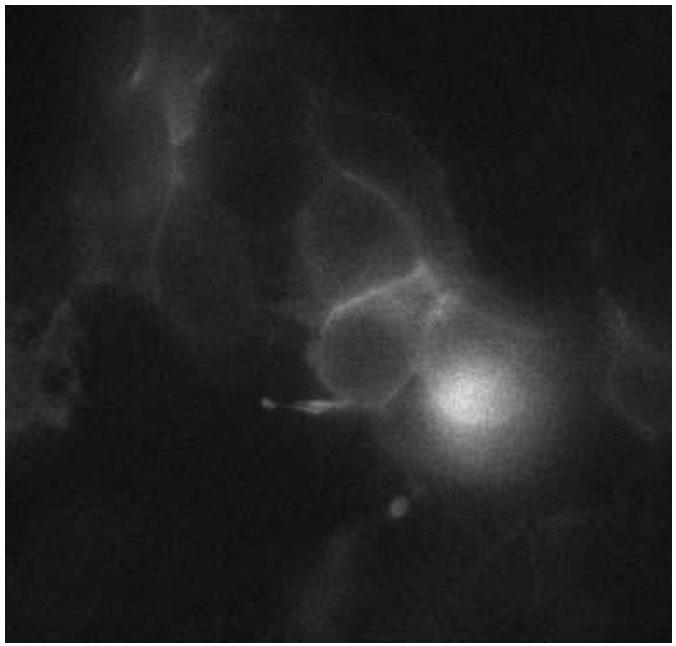
One photon microscopy

- Two wavelengths:
- 405 nm and 488 nm
- Zoom 60x.

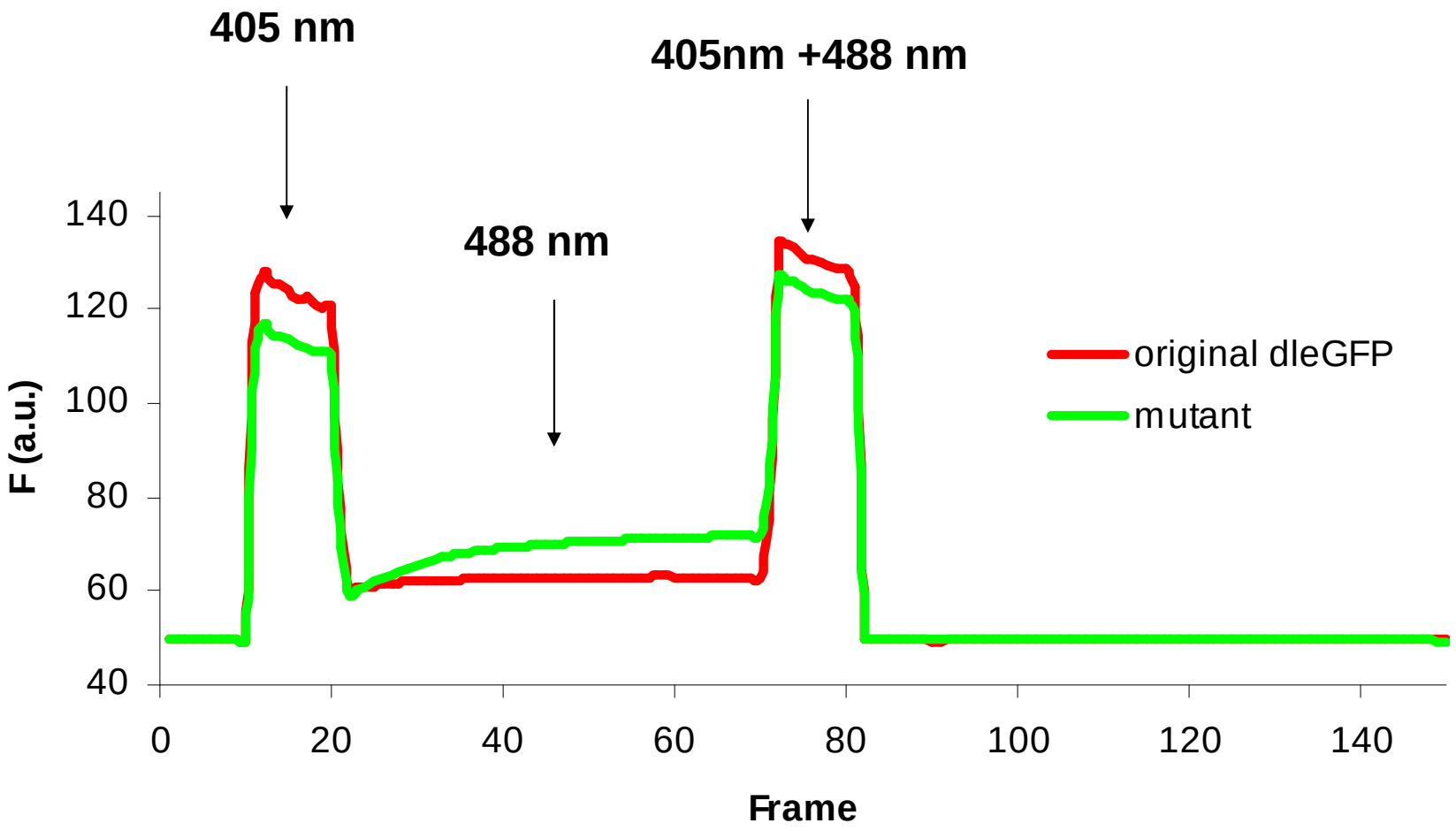
Original dleGFP



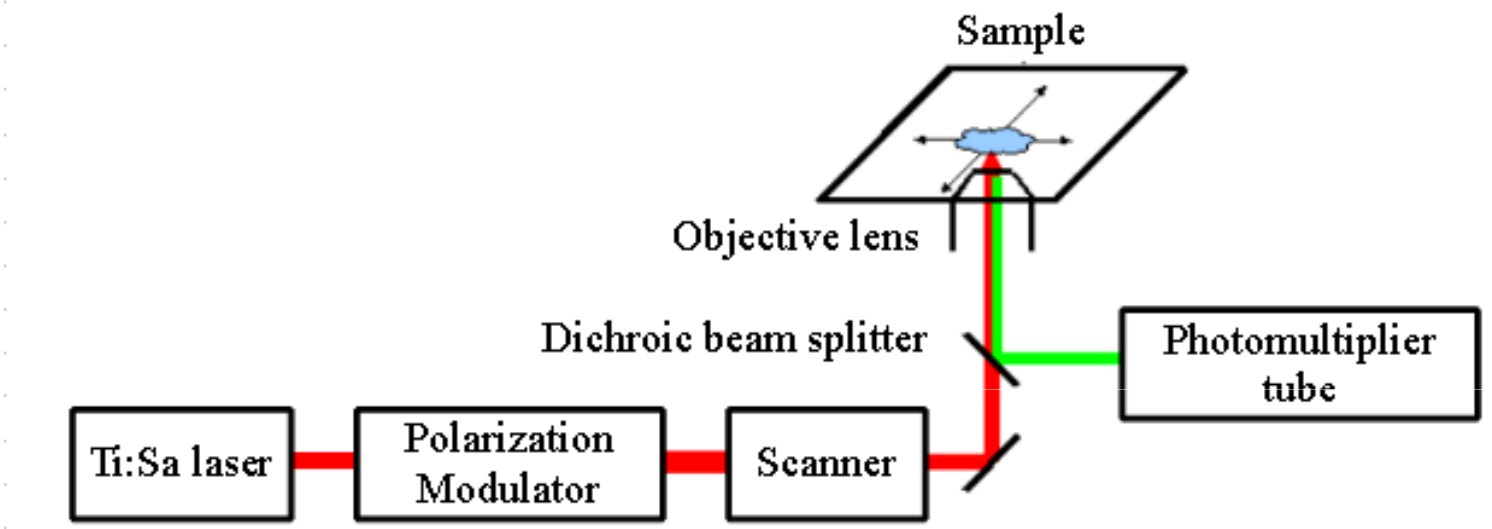
mutated dleGFP



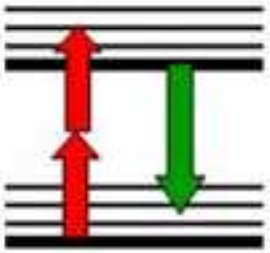
2.Testing the fluorescent properties



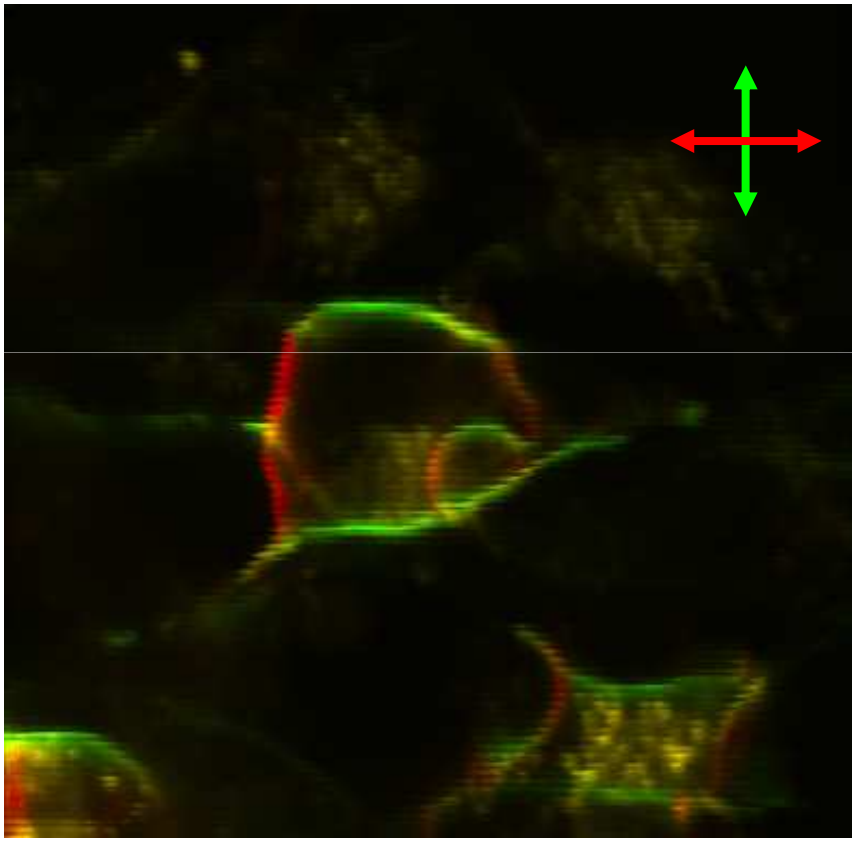
3.Two-photon polarization microscopy setup



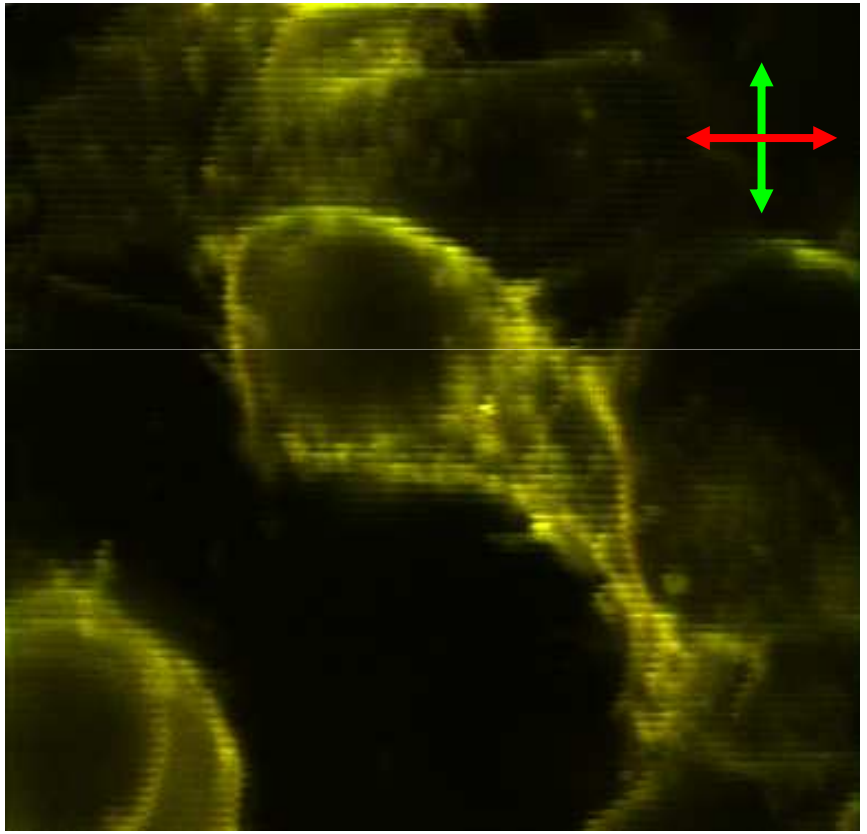
Two-photon excitation



2PPM of dleGFP and our mutant



dleGFP



T65A_G63A_dleGFP

Conclusions

- We introduced two point mutations (T65A and Q69L) in the dleGFP.
- Introduced mutations altered fluorescent properties of dleGFP and led to reduction of its linear dichroism due to changes of dleGFP fluorophore.
- Further work is required for introduction of two more mutations for improvement of photoswitching characteristics of dleGFP.

Acknowledgment

- Josef Lazar, PhD
- Alexey Bondar
- Valentyna Kuznetsova
- Karolina Tošnerová

http://www.eatingwell.com/food_news_origins/green_sustainable/green_up_kitchen_challenge


Thank you all for your attention.