



## Imaging Fluorescent Protein

### Background

The green fluorescent protein (wt GFP) from the jellyfish *Aequorea Victoria* (1) is widely used in fluorescence microscopy. Although the wild type form has undergone many mutations to optimise its properties as a tool for biological investigations, a process that still going on. One of those mutants is the commercially distributed EGFP which has a red-shifted excitation maximum and is brighter than wtGFP. To reach higher expression levels its cDNA codon usage has been optimised for the mammalian translation system.

Further mutations of GFP led to the generation of a colourful group of fluorescent proteins (referred to as XFPs) and there is a continuous effort to develop new improved variants.

Most commercially available XFPs can be ordered from BD Biosciences (formerly Clontech).

XFPs used today, an overview (there are more!)

order: increasing excitation wavelength

Name	Ex. Max. (nm)	Em. Max. (nm)	Comments
BFP			Low quantum yield (dim), strong photobleaching
CFP			Bright, very little photobleaching but: long maturing time
ECFP	439	476	
EGFP	484	510	Bright, very little photobleaching
YFP (1 <sup>st</sup> gen.)			High sensitivity towards pH and Cl- Photobleaching stronger than EGFP
YFP (2 <sup>nd</sup> gen.)			Less pH sensitive than 1 <sup>st</sup> gen. YFP
Yfp (3 <sup>rd</sup> gen.)			No Cl- sensitivity, little pH sensitivity, less photobleaching, faster maturation
EYFP	512	529	
RFP (DsRed)	563	582	tetrameric, wide excitation shoulder, long maturing time, partly folded protein fluoresces green, long emission, good for tissue labelling
Fluorescent Timer (DsRed variant, Clontech)			uses DsRed characteristic: during folding color changes from green to red to observe promoter activity over time
T1 (DsRed Express, Clontech)			maturing time only 10 min
mRFP1 (5)	500/584	607	new monomeric RFP

- Overview of spectral properties of XFPs. - Fluorescence filter information for XFPs from Chroma



A second group of fluorescent proteins is derived from reef corals and has consequently been named Reef Coral Fluorescent Proteins (RCFPs). Although deriving from a different class of organism, RCPs share structural homology with GFP. Unlike the color variants of Aequora GFP, which represent mutant variants of a single fluorescent protein, RCPs are unique proteins encoded by distinct genes. They are not generally recommended for use as protein tags. DsRed (from the list above) is one representative of this group. BD-Biosciences offers 6 spectrally distinct proteins from the family.

#### Recent developments

- **Photoactivatable YFP-mutant: PA-GFP (6)**
  - o undergoes a 100-fold increase in fluorescence at 488nm when illuminated before at 413nm
- **Color-changing protein Kaede (7)**
  - o converts from a green (475nm exc.) to a red (550nm exc.) fluorescent protein after irradiation with 350 – 400 nm light

Application for both: localized labeling of organelles or protein subpopulation which can then be followed in space and time (protein trafficking, turnover, etc.)

Destabilized XFPs (BD Bioscience/Clontech)

EGFP with half-lives of 1-4 hours

#### Applications:

- measure kinetics of transient mRNA transcription from regulated promoters
- monitor gene expression during development
- characterize cis-regulatory elements

#### Equipment needed for imaging fluorescent proteins in living cells

##### TILL Hardware

- Polychrome V
- DSP-control board
- condenser for your microscope
- fluorescence filter sets matching your XFPs
- camera
  - o Imago QE for imaging subcellular structures
  - o Imago VGA for imaging whole cells
- imaging workstation
- optional
  - o piezo-objective mover for z-stack acquisition
  - o Dual-view Microimager for simultaneous detection of two emission wavelengths
  - o UV-Flash for uncaging experiments



## TILL Software

- Software TILLvisION
- optional:
  - o Software extension Tracking
  - o Software extension Deconvolution

## Microscope equipment

- microscope frame with 100% port camera port
- high-NA objectives for observation of low light samples
- filter cubes for fluorescent filter sets
- optional:
  - o incubation chamber for long time recording (temperature and CO<sub>2</sub> control)

## Techniques / Protocols

### Molecular Biology

Clone your gene of interest into a (commercially available) XFP-containing vector (e.g. from Clontech). By doing so, generate either a vector for cotransfection of your gene of interest and GFP or a GFP-fusion protein or a fusion proteins. In case of a fusion protein the XFP-part can be linked N-terminally, C-terminally or be contained centrally in the protein sequence. Where you put it depends on the functional sides or your protein. Inserting the XFP at the wrong site may lead to a non-functional fusion protein. Check cloning result by sequencing.

Labelling of subcellular structures / organelles: pick a vector with a respective translocation sequence.

Promotor structure: do you want to have it inducible?

Vector: for transient or stable transfection? Selection marker for transfection?

### Transfection

CaPhosphate, Electroporation, Lipofectamin a.o.  
stable or transient? transgenic organisms?

### Imaging

Seed cells on coverslips (in tissue plates with coverslip bottom), coat coverslips with Gelatine, Poly-L-Lysin, Fibronectin if necessary.

Cell fixation: 2-3% Formaldehyde, Triton-X100, Methanol

Store fixed cells with an antibleaching agent!

It is important to optimize the fluorescent filters of your microscope according to the XFP you are using. Only this guarantees optimal background-to-noise signal.



When you are working with multiple labels in one cell, you can use double/trippel-filter sets. In combination with a monochromator as a light source you can very quickly image the different dyes without having to change the filter set.

## Applications

### XFPs as passive Indicators

Monitoring the appearance, degradation, protein turnover, location or translocation of your protein of interest with the help of an XFP protein partner.

### XFP as Marker of Gene Expression

- as a reporter gene for gene expression located behind the promoter of interest. Prerequisite are stably transfected cells or transgenic organisms. Method replaces enzymatic expression reporting systems base on  $\beta$ -galactosidase or  $\beta$ -lactamase.
- XFP as a marker in cotransfection in transiently transfected cells: transfected cell can be identified quickly before the experiment (not through an antibody stain afterwards), course of transfection (expression levels) can be followed easily

### XFP-fusion proteins to image protein of interest in living cells in real time

- Spatial marker for proteins in living cells: allows precise location of the protein of interest without the risk of artefacts due to fixation and/or staining procedures
- XFP as a tool to visualize intracellular organelles (or other subcellular structures) in living cells (multi-labeling):
  - o Clontech provides vectors targeted to: actin, tubulin, mitochondria, nucleus, endoplasmic reticulum, Golgi, PM, preoxisomes, endosomes (early, late): colocalization with protein of interest can be detected easily
- protein/organelle dynamic (multiple proteins with different XFPs) in real time
- Fluorescence speckle microscopy (8): monitoring cytoskeletal dynamics
- localization of intracellular messenger molecules (3-PIs, DAG & PA) (9, 10, 11)
- RNA localization (67-70)
- gene activity and change in chromatin structure (71-74)
- protein dynamics with temperature-sensitive GFP-mutants (12, 13): pulse-chase type experiments
- studies of protein dynamics with bleaching experiments:
  - o FRAP = fluorescence recovery after photobleaching (ref.)
  - o FLIP = fluorescence loss in photobleaching (ref.)



### XFPs as active Indicators

Quantitative measurements using changes in the fluorescence spectra/intensity of XFPs

- pH-measurements with engineered GFPs (14, 15)
- Cl<sup>-</sup> fluxes with halide-sensitive YFPs (16)
- Calcium-Sensors:
  - o Camgaroos: Calmodulin inserted into YFP => fluorescence intensity increases sevenfold on binding of Ca<sup>2+</sup>. Used to measure Ca<sup>2+</sup> - concentration inside Mitochondria (17).
- Pericams (18): circularly permuted GFP (N- and C-termini connected by flexible linker, new N- and C- termini introduced close to chromophore: higher sensitivity of fluorescence towards mechanical stresses) is inserted between calmodulin and M13 to yield Ca<sup>2+</sup> sensor. More sensitive than Camgaroos, some Pericams increase wavelength, some shift excitation wavelength: ratiometric dyes (Chroma filter set 71013)

XFP-Sensors based on intramolecular FRET for

- Ca<sup>2+</sup> (Cameleons)
- Indicators of protease activity
- other cellular parameters:
  - o cGMP
  - o phosphorylation
  - o membrane potential

XFP-sensors based on intermolecular FRET

- Indicators for cAMP
- Protein-Protein-Interaction:
  - o Description factors
  - o G-proteins

### **Why choosing TILL Photonics equipment to image your fluorescent proteins?**

#### Flexibility in the excitation wavelength

With the Polychrome V as excitation light source you have the full visible range available to choose your excitation wavelength. You can run a spectrum to determine the optimal excitation wavelength for your current combination of fluorescent protein and filter set. Exciting at the optimal wavelength leads to the best images in terms of signal-to-noise and intensity.

#### Simultaneous imaging of different colors

This flexibility of excitation wavelength makes the detection of multiple labels easy



and fast. With suitable labels and well-chosen dichroic mirrors not even a change of filter set is necessary to detect two or three different fluorescent proteins in your cell.

As the wavelength change requires less than 3 msec an almost simultaneous investigation of multiple labels is possible.

#### Bleaching protection

Your samples are optimally protected against bleaching due to the tight synchronisation between camera and illumination. Your cells only get illuminated during the exposure time of the camera. Not a msec longer. In addition allow the TILL

Imago cameras the use of very short exposure times, as they are optimized for sensitivity and a superb signal-to-noise ratio.

#### Fast time lapse

The TILL Imaging System has been designed for speed of image acquisition. The system can capture one image while simultaneously be reading the previous image to memory. This makes it the fastest system on the market.

#### Tracking of movement with tracking software

The tracking module (link) of the TILL Imaging software TILLvisION allows you to quantify the movement of subcellular structures, such as transport vesicles.

### **Links**

Most commercially available XFPs as cloning vectors, antibodies against XFPs, vectors for RCPs, and more:

- BD-Bioscience (formerly Clontech)

Fluorescent filters for your living color application:

- Chroma
- AHF
- Antibodies against GFP
- Molecular Probes



## References

- Ormo M, Cubitt AB, Kallio K, et al.: Crystal structure of the Aequorea victoria green fluorescent protein. *Science* 273, 1392-1395 (1996)
- Sawano A & Miyawaki A: Directed evolution of green fluorescent protein by a new versatile PCR strategy for site-directed and semi-random mutagenesis. *Nucleic Acids Res.* 28, E78 (2000)
- Sawano A & Miyawaki A: Directed evolution of green fluorescent protein by a new versatile PCR strategy for site-directed and semi-random mutagenesis. *Nucleic Acids Res.* 28, E78 (2000)
- Griesbeck O, Baird GS, Campbell RE, Zacharias DA & Tsien RY: Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *J. Biol. Chem.* 276, 29188-29194 (2001)
- Nagai T et al.: A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nature Biotechnol.* 20, 87-90 (2002)
- Campbell RE et al.: A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* 99, 7877-7882 (2002)
- Patterson, GH & Lippincott-Schwartz, J: A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* 297, 1873-1877 (2002)
- Ando R, Hama H, Yamamoto-Hino M, Mizuno H & Miyawaki A: An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. USA* 99, 12651-12656 (2002)
- Waterman-Storer CM, Desai A, Bulinski JC & Salmon ED: Fluorescent speckle microscopy, a method to visualize the dynamics of protein assemblies in living cells. *Curr. Biol.* 8 (22), 1227-1230 (1998)
- Servant G et al.: Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* 287, 1037-1040 (2000)
- Meili R et al.: Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in Dictyostelium. *EMBO J.* 18 (8), 2092-2105 (1999)
- Oancea E & Meyer T: Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. *Cell* 95, 307-318 (1998)
- Kaether C & Gerdes HH: Visualization of protein transport along the secretory pathway using green fluorescent protein. *FEBS Lett.* 369, 267-271 (1995)
- Rudolf R, Salm T, Rustom A & Gerdes HH: Dynamics of immature secretory granules. *Mol. Biol. Cell* 12 (12), 1353-1365 (2001)
- Kneen M, Farinas J, Li Y & Verkman AS: Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys. J.* 74, 1591-1599 (1998)