Apparent mechanisms of photolysis of HP1β-GFP in living cells as revealed with fluorescent confocal microscopy

Yuriy Glazachev[1], Darya Orlova[1,2], Petra Sehnalová [3] , Eva Bártová[3]

[1]*Institute of chemical kinetics and combustion, Novosibirsk, Russia*

[2]*Stanford University School of Medicine, Stanford CA, USA*

[3]*Institute of Biophysics, Brno, Czech Republic*

Summary

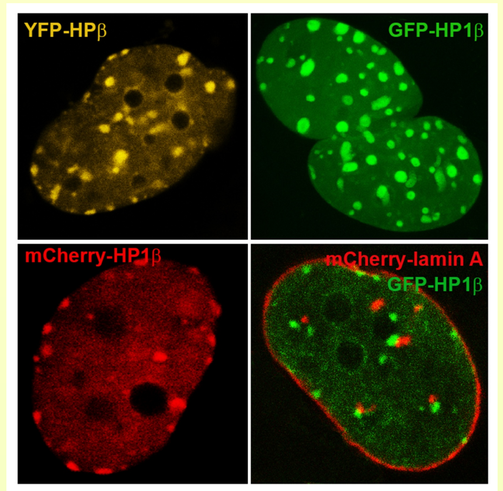
The presented work is dedicated to investigation of kinetics of photolysis of HP1β-GFP protein as observed in nucleus of living cells. For this purpose, we registered the Fluorescence Loss In Photobleaching of fluorophore under Pulse-Position Modulated (PPM-FLIP) laser irradiation with fluorescent confocal microscope. To describe the observed kinetics, we considered two models, which imply the existence of intermediate state and three effective rates constants: transition, recovery/relaxation and photodegradation.

The irradiation dependence of the rate constants as recovery and degradation were sigmoid-like. That point out to that mechanism of photolysis is even more complicated then considered. Anyway, such sigmoid like dependence allows to choose more appropriate model.

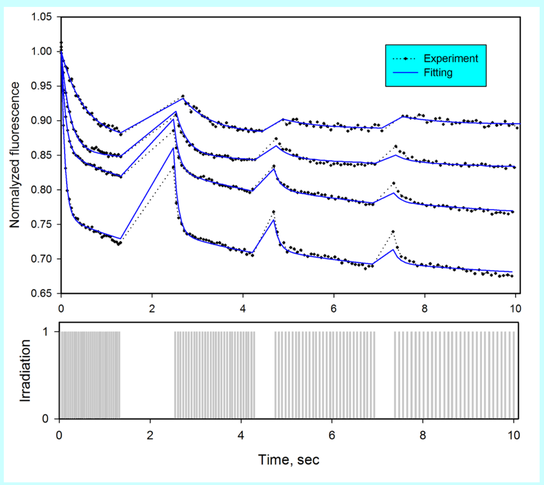
The experiments with HP1β-GFP in hypoxic condition did not result to any significant changes of kinetics and rate constants that demonstrated that oxygen does not act neither as relaxation not bleaching agent. The treatment of cells with actinomycin D, anyway, results in increase of rates constants leading to decrease of apparent photostability of HP1β-GFP.

Similar experiment were performed with fluorescent proteins as HP1β-YFP and HP1β-mCherry. Both of these species demonstrated the kinetics without well pronounced recovery kinetics.

Taking into account obtained results, we proposed the simple approach while applying FRAP method for more reasonable analysis of diffusion of HP1-GFP in living cells.

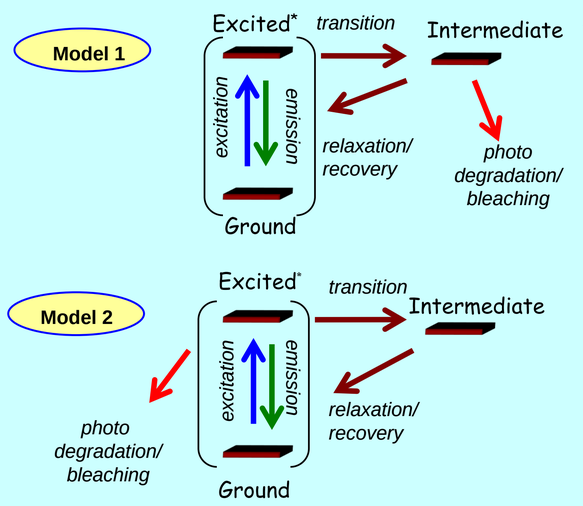


PPM-FLIP kinetics of HP1b-GFP at different irradiation power

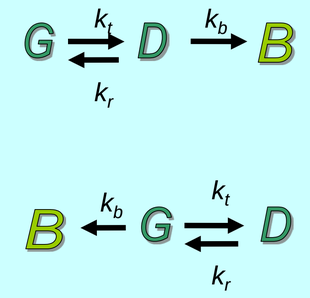


PPM approach implies the constant pulse width and varying pulse steps. Such approach allows to calculate both direct and reverse rate constants of reaction. Leico confocal microscope allows to apply in single kinetics four series, each of which can have its own pulse step. In this experiment, we used pulse width of 39 ms, and pulse steps of 50, 60, 70 and 80 ms Irradiation wavelength 476 and 488 nm

Models of kinetics of photolysis



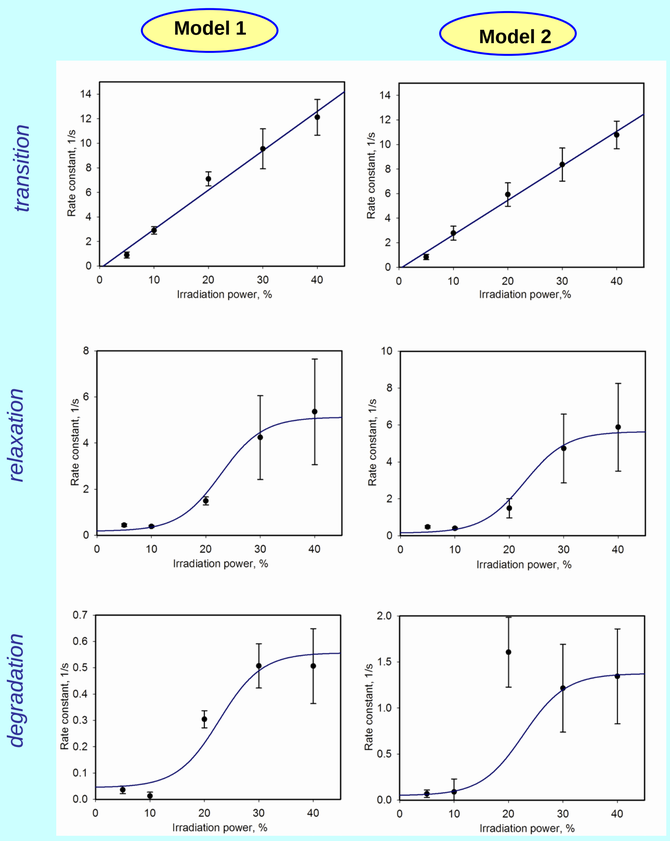
Reduced schemes



G – ground state, D – intermediate state, B – bleached state
k_t – transition rate constant
k_r – relaxation/recovery rate constant
k_b – photo degradation/bleaching rate constant

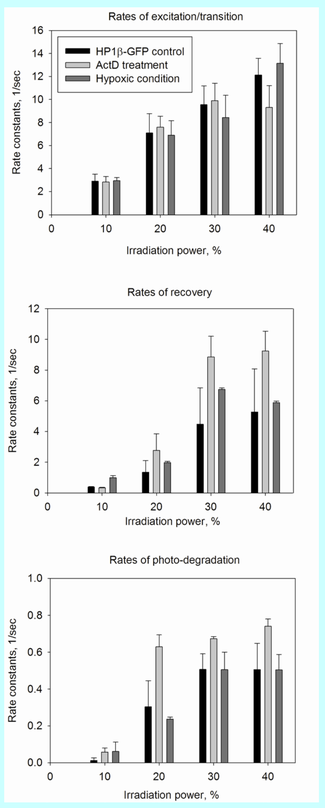
In Model 1, photo-degradation occurs from long lived intermediate state
In Model 2, it occurs from short lived (2.6 ns) excited state

Dependence of calculated rate constants vs irradiation power

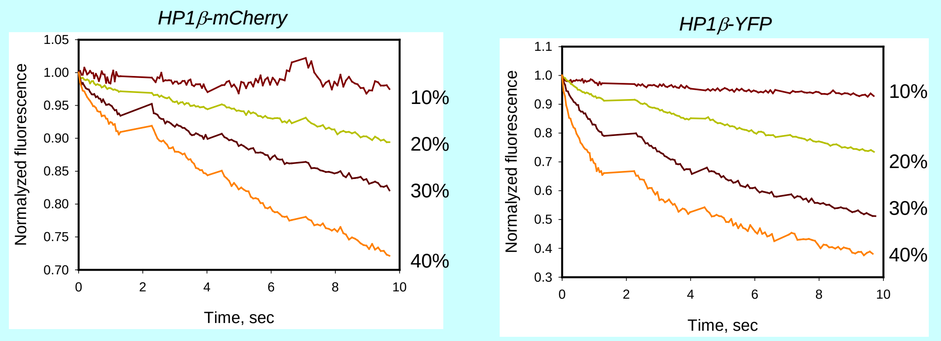


Sigmoid like dependence of rate constants demonstrates that both recovery and photo-degradation processes should proceed from some intermediate state. So Model 1 could be taken as a basic.

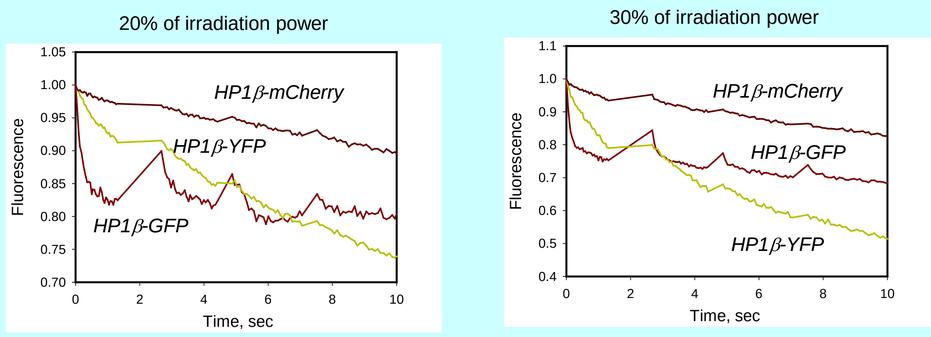
Comparison of the calculated rates constants (Model 1) for HP1β-GFP in hypoxic conditions and after actinomycin-D treatment



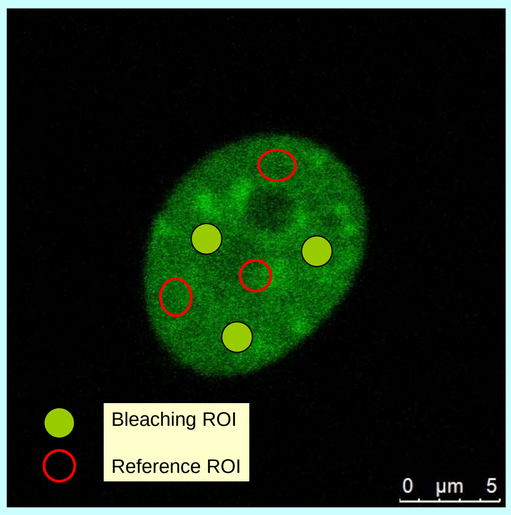
PPM-FLIP kinetics for HP1β-YFP and HP1β-mCherry at different irradiation power



Comparison of PPM-FLIP kinetics of HP1β–GFP , -YFP, -mCherry

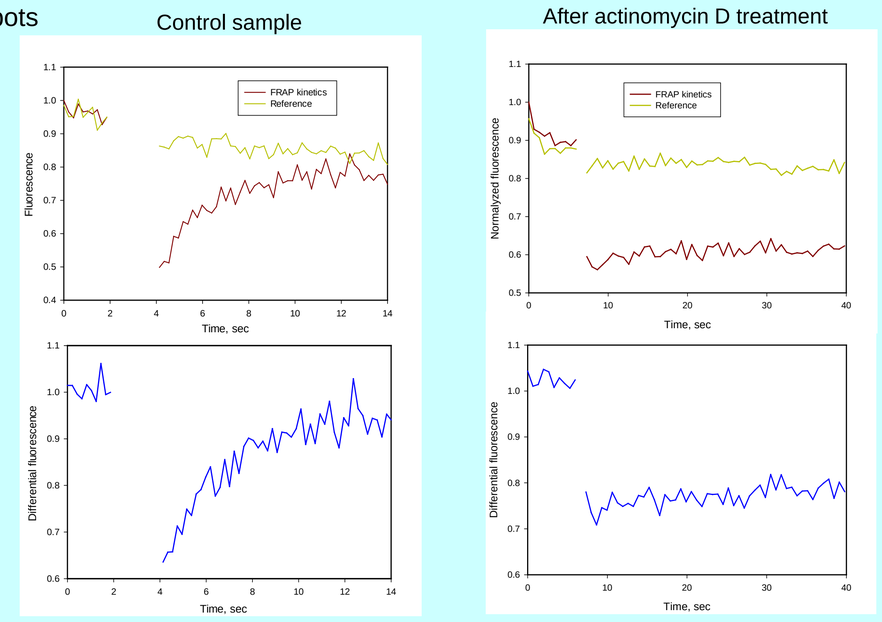


Demonstration of multi-spot FRAP with reference spots



Control sample

After actinomycin D treatment



Each kinetics are averaging over three spots (ROI) for better signal. Differential kinetics is a result of difference of reference and sample/bleaching kinetics plus a unit. Such approach allows to compensate the unwanted features of GFP photolysis during experiment

Conclusion

- PPM-FLIP approach can be applied for analysis of kinetics of photolysis of fluorescent proteins with routine confocal microscope
- The basic apparent mechanism of GFP photolysis implies the existence of intermediate state, from which both relaxation and photo-degradation occur.
- More detailed mechanism implies the existence additional states or/and non-linear effects
- Immobilization of HP1β-GFP on chromatin effects on photochemical rates constants, where as oxygen does not.
- To provide more reasonable FRAP experiments, the differentials kinetics versus reference ROIs are proposed for analysis.

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