Fluorescence photobleaching phenomenon in biological cell research

understanding and applications of phenomemon

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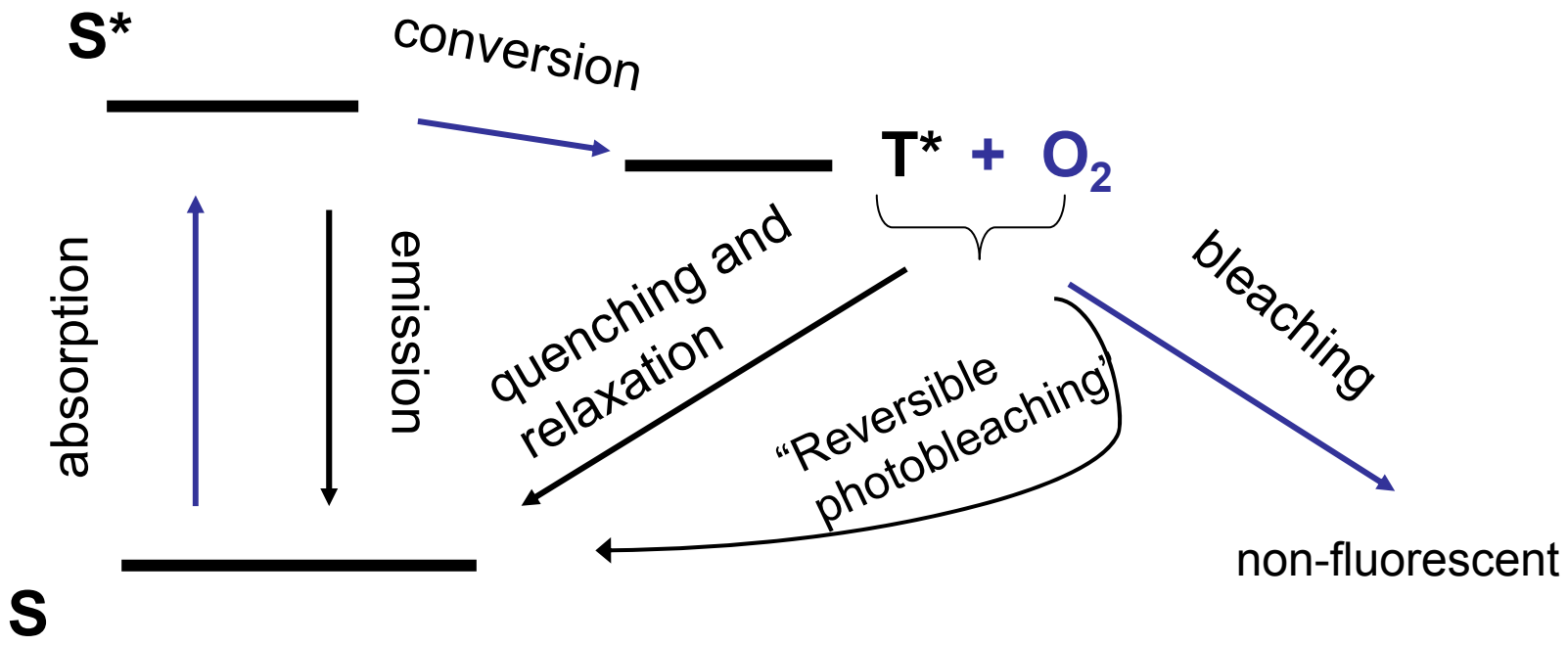
Main activity and field of interest

- Analysis of oxidative status of cellular liquids (plasma or cytoplasm) and living cells
  - Reactive oxygen spieces (ROS) formation including NO
  - Thiol disulfide status
  - Enzyme contents and activity
- EPR spectroscopy (spin probe and spin trap methods), optical, HPLC and biochemical methods
- Fluorescence probe methods (fluorescence microscopy and photometry)

What is photobleaching phenomenon as investigating tool

- Photobleaching results in loss of fluorofore while measurement of fluorescence
- It can create non equilibrium fluorofore concentration
- “two-in-one”: affection on investigated system and measurement the changes
- Parameters to measure: rate of photobleaching and rate of recovery of fluorescence

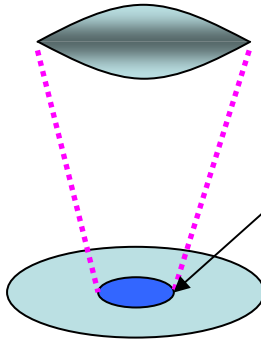
Simplified mechanism of photobleaching of fluorescein



Dominant mechanism is oxygen dependent (at normal oxygen tension)
Bleaching rate is proportional to intensity of irradiation and oxygen concentration

Fluorescence recovery after photobleaching , FRAP (first publications 1974-1976)

Geometry of measurements

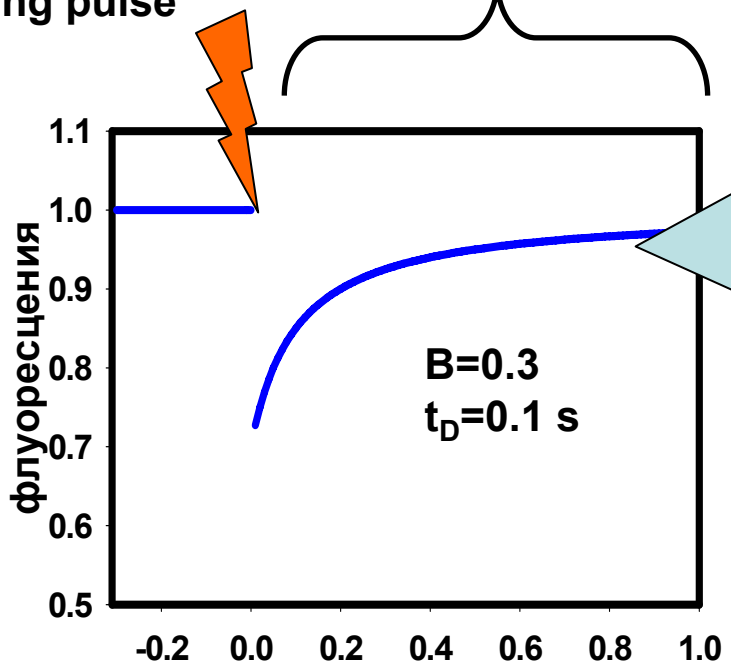


Photobleaching is used only once to create non-equilibrium concentration profile
Probing occurs under non-bleaching irradiation.

Pump-and-probe method

Fluorescence probing

Bleaching pulse



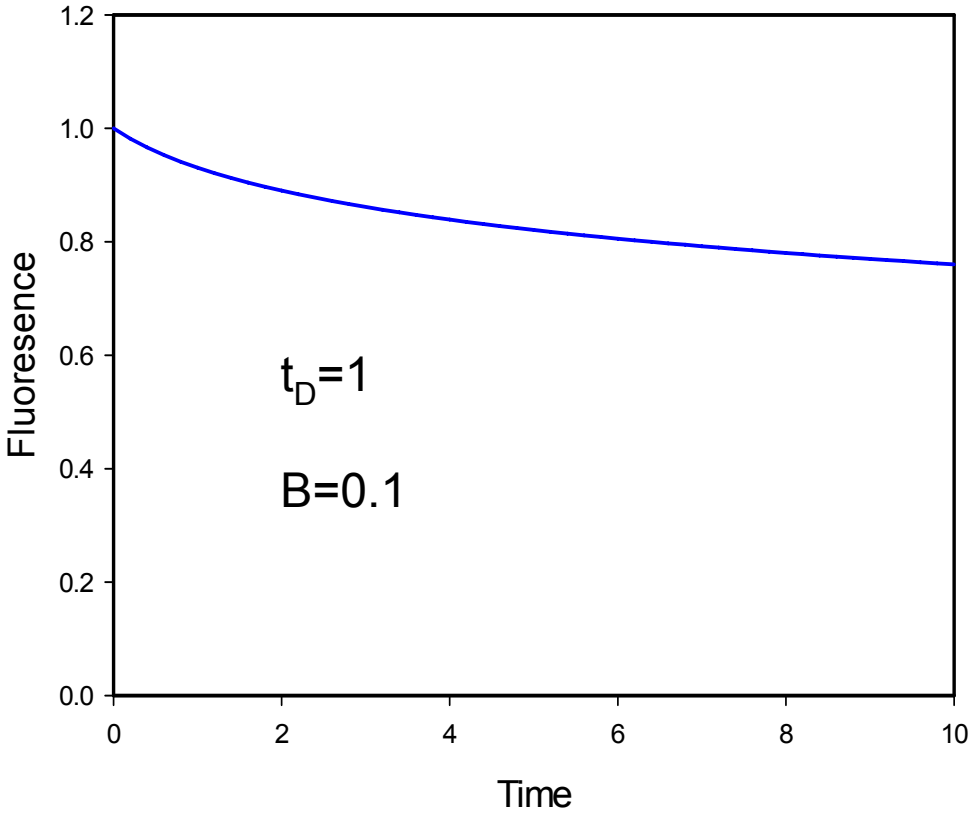
Kinetics of fluorescence recovery due to fluorophore diffusion

$$F(t) = F(0) \cdot \left(1 - \frac{B}{1 + \frac{t}{td}}\right)$$

$$D = \frac{\bar{r^2}}{4 \cdot td}$$

Continuous photobleaching

Pump-probe method. Observation of fluorescence kinetics under continuous irradiation of bleaching intensity



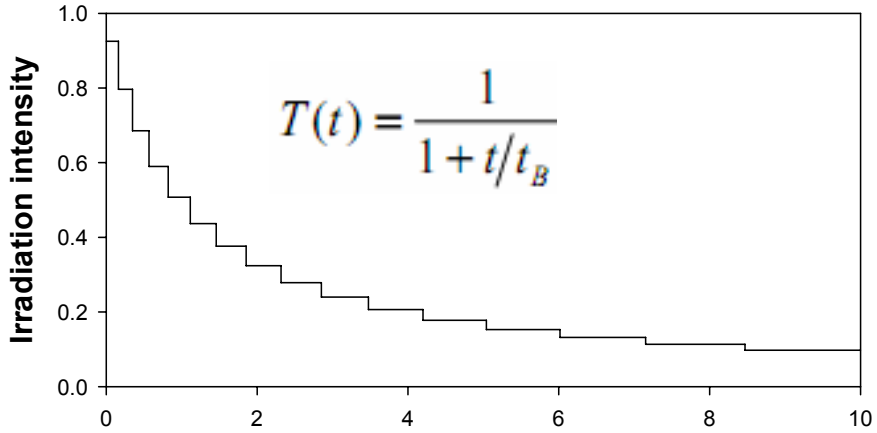
$$1 - B \cdot t_D \cdot \ln\left(1 - t/t_D\right)$$

Increasing of signal-to-noise ratio up to about 10 times

Low information, a lot of interfering factors, shot kinetics only

Pump-probe methods under decaying irradiation dose

1



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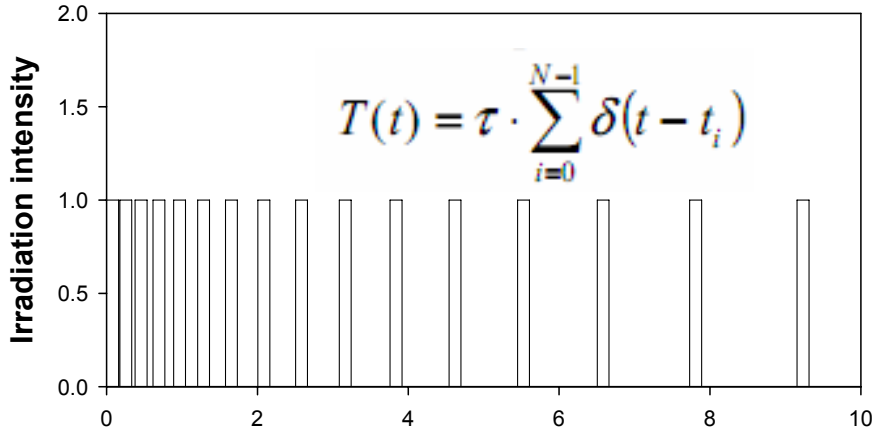
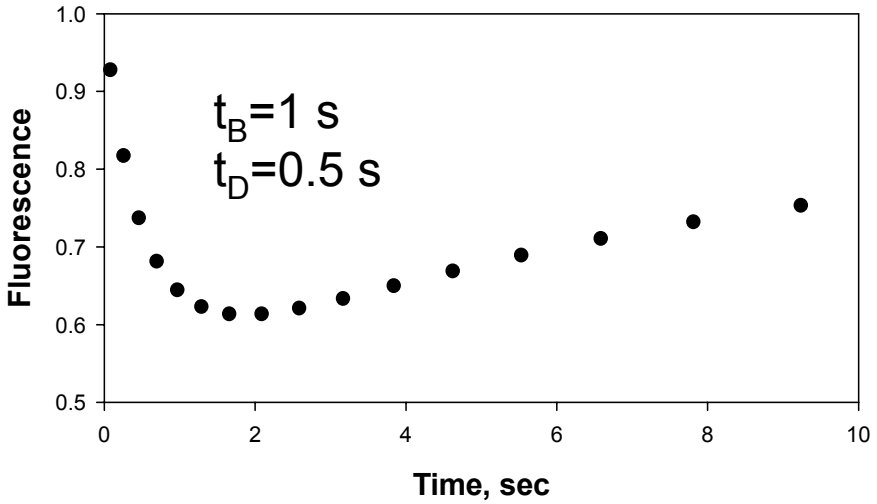




1. Amplitude modulation (Glazachev, Yu.I. Khramtsov V.V, J.Fluorescence,2006)

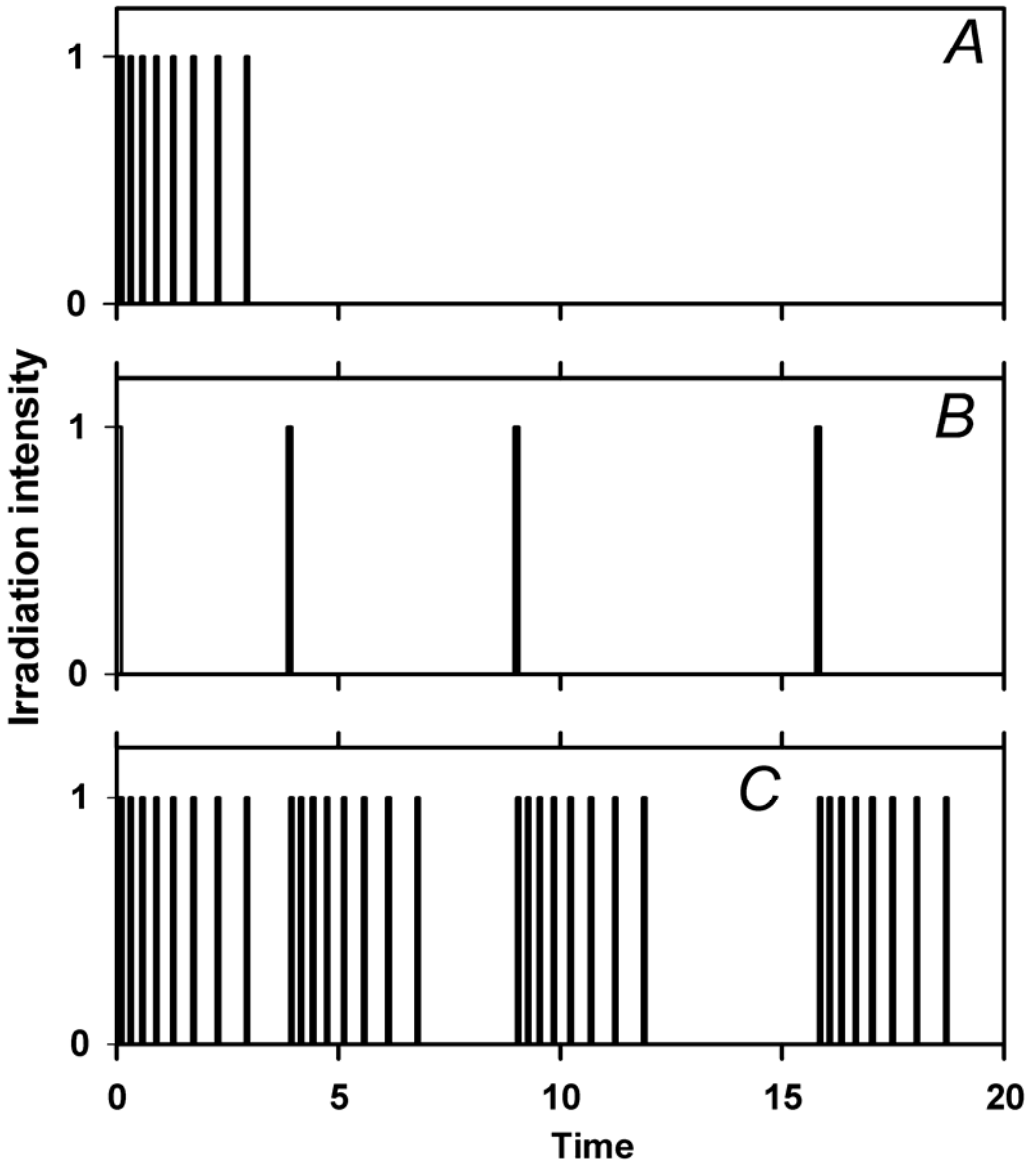
2. Pulse-position modulation, PPM-FPR (Glazachev Yu.I., J.Fluorescence, 2009)

Kinetics of fluorescence yield

1. $$1 - B \cdot \int_0^t \frac{T(x)}{1 + \frac{t - x}{t_D}} dx$$

2. $$1 - B \cdot \tau \cdot \left[\sum_{j=0}^{i-1} \frac{1}{1 + (t_i - t_j)/t_D} + \frac{1}{2} \right]$$

Advanced pulse series of PPM approach



Fast modulation

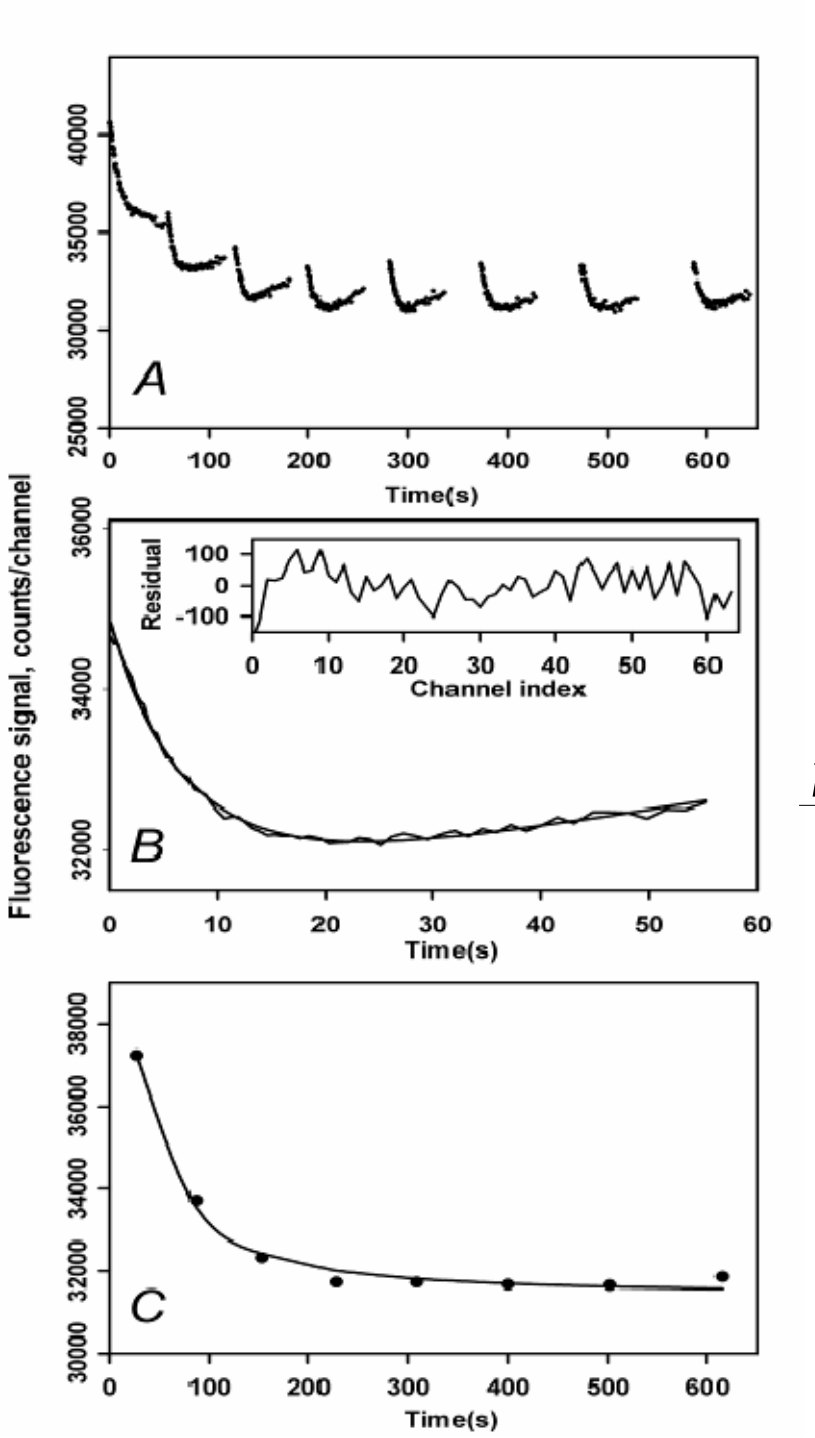
$$T^a(t) = \tau \cdot \sum_i \delta\left(t - t_i^a\right)$$

Slow modultion

$$T^b(t) = \tau \cdot \sum_j \delta\left(t - t_j^b\right)$$

Convolution

$$T(t) = \tau \cdot \sum_i \sum_j \delta\left(t - t_i^a - t_j^b\right)$$



FITC-labeled albumine in water/ glycerol mixture (80%). Reference sample ($D=5.6\times10^{-9}$ cm ^{2}/c)

A. Observed fluorescence kinetics. Modulation series 64x8

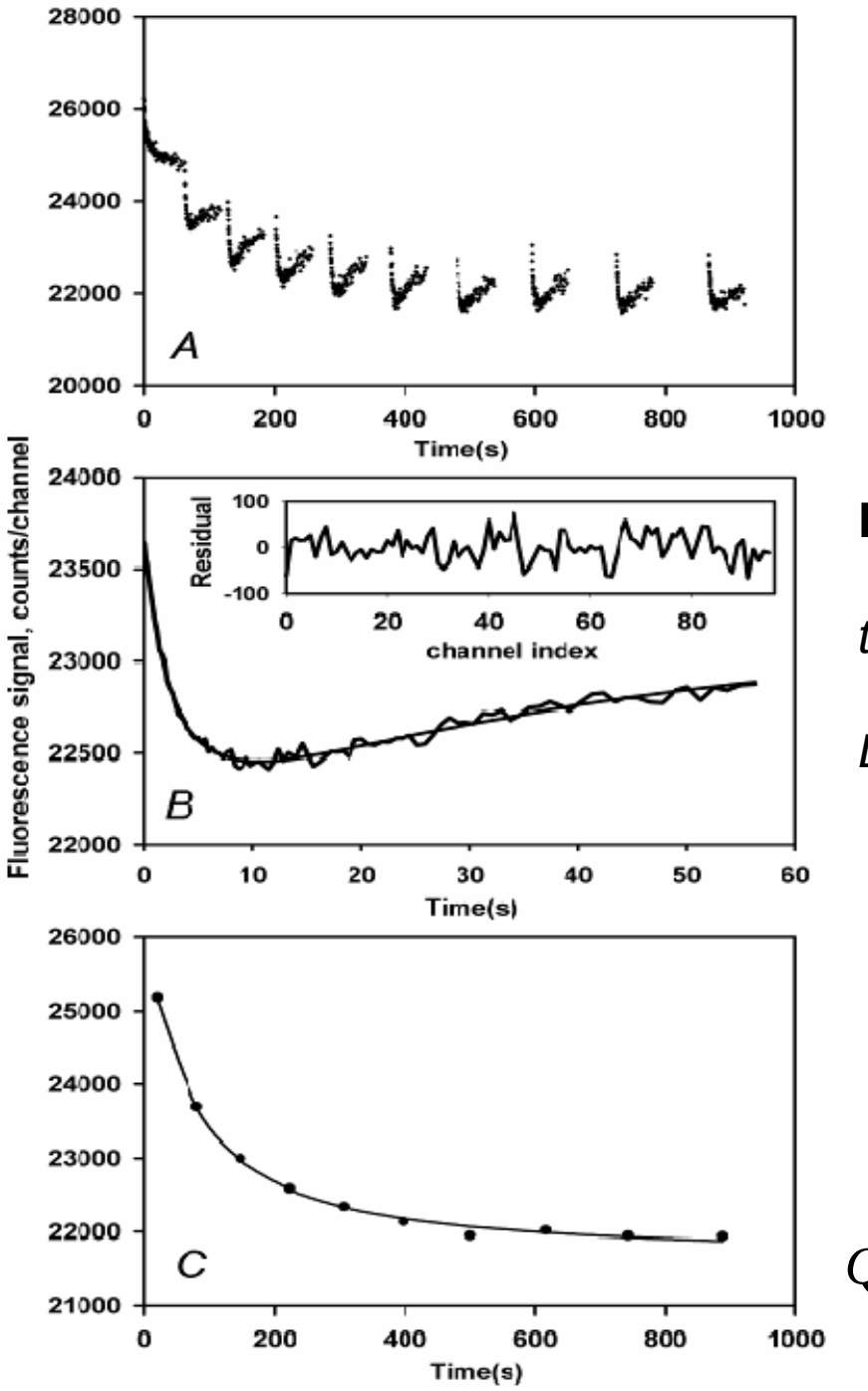
B. Averaging over fast sub kinetics – diffusion in solution

t_D =11±1 s; B=0.015±0.002 s^{-1}

$$\frac{f(t_i)}{f_0} = 1 - B\cdot\tau\left(\cdot\left[\sum_{j=0}^{i-1}\frac{1}{1+(t_i - t_j)/t_D} + \frac{1}{2}\right] + \alpha\cdot\left(i+\frac{1}{2}\right)\right)$$

C. Each point is mean of fast subkinetics. Bleaching of protein immobilized on glass surface. Fraction value ~ 8-10%

$$\left.\frac{f(t_j)}{f_0}\right|_{immobile} = \frac{1-\exp\{-2B\cdot N\cdot\tau(j+1/2)\}}{2B\cdot N\cdot\tau(j+1/2)}$$



FITC-DMPE in POPC liposomes (molar ratio 1:70000)

B. Fast subkinetics– diffusion in membrane.

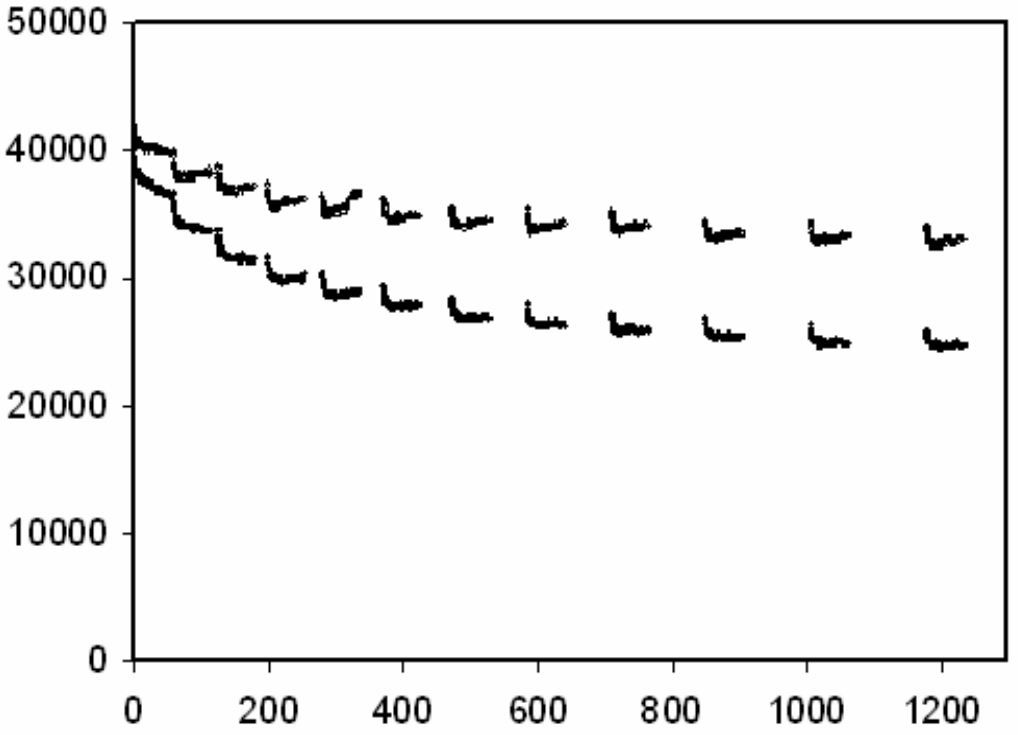
t_D = 1.2±0.2 s; B = 0.03±0.002 s^{-1}

D = (5±1)·10^{-8} cm^2/s

C. Border effect. Decreasing a total quantity of fluorophore in limited area due to photobleaching

$$Q(t_j) = Q_0 \frac{1-\exp\left\{-\gamma \cdot 2B \cdot N \cdot \tau (r/R)^2 (j+1/2)\right\}}{\gamma \cdot 2B \cdot N \cdot \tau (r/R)^2 (j+1/2)}$$

PPM-FPR kinetics with different liposome size



Size of liposome:

70-80 microns

50 microns

Bleaching spot ~ 10 microns

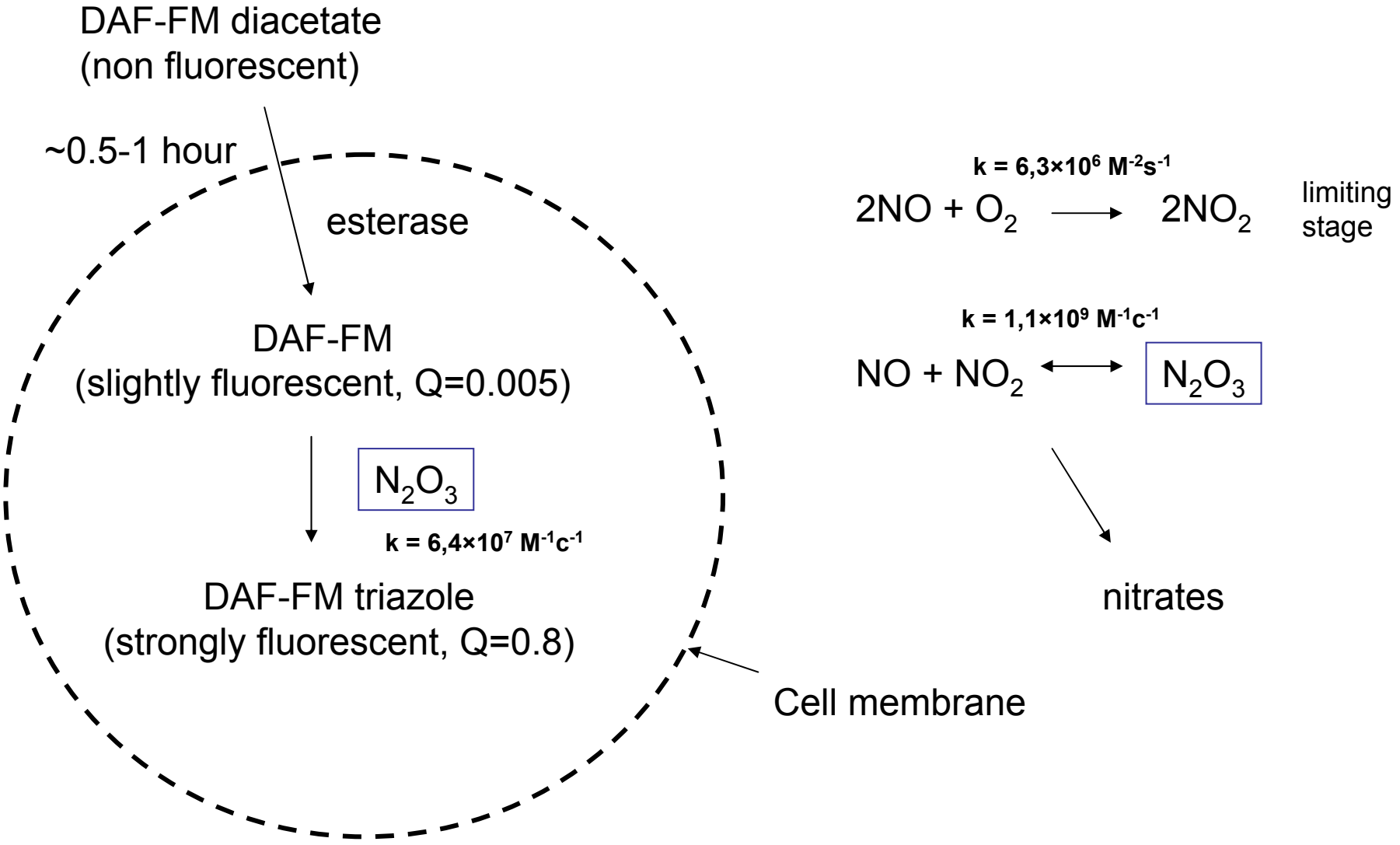
Results and conclusion on first part

- Pump-probe method results in 30-60 signal increasing over pump-and-probe. Decreasing of irradiation dose with time course allows kinetics measuring as long as reasonably necessary.

- Pulse-position modulation with series convolution allows simultaneous(!) measuring the fluorescence recovery/relaxation processes in the different time scale and of different nature: lateral diffusion, membrane penetration, sorption/desorption, chemical transformation etc. It allows to apply the routine fluorescent probes for multiple purpose.

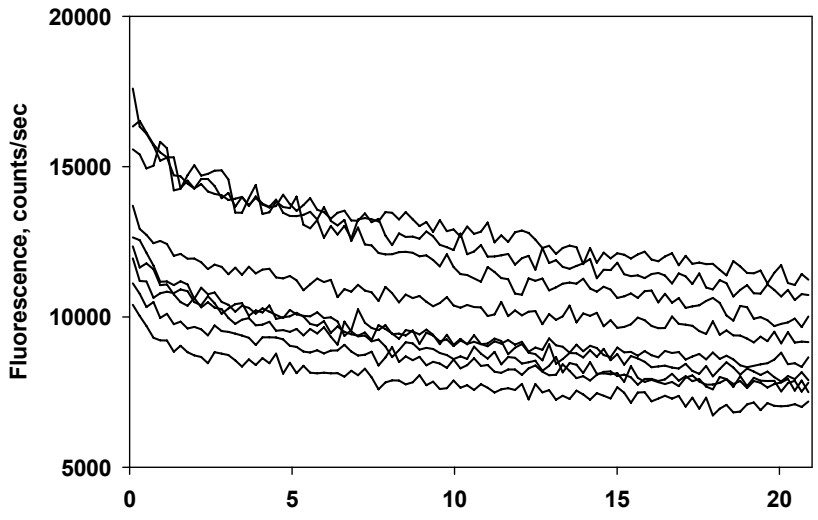
- Presented approach can be consider as express method for scanning and measurements of dynamics processes in living cells. In perspective, one may create a map of parameters distribution for a portion of investigated blood cells.

Method of determination of NO inside of living cells with fluorescence probe DAF-FM diacetate

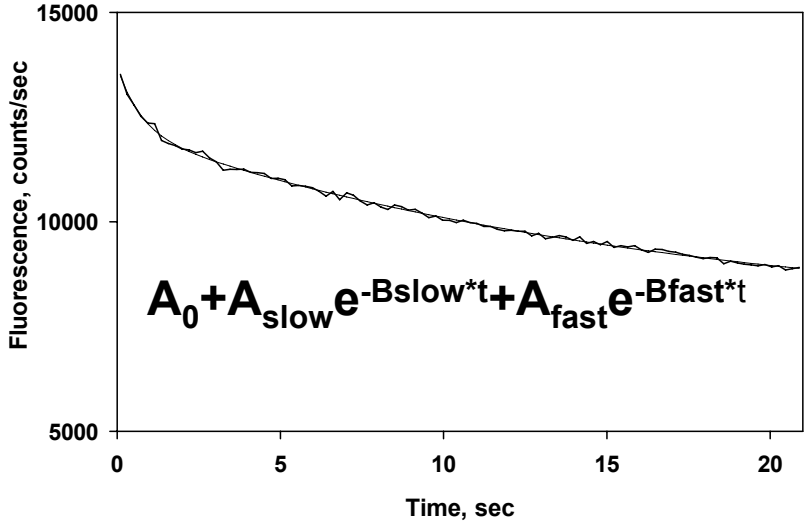


Kinetics of fluorescence photobleaching of cells incubated with DAF-FM diacetate with following addition of NONOate

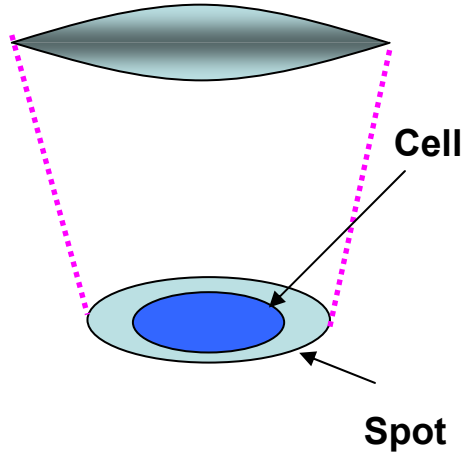
Each kinetics from single cell



Averaged kinetics and fitting with function



Geometry of measurements

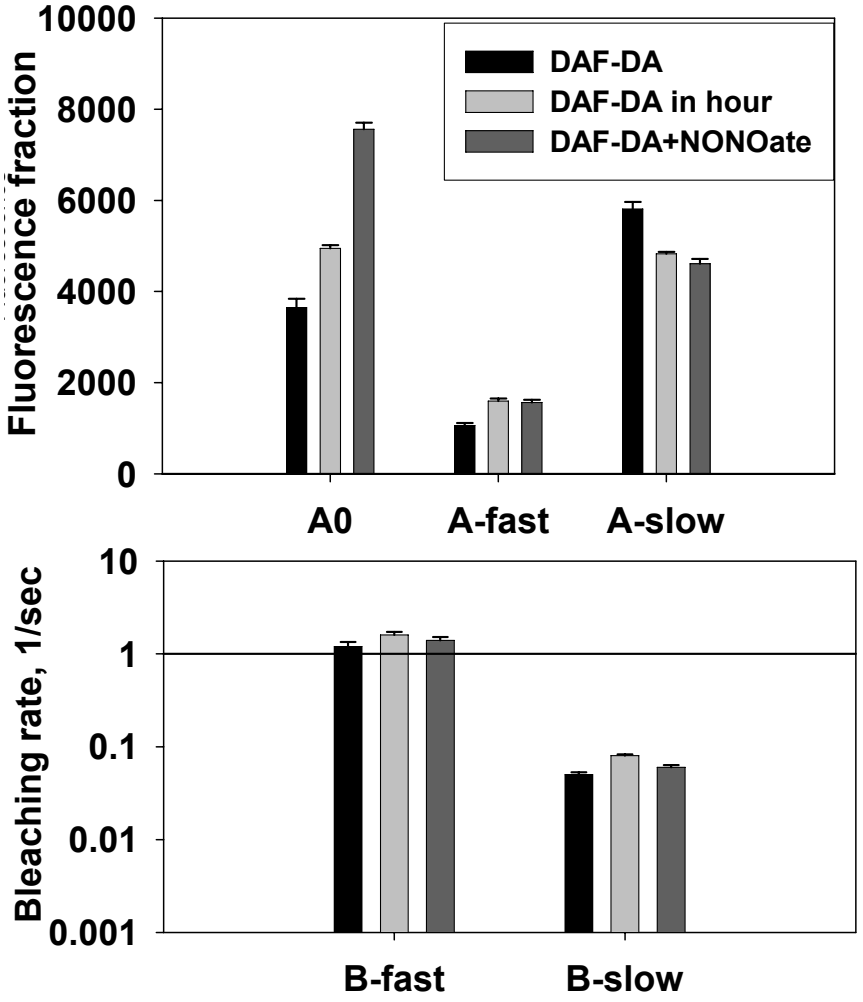


NONOate spontaneously decays with formation of NO
Rate of NO generation in experiments was 50 nM/min

$B_{slow}=0.06\ s^{-1}$
$B_{fast}=1.4\ s^{-1}$

Oxygen concentration:
in water 0.2-0.25 mM
In lipid 5-7 mM
(literature data)

Parameters of kinetics of photobleaching of DAF-FM diacetate
upon addition to cells, one hour later, and then after addition of NO-donor (NONOate)

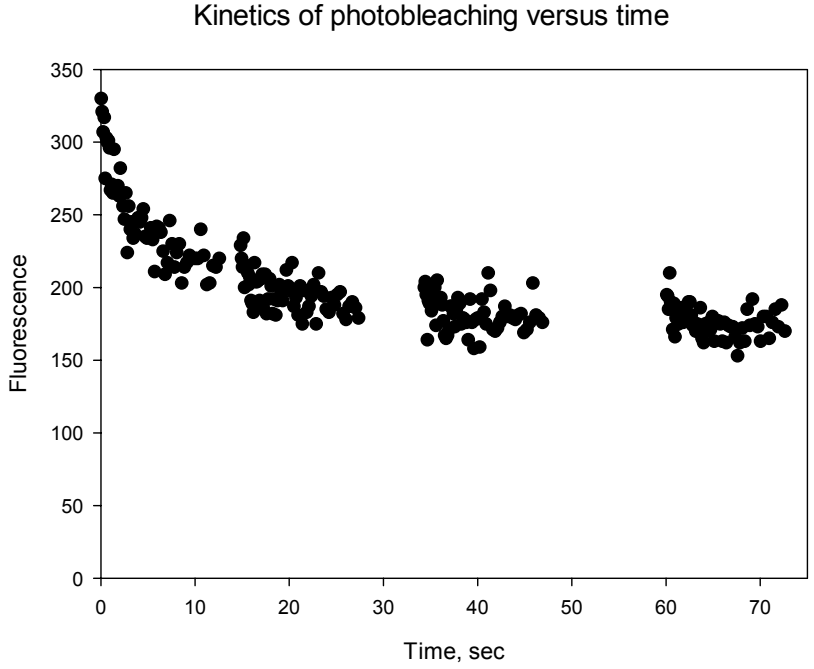


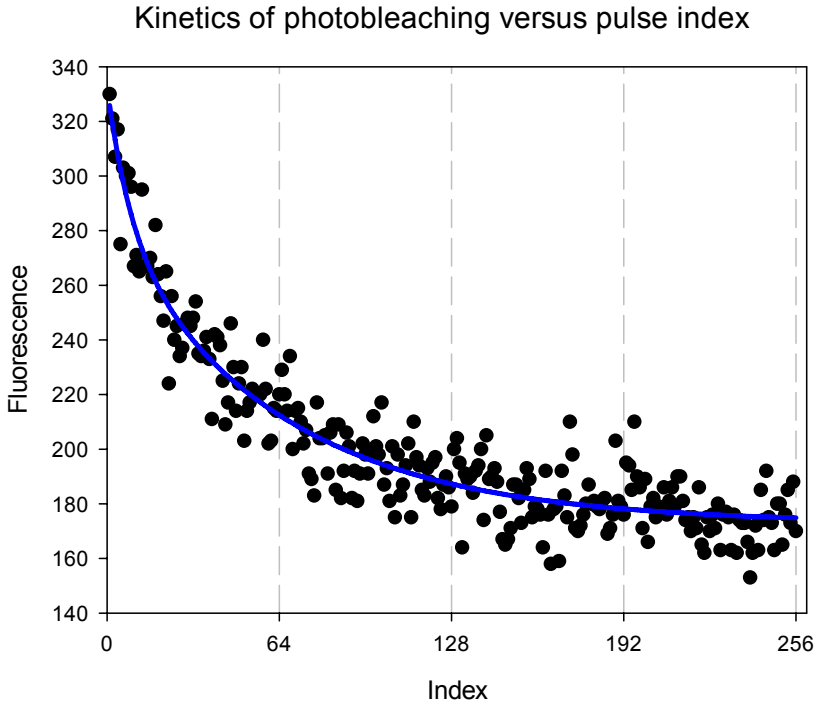
Data speculation

Fluorescence probe DAF-FM is localized in three region with different oxygen contents: lipid, aqueous and “oxygen free”.

Reaction with NO results in fluorescent increasing of only for probe localized in “oxygen free” region.

Kinetics of photobleaching of DAF-FM in single cell using PPM irradiation





The probe exchange between regions was not detected during experimental measurements.

Thanks for attention

Greeting from our team

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A.D. Ryazanova