# AN2: History of double-kinetic experiments

Fluorescence has found wide broad scope of different applications in life science and biotechnology that is based on the unique scope of fluorescence characteristics. Optical methods have an incredible sensitivity; act of photon emission by single molecule can be detected. Processes in femtosecond time scale can be observed using optical methods. Fluorescence is characterized by many parameters as excitation and emission spectra, the lifetime, anisotropy, and the quantum yield. The fluorescence parameters are sensitive to temperature, polarity and viscosity of solvents, and to various excited state reactions. Using intrinsic protein fluorophores like Tryptophan or fluorescent probes attached to proteins information about fluorophores environment could be obtained.

Due to the variety of processes affecting fluorescence probe emission, it is sometimes difficult to attribute observable changes in fluorescence intensity to specific mechanisms. For example, it is not possible to separate effects of static and dynamic quenching using only steady-state luminescence data. Very often only time-resolved information allows unambiguous interpretation of processes in a sample. In order to obtain this information a waveform of fluorescence decay after short excitation pulse should be measured. A typical time scale for these experiments is tens of nanosecond. It will be referred to us as “spectroscopy time scale”.

Another class of time-resolved optical experiments is performed to get information about kinetic of biochemical reaction. In this case fluorescence intensity is recorded after rapid change of some parameter like reagent concentration, temperature, pressure, etc. This class includes such techniques as stopped-flow, continues flow, temperature jump, flash photolysis, etc. The time scale of these experiments is often limited by used technique and usually lies in millisecond diapason. It is referred to us as “biochemical time scale”.

This is a very logical step to move from just intensity measuring during optical time-resolved experiments in biochemical time scale to fluorescence decays measuring. The term “double kinetic” is usually utilized to describe this sort of experiments. First experiments of this kind were performed in laboratory of L. Brand [1, 2]. They used TCSPC technique for fluorescence decays measuring. Because only excitation sources available at the time were low-kilohertz repetition rate flash lamps the time resolution in biochemical time scale was only minutes. Since then excitation sources and collection electronics were sufficiently improved and TCSPC technique was successfully used in double-kinetic experiments based on stopped-flow [3-7, 19], and continues flow [8]. The TCSPC method provides best available time resolution up to single picoseconds what very often exceed experiment needs. The principal rule of TCPSC requires keeping counting rate 10 times lower than rate of excitation pulses to avoid distortion caused by the occurrence of more than one photon per counting cycle. This means a data for several hundred stopped-flow mixes should be summated to get reasonable S/N ratio for tens of double-kinetic waveforms. Because sample is usually very valuable especially for biochemical experiments, an amount of sample for several hundred stopped-flow mixes not always available. Using of streak camera in combination with stopped-flow mixer for double-kinetic experiment was reported [9]. This combination requires only one stopped-flow mix for waveform what is still a lot if hundreds of

decays need to be measured. The dynamic range of steak camera is low what affects data precision. One more light detecting technique used for double-kinetic experiments is a transient digitizing. In this technique excitation pulse with energy conducive to produce number of photons from sample is used and information about luminescence lifetime can be determined from shape of measured waveform[10, 11]. This technique was successfully used in T jump [12-14] and stopped-flow [15-18] experiments.

Ballew *et. all* [12-14] used direct fluorescence decay waveform digitizing after temperature jump generated by powerful IR laser pulse. Fluorescence was excited by frequency tripled mode-locked titanium sapphire lased with repetition rate 71.4 MHz. Data were recorded by digital storage oscilloscope with sampling interval 0.5 ns. 50 blocks of data 40 us long with increasing time gaps between blocks were collected. This allow data limited by oscilloscope memory to 4 Mb to be collected for 20 ms after temperature generated laser flash by sacrificing data in a gaps. The performance of this instrument is limited to system with dyes with luminescence lifetime shorter than 5 ns and excitation wavelengths near 290 nm due to fixed wavelength and repetition rate of laser used for fluorescence excitation.

Ratner *et. all* [15-18] developed a time-resolved luminescence instrument for double-kinetic experiments based on stopped-flow technique. After stopped-flow mix fluorescence decays were excited by low repetition rate dye laser and digitized using digital storage oscilloscope. To improve time resolution in biochemical time scale series of experiments with different time shift between stopped-flow mix and first excitation laser pulse were carried out.


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