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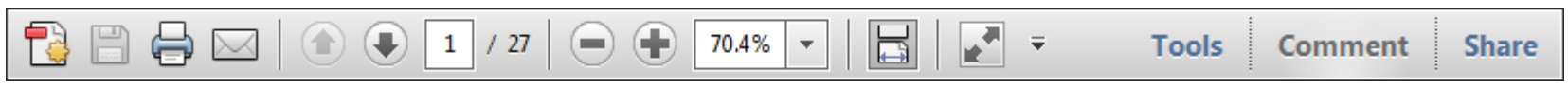

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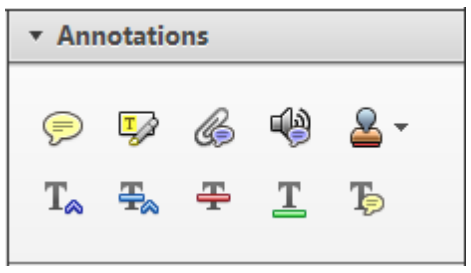

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Strikes a line through text and opens up a text box where replacement text can be entered.

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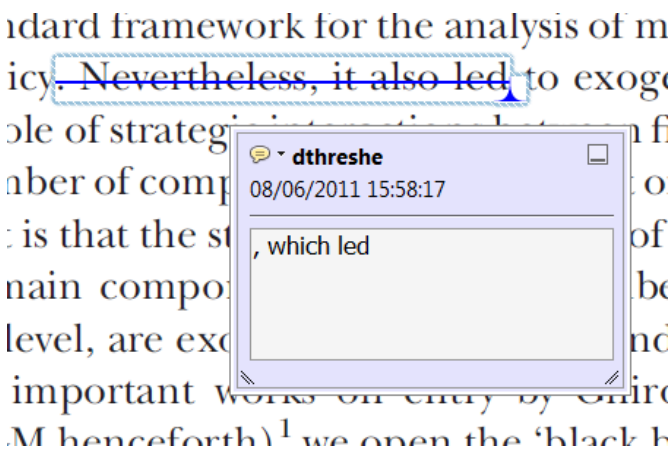

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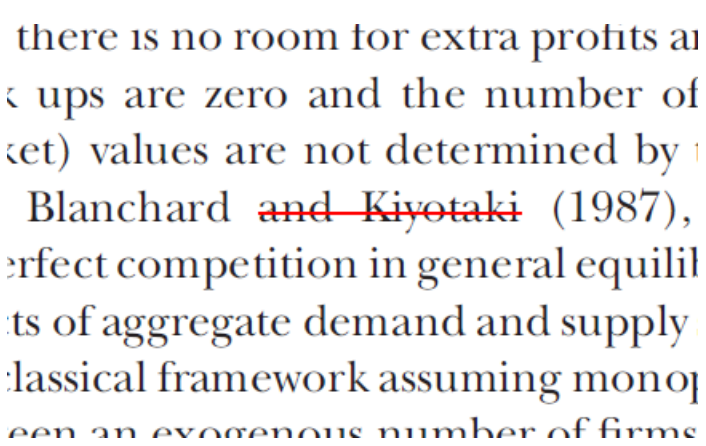

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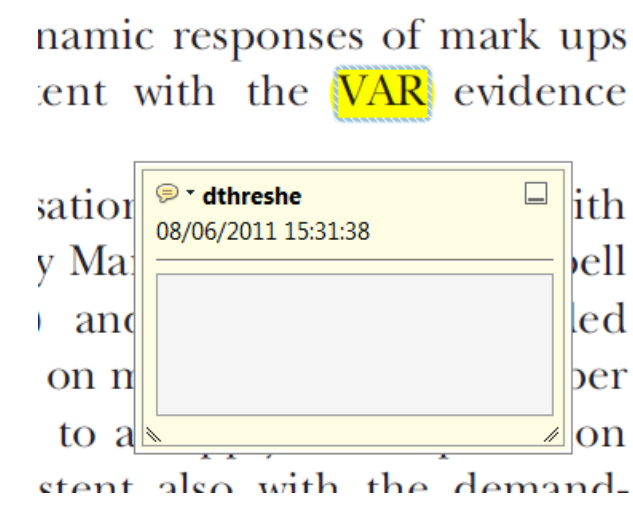

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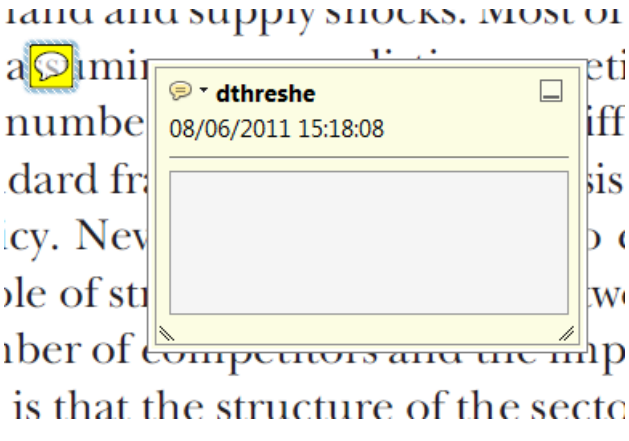

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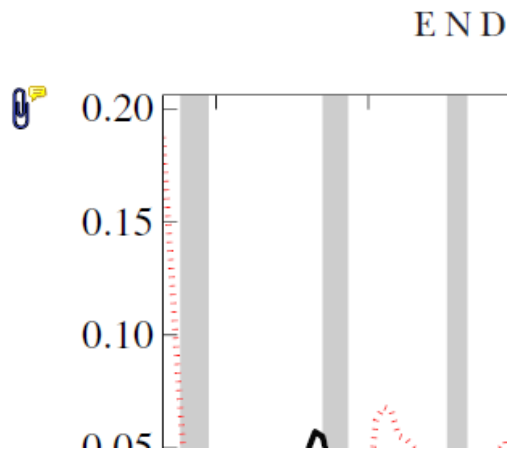
How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



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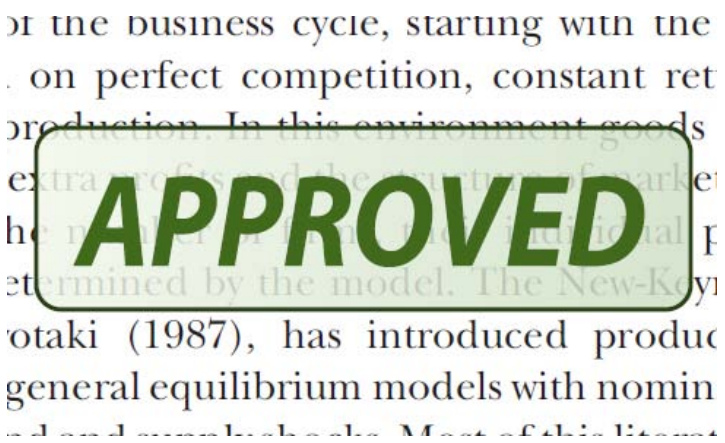

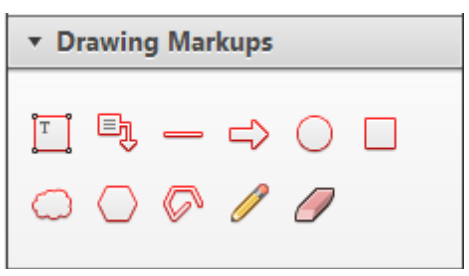

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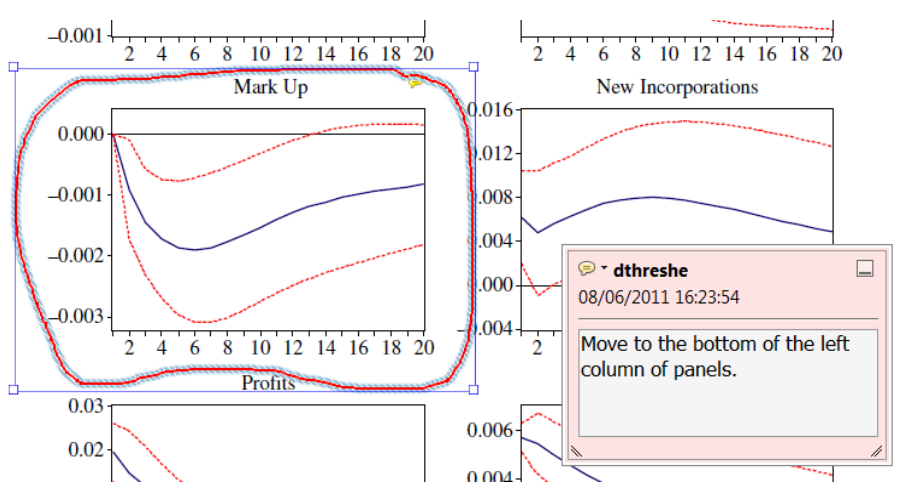

How to use it

- Click on one of the shapes in the Drawing Markups section.
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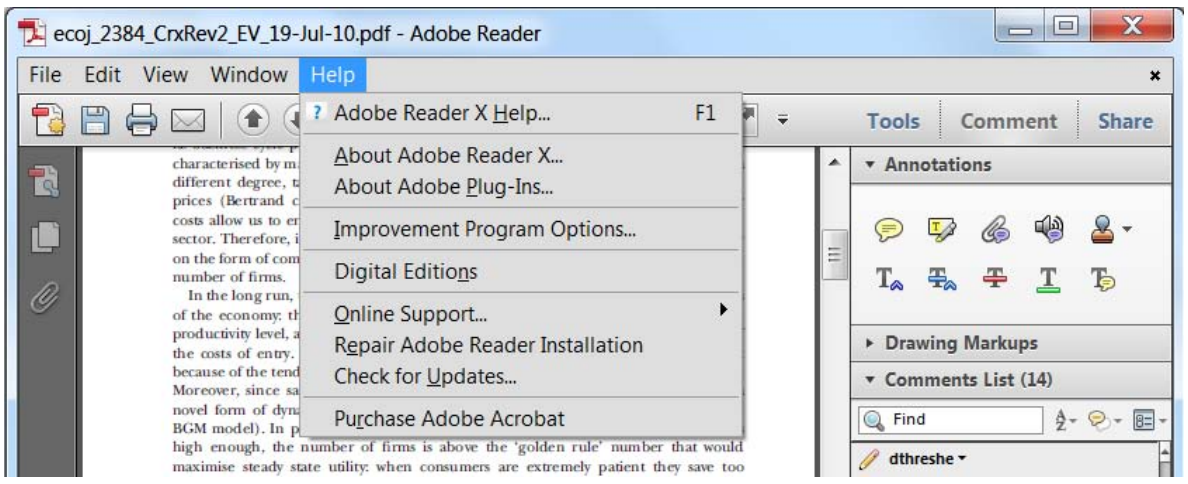

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The Other Side of the Coin: Time-Domain Fluorescence Lifetime in Flow

AQ7 László Bene,* László Damjanovich

• **Key terms**
fluorescence lifetime; flow cytometry; time-correlated single photon counting (TCSPC); burst integrated fluorescence lifetime (BIFL); time-tagged time-resolved (TTTR); fluorescence resonance energy transfer (FRET)

FLUORESCENCE lifetime is a central quantity of fluorescence spectroscopy reflecting fluorophore heterogeneity and the dynamics of fluorophore–environment interactions (1). It is increasingly used in molecular biology and biochemistry to monitor ion concentration, pH, polarity, enzyme-binding activities, fluorescence quenching and resonance energy transfer (FRET), temperature, and even refractive index on the nanoscale, to name some applications (2–5). In spite of its incredibly small value (ns), technical development in the field of light sources—short pulsed-lasers with high repetition rate—and detectors—avalanche photodiode—made feasible its direct determination first in bulk solution, then even at the levels of single cells and single-molecules (6,7).

Essentially two versions of the lifetime measurements have been developed with their own advantages and disadvantages. In the phase method, harmonic excitation (frequency-domain) is used when fluorescence is also harmonic but with a phase delay and demodulation as compared to the excitation, from which lifetime can be computed (8). The advantage of the method is its rapidity due to the efficient Fourier-algorithms, favoring multiplexing and applications for real time monitoring of dynamic events in video microscopy. The disadvantages are that relatively high signals are needed, and because lifetime is an indirectly measured parameter here, lifetime heterogeneity can not be revealed directly, albeit the recently introduced AB-plot technique helped a lot in this recpect (9). Another drawback is that small lifetime changes frequently occuring e.g. in FRET measurements cannot be measured readily. The other method, the time-domain or pulsed technique, directly samples the fluorescence decay curves elicited by very short light flashes via single photon counting or stroboscopic detection (10). The advantages of this approach are that it works the best for weekly fluorescing samples—to avoid photon pile-up—, and that lifetime heterogeneity can directly be revealed by the nature of the recorded decay curves, namely whether it is a single or a multiple exponential. The disadvantage of the method is its slow speed preventing its usage for tracking high speed processes.

Nowadays, lifetime technology culminated in the near-routine usage of commercially available versatile fluorescence lifetime imaging microscope (FLIM) systems in the time-domain (11,12) and in the frequency-domain (13). In contrast to the stationary samples in microscopy, the available detection time in flow conditions is limited which imposes a serious constraint on implementing direct lifetime measurements in flow. However, the ever-increasing speed of electronics made the implementation of the different lifetime measuring techniques also possible in flow cytometry, first the phase method pioneered by Steinkamp and coworkers (4), then the time-domain method pioneered by Condrau et al. (14) in the form of time gated detection of fluorescence of lanthanides. Also in flow, recently Cao et al. (15) reported on an attractive pulse-shape method situated between the pulsed and phase

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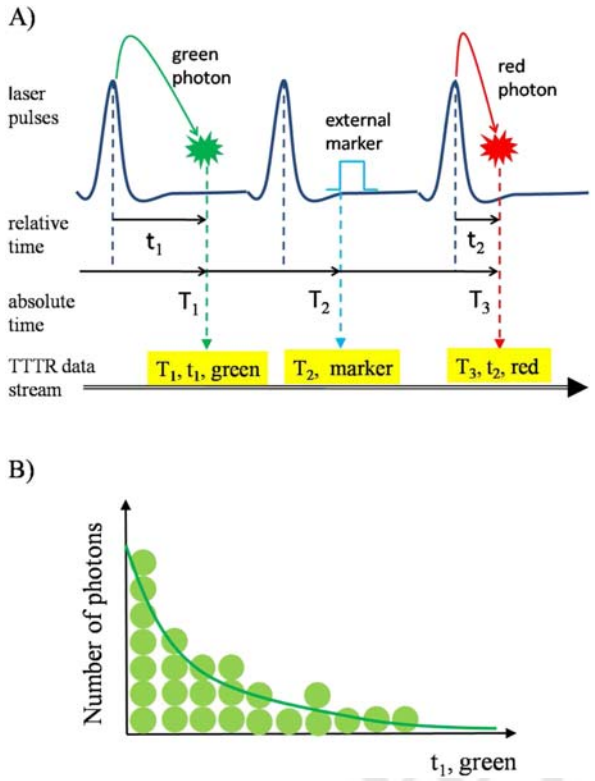

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Figure 1. Scheme of the "time-tagged time-resolved" (TTTR) principle of fluorescence data collection. Panel **A**: Events—measurements (photons at T_1, T_3) and marker signals (at T_2) for external interventions—are organized sequentially in a unified global time scale (T) realized by the exciting laser pulse train (blue signals) used as a clock signal (6,12). After each excitation pulse either the arrival time relative to the pulse time (t_1, t_2, 0–10 ns) and the channel (detected emission bands and/or polarization directions) of a first photon are registered, or the times of external interventions, such as a driving signal of a piezo-scanner marking the beginning of a line scan during a 2-dimensional raster scanning, i.e. recording an image in a confocal microscope. The registration of these numbers is in succession on the absolute time scale (T, ms s, covering 500–10^4 bursts) fixed by the train of laser pulses. The power of this data organization scheme lies in its flexibility for enabling multiplexing a very diverse range of parameters and data analysis methods, such as auto- and cross-correlations in time, intra-particle (burst) fluorescence decay curves and time-gated detection of correlations. Panel **B**: By binning according to the ns-arrival times relative to the pulse time—t_1 for the green photons—, photon-frequency histograms can be generated representing the fluorescence decay curves for the whole measurement. Besides, fine structure of the photon stream can be revealed by plotting photon frequency—obtained by distributing the photon macro-times into histogram bins (~20 μs)—as the function of the absolute time making possible the identification of individual particles as ms-crowdings of photons called "bursts" and subsequent thresholding on them. Alternatively, this segmentation of photon time trace can be achieved by plotting the inter-photon times (Panel B on Fig. 2) as the function of the absolute time and subsequent gating on the short inter-photon times. This can be done for each detection channel fixed by the detected wavelength range and polarization. Subsequently "burst"- or particle-level analyses can be performed and particle-level characteristics such as decay curves, average intensities, and decay-times in different detection channels can be determined amenable for constructing different bivariate scatter plots. This data organization scheme—originally introduced in the field of SMD (6)—well matches with the sequentially collected list mode data structure and the high degree multiplexing inherent in flow cytometry.

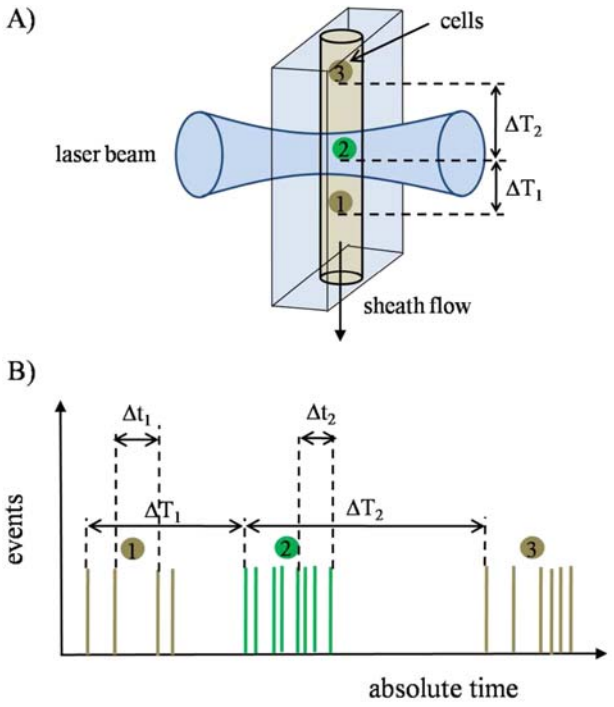

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Figure 2. Illustration of the particle–photon "burst" correspondence in flow cytometry. Panel **A**: In conventional flow cytometry, each cell is individually excited by an intersecting continuous laser beam, and several parameters are collected sequentially—i.e. in list mode—, if they obey some preadjusted thresholding and gating conditions. In the TTTR approach (6,12) the continuous laser is replaced by a pulsed one producing psec–fsec pulses at a high repetition rate (~100 MHz). Besides, to lengthen the pulse duration necessary for making time-correlated single-photon counting (TCSPC) (10) possible in flow, slowing down the particle flow is required. To this end the originally stream-in-air or flow chamber-based particle illumination arrangement has been replaced by a microfluidic chip controlling speed of sample delivery. Panel **B**: Each of the cells depicted in Panel A gives rise to a sequence of first photons, a "burst," the duration of which (~ms) is dictated by the flow speed, and the size of the cells and the beam waist. The frequency of photons in the "bursts" represents fluorescence intensity. The photon "bursts" from cells are distinguished from noise by gating on the high photon frequencies, i.e. large intensities or the small inter-photon times (Δt_1, Δt_2) (6). The larger inter-"burst" times (ΔT_1, ΔT_2) are determined by the flow speed and the cell concentration in the sample. In the illustrated case, the ascending order of cells according to brightness is 1-3-2.

methods with the capability for parallelism well fitting to the high-throughput multiplex nature of flow cytometry.

An inventive approach to time-domain flow cytometry has been presented by Nedbal et al. (in this issue, page XXX) AQ1 via converting a conventional inverted wide-field fluorescence microscope into a time-domain flow cytometer by placing a flow-chip (microfluidic chamber) on the sample stage and applying time-correlated single photon counting (TCSPC). With the flow-chip they adjusted the speed of cells to the speed of the data-acquisition. A spectacular feature of the arrangement is that the "trigger signal" of the conventional flow cytometer—synchronizing the different signal channels—has been replaced by the concept of "burst" analysis borrowed from the field of single-molecule detection (SMD). This approach

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has also been designated as "BIFL" (burst integrated fluorescence lifetime) or TTTR (time-tagged time-resolved), referring to the way of data collection (6,11,12). This type of data collection means that the different events are registered in parallel in two time scales: first photons are registered relative to the eliciting exciting pulses (micro-times) and also relative to the beginning of the experiment in a laboratory time frame (macro-time) fixed by the train of the exciting laser pulses used as a clock signal (Fig. 1). In addition to the arrival times of first photons, their color and polarization direction, as well as external marker signals such as those indicating spatial positions of the photon emissions in imaging—i.e. piezo-scanner driving signal—can also be registered on the absolute time-scale. This multiplexing capability of TTTR can advantageously be matched with that of flow cytometry. Signal-noise discrimination, i.e. identifying fluorescing particles, can be achieved by thresholding on the "inter-photon-period vs. macro-time," or the "time-binned integrated event number (i.e., fluorescence intensity) vs. macro-time" plots after registering the whole time history of the experiment, which is also the task of a trigger-signal (e.g., forward light scattering, FSC) in a conventional flow cytometer (Fig. 2) (6,11). This operation is analogous to the segmentation of spatial images. By applying this approach bursts of single particles can be identified efficiently, as indicated by the negligible 2.9%-contamination from the two-particle events. After segmentation of the time-trace, burst-level intensity decays, average lifetimes and intensities can be computed and organized into bivariate scatter plots to reveal possible dependencies, like in the case of the conventional flow cytometric dot-plots. Unique to the time-domain approach is that events can also be gated on the arrival times ("time-gating"), making possible distinguishing useful signals from the unwanted background, like autofluorescence and light scattering, based on the much smaller arrival times for background photons than for useful fluorescence. Furthermore, distinguishing between different fluorophore species or two forms of the same species can be done by gating on the different lifetimes (7,11).

The applicability of the method has been demonstrated by the authors in determining FRET applying CFP-RFP visible fluorescent protein rulers with well-defined spatial separations, in a GFP-Cy3 FRET-assay for EGFR tyrosine phosphorylation, and in measuring lifetimes of a two-state polarity-sensitive dye (di-4-ANEPPDHQ) in the lipid membrane phases. According to their data ~0.1 ns lifetime differences due to FRET, or due to environmental lifetime-heterogeneity can be resolved presently.

The method is inherently multiplexed by the nature of TTTR, and by including spatial marker signals it could also be applied for imaging in flow. Future developments are expected to aim at speeding up the data acquisition and processing to the running time of conventional flow cytometers (from ~50 events/s to ~10^2-10^3 events/s).

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