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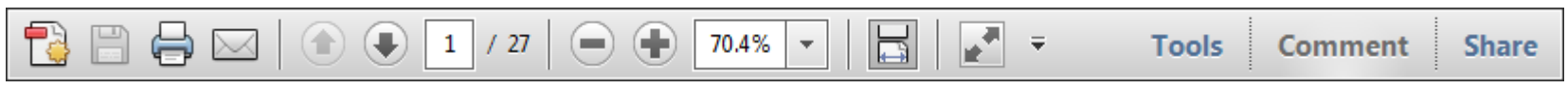
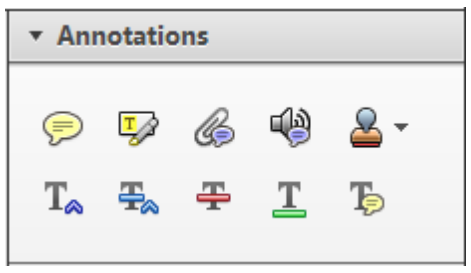



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

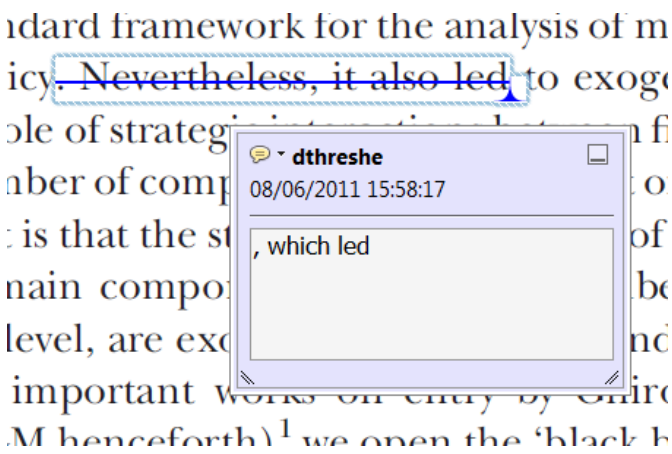



## 1. Replace (Ins) Tool – for replacing text.

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

**How to use it**

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

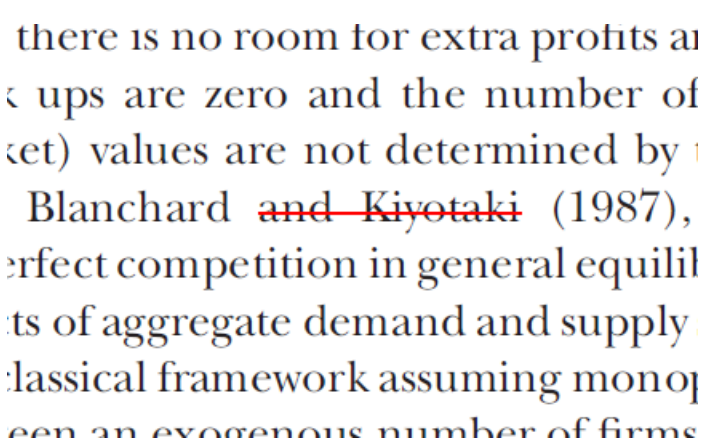



## 2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

**How to use it**

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

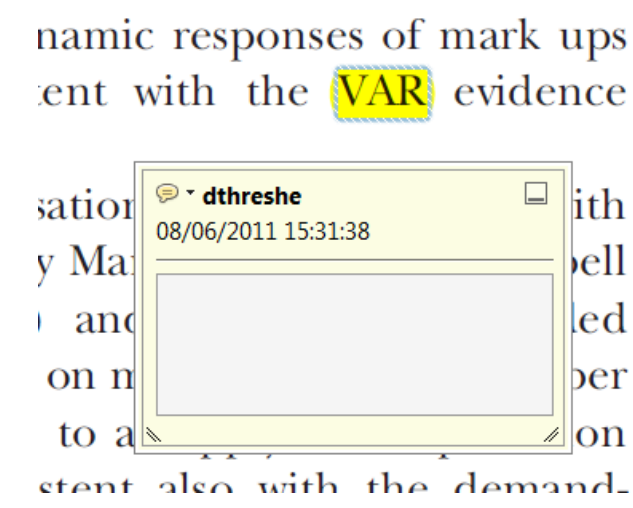


## 3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

**How to use it**

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

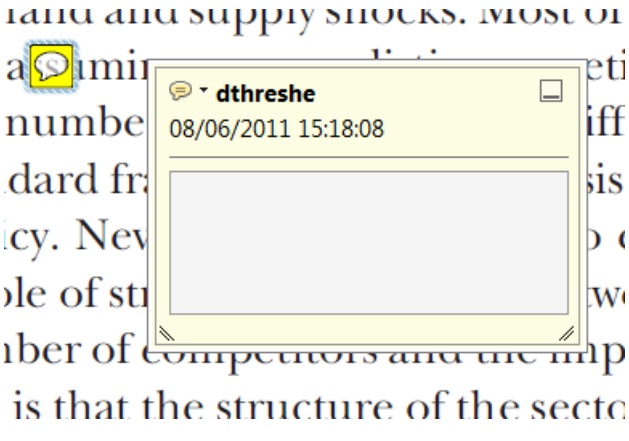



## 4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

**How to use it**

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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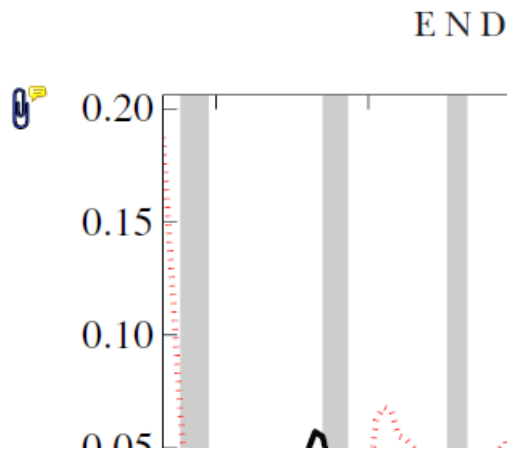
### 5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

**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.

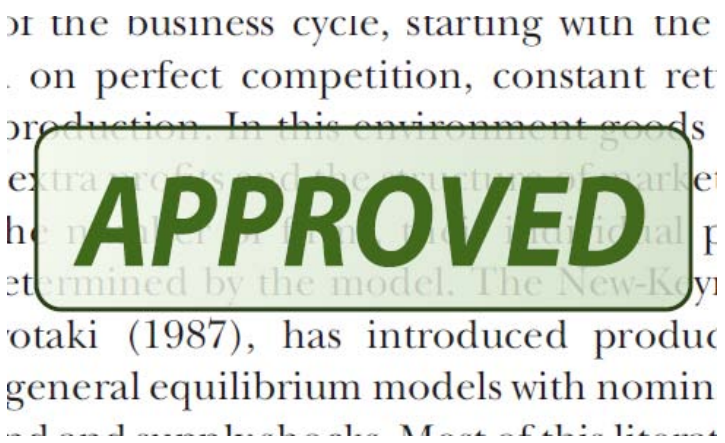


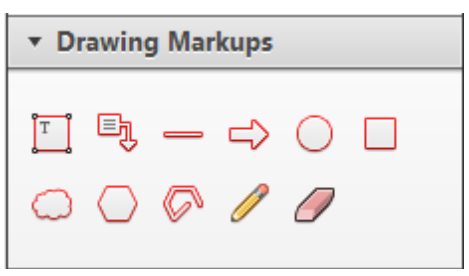

### 6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

**How to use it**

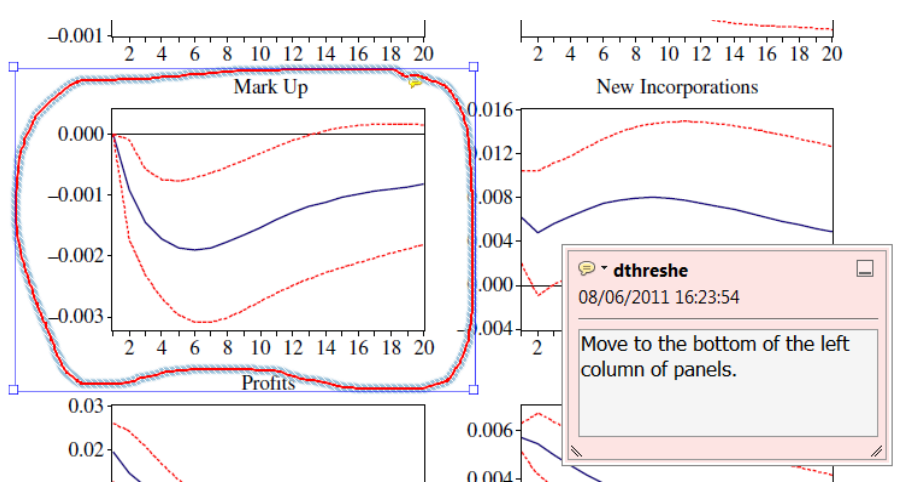
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).




### 7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

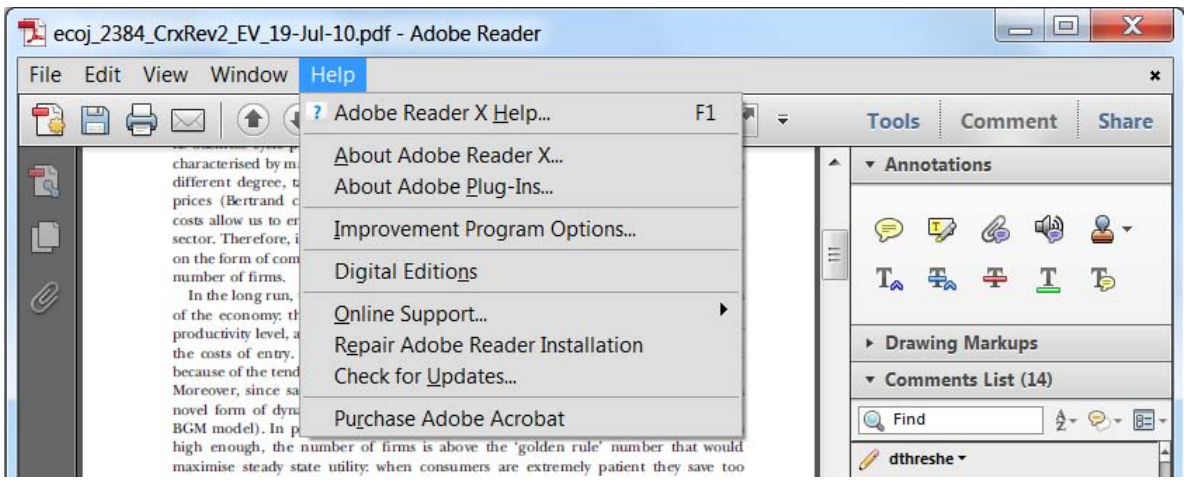
Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..




**How to use it**

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

**For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:**



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⌜ ⌝

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- ☐ B.S.
- ☐ Associate
- ☐ Other _____

**Field of Study**
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- ☐ Chemical Sciences
- ☐ Computer Sciences
- ☐ Engineering
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- ☐ Physical Sciences
- ☐ Other _____

**Research Areas**
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- ☐ Biophysics
- ☐ Biotechnology
- ☐ Cell & Molecular Biology
- ☐ Cell Physiology
- ☐ Clinical Lab Practice
- ☐ Clinical Research
- ☐ Flow & Image Cytometry
- ☐ Genetics
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- ☐ Other _____

**Interest Groups**

Check to be associated with one or more of the following groups:
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- ☐ Clinical Cytometry Group
- ☐ Cytometric Technology Group
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Cytometry PART A
Journal of the International Society for Advancement of Cytometry

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# À la Fizeau in Flow: Pulse Shape-Assisted Fluorescence Lifetime

AQ1 László Bene,[1]* János Szöllősi[2]

• Key terms
fluorescence lifetime; flow cytometry; phase-fluorometry; phase
AQ2 lifetime; modulation lifetime; convolution

FLUORESCENCE lifetime measurements are utilized more and more lately in molecular and cell biology partly due to its applicability for monitoring molecular dynamics and discrimination of molecular species, partly due to the commercial availability of microscopes and fluorimeters capable for fluorescence lifetime detection (1,2). Fluorescence lifetime is the average time an excited fluorophore spends in the excited state prior to returning to the ground state, via emission of light or other, non-radiative ways of de-excitation. Its widespread utility rests on the property that in addition to fluorescence, many other processes involving the excited fluorophore may exist which take place on the same time scale and may compete with fluorescence in the de-excitation and consequently may influence fluorescence lifetime (3–5). To name a few, these processes are: internal conversion, vibrational relaxation, intersystem crossing, Förster resonance energy transfer (FRET), dynamic quenching, solvent relaxation, charge transfer, and photolysis (photobleaching); and most of these processes involve interactions of the excited fluorophore with its local environment (1,3). For this reason fluorescence lifetime can be used to monitor locally the concentrations of ions and molecules, electric field and polarity, temperature, viscosity, and refractive index (1–3,6–9). By spanning a much larger range than the wavelength of the emitted fluorescence, it is more amenable for multiplexing than wavelength (8,10). It can be used as a contrast parameter, for example, for discriminating between emitters having overlapping emission spectra (9). It is also a state parameter, meaning that it is independent of conditions of excitation such as color, intensity, and polarization of the exciting light. Based on its independence of concentration and due to the fact that it does not necessitate calibration, it is a more sensitive indicator of FRET than the intensity alone (3,11). It can be changed only physically—termed radiative decay engineering—by influencing the mode structure of the random vacuum field fluctuations responsible for the stimulation of spontaneous emission, for example, by placing a metal mirror in the vicinity of the fluorophore or by embedding the fluorophore in a cavity resonator (12,13).

The detection of fluorescence lifetime rests on the "drag" and attenuation imposed on the reemission process, the fluorophore acting as a low pass filter, by cutting and smoothing out sharp features of the original time variation of the exciting light (Fig. 1) (3). This feature mathematically is expressed by F1 the operation on the time profile of excitation and pulse-response of fluorophore—called convolution—leading to a shift to the right on the time-axis and demodulation for the time profile of fluorescence.

Instead of using short excitation pulses the fluorescence lifetime can also be measured by a long lasting observation of fluorescence, which is periodically modulated at a frequency (~100 MHz) corresponding to the inverse lifetime, with a detector the gain of which is modulated in synchrony

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Received 3 October 2014; Accepted 7 October 2014

Grant sponsor: TÁMOP-4.2.2.A-11/1/KONV-2012-0045 project co-financed by the European Union and the European Social Fund

Grant sponsor: OTKA NK Research Grant, Grant number: 101337

Grant sponsor: OTKA Bridging Fund support OSTRAT/810/213 by University of Debrecen.

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Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.22583

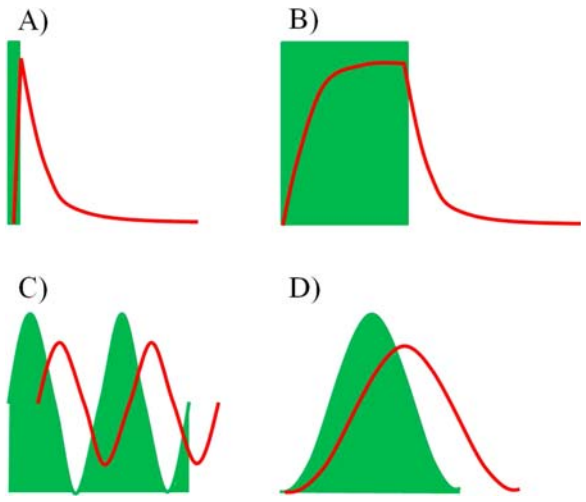


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**Figure 1.** Illustration of the low-path filtering effect of fluorophores. The sharp features of the excitation time profiles (*green shaded areas*) are smoothed out by the inertia against reemission (*red lines*) encoded in the excited state lifetime. The different lifetime measuring techniques are based on the detection of the relative distortion of the emission profile as compared to the excitating one and to determine the characteristics of the decay profile (the different lifetimes and the relative abundance of the different lifetimes). Panel A: The fluorescence profile elicited by an excitation pulse much shorter than fluorescence lifetime ("$\delta$-pulse") is the true decay profile of fluorescence ("impulse response"). If the excitation is with not a "$\delta$-pulse" but with an extended one (Panels B-D), then the emission intensity at any given time point is determined by the contributions of all the excitations happening before that given time point, analytically described by "convolution." Panel B: Upon excitation by a square pulse, both the appearance and the disappearance of fluorescence is not instantenous, but gradual, having rates dictated by the fluorescence lifetime. Panel C: Upon excitation by a sinusoidal profile, the emission is also sinusoid, but with a phase shifted to the right and with a reduced modulation (deviation of signal amplitude from the mean). Panel D: All the information of the decay profile is kept, when the excitation is with a single pulse of Gaussian profile, such as in flow cytometry. This situation can also be handled as the first peak of the excitation with a sinusoid wave-train (Panel C). Here the peak of the fluorescence pulse is shifted to the right and its amplitude is decreased as compared to those of the light scattering (forward: FSC, perpendicular: SSC) pulses. The decay profile of the fluorescence can be obtained in the knowledge of the excitation profile, encoded in the time profile of the light scattering—forward or perpendicular—signals. However to carry out this operation requires high speed electronics to precisely fix the timing of detection of the fluorescence pulse at the rising edge of the light scattering (triggering) and large number of sampling points during approximately 10 $\mu$s-pulse period. Complications can arise from, for example, photobleaching, saturation, and using light scattering as a reference of the excitation profile, because the cells are not perfect mirrors.

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(homodyne detection) or almost in synchrony (heterodyne detection) with that of the excitation, but shifted in phase electronically nowadays or by translating the detector itself in older equipments, in the principle of Fizeau's measurement of
F2 speed of light (Fig. 2) (5,7,8,10,13,14). As a result of the synchronous and near synchronous detections the detector signal is a DC one depending on the introduced phase step and observation time and an AC signal varying slowly (~100 kHz) at the difference frequency, respectively. However, measuring only the sample's signal is not adequate, because to take into account the retardation—that is, to fix the origin of the time of observation, which is the time of excitation—and demodulation introduced in the light path towards the detector, measurement of a sample emitting light with a known retardation in the same observation path as for fluorescence is also necessary. Scattering solution of metal powder, milk, or a light reflector mirror having zero retardance or a fluorophore of known lifetime introduced in the sample position can serve as reference. The unknown lifetime can be determined from the relative phase delay and relative demodulation of the sample as compared to the reference (1,3–11,14).

The above described phase modulation method is extensively used as a contrast modality in fluorescence imaging or in bulk cuvette measurements of biological samples in lifetime spectrometers (fluorometers), where the times of observations can be substantially long to achieve high signal-to-noise ratios. Compared to these techniques, the distinguishing feature of flow cytometry is the high throughput and the multiplexing ability, two properties deserving realization of the phase method also in flow. However, in a flow cytometer, the finite duration of illumination imposes a serious constraint on the adaptation of the method. In spite of this drawback, it has been successfully used recently for discriminating between different states of fluorophores also in flow showing that the high throughput property can be advantageously combined with the phase detection. Combination of multiplexing feature of flow cytometry with the phase method—that is, to measure the lifetimes in several fluorescence channels in parallel—may be hindered by the insufficient speed of data processing electronics and the substantial cost increase (e.g., each excitation beam should be modulated independently) (8,9).

To circumvent the above inconveniences of the phase-modulation method, Cao et al. (in this issue page XXX) have AQ3 decided to abandon the present paradigm of lifetime measurements of using long lasting fluorescence wave-trains for obtaining lifetime information, and instead concentrate on the information content of single fluorescence pulses. Theoretically the whole information content on lifetime could be gained by considering the fluorescence response elicited by only a single excitation pulse (Fig. 1). The need for the application of the endless periodic wave-trains can be rooted back from solely practical considerations such as ensuring high signal-to-noise ratios, the adequate speed of electronics to carry out deconvolution on a single fluorescence pulse, and the convenience of using Fourier transforms in order to use the same algorithm independently of the exact shape of the exciting periodic wave (3). In spite of using a single pulse, their method, named "non-modulated fluorescence lifetime cytometry" (nFLIC), is conceptually closely connected to the phase-modulation method, because—as shown by them—it can be considered as the limiting case a periodic excitation of infinite period (or zero repetition frequency), which practically implies omitting pulses after the first one. This approach has been made feasible by the recently appearing high-speed digital data processors (e.g., 50 Mega-sample/sec DAQ having a 20 ns-sampling time necessary for ~500 data points in

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~10 μs-pulse duration, high-resolution (ns-) storing oscilloscopes) and the high-memory computers.

Although the feasibility of the single-pulse approach has been demonstrated, further challenges await for the developers both technically and theoretically, such as the elaboration of parallel in situ deconvolution of several pulses in the approximately 10 μs-pulse length. Presently the lifetime is calculated from the time delay of fluorescence pulse relative to the forward (or side) scattering pulse, defined as the period between maxima of the fitting Gaussians or the maxima or medians (half-area points) of the signals themselves. These are rather crude parameters having also a noise and signal-shape sensitivity. The final aim would be to obtain the full fluorescence decay curve—impulse response function— per each cell by deconvoluting the fluorescence pulse shape from the exciting one encoded in the light scattering pulse profile. Having the full decay curve for each cell the frequency distribution curves of the pre-exponentials describing the amounts of two forms of a dye, or the amounts of a dye situated in two different environments (polar–apolar, bound–unbound) can be constructed (9).

Additional complications to be solved are, for example, to handle pulse shape distortions due to improper shape of excitation profile, possible occurrence of "slit scan" mode of illumination for large cells, inhomogenous dye distribution, photobleaching, and photon-saturation, and to toggle between side and forward scatters to choose the better as a light reflector. Rotational motion of dyes could also inflate fluorescence lifetime detection, in as much as magic angle detection or excitation is used (54.7° away from the vertical with a vertically polarized laser) (3).



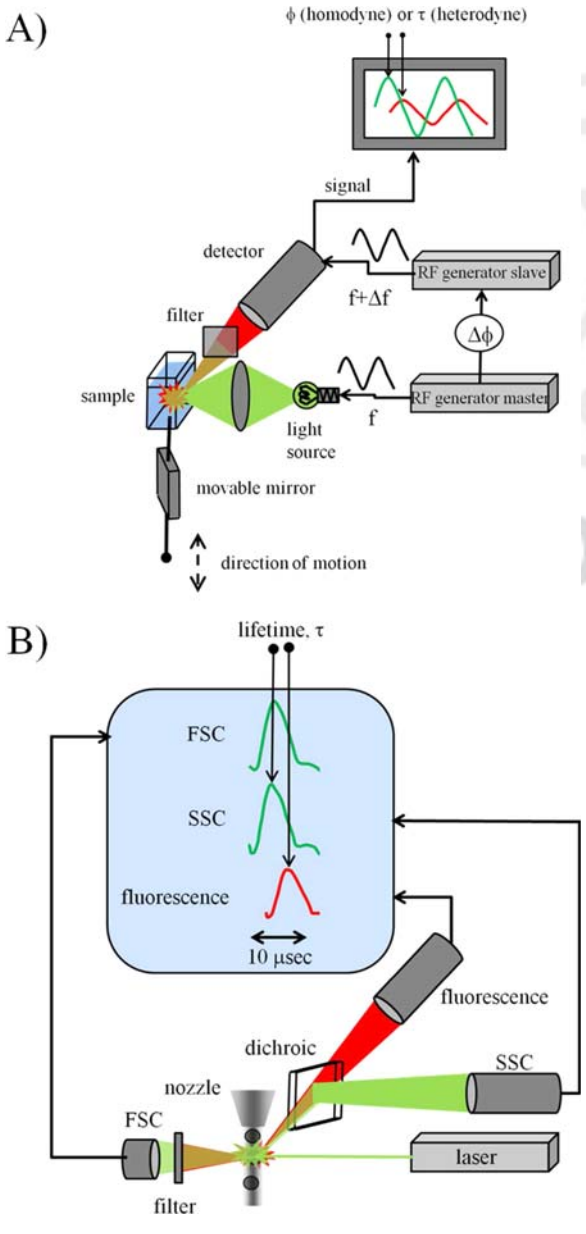


**Figure 2.** Schemes of lifetime measurements for a general phase-modulation arrangement and for flow cytometry. Panel A: The principle of "lock-in" detection. Both the excitation and detection are modulated by the radiofrequency (RF) "master" and "slave" signal generators, either phase-locked to each other with slightly different frequencies (Δf, heterodyne mode), or with the same frequency but with different phase steps ($\Delta\phi$, homodyne mode) between them. In each case the output of the detector is a sinusoid containing the same phase and modulation information as for the detected signal (fluorescence or scattering) (3,5). At the excitation side, modulated are either the power supply of the light source (for LED, arc lamps) or its emitted light (for lasers, halogen lamps) with accusto optic modulators (AOM), Pockels-cells, earlier with Kerr-cells (5,14), and even with simple mechanical shutters (Becquerel's phosphoroscope) (1,3). At the detection side, the gain of the detector (photomultiplyer gain, or image intensifier gain before CCD cameras in imaging) is modulated. These arrangements have two important aspects: (i) The first is the "lock-in" principle of detection—that is, detection in synchrony or with a fixed phase with the excitation—of short time intervals originally realized by Fizeau (Paris, 1849) in a "homodyne" experiment (14), when he measured the returning time of light via visual observation of the light source through a rotating cogs-wheel—serving as for both the "master" and "slave" signal generators—from which the determined the speed of light. (ii) The second is the need for an appropriate reference for the excitation profile, that is, an emitter having a known lifetime put precisely at the point of observation. This reference can be a solution of a dye having a known lifetime, or a reflecting mirror (e.g., in microscopes), and light scatterers such as metal powders, milk in solvents. The principle of synchronized detection is also used in other lifetime detection techniques such as time correlated single-photon counting (TSPC), time-gated fluorescence and fluorescence upconversion (10). Panel B: Flow cytometry is inherently pulsed and synchronized due to the cells' crossing the laser beam profiles and triggering. The individual pulses correspond to the first hill of the harmonic excitation—as shown in the paper of Cao et al. (page XXX)—obtainable at the limit of small lifetimes or infinitely large modulation periods (small modulation frequencies), implying correspondingly that lifetime can be obtained by simply measuring the time delay between the fluorescence and reference pulses, that is, the light scattering pulses (FSC, SSC). The time delay ($\tau$) between the fluorescence and light scatter pulses is grossly exaggerated in Panel B as compared to the 10 μs-signal duration. The success depends on how the time delay is determined, the degree to which light scattering mimics the excitation profile, and how the distorting effects of photobleaching and saturation are taken into account, to name a few possible obstacles, not withstanding "trivial" technical problems concerning the required high speed for the adequate sampling and precise timing of the pulses.



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This newly elaborated method can be used for many purposes such as genotyping and sorting according to lifetime and amplitude components of decay curves, the determination of FRET from lifetimes, or even rotational motion if the lifetimes of polarized intensity components are detected.

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