Opponent Review of the PhD thesis:

New Possibilities in Fluorescence Correlation Spectroscopy

by Aleš Benda

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The submitted thesis by Aleš Benda deals with fast developing field of fluorescence microscopy. The work was performed in the Heyrovsky Institute of Physical Chemistry, Czech Academy of Sciences in wide international collaboration.

The thesis contains a ***detailed introduction on 70 pages, followed by 6 papers*** (3 of them with A. Benda as the first author, 8 other papers co-authored by him are not included in the thesis). As stated by the author, his thesis contains several levels of work: development of Fluorescence Correlation Spectroscopy (FCS) methodology in both theoretical principles and practical setting-up of experiment. And finally, testing and application of these techniques in connection with other interesting techniques like ellipsometry.

The thesis is well organized, with an introduction comprehensibly describing principles of experimental techniques and studied systems. The six papers attached to the thesis passed a peer review process in renowned international journals (Langmuir, Review of Scientific Instruments, Biophysical Journal) which fact proves their high scientific standard.

English is very good. There are very little misprints or factual errors (e.g. on page 62 - the statement gives impression that all semiconductors absorb visible light, which is not the case, and ITO should be considered as degenerated semiconductor (n-type) with a wide band-gap).

Technical notice: The bookbinding was not taken into account when printing the text, so the internal margin was not large enough and it is sometimes almost impossible to read the text on left side.

Considering the whole work, I have to stress ***that the thesis represent an excellent piece of work, bringing important contribution to the field of fluorescence microscopy of biological systems.***

In my opinion, ***the main achievements*** are:

- introduction of the Z-scan technique, which is simple in principle but very useful
- implementation of the time-resolved FCS (TR-FCS) in the Confocor 1 microscope
- development of new algorithms for fast calculation of correlation in time-resolved-FCS
- combination of the FCS technique with ellipsometry
- surprising comparison of diffusion coefficients obtained with one- and two-photon excitation
- lifetime-tuning experiments etc.

Questions to be answered:

(1) *Pinhole size dependence* (page 29, Fig. 12): Why for small pinhole diameters the diffusion time is shorter and the count rate per molecule smaller? At these experimental conditions number of molecules in the focus approaches single-molecule level. Please, comment if it has some relation.

(2) *Single-molecule fluorescence*: The FCS apparatus used in the thesis is certainly able to detect single-molecule fluorescence. Could it have any interest for your studied systems to work with single-molecule signal? What problems or advantages you identify?

(3) *Contribution of A. Benda in the team work*: It is not clear what was your actual contribution in the team work (the papers have from five to nine co-authors). (Note, I have no objections against a broad collaboration, it is a necessity in high-level experimental science.) Please, specify your contribution.

Comments:

A Z-scan technique exists in non-linear optics for at least twenty years. Here, the sample is also scanned through the beam-waist and the signal passes through a pinhole, but a focusing/defocusing due to refraction index changes is measured.

Marginal terminological notices:

Why is the method called Fluorescence Correlation Spectroscopy - where is the spectrum? Probably there are some historical reasons, but better could be for example FPCS Fluorescence Photon Correlation Statistics.

I do not support the use of term "*in silico* testing", page. 54, even its is widely used. First, it is probably not correct Latin; it should be "in silicio". But most importantly, principle of testing is not *silicon* (even if it is the main building material of integrated circuits) but numerical models.

In conclusion, the submitted thesis clearly proves that Aleš Benda is able to carry high-level research in broad international collaboration, which brings very important contribution to the respective scientific field.
Therefore, I fully recommend accepting this work for conferment of doctoral degree.

Prague, 2006/09/03

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