

## **Multi-Wavelength TCSPC Fluorescence Lifetime Imaging**

**Wolfgang Becker, Axel Bergmann, Giovanni Biscotti**  
**Becker & Hickl GmbH, Nahmitzer Damm 30, D-12277 Berlin, Germany**  
**becker@becker-hickl.com**

**Iris Riemann, Karsten König**  
**Fraunhofer Institute for Biomedical Engineering, 66386 St. Ingbert, Germany**  
**Karsten.Koenig@ibmt.fraunhofer.de**

**Key Words:** Fluorescence Lifetime Imaging, FLIM, FRET, Autofluorescence

Fluorescence lifetime imaging (FLIM) is a direct approach to energy transfer processes in biological samples. Because the lifetime of a fluorophore is inherently ratiometric, i.e. does not directly depend on the concentration, it is used to probe ion concentrations, pH, or the binding to different lipid, Protein, or DNA structures. Fluorescence resonance energy transfer (FRET) can be used to probe the molecular distance between donor and acceptor molecules. Moreover, the fluorescence lifetime helps to distinguish between different fluorophores even if they are present in the same pixel of the image. The fluorescence lifetimes of typical fluorophores used in cell imaging are of the order of a few nanoseconds. However, the lifetimes in presence of strong fluorescence quenching, the lifetime of the quenched donor fraction in FRET experiments [1], and the lifetimes of short autofluorescence components [2], dye aggregates [3] and metal-dye complexes can be as short as 50 ps. Due to the mixture of different fluorophores and non-uniform quenching the fluorescence decay functions found in cells and tissue are normally multi-exponential. Therefore, a lifetime imaging technique should be able to resolve the components of multi-exponential fluorescence decay functions down to less than 50 ps.

We present an improved time-correlated single photon counting (TCSPC) multi-wavelength FLIM technique for two-photon or confocal laser scanning microscopes. The technique builds up a four-dimensional distribution of the photon density versus the time within the fluorescence decay, the x-y coordinates of the scanning area, and the wavelength. It avoids any time-gating or wavelength scanning and therefore works with near-ideal efficiency. Due to the large number of time channels, multi-exponential decay functions can be resolved down to 30 ps. A single TCSPC imaging channel works up to a photon count rate of about  $5 \cdot 10^6 \text{ s}^{-1}$ . Although this is more than typically obtained from living cells in two-photon microscopes higher photon rates may be available from larger tissue samples or in diffuse optical tomography experiments. Our imaging system can therefore be extended to four fully parallel TCSPC channels. The four-channel system can be operated at a total count rate of  $20 \cdot 10^6 \text{ s}^{-1}$  and is able to obtain double exponential lifetime images within an acquisition time of a few seconds.

[1] B.J. Bacskai, J. Skoch, G.A. Hickey, R. Allen, B.T. Hyman. Fluorescence resonance energy transfer determinations using multiphoton fluorescence lifetime imaging microscopy to characterize amyloid-beta plaques. *J. Biomed. Opt.* **8**, 368-375, 2003

[2] K. König, I. Riemann, High resolution optical tomography of human skin with subcellular resolution and picosecond time resolution. *J. Biomed. Opt.* **8**, 432-439, 2003

[3] L. Kelbauskas, W. Dietel, Internalization of aggregated photosensitizers by tumor cells: Subcellular time-resolved fluorescence spectroscopy on derivatives of pyropheophorbide-a ethers and chlorin e6 under femtosecond one- and two-photon excitation. *Photochem. Photobiol.* **76**, 686-694, 2002