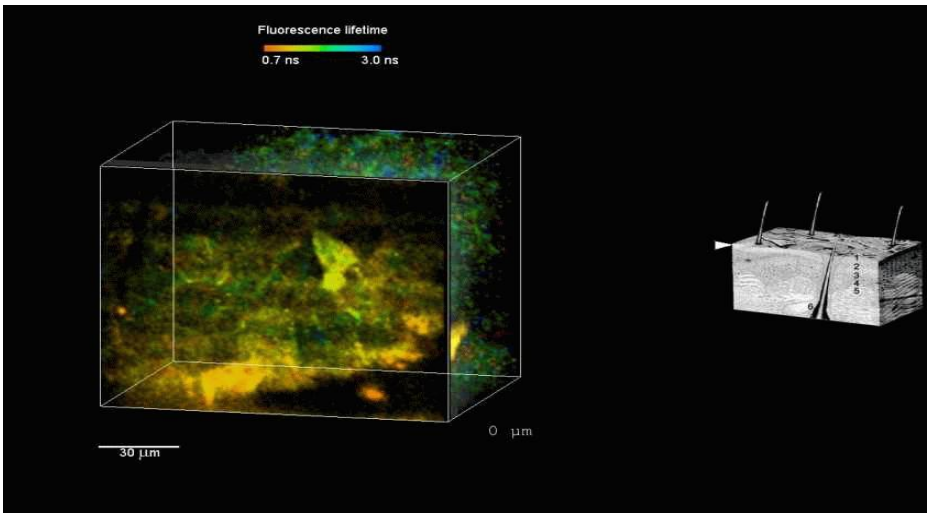
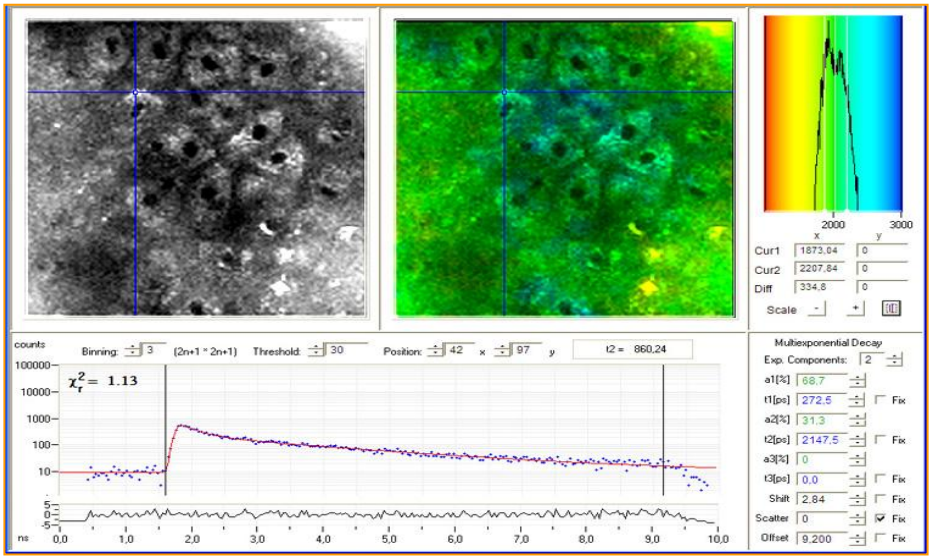
Fluorescence lifetime imaging or fluorescence lifetime microscopy

FLIM is defined as Fluorescence Lifetime Microscopy or Fluorescence Lifetime Imaging. FLIM images consist of spatially-resolved fluorescence lifetime τ data (τ=f(x,y)). Typical FLIM images are false-color coded where the colors reflect the τ values. FLIM images can be taken in the frequency mode (measurement of phase shift and demodulation) as well as in the time mode using streak cameras, time-gated cameras, and time-correlated single photon counting (TCSPC) units. Major FLIM applications include cell biology (e.g. FLIM-FRET to study protein-protein interactions), animal studies, artworks, microfluidics, forensic science, and clinical diagnostics. Clinical FLIM is currently applied in ophthalmology and dermatology based on TCSPC using picosecond laser diodes in the visible spectral range (one-photon excitation) and near infrared femtosecond Ti:sapphire lasers (two-photon excitation).



The colored FLIM cube depicts a stack of *in vivo* multiphoton FLIM sections from human skin with 250 ps temporal resolution and sub-micron spatial resolution. Red-blue: 0.7 ns-3 ns.



Two-photon FLIM image from *in vivo* human skin. The arrival times of fluorescence photons per pixel are depicted as fluorescence decay curve. Bi-exponential fitting provides amplitudes and lifetimes of two components. The fast, the slow, or the mean fluorescence lifetime can be added to the fluorescence intensity image (left) as false-color coded image (middle). The histogram shows the τ distribution from all 512x512 pixels.

History

FLIM in Life Sciences was introduced in Jena, East Germany, by König, Wabnitz, and Bugiel shortly before unification (1988, 1989). ZEISS Jena manufactured with the Friedrich Schiller University a first prototype of a confocal laser scanning microscope based on a mode-locked Ar^+ laser pumped tunable <10ps dye laser. The fluorescence was detected with the single photon counting unit SPC100 built by W. Becker. König et al. used this unique FLIM microscope to detect fluorescent porphyrin photosensitizers in living cells and in live tumor-bearing mice with sub-ns temporal resolution. Bi-exponential and global fitting provided τ data on intracellular and intratissue porphyrin monomers, dimers, and aggregates.

First FLIM images from humans were obtained 10 years later in 1998. The FLIM images from teeth and dental plaques of a volunteer were taken with a time-gated camera at the University of Applied Sciences in Aalen, Germany, by König and Schneckenburger.

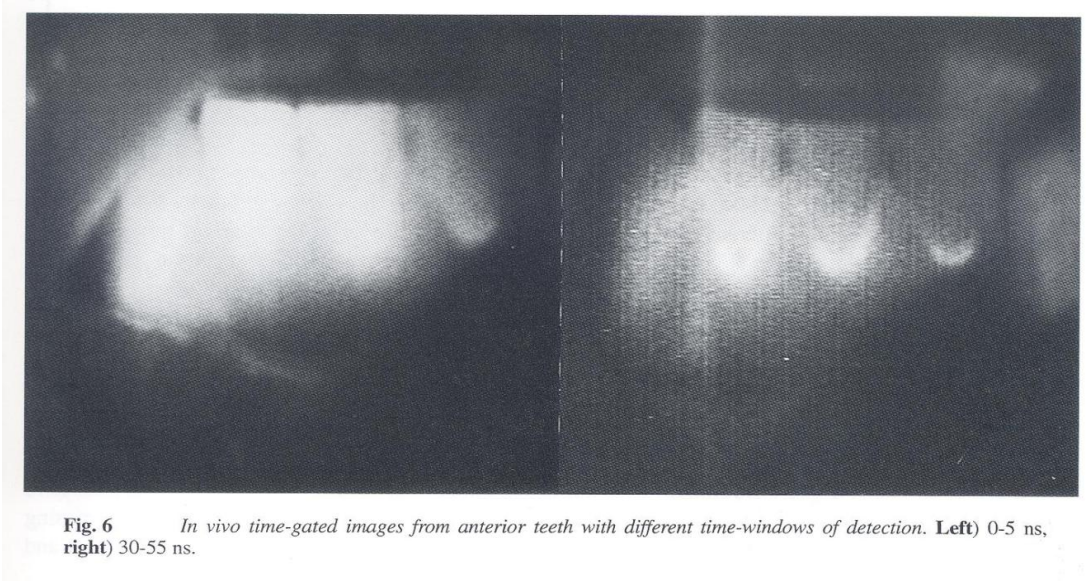


Fig. 6 *In vivo time-gated images from anterior teeth with different time-windows of detection.* **Left**) 0-5 ns, **right**) 30-55 ns.

Bacteria (*Actinomyces odontolyticus)* produce coproporphyrin and PP IX with long >10 ns fluorescence lifetimes. Therefore, time-windows with a long >10 ns delay compared to the excitation light exhibited the porphyrin autofluorescence in caries and dental plaque in this volunteer and not the short-lived tooth autofluorescence.

First two-photon FLIM data from mice and *in vivo* human skin (researchers arm) were taken by Masters et al. in 1999.

In 2000, the first clinical multiphoton tomograph was launched by the JenLab GmbH. König and coworkers used a tunable 80 MHz femtosecond Ti:sapphire laser as excitation source, galvoscanners, piezodriven NA1.3 focusing optics, and an tomograph-skin-interface. The autofluorescence and SHG was detected by photomultipliers in the analogue detection mode as well as by a second PMT with fast rise time in combination with TCSPC.

In 2004, first in vivo FLIM measurements in the human eye were taken by Schweitzer et al. using picosecond laser diodes in the VIS range to excite the ocular autofluorescence.

Clinical FLIM

Today's major clinical one-photon FLIM application is in the area of ophthalmology. *Heidelberg Engineering* produced the first FLIM prototypes for clinical use based on TCSPC. They are tested e.g. for the detection of macula degeneration. This pioneering FLIM work was performed by Schweitzer et al. in Jena, Germany.

Major applications of clinical two-photon FLIM include melanoma detection, diagnostics of dermatological disorders, cosmetic research, skin aging measurements, *in situ* drug monitoring, and tissue engineering. The most powerful advantage of clinical FLIM is the ability to provide label-free optical biopsies. FLIM can be used to differentiate between different fluorophores, to perform functional imaging, and to obtain information on the microenvironment, the binding behavior (e.g. NADPH-protein), and the modified metabolism in deceased areas. Meanwhile, high-resolution clinical multiphoton imaging of *in vivo* human skin have been performed on >2,000 patients/volunteers with certified multiphoton tomographs. Most of the clinical tomographs DermaInspect™, MPTflex™, and DermaInspect-CARS™ in Australia, Japan, and Europe are equipped with a TCSPC FLIM module.

References

Bugiel, I., König, K., Wabnitz, H., "Investigation of cells by fluorescence laser scanning microscopy with subnanosecond time resolution," Lasers Life Sciences 3, 1-7 (1989).

König, K. PhD thesis. 1989. Archive University Jena.

König, K. and Wabnitz, H., "Fluoreszenzuntersuchungen mit hoher zeitlicher, spektraler und räumlicher Auflösung," Labortechnik 23, 26-31 (1990).

Piston, D.W., Sandison, D.R., Webb, W.W., "Time-resolved fluorescence imaging and backgraound rejection by two-photon excitation in laser scanning microscopy," Proc. SPIE 1640, 379-389 (1992).

König, K., So, P.T.C., Mantulin, W.W., Tromberg, B.J., Gratton, E., "Two-photon excited lifetime imaging of autofluorescence in cells during UVA and NIR photostress," J Microsc. 183, 197-204 (1996).

So, P., König, K., Berland, K., Dong, C.Y., French, T., Buehler, C., Ragan, T., Gratton, E., "New time-resolved techniques in two-photon microscopy," Cell Mol Biol 44, 771-794 (1998).

König, K., Schneckenburger, H., and Hibst, R., "Time-gated in vivo imaging of dental caries," Cell. Mol. Biol. 45, 233-239 (1999).

Masters, B.R., So, P.T.C., and Gratton, E. "Multi-photon Excitation Microscopy and Confocal Microscopy of Human Skin In Vivo," Comments on Molecular and Cellular Biophysics 9, 379-405 (1999).

Becker, W., Bergmann, A., König, K., Tirlapur U., "Picosecond Fluorescence Lifetime Microscopy by TCSPC Imaging," Proc. SPIE 4262, 414-419 (2001).

Becker, W., Benndorf, K., Bergmann, A., Biskup, C., König, K., Tirlapur, U., Zimmer, T., "FRET measurements by TCSPC laser scanning microscopy," Proc. SPIE 4431, 249-245 (2001).

Schweitzer, D., Kolb, A., Hammer, M., "Autofluorescence lifetime measurement in images of the human ocular fundus,". Proc. SPIE 4432, 29-39 (2001).

König, K., Wollina, U., Riemann, I., Peuckert, C., Halbhuber, K.J., Fünfstück, V., Fischer, T.W., Elsner, P., "Optical tomography of human skin with subcellular spatial and picosecond time resolution," Proc. SPIE 4620, 191-201 (2002).

König, K. and Riemann, I., "High-resolution multiphoton tomography of human skin with subcellular spatial resolution and picosecond time resolution", J Biomed Opt 8, 432-439 (2003).

König, K., Riemann, I., Ehrlich, G., Ulrich, V., Fischer, P., "Multiphoton FLIM and Spectral Imaging of Cells and Tissues," Proc. SPIE 5323, 240-251 (2004).

Schweitzer, D., et al., "In vivo measurement of time resolved auto-fluorescence at the human ocular fundus," J Biomed Opt 9(6), 1214-1222 (2004).

Becker, W., "Advanced time-correlated single-photon counting techniques," Springer, Berlin, Heidelberg, New York, 2005

Ehlers, A., Riemann, I., Anhut, T., Kaatz, M., Elsner, P., König, K., "Fluorescence lifetime imaging of human skin and hair," Proc. SPIE 6089, 60890N (2006).

Festy, F., Ameer-Beg, S.M., Tony, N., Suhling, K., "Imaging proteins in vivo using fluorescence lifetime microscopy," Mol Bio Syst 3, 381-391 (2007).

Dimitrow, E., Riemann, I., Ehlers, A., Koehler, J.M., Norgauer, J., Elsner, P., König, K., Kaatz, M., "Spectral fluorescence lifetime detection and selective melanin imaging by multiphoton laser tomography for melanoma diagnosis," Exp. Dermatol. 18, 509-515 (2009).

König, K., Uchugonova, A., Gorjup E., "Multiphoton fluorescence lifetime imaging of 3D-stem cell spheroids during differentiation," Microsc Res Tech 74, 9-17 (2010).

Roberts, M. S., Dancik, Y., Prow, T. W., Thorling, C. A., Lin, L. L., Grice, J. E., Robertson, T. A., König, K., Becker, W., "Non-invasive imaging of skin physiology and percutaneous penetration using fluorescence spectral and lifetime imaging with multiphoton and confocal microscopy," Eur J Pharm Biopharm 77, 469-488 (2011).

Huck, V., Gorzelanny, C., Thomas, K., Niemeyer, V., Luger, T. A., König, K., Schneider, S. W., "Intravital multiphoton tomography as a novel tool for non-invasive in vivo analysis of human skin affected with atopic dermatitis," Proc. SPIE 7548, 75480B (2010).

Talbot, C.B., Patalay, R., Munro, I., Breunig, H.G., König, K., Alexandrov, Y., Warren, S., Chu, A., Stamp, G.W., Neil, M.A.A., French, P.M.W., Dunsby, C., "A multispectral FLIM tomograph for in-vivo imaging of skin cancer," Proc. SPIE 7903, 79032B (2011).

Patalay, R., Talbot, C., Munro, I., Breunig, H.G., König, K., Alexandrov, Y., Warren, S., Neil, M.A.A., French, P.M.W., Chu, A., Stamp, G.W., Dunsby, C., "Fluorescence lifetime imaging of skin cancer," Proc. SPIE 7883, 78830A.