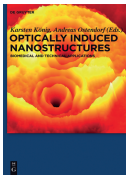
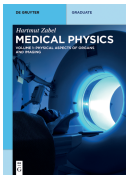
Karsten König (Ed.)
Multiphoton Microscopy and Fluorescence Lifetime Imaging

Also of Interest

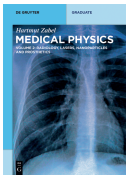


Optically Induced Nanostructures.
Biomedical and Technical Applications
Karsten König, Andreas Ostendorf, 2015
ISBN 978-3-11-033718-1, e-ISBN (PDF) 978-3-11-035432-4,
e-ISBN (EPUB) 978-3-11-038350-8

Biomedical Imaging.
Principles of Radiography, Tomography and Medical Physics
Tim Salditt, Timo Aspelmeier, Sebastian Aeffner, 2017
ISBN 978-3-11-042668-7, e-ISBN (PDF) 978-3-11-042669-4,
e-ISBN (EPUB) 978-3-11-042351-8



Medical Physics.
Volume 1: Physical Aspects of Organs and Imaging
Hartmut Zabel, 2017
ISBN 978-3-11-037281-6, e-ISBN (PDF) 978-3-11-037283-0,
e-ISBN (EPUB) 978-3-11-037285-4



Medical Physics.
Volume 2: Radiology, Lasers, Nanoparticles and Prosthetics
Hartmut Zabel, 2017
ISBN 978-3-11-055310-9, e-ISBN (PDF) 978-3-11-055311-6,
e-ISBN (EPUB) 978-3-11-055317-8
Volume 1 & Volume 2: also available as a set.
Set-ISBN 978-3-11-055957-6

Advances in Molecular Biophotonics
Yong Deng, Zhenli Huang, Yu Li, Da Xing, Zhihong Zhang, 2017
ISBN 978-3-11-030438-1, e-ISBN (PDF) 978-3-11-030459-6,
e-ISBN (EPUB) 978-3-11-038803-9

Multiphoton Microscopy and Fluorescence Lifetime Imaging

Applications in Biology and Medicine

Edited by
Karsten König

DE GRUYTER
UNIVERSITÄT DES SAARLANDES
JenLab

Editor
Prof. Dr. Karsten König
Saarland University
Department of Biophotonics and Laser Technology
66123 Saarbruecken
Germany
k.koenig@blt.uni-saarland.de

ISBN 978-3-11-043898-7
e-ISBN (PDF) 978-3-11-042998-5
e-ISBN (EPUB) 978-3-11-043007-3

Library of Congress Cataloging-in-Publication Data
A CIP catalog record for this book has been applied for at the Library of Congress.

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at http://dnb.dnb.de.

Cover image: Karsten König
Typesetting: PTP-Berlin, Protago-TEX-Production GmbH, Berlin
Printing and binding: CPI books GmbH, Leck
♾ Printed on acid-free paper
Printed in Germany

www.degruyter.com

Dedicated to Professor Wolfgang Kaiser
Pioneer of Laser Technology and Nonlinear Optics

Preface

The observation of two-photon excited fluorescence by Kaiser and Garrett and of second-harmonic generation (SHG) by Franken et al. in 1961 marked the birth of Nonlinear Optics (NLO). They used a ruby laser emitting in the red spectral region at 694.3 nm. This novel light source was invented just some months before.

The NLO theory of two-photon absorption was provided by the PhD student and later Nobel Prize winner named Maria Göppert in Göttingen in 1929. However, at that "pre-laser" time, there was no intense light source to prove her hypothesis of two-quantum transitions.

In 1961, three decades later, Wolfgang Kaiser called up Maria Goeppert-Mayer, who had married the chemist Joseph Edward Mayer and worked as professor in San Diego. He told that her theory of two-photon absorption was finally confirmed. In order to honor her outstanding PhD thesis, the unit of two-photon absorption cross sections was termed "GM".

When combined with an optical microscope, the laser beam can be confined to an intense sub-wavelength light spot. According to Abbe's diffraction law, the spot diameter can be as small as 200 nanometers when using visible light.

In 1962, intense ruby laser spots were used to generate a nonlinear induced micro-emission from materials. Laser-induced breakdown spectroscopy (LIBS) was born. Later on, Berns et al. used the laser microscope to perform intracellular laser surgery and Ashkin employed the laser microscope non-destructively to create optical traps and laser tweezers.

The pixel-by-pixel exposure across a sample with a focused laser spot by stage scanning or beam scanning in order to induce signals such as fluorescence led to the invention of laser scanning microscopy. Confocal laser scanning microscopes (CLSM) provide optical sections for three-dimensional (3D) imaging. Typically, non-pulsed (cw) visible laser beams are used.

The integration of ultrafast picosecond and femtosecond lasers into scanning microscopes was a milestone in optical microscopy. It allowed fluorescence lifetime imaging (FLIM) by time-correlated single-photon counting (TCSPC) and efficient non-linear excitation within the focal spot, in particular two-photon microscopy.

The first integration of an ultrafast laser with a confocal laser scanning microscope happened to my knowledge in the late 1980s in Jena in East Germany. The microscope manufacturer VEB Carl Zeiss Jena developed together with our Department of Physics at the Friedrich Schiller University a prototype of a picosecond laser scanning microscope for 4D microscopy with high spatial and temporal resolution based on a mode-locked argon ion laser and a TCSPC module. Using that unique prototype, we performed the first FLIM laser scanning microscopy in Life Sciences in 1988 on living cancer cells.

https://doi.org/10.1515/9783110429985-001

FLIM microscopy opened the possibility to introduce a further image contrast modality, to probe the microenvironment of the fluorophore, and to detect protein-protein interactions with high spatial resolution.

However, working in the NLO group under the guidance of the ultrashort laser pulse expert Bernd Wilhelmi, we missed the chance to detect two-photon excited fluorescence with this very first ultrafast laser scanning microscope.

One year later in 1989, Denk, Strickler, and Webb from Cornell University used a sub-picosecond dye laser microscope to perform two-photon imaging and two-photon photochemistry. Cornell University filed a patent on November 14, 1989 on two-photon laser microscopy. Licensed to the UK company Bio-Rad, this patent started a major legal fight between the major microscope producers. In 2004, ZEISS acquired the Cell Science Business of Bio-Rad to get access to the worldwide exclusive rights of multiphoton microscopy.

Femtosecond near-infrared (NIR) laser two-photon microscopes became the major tool for live cell imaging. Not only intracellular exogenous fluorophores and fluorescent proteins in transfected cells have been visualized over longer periods of time noninvasively. Also a variety of endogenous fluorophores has been imaged. Piston and König et al. introduced two-photon autofluorescence FLIM on live cells.

Two-photon optical sections were performed on tissue biopsies, and later mounted in the skull through a glass window within the brain of live transgenic mice. Two-photon fluorescence microscopes thus became now unique 3D imaging systems to study intratissue neurons in situ.

In 1998, Masters et al. used a home-built two-photon microscope to image the skin of the author's arm noninvasively and label-free. They were able to monitor the complete skin architecture of the epidermis and upper dermis.

Finally in 2003, König and coworkers introduced the first certified in vivo multiphoton tomograph for clinical imaging based on two-photon excited autofluorescence, SHG, and FLIM. Meanwhile the skin of thousands of volunteers and patients has been examined with these multiphoton tomographs to detect skin cancer and skin aging signs, to trace cosmetic and pharmaceutical compounds, and even to measure skin alterations of astronauts after long-term space trips.

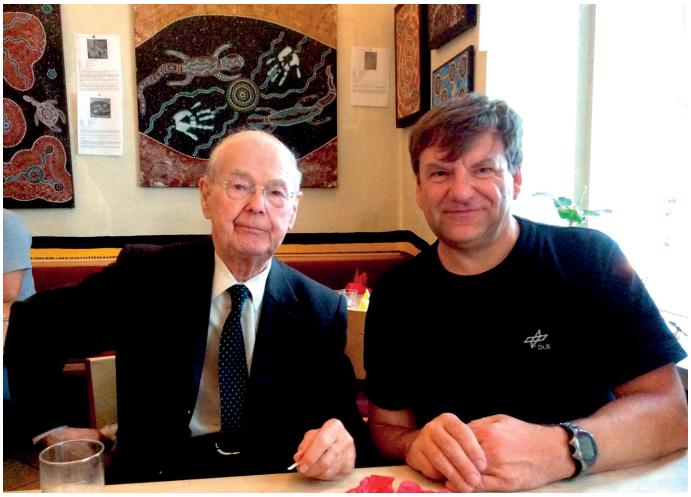
Instead of using a single femtosecond laser beam, Stefan Hell added a second optically shaped laser beam to come up with the Nobel Prize winning STED microscopy. STED is the abbreviation for *stimulated emission depletion microscopy*, which opened the door for super-resolution microscopy far beyond Abbe's diffraction limit, and is also called optical nanoscopy.

Two ultrafast laser beams were also employed to realize 3D microscopy with chemical fingerprints based on *Coherent Anti-Stokes Raman Spectroscopy* (CARS). Meanwhile, CARS tomographs are in clinical use in hospitals in Germany, USA, and China.

Besides imaging, nonlinear microscopes became novel micromachining tools in material production and refractive eye surgery. Within the last ten years, femtosecond laser nanoscopes have "turned" micromachining into nanomachining. When using one-beam NIR nanoscopes, only the central part of the intense laser spot is employed to create sub-100 nm structures based on two-photon lithography, multiphoton ionization, and plasma formation. In bulk 3D nanoprocessing became feasible. When using two-beam nanoscopes, the 3D nanostructures with a feature size down to 9 nm have been produced with nonlinear lithography where one beam is used for photoinduction and the other one for photoinhibition.

Undoubtedly, nonlinear microscopy/nanoscopy and multiphoton tomography have revolutionized the imaging of live cells and tissues. It all started with the pioneering work of Kaiser and Garrett who demonstrated the two-photon fluorescence for the first time. It is a great pleasure to dedicate this book to Professor Wolfgang Kaiser on his 92nd birthday, which is on July 17, 2017.

Berlin, July 2017
Karsten König
Department of Biophotonics and Laser Technology, Saarland University, Germany



Wolfgang Kaiser with Karsten König at the *10th International Workshop and Conference on Advanced Multiphoton and Fluorescence Lifetime Imaging Techniques FLIM2015* on June 17, 2015.

Foreword

The publication of this book is timely, because it is likely that multiphoton imaging is about to make a serious impact in clinical diagnosis, after more than two decades of successful applications in research in the life sciences. And who better to edit the book than Karsten König, who has been active in the field almost since its experimental demonstration?

Two-photon absorption was first proposed by Maria Göppert in 1929. It is interesting to recall that Paul Dirac spent three months in Göttingen in early 1927, visiting Max Born, Göppert's supervisor. In this same period he submitted two papers, 'The quantum theory of dispersion', and 'The quantum theory of emission and absorption of radiation', which introduce the quantum theory of creation and annihilation of photons. The simultaneous absorption of multiple photons was predicted by his theory, but he rejected this result as 'These terms correspond to processes in which two light-quanta are emitted or absorbed simultaneously, and cannot rise in a light-quantum theory in which there are no forces between the light quanta. The effects of these terms will be found to be negligible, so that the disagreement with the light-quantum theory is not serious.' Later, Dirac made reference to multiphoton absorption in his book *The Principles of Quantum Mechanics*.

Various different nonlinear processes were observed experimentally soon after the invention of the laser. In a paper submitted in 1976, we (Sheppard and Kompfner: Resonant optical scanning microscope. Appl Opt. 1978;17:2879–2882) proposed that the high field strength in a tightly focused laser beam could be used to excite nonlinear effects and produce images by scanning of the laser spot. Harmonic generation, two-photon fluorescence, and coherent Raman scattering were processes that were specifically mentioned. Second-harmonic images were presented at a conference in 1977 (Sheppard et al. The second harmonic generation (SHG) microscope. IEEE J Quant Electron. 1977;QE13:100D, post-deadline). Although the advantage of using short pulses was mentioned in the paper, the harmonic images were, in fact, produced using a continuous wave laser. We had also experimented on using picosecond pulses from a mode-locked argon ion laser. We thought that we had observed a second-harmonic signal, but further investigation showed it was fluorescence in the infrared. The patent granted to Denk, Strickler, and Webb (US5034613) was for a two-photon fluorescence microscope, specifically one using pulses with a pulse length shorter than one picosecond. Interestingly, a patent was also granted to Hänninen and Hell (WO1995030166) for a microscope system using pulses longer than one picosecond. These patents have now expired, and several companies manufacture multiphoton microscopes.

https://doi.org/10.1515/9783110429985-002

The scope of the present book includes multiphoton manipulation and material processing, and also fluorescence lifetime imaging. These associated methods are also very useful in biological and medical applications. The book satisfies a need for an up-to-date treatment of all these related techniques.

Wollongong, NSW, Australia, August 2017
Colin Sheppard

Contents

Preface —— vii

Foreword —— xi

List of contributing authors —— xxiii

Part I: **Basics**

Karsten König

1 Brief history of fluorescence lifetime imaging —— 3

1.1 Introduction —— **3**

1.2 Time-resolved spectroscopy and first time-resolved fluorescence microscopes —— **4**

1.3 First FLIM laser scanning microscope —— **4**

1.4 Two-photon FLIM microscopy —— **7**

1.5 First wide-field FLIM in humans —— **7**

1.6 Clinical FLIM tomography —— **8**

1.7 One-photon FLIM in ophthalmology —— **10**

1.8 Endoscopic FLIM —— **11**

1.9 FLIM-FRET —— **11**

1.10 Conclusion —— **13**

Wolfgang Kaiser

2 The long journey to the laser and its use for nonlinear optics —— 17

Wolfgang Becker

3 Advanced TCSPC-FLIM techniques —— 23

3.1 The fluorescence decay function as an indicator of molecular parameters —— **23**

3.1.1 Ion concentrations —— **24**

3.1.2 pH sensors —— **25**

3.1.3 Binding to proteins, protein configuration —— **25**

3.1.4 Förster Resonance Energy Transfer: FRET —— **25**

3.1.5 Endogenous fluorophores, effect of metabolic activity —— **26**

3.1.6 Oxygen —— **26**

3.1.7 Redox potential —— **27**

3.1.8 Electron transfer —— **27**

3.1.9 Other parameters influencing fluorescence lifetime —— **27**

3.1.10 Requirements for a FLIM technique in biology —— **27**

3.2 TCSPC-FLIM with laser scanning systems — **28**
3.2.1 The advantage of scanning — **28**
3.2.2 FLIM by multidimensional TCSPC — **29**
3.3 Combination with different optical scanning techniques — **33**
3.3.1 One-photon excitation confocal FLIM — **33**
3.3.2 Multiphoton FLIM — **33**
3.3.3 FLIM with excitation wavelength multiplexing — **34**
3.3.4 Near-infrared FLIM — **35**
3.3.5 STED-FLIM — **36**
3.3.6 Multiphoton tomography of human skin — **37**
3.3.7 Ophthalmic FLIM — **37**
3.3.8 TCSPC-FLIM with other scanning techniques — **38**
3.4 Recent advances in TCSPC-FLIM — **39**
3.4.1 Megapixel FLIM — **39**
3.4.2 Multiwavelength FLIM — **40**
3.4.3 *X*-*Y* mosaic FLIM — **41**
3.4.4 *Z*-stack FLIM by mosaic recording — **42**
3.4.5 Temporal mosaic FLIM — **43**
3.4.6 Simultaneous fluorescence and phosphorescence lifetime imaging — **45**
3.5 Summary — **47**

Arnd Krueger
4 Ultrafast lasers in biophotonics — 53
4.1 Introduction — **53**
4.2 Fundamentals of ultrafast pulse generation and propagation — **54**
4.2.1 Modelocking — **54**
4.2.2 The effect of dispersion on ultrafast pulses — **60**
4.3 Types of ultrafast lasers used in biophotonics — **64**
4.3.1 Femtosecond Ti:sapphire lasers — **64**
4.3.2 Other ultrafast diode-pumped solid state lasers — **67**
4.3.3 Ultrafast fiber lasers — **67**
4.3.4 Ultrafast optical parametric oscillators — **68**
4.4 Applications of ultrafast lasers in biophotonics — **70**
4.4.1 Overview — **70**
4.4.2 Femtosecond lasers in vision correction — **70**
4.4.3 Ultrafast lasers in microscopy — **72**
4.5 Summary and outlook — **77**

Part II: **Modern nonlinear microscopy of live cells**

Luca Lanzano, Giuseppe Vicidomini, Lorenzo Scipioni, Marco Castello, and Alberto Diaspro

5 STED microscopy: exploring fluorescence lifetime gradients for super-resolution at reduced illumination intensities — 85

5.1 Introduction — **85**
5.2 Gated- and SPLIT-STED theory — **88**
5.2.1 Temporal point spread function — **88**
5.2.2 Gated-STED microscopy — **90**
5.2.3 SPLIT-STED microscopy — **91**
5.3 Gated- and SPLIT-STED comparison — **96**
5.4 Discussion and conclusions — **98**

Peter T. C. So, Heejin Choi, Elijah Yew, and Christopher Rowlands

6 Principles and applications of temporal-focusing wide-field two-photon microscopy — 103

6.1 Introduction — **103**
6.2 Invention of temporal focusing two-photon microscopy and basic operating principle — **105**
6.3 Image formation theory for temporal focusing microscopes — **107**
6.4 Remedying the poor axial resolution of temporal focusing 2P microscopy — **112**
6.5 Characterizing performance of temporal focusing 2P microscope for deep tissue imaging — **118**
6.6 Application 1: Functional imaging of neuronal network using TFM — **121**
6.7 Application 2: Fluorescence and phosphorescence lifetime imaging using TFM — **125**
6.8 Application 3: Cell-selective optogenetics using TFM — **130**
6.9 Application 4: Cell selective photodynamic therapy using TPM — **133**
6.10 Conclusion — **135**

Shagufta Rehman Alam, Meghan J. O. Melia, Horst Wallrabe, Zdenek Svindrych, Dhyan Chandra, Suchitra Joshi, Jaideep Kapur, and Ammasi Periasamy

7 FLIM-FRET microscopy — 141

7.1 Introduction — **141**
7.1.1 Tissue autofluorescence as a biomarker — **141**
7.1.2 Metabolic coenzymes in cellular metabolism — **142**
7.1.3 Tryptophan — **145**
7.2 Examples of some applications of NAD(P)H and tryptophan lifetime and intensity imaging to diseases — **146**
7.2.1 Prostate cancer and treatment — **147**

7.2.2 Doxorubicin —— **148**
7.3 Multiphoton NAD(P)H and Trp FLIM-FRET microscopy in prostate cancer —— **148**
7.3.1 Cell culture —— **148**
7.3.2 Basics of FRET —— **149**
7.3.3 Multiphoton FLIM-FRET microscopy —— **150**
7.3.4 Image processing and analysis —— **152**
7.4 Research applications —— **153**
7.4.1 Effect of glucose stimulation on metabolic activity in normal and prostate cancer cells —— **153**
7.4.2 Effect of doxorubicin treatment on metabolic activity in prostate cancer cells —— **155**
7.5 Multiphoton NAD(P)H and Trp FLIM-FRET microscopy of hippocampal tissue in an in vitro KCl induced seizure model —— **156**
7.5.1 FLIM-imaging of hippocampal tissue in an in vitro KCl induced seizure model —— **156**
7.5.2 Effect of KCl treatment on metabolic activity in mouse hippocampal slices —— **157**
7.6 Conclusion —— **158**

Angelika Rück, Jasmin Breymayer, and Sviatlana Kalinina
8 TCSPC FLIM and PLIM for metabolic imaging and oxygen sensing —— 163
8.1 Cellular energy metabolism in different environments —— **163**
8.2 Cellular energy metabolism and FLIM of autofluorescent coenzymes —— **165**
8.3 FLIM of NADH and NAD(P)H —— **168**
8.4 PLIM of oxygen sensors —— **169**
8.5 TCSPC FLIM and PLIM —— **170**

Karsten König
9 Laser tweezers are sources of two-photon effects —— 177
9.1 Introduction —— **177**
9.2 Experimental setup —— **180**
9.3 Materials and methods —— **182**
9.4 Determining the trapping force —— **182**
9.5 Determining the motility force —— **182**
9.6 Fluorescence imaging of trapped spermatozoa —— **183**
9.7 Trap-induced two-photon fluorescence —— **184**
9.8 Trap-induced nonlinear phototoxic effects —— **184**
9.9 Photodamage effects as a result of mode-beating phenomena —— **185**
9.10 Conclusion —— **186**

Marina Shirmanova, Tatiana Sergeeva, Irina Druzhkova, Aleksandra Meleshina, Maria Lukina, Varvara Dudenkova, Vladislav Shcheslavskiy, Wolfgang Becker, Vsevolod Belousov, Nataliya Mishina, and Elena Zagaynova

10 Metabolic shifts in cell proliferation and differentiation — 189

10.1 Introduction — **189**

10.2 Materials and methods — **190**

10.2.1 Cancer cells and tumor model — **190**

10.2.2 Stem cells and adipogenic differentiation — **191**

10.2.3 Two-photon fluorescence microscopy and FLIM — **191**

10.3 Metabolic shifts in cancer — **192**

10.3.1 Metabolic interaction of cancer cells and fibroblasts — **192**

10.3.2 Metabolic heterogeneity of tumors — **197**

10.4 Metabolic shifts in stem cells — **199**

10.5 Conclusions — **203**

Karsten König

11 Femtosecond laser nanoprocessing — 209

11.1 Laser microscopes for material processing and analysis — **209**

11.2 The sub-20 femtosecond laser scanning microscope for nanoprocessing and two photon imaging — **215**

11.3 Two-photon lithography with broadband pulses — **216**

11.4 Nanowire production by laser-assisted etching — **218**

11.5 Optical cleaning — **218**

11.6 Targeted transfection — **218**

11.7 Optical reprogramming — **219**

11.8 Outlook — **221**

Hans Georg Breunig and Karsten König

12 Cryomultiphoton imaging — 227

12.1 Introduction — **227**

12.2 Materials and methods — **228**

12.2.1 Heating and cooling stage — **228**

12.2.2 Multiphoton imaging — **230**

12.2.3 Imaging systems — **230**

12.2.4 Sample preparation — **232**

12.3 Results and discussion — **233**

12.3.1 Cell monolayer imaging — **233**

12.3.2 In situ imaging of plants — **236**

12.4 Conclusion — **240**

Part III: **Nonlinear tissue imaging**

Karsten König
13 Multiphoton Tomography (MPT) —— 247
13.1 Introduction —— **247**
13.2 Principle of Multiphoton Tomography (MPT) —— **250**
13.3 Multiphoton tomographs —— **253**
13.4 In vivo histology based on MPT —— **254**
13.5 Optical Metabolic Imaging (OMI) based on two-photon FLIM —— **256**
13.6 Multimodal imaging (AF, SHG, FLIM, CARS) —— **258**
13.7 Skin cancer detection with MPT —— **258**
13.8 The skin ageing index SAAID —— **260**
13.9 MPT in space medicine —— **261**
13.10 Multiphoton tomography of in vivo human brain —— **261**
13.11 Two-photon imaging of cornea transplants —— **262**
13.12 Watching stem cells at work in transgenic mice —— **262**
13.13 Conclusion —— **263**

Martin Weinigel, Hans Georg Breunig, and Karsten König
14 Clinical multimodal CARS imaging —— 269
14.1 Introduction —— **269**
14.1.1 CARS —— **269**
14.1.2 CARS microscopy —— **271**
14.2 Setup of CARS tomographs —— **272**
14.2.1 Requirements for CARS imaging —— **274**
14.2.2 First clinical CARS tomographs —— **274**
14.2.3 The flexible multimodal CARS tomograph —— **276**
14.2.4 Multichannel-detection —— **278**
14.3 Results and Discussion —— **279**
14.3.1 Demonstration of the spatio-temporal overlap —— **279**
14.3.2 Ex vivo imaging —— **281**
14.3.3 In vivo human skin imaging —— **282**
14.3.4 Chemical contrast —— **283**
14.4 Conclusion —— **283**

Mihaela Balu, Kristen M. Kelly, Ronald M. Harris, Karsten König, Christopher B. Zachary, and Bruce J. Tromberg
15 In vivo multiphoton microscopy of human skin —— 287
15.1 Introduction —— **287**
15.2 MPM technology and translation into the clinic —— **288**
15.3 Applications of MPM-based clinical tomographs in dermatology —— **289**
15.3.1 In vivo MPM imaging of normal skin —— **289**

15.3.2 In vivo MPM imaging can determine the depth dependent sensitivity of human epidermis to vascular oxygen supply — **290**
15.3.3 In vivo MPM imaging of melanoma — **294**
15.3.4 In vivo MPM imaging of basal cell carcinoma (BCC) — **294**
15.3.5 Other applications of MPM in dermatology — **296**
15.4 Discussion — **296**

Ana Batista, Hans Georg Breunig, Christoph Donitzky, and Karsten König
16 Two-photon microscopy and fluorescence lifetime imaging of the cornea — 301
16.1 Cornea anatomy, histology, and physiology — **301**
16.1.1 Epithelium — **302**
16.1.2 Bowman's layer — **302**
16.1.3 Stroma — **303**
16.1.4 Descemet's membrane — **303**
16.1.5 Endothelium — **303**
16.1.6 Corneal nourishment and cells metabolism — **304**
16.2 Current clinical corneal imaging methodologies — **304**
16.3 Two-photon corneal imaging — **305**
16.3.1 Autofluorescence intensity imaging — **306**
16.3.2 Autofluorescence lifetime imaging — **307**
16.3.3 Second-harmonic generation imaging (SHG) — **308**
16.3.4 Two-photon instrumentation — **309**
16.4 Two-photon imaging of the human cornea — **310**
16.4.1 Viability for corneal transplantation — **313**
16.4.2 Corneal pathologies evaluation — **314**
16.5 Conclusions and outlook — **316**

Anna Letizia Allegra Mascaro, Ludovico Silvestri, Leonardo Sacconi, and Francesco S. Pavone
17 Multiscale correlative imaging of the brain — 321
17.1 Introduction — **321**
17.2 Brain anatomy — **322**
17.2.1 Serial two-photon sectioning — **322**
17.2.2 Micro-optical sectioning tomography — **323**
17.2.3 Light sheet microscopy — **323**
17.3 Structural plasticity of cortical neurons: from an historical perspective to recent advances in fluorescence imaging in vivo — **325**
17.4 Functional imaging and stimulation of neural circuits — **329**
17.4.1 Optical recording of neuronal activity — **329**
17.4.2 Optical stimulation of neurons — **330**
17.5 Correlative microscopy — **333**

17.5.1 Understanding brain machinery requires multilevel investigation —— **333**
17.5.2 Correlative imaging overcomes the limitation of single techniques —— **335**
17.5.3 Fusing multiple levels of investigation might boost our understanding of the brain —— **337**

Amy Holmes, Camilla Thorling, Xin Liu, Xiaowen Liang, Haolu Wang, Hans G. Breunig, Karsten König, Hauke Studier, and Michael S. Roberts
18 Revealing interaction of dyes and nanomaterials by multiphoton imaging —— 345
18.1 Introduction —— **345**
18.2 Multiphoton imaging of nanomaterials within tissue —— **347**
18.3 Monitoring zinc oxide nanoparticle (ZnO NPs) penetration into human skin —— **349**
18.4 Monitoring silver nanoparticles (Ag NPs) penetration into human skin —— **355**
18.5 Detection of dyes within the liver of rodent models —— **357**
18.6 Detection of nanoparticles within the liver of rodent models —— **361**

Ana-Maria Pena, Etienne Decencière, Sébastien Brizion, Steeve Victorin, Serge Koudoro, Thérèse Baldeweck, and Emmanuelle Tancrède-Bohin
19 Multiphoton FLIM in cosmetic clinical research —— 369
19.1 Multiphoton fluorescence lifetime imaging of *in vivo* human skin —— **369**
19.2 Clinical multiphoton FLIM systems —— **373**
19.3 Quantitative data afforded by multiphoton imaging of human skin *in vivo* —— **376**
19.3.1 Automatic 3D segmentation of skin layers —— **376**
19.3.2 Pseudo-FLIM specific melanin detection —— **377**
19.3.3 Quantitative parameters —— **380**
19.4 Cosmetic applications —— **381**
19.4.1 Photo-aging —— **381**
19.4.2 Study of constitutive pigmentation —— **384**
19.4.3 Efficacy assessment of anti-aging or whitening cosmetic ingredients —— **386**
19.5 Conclusion —— **388**

Sven R. Kantelhardt
20 Multiphoton microscopy and fluorescence lifetime imaging for resection guidance in malignant glioma surgery —— 395
20.1 Some facts about malignant glioma —— **396**

20.2 Standard therapy and prognosis — **397**
20.3 Limitations for surgical resection — **398**
20.4 Fluorescence imaging for resection guidance in glioma surgery — **399**
20.5 Multiphoton intensity imaging of native (murine) brain tissue — **399**
20.6 Multiphoton fluorescence lifetime (FLIM) imaging of native (murine) brain tissue — **401**
20.7 Multiphoton microscopy of human cell line derived GBM tissue in an orthotopic mouse glioma model — **402**
20.8 Multiphoton microscopy of 5-ALA-stained experimental gliomas — **404**
20.9 Multiphoton microscopy of human glioma tissue ex vivo — **404**
20.10 Multiphoton microscopy of human glioma tissue in vivo and outlook — **406**

Aisada Uchugonova and Robert M. Hoffman
21 Non-invasive single-photon and multi-photon imaging of stem cells and cancer cells in mouse models — 411
21.1 Fluorescent proteins and non-invasive single-photon imaging in live mice — **411**
21.2 Advantages of multiphoton imaging of stem cells and cancer cells in live mice — **415**
21.2.1 Real-time imaging of stem cells and their dynamics with subcellular resolution — **416**
21.2.2 Multiphoton surgery of stem cells — **418**
21.2.3 Imaging of GFP-labeled and unlabeled stem cells — **419**
21.2.4 High-resolution non-invasive multi-photon tomographic cancer-cell imaging in living animals — **419**
21.2.5 Multi-photon imaging of tumor-targeting by *Salmonella typhimurium* A1-R — **419**
21.2.6 Prospects and limitations of multiphoton tomography — **421**

Christian Mess and Volker Huck
22 Bedside assessment of multiphoton tomography — 425
22.1 Multiphoton gleaming – endogenous fluorophores in human skin — **425**
22.2 From morphology to biochemical state – pathophysiological characterization of human skin — **426**
22.3 Hands-on clinical multiphoton tomography — **427**
22.4 The building blocks of intravital multiphoton tomographs — **428**
22.5 Alignment of intravital multiphoton tomographic data with classical skin analysis — **429**
22.6 Morphological analysis of the cellular mitochondrial distribution — **432**

22.7 Fluorescence lifetime imaging – foundation, calculation, clinical application — **435**
22.8 MPT-FLIM provides evidence of disease-related alteration of cellular metabolism — **437**
22.9 Outlook — **439**

Index — 445

List of contributing authors

Thérèse Baldeweck
L'Oréal Research and Innovation
1 avenue Eugène Schueller
93601 Aulnay-sous-Bois
France
tbaldeweck@rd.loreal.com

Mihaela Balu
Laser Microbeam and Medical Program
Beckman Laser Institute
University of California
Irvine, CA, 92612
USA
mbalu@uci.edu

Ana Batista
JenLab GmbH
Schillerstr. 1
07745 Jena
Germany
batista@jenlab.de

Wolfgang Becker
Becker & Hickl GmbH
Nahmitzer Damm 30
12277 Berlin
Germany
becker@becker-hickl.com

Vsevolod V. Belousov
Shemyakin-Ovchinnikov
Institute of Bioorganic Chemistry RAS
16/10 Miklukho-Maklaya St.
Moscow 117997
Russia
vsevolod.belousov@gmail.com

Hans Georg Breunig
JenLab GmbH
Schillerstrasse 1
07745 Jena
Germany
breunig@jenlab.de

Jasmin Breymayer
Ulm University
Core Facility Confocal and Multiphoton
Microscopy
Albert-Einstein-Allee 11
89081 Ulm
Germany
jasmin.breymayer@uni-ulm.de

Sébastien Brizion
L'Oréal Research and Innovation
1 avenue Eugène Schueller
93601 Aulnay-sous-Bois
France
sbrizion@rd.loreal.com

Marco Castello
Molecular Microscopy and Spectroscopy,
Nanophysics, Istituto Italiano di Tecnologia
via Morego 30
16163, Genoa
Italy
Marco.Castello@iit.it

Dhyan Chandra
Department of Pharmacology and Therapeutics
BLSC-Centre for Genetics and Pharmacology
Roswell Park Cancer Institute
Buffalo, NY 14263
USA
dhyan.chandra@roswellpark.org

Heejin Choi
Department of Mechanical and Biological
Engineering
Massachusetts Institute of Technology
Cambridge, MA 02139
USA
choihj@mit.edu

Etienne Decencière
MINES ParisTech, PSL Research University
CMM - Centre for Mathematical Morphology
Fontainebleau
France
Etienne.Decenciere@ensmp.fr

Alberto Diaspro
Nanoscopy, Nanophysics, Istituto Italiano
di Tecnologia
via Morego 30
16163, Genoa
Italy
alberto.diaspro@iit.it

Department of Physics
University of Genoa
via Dodecaneso 33
16146 Genoa
Italy

Nikon Imaging Center
Istituto Italiano die Tecnologia
via Morego 30
16163 Genoa
Italy

Christoph Donitzky
JenLab GmbH
Schillerstr. 1
07745 Jena
Germany
donitzky@jenlab.de

Varvara V. Dudenkova
Institute of Biomedical Technologies
Nizhny Novgorod State Medical Academy
Minin and Pozharsky Square 10/1
Nizhny Novgorod, 603005
Russia
orannge@mail.ru

Institute of Biology and Biomedicine
Nizhny Novgorod State University
Gagarin Avenue 23
Nizhny Novgorod, 603950
Russia

Irina N. Druzhkova
Institute of Biomedical Technologies
Nizhny Novgorod State Medical Academy
Minin and Pozharsky Square 10/1
Nizhny Novgorod, 603005
Russia
danirin@yandex.ru

Ronald M. Harris
Department of Dermatology
University of California
Irvine, CA, 92697
USA
rmharris@uci.edu

Robert M. Hoffman
AntiCancer Inc.
7917 Ostrow St
San Diego, CA 92111
USA
all@anticancer.com

University of California San Diego
Department of Surgery
California
USA

Amy Holmes
School of Pharmacy and Medical Sciences
The University of South Australia
5001 Adelaide
Australia
Amy.Holmes@unisa.edu.au

Volker Huck
University Medical Center Hamburg-Eppendorf
Center for Internal Medicine
SWG Multiphoton Tomography
Martinistr. 52
20246 Hamburg
Germany
v.huck@uke.de

Heidelberg University
Medical Faculty Mannheim
Experimental Dermatology
Theodor-Kutzer-Ufer 1–3
68167 Mannheim

Suchitra Joshi
Department of Neurology
University of Virginia
Charlottesville, VA 22908
USA
sj9z@virginia.edu

Wolfgang Kaiser
Technische Universität München
Fakultät für Physik
James-Franck-Str. 1
85748 Garching
Germany
wkaiser@tum.de

Sviatlana Kalinina
Ulm University
Core Facility Confocal and Multiphoton Microscopy
Albert-Einstein-Allee 11
89081 Ulm
Germany
Sviatlana.Kalinina@uni-ulm.de

Sven R. Kantelhardt
Dept. of Neurosurgery
Johannes-Gutenberg-University Mainz
55101 Mainz
Germany
sven.kantelhardt@unimedizin-mainz.de

Jaideep Kapur
Department of Neurology
University of Virginia
Charlottesville, VA 22908
USA
jk8t@virginia.edu

Kristen M. Kelly
Department of Dermatology
University of California
Irvine, CA, 92697
USA
kmkelly@uci.edu

Karsten König
Saarland University
Department of Biophotonics and Laser Technology
Campus A5.1
66123 Saarbruecken
Germany
k.koenig@blt.uni-saarland.de

JenLab GmbH
Schillerstr. 1
07745 Jena
Germany
koenig@jenlab.de

Serge Koudoro
MINES ParisTech, PSL Research University
CMM - Centre for Mathematical Morphology
Fontainebleau
France
serge.koudoro@mines-paristech.fr

Arnd Krueger
MKS Instruments, Inc.
Newport Spectra-Physics GmbH
Guerickeweg 7
64291 Darmstadt
Germany
arnd.krueger@newport.com

Luca Lanzano
Nanoscopy, Nanophysics, Istituto Italiano di Tecnologia
via Morego 30
16163, Genoa
Italy
Luca.Lanzano@iit.it

Xiaowen Liang
Therapeutics Research Centre
School of Medicine
The University of Queensland
Princess Alexandra Hospital
QLD 4102 Woolloongabba
Australia
x.liang@uq.edu.au

Xin Liu
Therapeutics Research Centre
School of Medicine
The University of Queensland
Princess Alexandra Hospital
QLD 4102 Woolloongabba
Australia
xin.liu@uq.edu.au

Maria M. Lukina
Institute of Biomedical Technologies
Nizhny Novgorod State Medical Academy
Minin and Pozharsky Square 10/1
Nizhny Novgorod, 603005
Russia
kuznetsova.m.m@yandex.ru

Anna Letizia Allegra Mascaro
European Laboratory for Nonlinear Spectroscopy (LENS)
Via Nello Carrara, 1
50019 Sesto Fiorentino (FI)
Italy
allegra@lens.unifi.it

Aleksandra V. Meleshina
Institute of Biomedical Technologies
Nizhny Novgorod State Medical Academy
Minin and Pozharsky Square 10/1
Nizhny Novgorod, 603005
Russia
almele@ya.ru

Institute of Biology and Biomedicine
Nizhny Novgorod State University
Gagarin Avenue 23
Nizhny Novgorod, 603950
Russia

Meghan J. O. Melia
The W.M. Keck Center for Cellular Imaging
Departments of Biology and Biomedical Engineering
Physical and Life Sciences Building
University of Virginia
Charlottesville,VA 22904
USA
mjo3vp@virginia.edu

Christian Mess
University Medical Center Hamburg-Eppendorf
Center for Internal Medicine
SWG Multiphoton Tomography
Martinistr. 52
20246 Hamburg
Germany
c.mess@uke.de

University of Münster
Department of Dermatology
Von-Esmarch-Str. 58
48148 Münster
Germany

Nataliya M. Mishina
Shemyakin-Ovchinnikov
Institute of Bioorganic Chemistry RAS
16/10 Miklukho-Maklaya
St., Moscow 117997
Russia
natsafr@yandex.ru

Francesco S. Pavone
European Laboratory for Non Linear Spectroscopy (LENS)
Via Nello Carrara 1
50019 Sesto Fiorentino
Italy
francesco.pavone@unifi.it

Ammasi Periasamy
The W.M. Keck Center for Cellular Imaging
Departments of Biology and Biomedical Engineering
Physical and Life Sciences Building
University of Virginia
Charlottesville,VA 22904
USA
ap3t@virginia.edu

Shagufta Rehman Alam
The W.M. Keck Center for Cellular Imaging
Departments of Biology and Biomedical Engineering
Physical and Life Sciences Building
University of Virginia
Charlottesville, VA 22904
USA
sr9zz@virginia.edu

Michael S. Roberts
School of Pharmacy and Medical Sciences
The University of South Australia
5001 Adelaide
Australia
m.roberts@uq.edu.au

Therapeutics Research Centre
School of Medicine
The University of Queensland
Princess Alexandra Hospital
Woolloongabba
Australia

Ana-Maria Pena
L'Oréal Research and Innovation
1 avenue Eugène Schueller
93601 Aulnay-sous-Bois
France
ampena@rd.loreal.com

Christopher Rowlands
Department of Bioengineering
Imperial College London
South Kensington
SW7 2AZ
United Kingdom
c.rowlands@imperial.ac.uk

Angelika Rück
Ulm University
Core Facility Confocal and Multiphoton Microscopy
Albert-Einstein-Allee 11
89081 Ulm
Germany
angelika.rueck@uni-ulm.de

Leonardo Sacconi
European Laboratory for Nonlinear Spectroscopy (LENS)
Via Nello Carrara, 1
50019 Sesto Fiorentino (FI)
Italy
sacconi@lens.unifi.it

Vladislav I. Shcheslavskiy
Becker & Hickl GmbH
Nahmitzer Damm 30
12277 Berlin
Germany
vis@becker-hickl.de

Lorenzo Scipioni
Nanoscopy, Nanophysics, Istituto Italiano di Tecnologia
via Morego 30
16163, Genoa
Italy
Lorenzo.Scipioni@iit.it

Department of Computer Science, Bioengineering, Robotics and Systems Engineering
University of Genoa
Italy

Tatiana F. Sergeeva
Institute of Biomedical Technologies
Nizhny Novgorod State Medical Academy
Minin and Pozharsky Square 10/1
Nizhny Novgorod, 603005
Russia
tatiana.f.sergeeva@gmail.com

Colin Sheppard
Honorary Professoral Fellow
School of Chemistry
Faculty of Science, Medicine and Health
University of Wollongong
Northfields Avenue
NSW2522
Australia

Marina V. Shirmanova
Institute of Biomedical Technologies
Nizhny Novgorod State Medical Academy
Minin and Pozharsky Square 10/1
Nizhny Novgorod, 603005
Russia
shirmanovam@mail.ru

Institute of Biology and Biomedicine
Nizhny Novgorod State University
Gagarin Avenue 23
Nizhny Novgorod, 603950
Russia

Ludovico Silvestri
European Laboratory for Nonlinear Spectroscopy (LENS)
Via Nello Carrara, 1
50019 Sesto Fiorentino (FI)
Italy
silvestri@lens.unifi.it

Peter T. C. So
Department of Mechanical and Biological Engineering
Massachusetts Institute of Technology
Cambridge, MA 02139
USA
ptso@mit.edu

Hauke Studier
School of Pharmacy and Medical Sciences
The University of South Australia
5001 Adelaide
Australia

Becker & Hickl GmbH
Nahmitzer Damm 30
12277 Berlin
Germany
studier@becker-hickl.de

Zdenek Svindrych
The W.M. Keck Center for Cellular Imaging
Departments of Biology and Biomedical Engineering
Physical and Life Sciences Building
University of Virginia
Charlottesville,VA 22904
USA
zs4d@virginia.edu

Emmanuelle Tancrède-Bohin
L'Oréal Research and Innovation
1 avenue Eugène Schueller
93601 Aulnay-sous-Bois
France
etancrede@rd.loreal.com

Camilla Thorling
Therapeutics Research Centre
School of Medicine
The University of Queensland
Princess Alexandra Hospital
QLD 4102 Woolloongabba
Australia
camilla.thorling@gmail.com

Bruce J. Tromberg
Laser Microbeam and Medical Program
Beckman Laser Institute
University of California
Irvine, CA, 92612
USA
bjtrombe@uci.edu

Aisada Uchugonova
Saarland University
Department of Biophotonics & Laser Technology
Campus A5.1
66123 Saarbruecken
Germany
a.uchugonova@blt.uni-saarland.de

Giuseppe Vicidomini
Molecular Microscopy and Spectroscopy,
Nanophysics, Istituto Italiano di Tecnologia
via Morego 30
16163, Genoa
Italy
giuseppe.vicidomini@iit.it

Steeve Victorin
L'Oréal Research and Innovation
1 avenue Eugène Schueller
93601 Aulnay-sous-Bois
France
svictorin@rd.loreal.com

Horst Wallrabe
The W.M. Keck Center for Cellular Imaging
Departments of Biology and Biomedical Engineering
Physical and Life Sciences Building
University of Virginia
Charlottesville, VA 22904
USA
hw5m@virginia.edu

Haolu Wang
Therapeutics Research Centre
School of Medicine
The University of Queensland
Princess Alexandra Hospital
QLD 4102 Woolloongabba
Australia
h.wang21@uq.edu.au

Martin Weinigel
JenLab GmbH
Schillerstrasse 1
07745 Jena
Germany
weinigel@jenlab.de

Elijah Yew
Department of Mechanical and Biological Engineering
Massachusetts Institute of Technology
Cambridge, MA 02139
USA
elijah.yew@gmail.com

Christopher B. Zachary
Department of Dermatology
University of California
Irvine, CA, 92697
USA
czachary@uci.edu

Elena V. Zagaynova
Institute of Biomedical Technologies
Nizhny Novgorod State Medical Academy
Minin and Pozharsky Square 10/1
Nizhny Novgorod, 603005
Russia
ezagaynova@gmail.com

Institute of Biology and Biomedicine
Nizhny Novgorod State University
Gagarin Avenue 23
Nizhny Novgorod, 603950
Russia

Part I: **Basics**

Karsten König

1 Brief history of fluorescence lifetime imaging

Abstract: This review gives an overview of the history of fluorescence lifetime imaging (FLIM) in life sciences. FLIM microscopy based on an ultrafast laser scanning microscope and time-correlated single photon counting (TCSPC) was introduced in Jena/Germany in 1988/89. FLIM images of porphyrin-labeled live cells and live mice were taken with an unique ZEISS confocal picosecond laser microscope. Five years later, the first *in vivo* FLIM on human volunteers started with time-gated cameras to detect dental caries based on one-photon wide-field pulsed laser excitation of autofluorescent bacteria. Another five years later, two-photon FLIM of autofluorescent skin was performed on a volunteer with a lab microscope in the frequency domain. The first clinical non-invasive optical, two-photon 3D FLIM biopsies were obtained fifteen years ago in patients with dermatological disorders using a certified clinical multiphoton tomograph based on a tunable femtosecond titanium:sapphire laser and TCSPC.

A current major FLIM application in cell biology is the study of protein-protein interactions in transfected cells by FLIM-FRET microscopy. Clinical FLIM applications are still on a research level and include preliminary studies on (i) one-photon FLIM autofluorescence microscopy of patients with ocular diseases using picosecond laser diodes, (ii) time-gated imaging in brain surgery using a nanosecond nitrogen laser, and (iii) two-photon clinical FLIM tomography of patients with skin cancer and brain tumors with near-infrared femtosecond lasers and TCSPC.

1.1 Introduction

FLIM is an imaging methodology with high specificity and high sensitivity to the nano- and microenvironment. It is relatively insensitive to concentration and signal intensity artefacts. The acronym FLIM stands for *Fluorescence Lifetime Imaging* (sometimes also for fluorescence lifetime microscopy) and means that the fluorescence lifetime τ is depicted with spatial resolution. FLIM images are typically generated with microscopes, endoscopes, and tomographs but can be also acquired on a macroscopic level. FLIM images can be taken in the frequency domain (measurement of phase shift and demodulation with cw or pulsed laser) as well as in the time domain using time-gated cameras, streak cameras, and time-correlated single photon counting (TCSPC) units [1]. They are often false-color coded where the colors reflect certain τ *values*.

FLIM applications in cell biology are based on endogenous (intrinsic) and exogenous fluorophore detection in a specific nano- and microenvironment. FLIM can be used to probe intermolecular interactions within a 10 nm distance, such as bindings, by Förster resonance energy transfer (FRET) and anisotropy measurements. Animal

https://doi.org/10.1515/9783110429985-003

studies, artworks, microfluidics, and forensic science such as fingerprint detection are other applications of FLIM [2–8].

However, the most challenging and exciting FLIM application is *in vivo* clinical imaging of patients. Clinical FLIM with several hundreds of patients is currently based on TCSPC using

(i) picosecond laser diodes in the visible spectral range (one-photon excitation) and
(ii) near-infrared (NIR) femtosecond Ti:sapphire lasers (two-photon excitation).

1.2 Time-resolved spectroscopy and first time-resolved fluorescence microscopes

For decades, time-resolved fluorescence spectroscopy has been employed to investigate photophysical, photochemical, and photobiological processes of fluorescent molecules in solution, such as the primary steps of vision and photosynthesis (e.g. [9]). In order to gain time-resolved fluorescence information on living cells, nanosecond and sub-nanosecond fluorometers were combined with microscopes. Typically, a fixed laser beam was focused to a micrometer-sized laser spot [10–17]. Applications of these time-resolved spectrometers with some spatial information included the analysis of chlorophyll fluorescence from single chloroplasts, the coenzyme fluorescence in bacteria, and the porphyrin fluorescence in cells after administration of fluorescent photosensitizers.

1.3 First FLIM laser scanning microscope

In 1988, a real breakthrough was obtained by the combination of an ultrafast pulsed laser in the picosecond range with a laser scanning microscope. Now the object was illuminated point-by-point by the focused pulsed laser beam and the corresponding time-resolved fluorescence response was detected at each point by time-correlated single photon counting (TCSPC). The fluorescence intensity I_F and the fluorescence decay time τ were used as parameters to generate FLIM images. This first prototype of the FLIM laser scanning microscope became operational in the Department of Physics at the Friedrich Schiller University Jena in 1988 [18], shortly before the unification of Germany. Two years earlier, the patent on time-resolved pulsed laser scanning microscopy had been filed by Gröbler from the company VEB Carl Zeiss Jena [19].

This unique FLIM system was based on a confocal laser scanning microscope (scanning stage with 0.5 µm steps) equipped with a 120 MHz mode-locked argon ion laser (100 ps). The fluorescence was detected with photomultipliers (FEU-77) with short picosecond rise time from the Soviet Union in combination with the time-resolved single photon counting unit SPC100 (ZOS Berlin, Fig. 1.1).

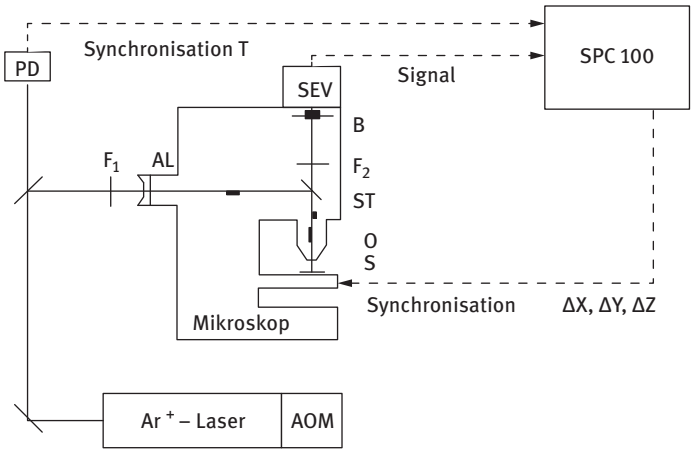


Fig. 1.1: First FLIM laser scanning microscope. The apparatus is based on a ZEISS confocal stage scan fluorescence microscope equipped with a mode-locked 120 MHz argon ion laser and the TCSPC module SPC100 [20–22]. Single photon events are accumulated in a 256-channel memory, maximum 65 535 counts per channel, channel width 48 ps or 20 ps, with a maximum count rate of 2×10^5/s. Temporal resolution: 300 ps, 50× NA 0.9 objective. AOM: acousto-optic mode-locker, PD: photodiode, F_1: attenuator, F_2: blocking filter, ML: matching lens, DM: dichroic mirror, O: objective, S: scanning stage with 0.5 µm steps, SPC: single photon counting.

König et al. used this unique novel TCSPC-FLIM tool to perform the first laser scanning FLIM in life science [20–22]. In 1988, our group generated FLIM images with submicron resolution (50×, NA 0.95) from living cancer cells. In particular, red-emitting intracellular fluorescent porphyrin photosensitizers were imaged as line scans and 2D plots with sub-nanosecond temporal resolution. Bi-exponential as well as global fitting was performed to obtain data on porphyrin monomers, dimers, and higher aggregates as well as to study photodynamic reactions (Fig. 1.2 and 1.3).

The mode-locked 120 MHz argon ion laser was also used to pump a tunable dye laser. Unfortunately, no two-photon excited fluorescence images (that would require the use of short pass filters) were taken at that time in Jena with this remarkable tunable picosecond laser scanning microscope working even in the red spectral range.

In 1989, Wang et al. reported on fluorescence lifetime distribution measurements by phase-resolved detection with an image dissector tube [23] and one year later on time-resolved fluorescence microscopy by multichannel photon counting [24].

Denk, Strickler, and Webb realized the two-photon microscope based on a subpicosecond dye laser in 1989/1990 [25, 26]. With the availability of the more stable and user-friendly femtosecond titanium:sapphire laser, two-photon laser scanning microscopes with their inherent optical sectioning rapidly became a major tool for cell biologists. Since these microscopes already have the expensive laser source, it is relatively simple to add on a TCSPC module in order to perform FLIM.

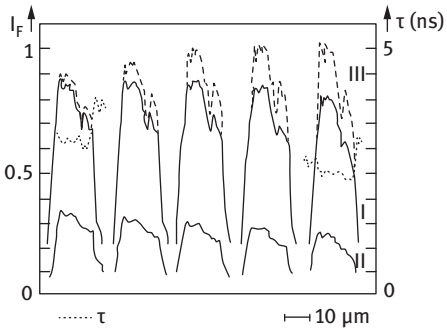


Fig. 1.2: Subsequent line scans (5 times the same cell, step width: 0.5 µm, laser spot size: 0.6 µm) with the FLIM microscope were taken to study laser-induced bleaching and photoproduct formation in a live cancer cell labeled with the photosensitizer HpD (mean power: 7 µW, interval I: 0.7–3.6 ns, interval II: 3.6–6.5 ns, interval III: whole excitation pulse period). The intensity increase in interval III and the decrease of the mean fluorescence lifetime reflect the formation of a short-lived porphyrin photoproduct during five laser scans [20–22].

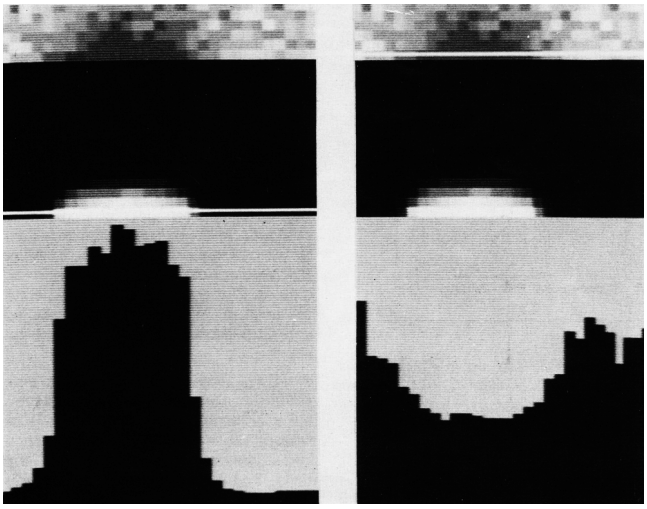


Fig. 1.3: First FLIM images using a confocal picosecond laser scanning microscope. The figure shows a 2D scan of the fluorescence of the intracellular photosensitizer HpD in a part of an *Ehrlich Ascites Carcinoma* (EAC) cell. Upper part: $\tau(x, y)$ values (1 µm steps), middle: fluorescence intensity values, lower part: histograms along the lines [20–22].

1.4 Two-photon FLIM microscopy

A drawback of one-photon FLIM with visible light sources is its inability to image the most important intracellular fluorescent biomolecule, the coenzyme NAD(P)H in the mitochondria with its absorption maximum around 340 nm. Furthermore, problems occur due to out-of-focus fluorescence excitation and the limited light penetration depth in most tissues. These drawbacks can be overcome when using multiphoton imaging with NIR lasers based on two-photon excited fluorescence, second harmonic generation (SHG), and coherent anti-Stokes Raman spectroscopy (CARS).

Some years after the invention of the two-photon microscope, Piston et al. [27], So et al. [28], and König et al. [29] performed two-photon FLIM microscopy on living cells. Interestingly, a variety of first two-photon FLIM microscopes used a frequency domain approach whereas the majority of today's two-photon FLIM devices are employing fast TCSPC. Soon after their introduction, the first two-photon FLIM images from animal tissues were taken and finally from the fingers of a microscope user. Masters et al. used a lab two-photon microscope operating in the frequency domain in 1997/1998 to obtain the first two-photon FLIM data from *in vivo* human skin [30–32].

1.5 First wide-field FLIM in humans

Some years earlier in the mid-1990s, the first wide-field macroscopic FLIM images from humans were obtained by König and Schneckenburger. *In vivo* autofluorescence images of the tooth region of two volunteers with carious lesions and dental plaque were taken. In particular, the distribution of the red-emitting, porphyrin-producing bacteria *Actinomyces odontolyticus* with fluorescence lifetimes of 10 ns and longer has been studied in non-healthy teeth of volunteers with wide-field FLIM [33, 34]. A time-gated camera and wide-field illumination with a frequency-doubled two nanosecond Nd:YAG laser was employed. Time-gated "snapshots" were acquired at various time delays after the excitation. When using "snapshots" several nanoseconds after the excitation pulse, dental plaque could be clearly detected due to the suppression of the short-lived autofluorescence of normal tooth material and the scattered laser light (Fig. 1.4).

Today, clinical wide-field macroscopic FLIM is employed to detect tumor borders during brain surgery. A nanosecond nitrogen laser at 337 nm is used to excite the fluorescence of the tissue and to allow "snapshots" for the identification of the tumor [35].

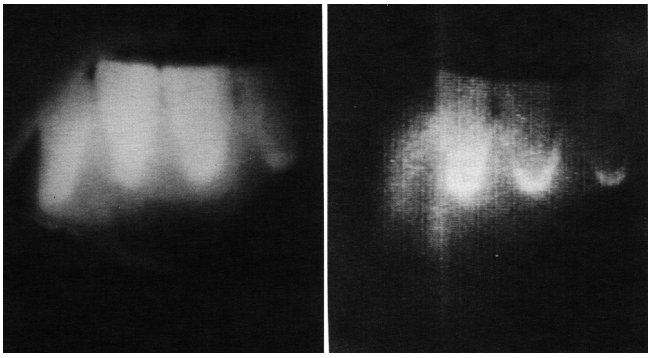


Fig. 1.4: *In vivo* time-gated autofluorescence images from anterior teeth of a volunteer with dental plaque and caries. Bacteria (*Actinomyces odontolyticus*) produce coproporphyrin and PP IX with long > 10 ns fluorescence lifetimes. Therefore, time windows with a long >10 ns delay compared to the excitation light exhibited the porphyrin autofluorescence in caries and dental plaque. The short-lived tooth autofluorescence is suppressed. Left: time gate: 0–5 ns, right: 30–55 ns [34].

1.6 Clinical FLIM tomography

A patent on a device and a method for clinical time-resolved two-photon imaging and treatment of skin disorders was filed by König in 2000 [36]. The first prototype of a medical two-photon tomograph "DermaInspect" with picosecond temporal resolution was realized based on a tunable Ti:sapphire laser, x/y-galvoscanners, a piezodriven NA 1.3 objective, and a TCSPC module in 2002 [37, 38]. Soon after it became CE-marked for clinical use and commercialized by JenLab GmbH, Jena, Germany. The first studies were performed on patients with skin cancer at the University Hospital Jena.

Today, the portable certified multiphoton tomographs "MPTflex" and "MPTflex-CARS" with their optomechanical arms are in clinical use in Australia, Japan, Russia, US, and Europe. These medical tomographs simultaneously depict FLIM images, SHG images, two-photon fluorescence intensity images, and CARS images, respectively (Fig. 1.5 and 1.6). Clinical FLIM applications include early detection of skin cancer, tumor border recognition during brain surgery, and detection of intratissue inflammation sites (e.g. [39–56]).

▸ **Fig. 1.6:** Clinical two-photon FLIM images from human skin are based on two-photon excitation of endogenous fluorophores using TCSPC. The arrival times of some fluorescence photons per pixel are depicted as fluorescence decay curve (lower part). Bi-exponential fitting provides amplitudes and lifetimes of two components as well as the mean fluorescence lifetime per pixel.

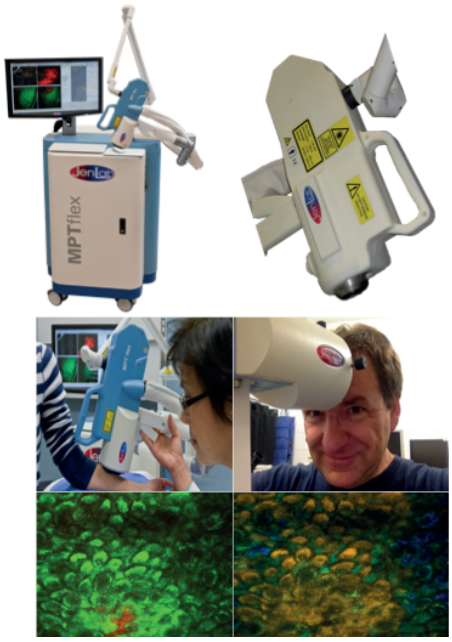
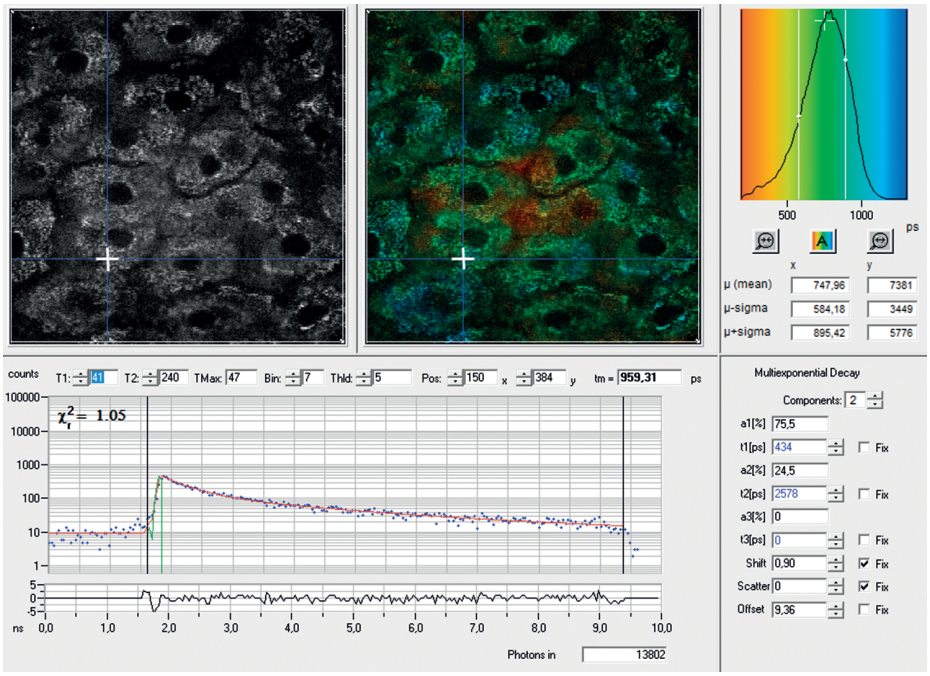
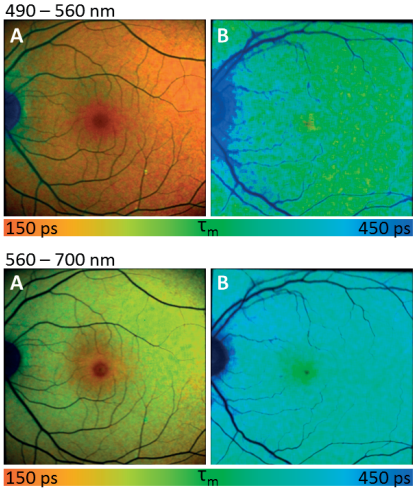


Fig. 1.5: The Prism Award winning certified clinical multiphoton FLIM tomograph *MPTflex*™ with its flexible optomechanical arm with active beam stabilizer, the compact scan/detection head, the tunable femtosecond laser, and the TCSPC module. The head consists of a 3D scanning system, the high NA focusing optics, and two single photon counting sensors. Wide-field (up to 5 × 5 mm^2) images can be taken by mosaic scanning. Horizontal, vertical and diagonal FLIM sections are possible. The lower image left shows an emission intensity image based on autofluorescence AF (green) and SHG (red). The right image shows the time-resolved AF image with pseudocolors representing the fluorescence lifetimes. FLIM of tissue AF provides a significantly better contrast than intensity images.



1.7 One-photon FLIM in ophthalmology

The major clinical one-photon FLIM application today is in the field of ophthalmology. Picosecond laser diodes in the visible range are employed to excite the ocular autofluorescence. FLIM images are taken by the use of TCSPC units (Fig. 1.7 and 1.8). The company *Heidelberg Engineering* has produced the first prototypes for clinical use. They are being tested, e.g., for the detection of macular degeneration and other diseases. This pioneering FLIM work for the field of ophthalmology was performed by Schweitzer and coworkers in Jena, Germany [57–59].



τ_1 → retinal pigmented epithelium
τ_2 → neural retina
τ_3 → influence of lens fluorescence

		a_1 [%]	τ_1 [ps]	a_2 [%]	τ_2 [ps]	a_3 [%]	τ_3 [ps]
490 - 560 nm	*A*	85.3	87.7	11.5	388.4	2.7	2691.7
	B	84.5	137.8	11.5	586.8	4	3767.9
560 - 700 nm	*A*	72.3	83.7	24.8	320.6	2.9	1723.1
	B	74.8	128.5	22.3	475.1	2.9	2388.2

Fig. 1.7: *In vivo* FLIM images from the eyes of two volunteers. Clinical one-photon FLIM is based on picosecond laser excitation (473 nm, 80 MHz) and TCSPC in two channels (498–560 nm and 560–720 nm). FLIM images of the editor (B) and a younger female student (A) were taken under the same conditions. Note the longer lifetimes in the case of the older volunteer. Image acquisition: 1.5 min.

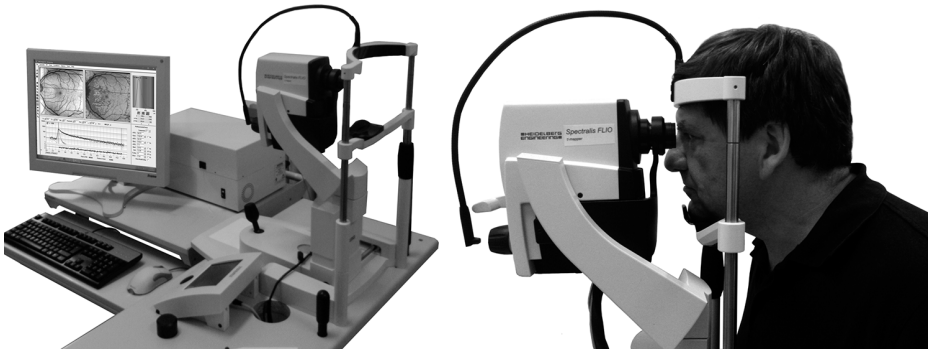


Fig. 1.8: The fluorescence lifetime ophthalmoscope FLIO.

1.8 Endoscopic FLIM

In the late 1990s, the first FLIM endoscopes, such as the system by Mizeret et al. that worked in the frequency domain [60], were also developed. Later on in 2004, video-rate FLIM with a potentially portable flexible one-photon FLIM endoscope was reported based on gated optical image intensifier technology [61].

Already in 2002, a compact two-photon fluorescence microscope based on a single-mode fiber coupler was reported and one year later, two-photon low-weight endoscopic systems were employed to study brain tissue in live mice [62].

Finally in 2007, the first clinical time-resolved two-photon fluorescence endoscopy was performed using special high NA GRIN microendoscopes on patients with chronic and acute wounds ([63], Fig. 1.9).

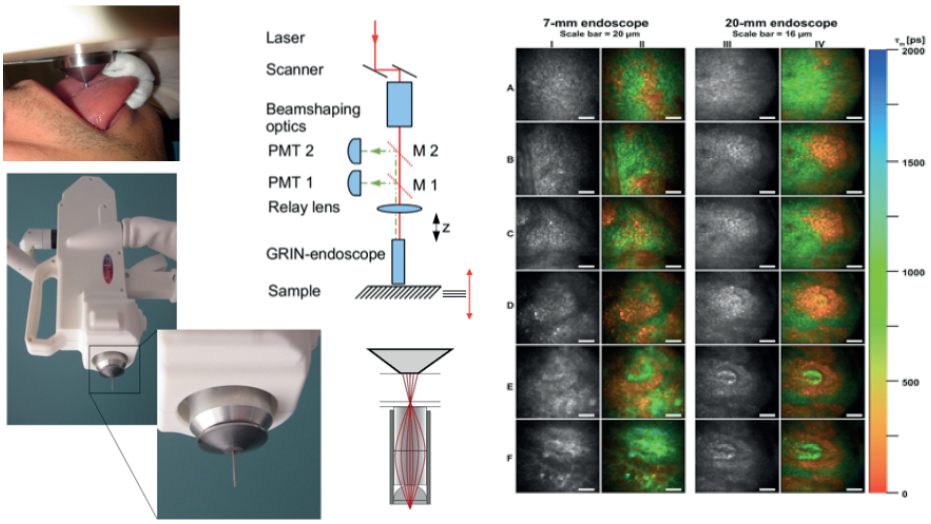


Fig. 1.9: Clinical two-photon GRIN microendoscope with high NA (0.8). The endoscope is connected to a multiphoton tomograph. The images show two-photon sections of the lip of a volunteer.

1.9 FLIM-FRET

One current major application of FLIM microscopes in cell biology is FLIM-FRET. Protein-protein interactions and the micro- and nano-environment of a molecule are studied by the detection of the shortening of the fluorescence lifetime of a donor molecule during Förster resonance energy transfer (FRET). During that transfer, the energy of the light-excited fluorescent donor is transferred non-radiatively to a nearby molecular chromophore in the ground state. A major condition is the spectral overlap

of the donor's emission with the acceptor's absorption. The probability of fluorescence is thereby diminished and as a result, the fluorescence lifetime is shortened. Förster showed in the 1940s that the rate of energy transfer is proportional to R^{-6}, where R is the distance between the chromophore centers [64]. Practically, it means that the distance should be less than 10 nanometers and binding should occur.

FRET can be measured with a normal steady-state fluorescence microscope. However, it is difficult to quantify the FRET measurements because standards must be used for calibration of the fluorescence intensities and problems are faced due to concentration gradients and dynamics of the proteins within the cell as well as due to photobleaching phenomena. FLIM can overcome these difficulties in making reliable FRET studies independent of fluorescence intensities by measuring the modifications of the fluorescence decay of the donor [5, 6, 65].

Most FLIM-FRET studies are performed on green fluorescent proteins (GFP) and other genetic constructs (Fig. 1.10). In some cases, FRET between natural fluorescent chromophores such as NADH and tryptophan has also been studied. Often FLIM-FRET is performed with one-photon donor excitation such as UV and blue light. Two-photon excitation may cause a minor problem because of the broad two-photon excitation spectra and the subsequent excitation of the donor as well as the non-desired excitation of the acceptor. However, this problem can be solved by deconvolution methods (e.g., global fitting). More information on FLIM-FRET can be found in [66–68].

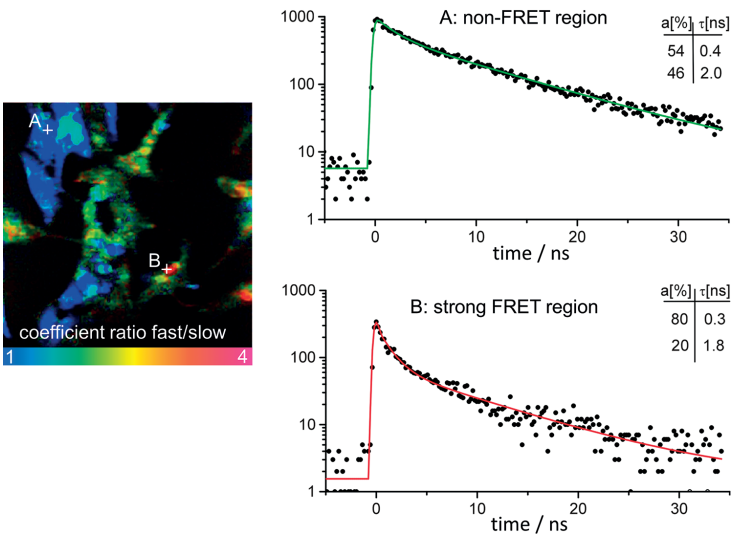


Fig. 1.10: FLIM-FRET measurement from 2001. Our group observed FRET in Vero cells containing green fluorescent protein (GFP, acceptor) and yellow fluorescent protein (YFP, donor). The shortening of the YFP lifetime and the increase of the ratio fast to slow component a_1/a_2 indicated FRET. ZEISS LSM-410, Multichannel plate detector, SPC-730 TCSPC module [5].

1.10 Conclusion

Since its introduction in life science in the late 1980s, FLIM scanning microscopy has become an important tool for cell biologists. In particular, FLIM microscopy gained a significant boost with the introduction of two-photon femtosecond laser scanning microscopes in the 1990s. Nowadays, TCSPC-FLIM add-on modules can easily be implemented in commercial ultrashort laser scanning microscopes.

Ten years after the invention of the two-photon microscope, the first commercial certified clinical FLIM tomographs ("DermaInspect") for medical diagnostics became available [36–38].

Current FLIM microscopes, FLIM tomographs, and FLIM endoscopes still have the drawback of being expensive and relatively bulky systems. However, with the availability of ultracompact low cost femtosecond laser sources within the next years, FLIM devices will likely become a common tool in research and application labs. Still, it remains a major goal to identify promising applications in the medical field [69].

References

[1] O'Connor DV, Phillips D, editors. Time-correlated single photon counting. Academic Press; 1984.
[2] Schneckenburger H, König K. Fluorescence decay kinetics and imaging of NAD(P)H and flavins as metabolic indicators. Opt Eng. 1992;31:1447–1451.
[3] So P, König K, Berland K, Dong CY, French T, Buehler C, Ragan T, Gratton E. New time-resolved techniques in two-photon microscopy. Cell Mol Biol. 1998;44:771–794.
[4] Bastiaens PIH, Squire A. Fluorescence lifetime imaging microscopy: spatial resolution of biochemical processes in the cell. Trends Cell Biol. 1999;9:48–52.
[5] Becker W, Benndorf K, Bergmann A, Biskup C, König K, Tirlapur U, Zimmer T. FRET measurements by TCSPC laser scanning microscopy. Proc SPIE 4431; 2001.
[6] Becker W, Bergmann A, König K, Tirlapur U. Picosecond Fluorescence Lifetime Microscopy by TCSPC Imaging. Proc SPIE 4262; 2001.
[7] Cunall RB, Dale RE, editors. Time-resolved spectroscopy in biochemistry and biology. New York, London: Plenum Press; 1983.
[8] Suhling K, et al. Fluorescence lifetime imaging (FLIM). Basic concepts and some recent developments. Medical Photonics. 2015;27:3–40.
[9] Venetta BD. Microscope phase fluorometer for determining the fluorescence lifetimes of fluorochromes. Rev Sci Instr. 1959;6:450–457.
[10] Docchio F, Ramponi R, Sacchi CA, Bottiroli G, Freita. An automatic pulsed laser microfluorometer with high spatial and temporal resolution. J Microsc. 1984;134:151–160.
[11] Bottiroli G, Cionini PG, Dicchio F, Sacchi CA. "In situ" evaluation of the functional state of chromatin by means of quinacrine mustard staining and time-resolved fluorescence microscopy. Histochem J. 1984;16:223–233.
[12] Schneckenburger H. Time-resolved microfluorescence in biomedical diagnosis. Opt Engineering. 1985;24:1042–1044.
[13] Rodgers MAJ, Firey PA. Instrumentation for fluorescence microscopy with picosecond time resolution. Photochem Photobiol. 1985;42:613–616.

[14] Minami T, Kawahigashi M, Sakai Y, Shimamoto K, Hirayama S. Fluorescence lifetime measurements under a microscope by the time-correlated single photon counting technique. J Luminescence. 1986;35:247–253.
[15] Pamponi R, Rodgers MAJ. An instrument for simultaneous acquisition of fluorescence spectra and fluorescence lifetimes from single cells. Photochem Photobiol. 1987;45:161–165.
[16] Schneckenburger H, Frenz M, Tsuchiya Y, Denzer U, Schleinkofer L. Picosecond fluorescence microscopy for measuring chlorophyll and porphyrin components in conifers and cultured cells. Lasers in the Life Sciences. 1987;1:229–307.
[17] Kinoshita S, Seki T, Fu Liu T, Kushida T. Fluorescence of hematoporphyrin in living cells and in solution. J Photochem Photobiol B. 1988;2:195–208.
[18] Gärtner W, Gröbler B, Schubert D, Wabnitz H, Wilhelmi B. Fluorescence scanning microscopy combined with subnanosecond time resolution. Experimental Techniques in Physics. 1988;36:443–451.
[19] Gröbler B. Apparatus for the graphic representation and analysis of fluorescence signals. Vorrichtung zur Analyse und bildlichen Darstellung des bei einer punktweisen Anregung eines Präparates durch Laserlicht entstehenden zeitlichen Intensitätsverlaufes der Fluoreszenzstrahlung. German patent DE 3614359C2, United States patent US4786170A. Filed April 28, 1986 (July 26, 1985)
[20] König K. Beiträge zur Photochemotherapie und optischen Diagnostik von Tumoren [PhD thesis]. Translation: Contributions to photochemotherapy and optical diagnostics of tumors. Jena, Germany: University of Jena Archive, Germany; 1989.
[21] Bugiel I, König K, Wabnitz H. Investigation of cells by fluorescence laser scanning microscopy with subnanosecond time resolution. Lasers Life Sciences. 1989;3:1–7.
[22] König K, Wabnitz H. Fluoreszenzuntersuchungen mit hoher zeitlicher, spektraler und räumlicher Auflösung. Labortechnik. 1990;23:26–31.
[23] Wang XF, Uchida T, Minami S. A fluorescence lifetime distribution measurement system based on phase-resolved detection using an image dissector tube. Appl Spectrosc. 1989;43:840–845.
[24] Wang XF, Kitajima S, Uchida T, Coleman DM, Minami S. Time-resolved fluorescence microscopy using multichannel photon counting. Appl Spectrosc. 1990;44:25–29.
[25] Denk W, Strickler JP, Webb WW. Two-photon laser microscopy. United States patent Patent US 5,034,613. Filed Nov 14, 1989.
[26] Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. Science. 1990;248:73–76.
[27] Piston DW, Sandison DR, Webb WW. Time-resolved fluorescence imaging and background rejection by two-photon excitation in laser scanning microscopy. Proc SPIE 1640; 1992.
[28] So PTC, French T, Yu WM, Berland K, Dong CY, Gratton E. Time-resolved fluorescence microscopy using two-photon excitation. Bioimaging. 1995;3:49–63.
[29] König K, So PTC, Mantulin WW, Tromberg BJ, Gratton E. Two-photon excited lifetime imaging of autofluorescence in cells during UVA and NIR photostress. J Microsc. 1996;183:197–204.
[30] Masters BR, So PTC, Gratton E. Multiphoton excitation fluorescence microscopy and spectroscopy of in vivo human skin. Biophys J. 1997;72:2405.
[31] Masters BR, So PTC, Gratton E. Multiphoton excitation microscopy of in vivo human skin. Functional and morphological optical biopsy based on three-dimensional imaging, lifetime measurements and fluorescence spectroscopy. Ann NY Acad Sci. 1998;838:58–67.
[32] Masters BR, So PTC, Gratton E. Optical biopsy of in vivo human skin: multi-photon excitation microscopy. Laser Med Sci. 1998;13:196–203.

[33] König K, Schneckenburger H. Laser-induced dental caries and plaque diagnosis on patients by sensitive autofluorescence spectroscopy and time-gated video imaging. Preliminary studies. Proc SPIE 2128: Lasers in Surgery. 1994;403–408.
[34] König K, Schneckenburger H, Hibst R. Time-gated in vivo imaging of dental caries. Cell Mol Biol. 1999;45:233–239.
[35] Sun Y, Hatami N, Yee M, Phipps J, Elson DS, Gorin F, Schrot RJ, Marcu L. Fluorescence lifetime imaging microscopy for brain tumor image-guided therapy. J Biomed Opt. 2010;15:056022.
[36] König K. Verfahren und Anordnung zur nicht-invasiven dreidimenisonalen optischen Untersuchung und Therapie der Haut. German patent DE 10065146A1. Filed December 22, 2000.
[37] König K, Wollina U, Riemann I, Peuckert C, Halbhuber KJ, Fünfstück V, Fischer TW, Elsner P. Optical tomography of human skin with subcellular spatial and picosecond time resolution. Proc SPIE 4620; 2002.
[38] König K, Riemann I. High-resolution multiphoton tomography of human skin with subcellular spatial resolution and picosecond time resolution. J Biomed Opt. 2003;8:432–439.
[39] König K, Riemann I, Ehrlich G, Ulrich V, Fischer P. Multiphoton FLIM and spectral imaging of cells and tissues. Proc SPIE 5323; 2004.
[40] König K. Clinical multiphoton tomography. J Biophotonics. 2008;1:13–23.
[41] Dimitrow E, Riemann I, Ehlers A, Koehler JM, 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. 2009;18:509–515.
[42] Huck V, Gorzelanny C, Thomas K, Niemeyer V, Luger T A, König K, Schneider SW. Intravital multiphoton tomography as a novel tool for non-invasive in vivo analysis of human skin affected with atopic dermatitis. Proc SPIE 7548; 2010.
[43] Talbot CB, Patalay R, Munro I, Breunig HG, König K, Alexandrov Y, Warren S, Chu A, Stamp GW, Neil MAA, French PMW, Dunsby C. A multispectral FLIM tomograph for in-vivo imaging of skin cancer. Proc SPIE 7903; 2011.
[44] Patalay R, Talbot C, Munro I, Breunig HG, König K, Alexandrov Y, Warren S, Neil MAA, French PMW, Chu A, Stamp GW, Dunsby C. Fluorescence lifetime imaging of skin cancer. Proc SPIE 7883; 2011.
[45] Benati E, Bellini V, Borsari S, Dunsby C, Ferrari C, French P, Guanti M, Guardoli D, König K, Pellacani G, Ponti G, Schianchi S, Talbot C, Seidenari S. Quantitative evaluation of healthy epidermis by means of multiphoton microscopy and fluorescence lifetime imaging microscopy. Skin Res Technol. 2011.
[46] Seidenari S, Arginelli F, Bassoli S, Cautela J, French PMW, Guanti M, Guardoli D, König K, Talbot C, Dunsby C. Multiphoton laser microscopy and fluorescence lifetime imaging for the evaluation of the skin. Dermatol Res Pract. 2012;810749.
[47] Manfredini M, Arginelli F, Dunsby C, French PMW, Talbot C, König K, Ponti G, Pellacani G, Seidenari S. High-resolution imaging of basal cell carcinoma: a comparison between multiphoton microscopy with fluorescence lifetime imaging and reflectance confocal microscopy. Skin Res Techn. 2013;19:e433–e443.
[48] Arginelli F, Manfredini M, Bassoli S, Dunsby C, French PMW, König K, Magnoni C, Ponti G, Talbot C, Seidenari S. High resolution diagnosis of common nevi by Multiphoton Laser Tomography and Fluorescence Lifetime Imaging. Skin Res Tech. 2013;19:194–204.
[49] Seidenari S, Arginelli F, Dunsby C, French PMW, König K, Magnoni C, Manfredini M, Talbot C, Ponti G. Multiphoton laser tomography and fluorescence lifetime imaging of basal cell carcinoma: morphological features for non-invasive diagnostics. Exp Dermatol. 2012;21:831–836.
[50] Koehler MJ, Preller A, Elsner P, König K, Hipler UC, Kaatz M. Non-invasive evaluation of dermal elastosis by in vivo multiphoton tomography with autofluorescence lifetime measurements. Exp Dermatol. 2012;21:48–51.

[51] Patalay R, et al. Quantification of cellular autofluorescence of human skin using multiphoton tomography and fluorescence lifetime imaging in two spectral detection channels. Biomed Opt Exp. 2011;2:3295–3308.
[52] Patalay R, Talbot C, Alexandrov Y, Lenz MO, Kumar S, Warren S, Munro I, Neil MAA, König K, French PMW, Chu A, Stamp GW, Dunsby C. Multispectral fluorescence lifetime imaging using multiphoton tomography for the evaluation of basal cell carcinomas. Plos One. 2012
[53] König K. Hybrid multiphoton multimodal tomography of in vivo human skin. IntraVital. 2012;1:11–26.
[54] Roberts MS, Dancik Y, Prow TW, Thorling CA, Lin LL, Grice JE, Robertson TA, 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. 2011;77:469–488.
[55] Huck V, Gorzelanny C, Thomas K, Getova V, Niemeyer V, Zens K, Unnerstall TR, Feger JS, Fallah MA, Metze D, Ständer S, Luger TA, König K, Mess C, Schneider SW. From morphology to biochemical state-intravital multiphoton fluorescence lifetime imaging of inflamed human skin. Sci Rep. 2016;6:22789.
[56] Kantelhardt S, Kalasauskas D, König K, Kim E, Weinigel M, Uchugonova A, Giese A. In vivo multiphoton tomography and fluorescence lifetime imaging of human brain tumor tissue. J Neurooncol. 2016;127:473–482.
[57] Schweitzer D, Kolb A, Hammer M, Thamm E. Tau-mapping of the autofluorescence of the human ocular fundus. Proc SPIE 4164; 2000.
[58] Schweitzer D, Kolb A, Hammer M. Autofluorescence lifetime measurement in images of the human ocular fundus. Proc SPIE 4432; 2001.
[59] Schweitzer D, Hammer M, Schweitzer F, Anders R, Doebbecke T, Schenke S, Gaillard ER. In vivo measurement of time resolved auto-fluorescence at the human ocular fundus. J Biomed Opt. 2004;9(6):1214–1222.
[60] Mizeret J, Stepinac T, Hansroul M, Studzinski A, van den Bergh H, Wagnières G. Instrumentation for real-time fluorescence lifetime imaging in endoscopy. Rev Sci Instrum. 1999;70:4689.
[61] Bird D, Gu M. Compact two-photon fluorescence microscope based on a single-mode fiber coupler. Optics Letters. 2002;27:1031–1033.
[62] Jung JC, Schnitzer MJ. Multiphoton endoscopy. Optics Letters. 2003;28:902–903.
[63] König K, Ehlers A, Riemann I, Schenkl S, Bückle R, Kaatz M. Clinical two-photon microendoscopy. Microscopy Research and Technique. 2007;70(5):398–402.
[64] Förster T. Energiewanderung und Fluoreszenz. Energy migration and fluorescence. Naturwissenschaften. 1946;6:166–175.
[65] Wouters FS, Bastiaens PIH. Fluorescence lifetime imaging of receptor tyrosine kinase activity in cells. Current Biol. 1999;9:1127–1130.
[66] Becker W. Advanced time-correlated single-photon counting techniques. Berlin, Heidelberg, New York: Springer; 2005.
[67] Periasamy A, Clegg RM, editors. FLIM in Biology and Medicine. Taylor & Francis (CRC Group); 2009
[68] Becker W. Advanced time-correlated single photon counting applications. Springer; 2015.
[69] König K, Uchugonova A. Multiphoton fluorescence lifetime imaging at the dawn of clinical application. In: Periasamy A, Clegg RM, editors. FLIM in biology and medicine. Taylor & Francis (CRC Group); 2009. p. 165–187.

Wolfgang Kaiser

2 The long journey to the laser and its use for nonlinear optics

Abstract: Einstein introduced the basic principle of the laser in 1917. However, the possibility of light amplification was not recognized for decades. Eventually, Maiman from the Hughes Aircraft Co. realized the first laser based on ruby as active medium. This unexpected result excited our group at the Bell Labs and we realized the first intense and highly directional (< 1 degree) laser beam. On October 5, 1960 we demonstrated the red laser beam fired from the radar tower in New Jersey and its detection 25 miles away. The new light source initiated the field of Nonlinear Optics (NLO). We realized the first two-photon fluorescence in 1961.

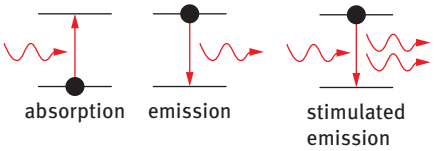
The title of this article suggests a substantial history of the laser. For you the laser is a well-known and well-established optical light source. But please note, that the laser started its existence just 55 years ago.

In 1900, Max Planck published a theory about the experimentally known black body radiation [1]. In his derivation he introduced the light quantum $E = hf$ for the transition between two energy states. This revolutionary idea was used by the – then young – Einstein in 1917 in a completely different derivation of the same black body radiation [2]. In this paper the basic principle of the laser was introduced: the stimulated emission of light. An excited quantum state can be de-excited by a passing light quantum of the proper frequency, i.e., two photons leave the system or – in other words – the stimulating photon is amplified. During the following decades the process of stimulated light emission received surprisingly little attention in the scientific community. In a few papers the dispersion of light in excited gases was investigated and named negative dispersion. The possibility of light amplification was not recognized for 34 years.

Finally, in 1951, Charles Townes, a professor at Columbia University in New York and consultant at the Bell Laboratories had the ingenious idea to build a microwave amplifier (with ammonia gas at 1.25 cm) using the stimulated excitation process. It is interesting to read in the biography of Townes how he found the maser (microwave amplifier by stimulated emission of radiation). He was on his way to a meeting concerning new microwave amplifiers. He knew that no proposals existed. Under a certain pressure the idea occurred to him to study the stimulated light emission process quantitatively for the application in a new device. In fact, in 1953 the first maser was in operation. The desirable extension to higher frequencies – preferentially to the visible – was first discussed theoretically by Schawlow and Townes in 1958 [3]. Tab. 2.1 provides an overview of the historical development of the maser and laser.

https://doi.org/10.1515/9783110429985-004

Tab. 2.1: Overview of the historical development of the maser and laser.

1900	Planck	Quantum of radiation $E = hf$
1917	Einstein	
1923	Tolman	
1927	Ladenburg	
1941	Fabrikant	
1951	Townes	
1952–1955	Townes	MASER
(1955)	Basov	Microwave Amplification by Stimulated
	Prokhorov	Emission of Radiation
1958	Schawlow	LASER
	Townes	Theory: *Physical Review* 112, 1940 (1958)

T. H. Maiman [4], a physicist at the Hughes Aircraft Co., realized the first laser based on ruby as active medium. He talked on the new light source during a press conference in New York on July 7, 1960. The newspaper *New York Times* reported on it one day later. The first scientific paper "Stimulated optical radiation in ruby" was published in *Nature* in August 6, 1960. Maiman pumped a ruby crystal with a powerful flash lamp and observed narrowing of the emitting beam to 55 degrees. The beam was delivered through a hole in the silver coating.

This unexpected result excited our group at the Bell Laboratories to repeat the ruby experiment. After three weeks we saw the expected extremely intense, highly directional (<1 degree) beam of coherent highly narrowed radiation and sent a manuscript on our findings to the journal *Physics Review Letters* on August 26, 1960 ([5], Fig. 2.1).

There was no doubt: This observation was a true laser emission as expected from theory. We used a semitransparent silver coating instead of a hole for out-coupling. On October 5, 1960 we gave a press conference (Fig. 2.2) and demonstrated a red beam of the new light source, fired from the radar tower in New Jersey and detected 25 miles away at Crawfort Hill using a photomultiplier (Fig. 2.3 and 2.4).

The same year, the He:Ne gas laser became operational at Bell [6] and the 4-level-laser was introduced [7, 8].

The very strong light emission by the new light source laser initiated the field of Nonlinear Optics (NLO). In 1961, second harmonic radiation was found in non-centrosymmetric crystals (e.g. quartz) by Franken et al. at the University of Michigan [9] and by Giordmaine at Bell [10]. First two-photon fluorescence was seen in $Eu^{2+}CaF_2$ by Kaiser and Garrett at Bell [11].