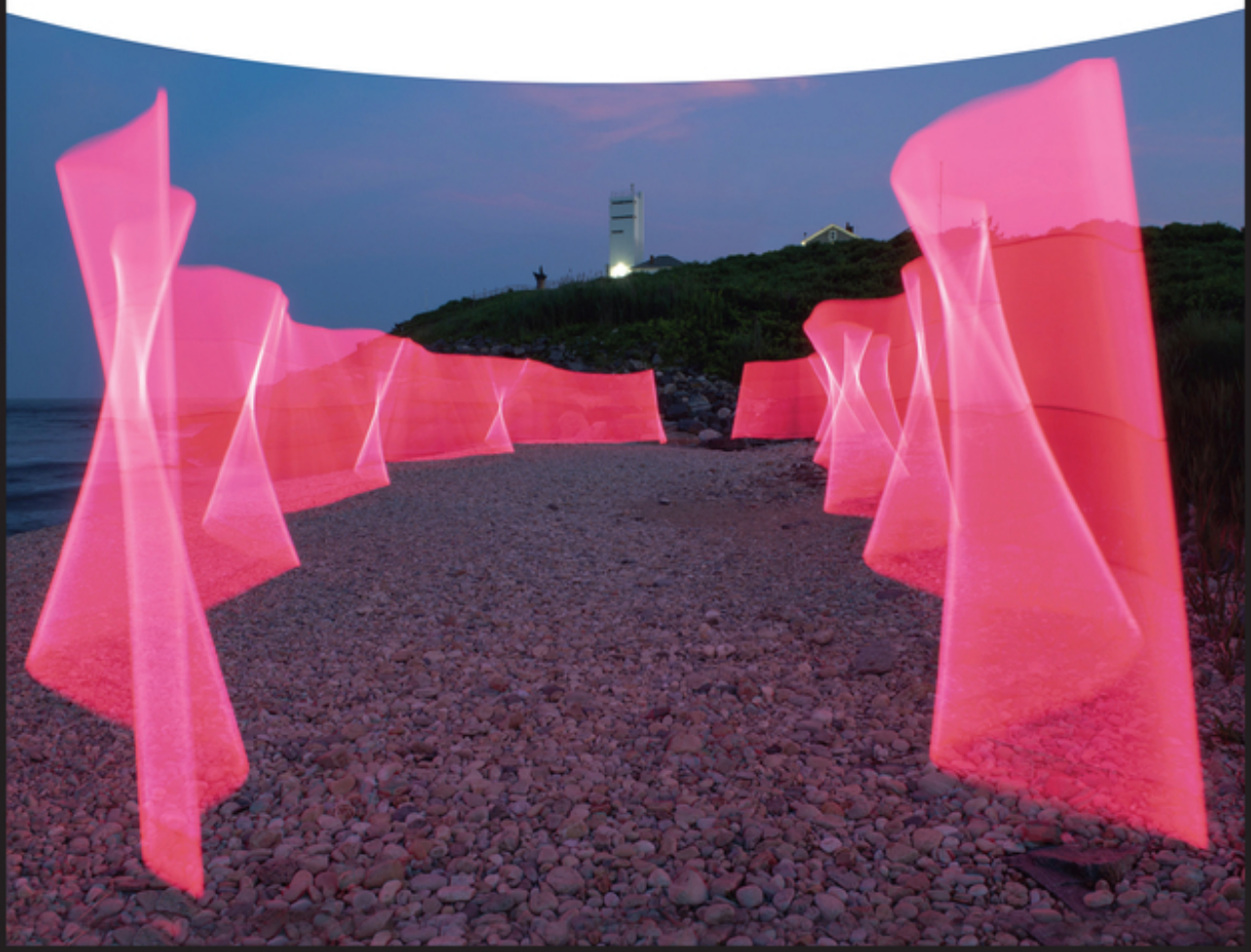
WILEY VCH

Edited by
Emmanuel G. Reynaud and Pavel Tomančák

Light Sheet Fluorescence Microscopy



Light Sheet Fluorescence Microscopy

Light Sheet Fluorescence Microscopy

Edited by Emmanuel G. Reynaud and Pavel Tomančák

WILEY VCH

Editors

Dr. Emmanuel G. Reynaud
UCD Conway Institute
University College Dublin
School of Biomolecular and Biomedical Sciences
Belfield, Dublin 4
Ireland

Dr. Pavel Tomančák
Max Planck Institute of Molecular Cell Biology and Genetics
Pfotenhauerstrasse 108
01307 Dresden
Germany

and

Central European Institute of Technology (CEITEC) consortium
Žerotínovo nám. 617/9
Brno, 601 77
Czech Republic

Cover Image: VickyDaSilva

Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

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.d-nb.de>.

Print ISBN: 978-3-527-34135-1
ePDF ISBN: 978-3-527-80390-3
ePub ISBN: 978-3-527-80393-4
oBook ISBN: 978-3-527-80391-0

Cover Design: ADAM DESIGN, Weinheim, Germany
Typesetting: Straive, Chennai, India

To the two Jeans,
Humanity and kindness
In the flesh

Contents

Foreword by Ernst H. K. Stelzer

In light sheet-based fluorescence microscopy (LSFM), optical sectioning in the excitation process minimizes fluorophore bleaching and phototoxic effects. This allows biological specimens to survive long-term three-dimensional multi-spectral imaging at high spatiotemporal resolution along multiple directions. The illumination of an entire plane allows the recording of images with a camera. Hence, millions of pixels are recorded in parallel, while several tens or hundreds of image are recorded within a few seconds.

LSFM has revolutionized fluorescence microscopy as it allows scientists to perform experiments in an entirely different manner and to record data that had not been accessible before. LSFM is a disruptive technology, because it forces the scientific community to re-think the manner in which it performs its experiments, to avoid flat and hard surfaces, to reconsider quality criteria, and to study as well as maintain the three-dimensional dynamic multicellular structure of essentially all biological specimens.

LSFM is based on an extremely simple and yet ingenious optical arrangement that provides true optical sectioning over an extended field of view. In contrast to epi-fluorescence microscopy, LSFM refers to a technology that observes a specimen with at least one microscope objective lens whose focal plane is illuminated azimuthally by at least one sheet of light. In LSFM, only a thin volume wrapped around the focal plane of the detection microscope objective lens is illuminated. Therefore, endogenous organic molecules or fluorophores in the volumes in front and behind the light sheet do not receive any light and are not subject to photo-damage. These fluorophores cannot contribute to image blurring by out-of-focus light. Three-dimensional image stacks are generated by moving the light sheet and the specimen relative to each other.

In physical terms, LSFM provides true optical sectioning and, therefore, a three-dimensional resolution. Hundreds of planes in different locations along the optical axis of the detection lens are independently illuminated. Since, in to contrast to confocal fluorescence microscopy, the illumination process provides the optical sectioning capability of LSFM, it exposes specimens to 3–5 orders of magnitude less energy than confocal microscopy.

In general, optical sectioning and no phototoxic damage or photo bleaching outside a small volume close to the focal plane are intrinsic properties of LSFM. The

two canonic implementations are selective/single plane illumination microscopy (SPIM) [1] and digital-scanned laser light sheet microscopy (DSLM) [2], which provide a coherent and an incoherent illumination, respectively. In particular, DSLM has become an indispensable tool in developmental biology, three-dimensional cell biology, and plant biology as well as for cleared and expanded specimens. DSLM is also the basis for most LSFM-derived technologies that try to improve spatial resolution.

Light sheets have been known for more than 100 years, but so have light spots. However, until lasers became available in 1960 [3], neither light spots nor light sheets were diffraction limited. Hence, optically sectioning instruments [4] could not be built. A confocal fluorescence microscope, which illuminates a specimen sequentially with a diffraction-limited spot of light, requires a laser as its light source. LSFM, which illuminates a specimen sequentially plane by plane with a diffraction-limited light sheet, also requires a laser as its light source.

Laser light sheet-based macroscopic devices [5] had been built several times, but their capability to perform at a microscopic level was not known until, starting around 2002, my group (Light Microscopy Group [LMG]), then at the European Molecular Biology Laboratory (EMBL) in Heidelberg, built, patented, and applied the SPIM. LSFM was used to observe live biological specimens, assessed for its optical properties [6], evaluated for its applicability for multiple-views imaging [7, 8] and considered for its applicability at the molecular level with fluorescence lifetime imaging microscopy (FLIM)/fluorescence resonance energy transfer (FRET) [9] as well as fluorescence correlation spectroscopy (FCS)-selective plane illumination microscopy (SPIM) [10]. EMBL's LMG had systematically evaluated diffraction-limited microscopes with two to four lenses, both in theory and in practice, since the early 1990s. The disruptive impact of LSFM was recognized in 2015, when Nature Methods announced "Light Sheet-based Fluorescence Microscopy" Method of the Year 2014 [11].

LSFM is the result of developing confocal [12], 4Pi [13], and Theta [14] fluorescence microscopies for about 20 years. It is an excellent example for the achievements of scientists with an interdisciplinary spirit. Patents and papers published since 1993 and seminal papers published since 2004 document the intensity and the determination that are necessary to make the scientific community aware of a disruptive technology. Much further work and the refinements of LSFM enable the imaging of live biological samples under close-to-natural conditions for several days, leading to breakthroughs in, among other fields, developmental biology, neurobiology, and histopathology as well as drug development. The initial work was performed at EMBL. However, many brilliant people have independently pushed the technology to new levels all around the world.

My scientific profile describes a physicist who worked in interdisciplinary environments for more than 35 years. I was able to bridge gaps between physics, optics, and instrumentation on one side and molecular, cell, plant, and developmental biology on the other side. The many steps that are required for reasonable biological systems, excellent data and well-defined mathematical-physical interpretations of biological processes have guided me in my decisions during many seemingly different projects.

During my PhD thesis (1983–1987), I worked on confocal transmission, reflection, and fluorescence microscopy. I developed confocal 4Pi fluorescence microscopy during 1990–1993 and introduced orthogonal and multi-lens detection schemes commencing with confocal theta fluorescence microscopy around 1993. The latter led to the development of the tetrahedral microscope in 1999, which in turn triggered the development of LSFM in 2001. Some of my other contributions include the optical tweezers-based photonic force microscope in 1993 and a novel and very successful approach to laser-based cutting devices in 1999.

I am extremely grateful that I had, and still have, the opportunity to work with many brilliant people who have contributed enormously to the topics that I mentioned before. I seriously hope that I inspire them because I can assure you that they certainly inspired me.

December 2019

Ernst H. K. Stelzer,
Goethe-Universität, Frankfurt am Main, Germany

References

1 Huisken, J., Swoger, J., Del Bene, F. et al. (2004). Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* 305: 1007–1009.
2 Keller, P.J., Schmidt, A.D., Wittbrodt, J., and Stelzer, E.H.K. (2008). Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science* 322: 1065–1069.
3 Maiman, T.H. (1960). Stimulated optical radiation in ruby. *Nature* 187: 493–494.
4 Cox, I.J. (1984). Scanning optical fluorescence microscopy. *Journal of Microscopy* 133: 149–154.
5 Voie, A.H., Burns, D.H., and Spelman, F.A. (1993). Orthogonal-plane fluorescence optical sectioning: three-dimensional imaging of macroscopic biological specimens. *Journal of Microscopy* 170: 229–236.
6 Engelbrecht, C.J. and Stelzer, E.H.K. (2006). Resolution enhancement in a light-sheet-based microscope (SPIM). *Optics Letters* 31: 1477–1479.
7 Swoger, J., Verveer, P., Greger, K. et al. (2007). Multi-view image fusion improves resolution in three-dimensional microscopy. *Optics Express* 15: 8029–8042.
8 Verveer, P.J., Swoger, J., Pampaloni, F. et al. (2007). High-resolution three-dimensional imaging of large specimens with light sheet-based microscopy. *Nature Methods* 4: 311–313.
9 Greger, K., Neetz, M.J., Reynaud, E.G., and Stelzer, E.H.K. (2011). Three-dimensional fluorescence lifetime imaging with a single plane illumination microscope provides an improved signal to noise ratio. *Optics Express* 19: 20743–20750.
10 Wohland, T., Shi, X., Sankaran, J., and Stelzer, E.H.K. (2010). Single plane illumination fluorescence correlation spectroscopy (SPIM-FCS) probes inhomogeneous three-dimensional environments. *Optics Express* 18: 10627–10641.

11 Stelzer, E.H.K. (2015). Light-sheet fluorescence microscopy for quantitative biology. *Nature Methods* 12: 23–26.

12 Wijnaendts van Resandt, R.W., Marsman, H.J.B., Kaplan, R. et al. (1985). Optical fluorescence microscopy in three dimensions: microtomoscopy. *Journal of Microscopy* 138: 29–34.

13 Hell, S. and Stelzer, E.H.K. (1992). Properties of a 4Pi confocal fluorescence microscope. *Journal of the Optical Society of America A: Optics, Image Science, and Vision* 9: 2159–2166.

14 Stelzer, E.H.K. and Lindek, S. (1994). Fundamental reduction of the observation volume in far-field light microscopy by detection orthogonal to the illumination axis: confocal theta microscopy. *Optics Communications* 111: 536–547.

Preface

In the *Science* summer of 2004 issue 5686, a 90° shift in illumination was proposed to establish a new approach to optical sectioning in 3D light microscopy that reduces the phototoxicity of laser illumination and promotes long-term imaging of living samples. The divorce of illumination and detection axes of a microscope dates to 1904 when it was used to visualize colloidal samples. More recently this arrangement was deployed to image the anatomy of the inner ear, but the selective plane illumination microscopy (SPIM) gave the idea a new life and unleashed it to conquer the vast realms of live cell and organism imaging. In the late 2008, we organized a small and rather secretive meeting of the practitioners of the young and still relatively obscure art of light sheet imaging. The small light sheet workshop grew bigger every year, and in 2018 we celebrated 10 years of building an amazing community of light sheet aficionados. The light sheet community diversified, adopting not only microscopy technology developers but also image analysis wizards, Big Data management geeks, and, most importantly, armies of biologists increasingly fond of illuminating their samples with light sheets. The small 90° repositioning of illumination triggered a chain reaction of publications in high-impact journals, generated new commercial opportunities, and left us with more than 100 light sheet designs (and their associated acronyms). We learned to deal not only with various realizations of light sheet hardware but also with the humongous waves of data that demand new computational approaches as well as with creative sample preparations for very long-term imaging sessions where the sample is expected to thrive even after the photoshoot! We have been helping as much as being helped in the quest of building a vibrant light sheet community by organizing practical courses, workshops, and conferences, pushing more and more researchers to jump onto the light sheet bandwagon. Especially fruitful has been the collaboration with companies who generously sponsored many of the events and steadily transformed the optical table light sheet monstrosities into slick automated microscopes which anyone can buy and use. We have been supported from the start by Carl Zeiss Microscopy GmbH, and this book has received financial support from Leica Microimaging GmbH and Cairn Research Ltd.

It has been a long journey to make this book a reality – more than 10 years with many hiccups and false starts. Many people helped along the way, some, given the decade-long gestation, have moved on in their lives past the light sheet addiction. Thanks to Klaus, Urŏs, and Ernst for getting this project off the ground. We also want

to thank all the people who helped us finalize this book by reviewing, commenting, and correcting such a diverse list of topics. Thanks to Hari Shroff, Alberto Diaspro, Philippe Laissue, Nicola Maghelli, Jérémie Capoulade, Olaf Selchow, and Olivier Burri for providing their expert advice and comments. And finally, we are always grateful to be part of such a vibrant community!

In between Dublin and Dresden
September 2023

Emmanuel and Pavel

1

Let There be Light Sheet

Pavel Tomančák[1,2] and Emmanuel G. Reynaud[3]

[1] Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany
[2] Central European Institute of Technology (CEITEC) consortium, Žerotínovo nám. 617/9, Brno, 601 77, Czech Republic
[3] UCD Conway Institute, University College Dublin, School of Biomolecular and Biomedical Sciences, Belfield, Dublin 4, Ireland

The central and essential element of light sheet fluorescence microscopy is the use of a plane of light AKA light sheet to illuminate one plane of the sample at a time. Interestingly, a sheet of light creates not only a plane of light that optically slices the space but also a line of light when it interacts with surfaces (Figure 1.1). This second property of light sheets is extensively used to scan surfaces in industry (e.g. line scan cameras), commerce (e.g. bar code scanners), cleaning (e.g. Dyson V15 Dyson Absolute), architecture and art (e.g. 3D photogrammetry of buildings and sculptures). It also finds practical everyday applications in levelling systems for construction. However, this book revolves around the use of light sheets in microscopy.

1.1 Historical Context of Light Sheet Microscopy – Ultramicroscopy

The use of a plane of light in Selective Plane Illumination Microscopy (SPIM) was a great idea that resonated with the needs of biological imaging community. However, it was not new. In fact, already in 1903, Henry Siedentopf and Richard Zsigmondy utilized a planar sheet of light to image colloids in solution. They called their instrument the Ultramicroscope presumably to reflect its superiority over a conventional microscope.

Richard Zsigmondy worked as a scientific researcher at the Schott AG, a well-known glass manufacturing company in the German town Jena. Schott was a long-time partner providing optical glass to the famous Zeiss Werke, established just on the other side of the town. Zsigmondy apparently grew tired of studying colored and opaque glass and after three years left Schott. He however stayed in Jena and became a private teacher, a profitable position at the time. He married

Light Sheet Fluorescence Microscopy, First Edition. Edited by Emmanuel G. Reynaud and Pavel Tomančák.

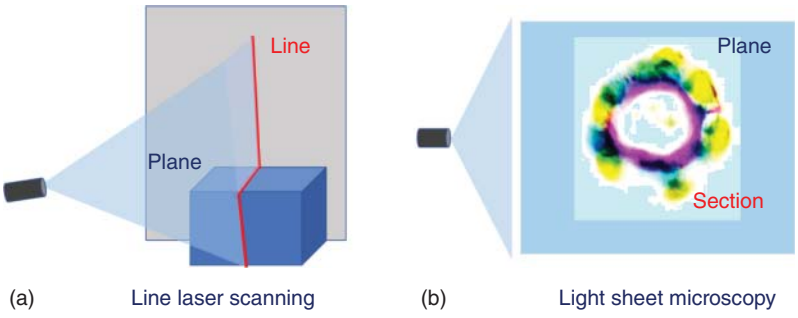


Figure 1.1 A plane of light. (a) can scan a surface or (b) scan a volume.

the daughter of a professor of anatomical pathology and established himself as a free, institutionally independent colloid chemist in Jena. Jena was a well-connected academic town with industries such as Zeiss Werke and Schott alongside a well-known university. Zsigmondy was looking for a way to see microscopic gold particles in colloid solutions (e.g. ruby glass). Colloid chemistry was seen as a very dynamic research field with far-reaching potential to understand the foundations of matter and life.

Zsigmondy managed to get recruited in the Zeiss Werke where he met Henry Siedentopf, a physicist with whom he worked on the development of the Ultramicroscope [1, 2]. They discovered that it was possible to observe very small particles by illuminating the preparation being studied in a direction that is perpendicular to the viewing angle on a dark background (i.e. in a dark field) [3].

The instrument allowed Richard Zsigmondy to prove the heterogeneous nature of colloidal solutions for which he was awarded the Nobel prize in Chemistry in 1925 [4]. Moreover, the Ultramicroscope helped establish the experimental foundations of the kinetic theory (and hence of atomism) by allowing studies of Brownian motion. The advances in colloid chemistry brought about by Ultramicroscopy meant that it played an indirect role in the awarding of the Nobel Prize to Einstein (Physics, 1921), Perrin (Physics, 1926), and Svedberg (Chemistry, 1926).

Henry Siedentopf was appointed Director of the Zeiss Werke' microscope laboratory until he retired in 1938. Together with French physiologist Jean Commandon he modified the Ultramicroscope to enable time-lapse imaging at the time referred to as "Ultramicroscopy microcinematography" [5]. The ultramicroscope was then extensively used as a dark field microscope in microbiology, parasitology, and plant biology until 1935, but then the instrument vanished form the Zeiss Werke catalogue and consequently from the radar of researchers in life sciences.

Interestingly, the term Ultramicroscopy was first revived at the end of 1960 and early 1970 to describe electron microscopy technologies. A journal titled *Ultramicroscopy* was established in 1975 and is defined as *ultramicroscopy deals with the application of all manner of radiation and utilization of any new principles that results in an improved state of the art*.

1.2 Light Sheet Imaging Across the Twentieth Century

The use of light sheet for imaging however did not disappear with the demise of the Ultramicroscope. It moved to different fields. Photosculpture, a technology developed in between 1859 and 1861 by the French sculptor and photograph François Willème (1830 and 1905), used a series of pictures of a subject to create a series of silhouettes to accurately reproduce it in plaster. The principle is rather like the modern Optical Projection Tomography. In 1935, two British Jeffreys Sidney Thomas and Petty Henry John also of Photosculpture Ltd, patented a new photosculpture system using a plane of light to scan the surface of the person and reproduce it with a better precision. It was the first use of line scanning to create a 3D model of the human subject in pictures and reality (plaster) [6]. This use of light sheet imaging for surface monitoring and generation of 3D volumes was further refined during the next 80 years and is now a main element of 3D scanning technology in industry, art, and architecture as well as mundane tasks such as bar code scanning. It finds however also uses in science for instance for macroscopy imaging of insects (3DLSM; [7]).

In the 1970s, groups of researchers investigated the possibility of using the method of laser speckle already developed in solid mechanics and showed that it could be applied to the measurement of fluid velocity fields. In 1977, three different research groups [8–10] independently demonstrated the feasibility of applying the laser speckle phenomenon to fluid flow by measuring the parabolic profile in laminar tube flow. They used a light sheet and established Particle Image Velocimetry (PIV) as an optical method to visualize flow dynamics not only in water but also in gas. Fundamentally, the system used a single fixed illumination plane without 3D capacity, an approach also used in modern Light Sheet Fluorescence Microscopy [11]. PIV became a dynamic field and introduced also 3D imaging (e.g. 3D PIV). Its simplicity made it suitable for extreme environments, for example in underwater systems capable of imaging sea creatures such as larvaceans at great depths (e.g. DeepPIV).

1.3 And here Comes the Flood

The light sheet microscopy winter ended in the last decade of the twentieth century. The revival began with relatively modest contributions whose impact remained confined to the microscopy technology development community. In 1993, light sheet microscopy was for the first time applied to 3D imaging of biological samples - the mouse cochlea [12]. Meanwhile, Ernst H.K. Stelzer and colleagues were developing a theta confocal microscope which used an orthogonal light sheet to illuminate specimens. They published their investigations in two papers that cited the historical Siedentopf [13] and the more recently published Voie [14] work. A few years later, Huber and colleagues presented a portable light sheet-based system for the non-invasive digital and photographic documentation of small objects such

as complete insects without loss of texture, color, or transparency – the 3D light scanning macrophotography (3DLSM) [7]. Building on the use of light sheet-based instruments in fluid mechanics studies, the Jaffe group designed a Thin Light Sheet Microscope (TLSM) for the studies of marine bacteria and larger particles [15].

However, the true revival of the light sheet technique came with the spectacular demonstration of the capabilities of SPIM (Selective and sometimes Single Plane Illumination Microscopy) in imaging of living model organism embryos [16]. It was soon followed by an impressive demonstration of *in toto* recording of an early development of zebra fish [17]. It opened the floodgates. Microscopy is an old and mature research field, and the introduction of a conceptually new design is a rare occurrence. When it happens, the new space becomes quickly populated by the microscopy technology developers who apply older concepts to the new paradigm. This happened in a big way with light sheet microscopy. Since 2004, every component of the light sheet microscope has been re-imagined. Innovation in light sheet formation, scanning, sample mounting, realization of multi angular imaging, and signal detection has led to an explosion of new microscopes [18]. This rapid evolution changed almost everything about the original SPIM set-up. The remaining synapomorphy, a feature shared by all evolutionary descendants of an ancestral species, was the use of light sheet and the physical separation of the illumination and detection. Even systems that rely on a single lens use light sheet and implement this separation [19, 20]. The downside of this Cambrian explosion in the light sheet field was the overproliferation of impenetrable acronyms for the various flavors of the light sheet technology. The paragraph below lists the ever-growing line-up of different realizations of the light sheet microscopes and the associated abbreviations. For a novice consumer of the technology, this menu can be daunting, and it is one of the reasons for putting together this book.

1903 – Ultramicroscope; **1960 – LSP** (Light Scanning Photomacrography); **1980s – PIV** (Particle Image Velocimetry); **1993 –** orthogonal-plane fluorescence optical sectioning (OPFOS); **2002 – TLSM** (Thin Light Sheet Microscopy); **2004 – SPIM** (Selective Plane Illumination Microscopy) or **SPIM (**Single Plane Illumination Microscopy); **2007 – UM** (Ultramicroscope), **mSPIM** (Multidirectional Selective Plane Illumination Microscopy); **2008 – DSLM** (Digital Scanned Laser Light Sheet Fluorescence Microscopy), **OCPI** (Objective Coupled Planar Illumination), **OPM** (Oblique Plane Microscopy), **HILO** (Highly Inclined Laminated Optical); **2009 – TSLIM** (Thin Sheet Laser Imaging Microscopy), **PLIF** (Planar Laser Induced Fluorescence); **2010 – DSLM-SI** (DSLM-Structured Illumination), **MISERB** (Microscopy Self Reconstructing Beam), **SPIM-FCS** (SPIM-Fluorescence Correlation Spectroscopy), **miniSPIM** (obvious!); **2011 – LSFM** (Light Sheet Fluorescence Microscopy), **BBPI** (Bessel Beam Plane Illumination), **iSPIM** (Inverted SPIM), **2P-SPIM** (2 photon SPIM), **IML-SPIM** (Individual Molecule Localization SPIM), **FLIM-SPIM** (Fluorescence Lifetime Imaging Microscopy SPIM); **2012 – 2P-DSLM** (2 photon DSLM), **CSLM** (Confocal Light Sheet Microscopy), **SiMView** (Simultaneous MultiView imaging), **Lightsheet Z.1** (Carl Zeiss GmbH), **OLSM** (Oblique Light Sheet Microscope), **iSPIM** (Inclined SPIM), **MuViSPIM** (Multi View SPIM, Luxendo GmbH), **WAO-SPIM** (Wavefront sensor Adaptive

Optics SPIM), **AO-SPIM** (Adaptive Optics SPIM); **2013 – RSLM** (Reflected Light Sheet Microscopy), **LSBM** (Light Sheet Bayesian Microscopy), **PCLSM** (Prism-Coupled Light Sheet Microscopy), **LST** (Light Sheet Tomography), **OpenSpin**, **OpenSPIM**, **2PE-SPIM** (Two Photon Excitation SPIM), **diSPIM** (Dual view inclined SPIM); **2014 – 2P3A-DSLM** (Two Photon 3 Axis DLSM), **COLM** (Clarity Optimized Light-sheet Microscopy), **MSLM** (Multiple Light Sheet Microscopy), **APOM** (Axial Plane Optical Microscopy), **TC-LSFM** (Tissue culture – LSFM), **LLS** (Lattice Light Sheet), **BTLSM-II** (Bi-directional Triple Light Sheet Microscopy), **SPIM-FCCS** (SPIM Fluorescence Cross Correlation Spectroscopy), **OPTiSPIM** (Optical projection Tomography I SPIM); **2015 – ASLM** (Axially Swept Light Sheet Microscopy), **LEGOLish** (LEGO Light Sheet), **SCAPE** (Swept Confocally-aligned planar excitation), **mu-SPIM**, **oSPIM** (Oblique SPIM), **AdaptiveSPIM** (Obvious!), **doSPIM** (Dual Oblique SPIM), **LatticeSPIM**, **OpenTopSPIM**, **SPIM-Fluid** (Well!); **2016 – diaSLM** (diagonally Swept Light-sheet Microscopy), **CSLM** (Curtailed light sheet microscopy), **RESOLFT** (REversible saturable/Switchable Optical Fluorescence Transitions light-sheet nanoscope), **2PLS-SOFI** (two-Photon super-resolution Light-Sheet imaging via Stochastic Optical Fluctuation Imaging), **STED MISERB** (Another obvious one!), **soLSM** (Single Objective Light-Sheet Microscopy), **cLSFM** (Cardiac LSFM), **PLST** (Polarized Light Sheet Tomography), **PIP** (Plane Illumination Plugin), **SVI-LSM** (Selective Volume Illumination – Light Field Microscopy), **SPIDDM** (Selective Plane Illumination Differential Dynamic Microscopy), **eduSPIM** (Educational SPIM), **2PE-iSPIM** (Two-Photon inverted Selective Plane Illumination Microscopy), **SPIM**, **QuviSPIM** (Quantitative View SPIM, Luxendo GmbH), **TLS-SPIM** (Tiling Light-Sheet SPIM); **2017 – FL-DSLM** (Frequency domain-FLIM DSLM), **HT LSFM** (High Throughput LSFM), **csiLSFM** (coherent structured illumination LSFM)**, aLSFMM** (Augmented Line-Scan Focal Modulation Microscope), **OLST** (Oblique Light Sheet Tomography), **pLSFM** (parallelized LSFM), **sideSPIM** (Easy!); **2018 – 4D LSFM** (like x, y, z, and t?), **3p LSFM** (how many photons?), **3P BB LSFM** (BB for Bessel Beam), **3D LSM** (Why?), **HILO fCT** (fluorescence computed tomography), **HILO LCCT** (HILO Live-Cell CT), **hLCTT** (HILO LCCT), **eLCCT** (epi Live-Cell CT), **HLTP** (high-throughput *light-sheet* tomography platform), **SOPI** (scanned oblique plane illumination), **TILT3D** (*Tilted light sheet* microscopy with 3D point spread functions), **LEMOLish** (LEGO Motorized Light Sheet), **LCS-SPIM** (Large cleared sample SPIM), **SPIM-mPIV** (SPIM-micro Particle Image Velocimetry), **socSPIM** (single objective cantilever SPIM), **SLM-SPIM** (Spatial Light Modulator-SPIM), **2019 – AFM-LS** (single objective cantilever SPIM), **AO-LSFM** (single objective cantilever SPIM), **APOM**, **DOPM** (single objective cantilever SPIM), **LSLFM** (single objective cantilever SPIM), **OTLS** (single objective cantilever SPIM), **SCAPE 2.0**, **eSPIM** (SPIM), **Mars-SPIM** (SPIM), **2020 – dOPM** (Dual-view OPM), **LLSM** (Live-cell Lattice *light-Sheet* Microscopy), **LLSDM** (Lattice *Light-Sheet* Difference *Microscopy*), **compactLSFM** (No comment!), **di2CLSFM** (dual-view inverted confocal *light sheet* fluorescence *microscope*), **AFM-SPIM** (Yes!), **2021 – 4D CMLS** (four-Dimensional Cuboid Multiangle illumination-based Light-sheet Super-resolution), **3D LSRM** (*cubic spline algorithm-based depth-dependent fluorescence-free three-dimensional*

light-sheet super-resolution microscopy), **daoSPIM** (Dual-view adaptive optics SPIM), **DMx-LSFM** (Dual arm Multi-level magnification Light Sheet Fluorescence Microscopy), **IHLLS 1L** (Incoherent Holography Lattice *Light-Sheet Single Lens*), **IHLLS 2L** (Incoherent Holography Lattice *Light-Sheet Dual Lens*), **ICHLLS** (Incoherent Color Holography Lattice Light-Sheet), **DO-DSLM** (Deep-learning On-chip-DSLM), **NIR-II-SIM** (Near-InfraRed II Structured-Illumination light-sheet Microscopy), **COMPASSLSM** (Compact Axially Swept Scanned Light Sheet Microscope), **LIC** (Light sheet Imaging Cytometry), **iLIFE** (Integrated Light-sheet Imaging Flow-based Enquiry), **OTAS-LSM** (Open-Top Axially Swept LSM), **sLSM** (Scattering-based LSM), **BLIM** (Bi-directional *Light-sheet* Illumination Microscope), **DC-APOM** (Digital Confocal-APOM), **3D-iLLS** (3D interferometric lattice *light-sheet*), **MT-SPIM** (Multiview Tiling SPIM), **ldSPIM** (local-delivery SPIM), **M-SPIM** (Multi-view SPIM), **2022 – Flexi-SPIM** (So good!), **OPSIM** (OPM with SIM), **IDDR-SPIM** (Isotropic Divide stages-to-process [IDSP] double-ring [DR] modulation-SPIM), **NIR-II-ASLM** (Near-InfraRed II-ASLM), **LLSDM** (Lattice Light-Sheet by fluorescence Differential Detection), **ctASLM** (cleared-tissue ASLM), **SOLEIL** (Single Objective Lens Inclined Light sheet localization microscopy), **AO-LLSM** (Adaptive Optics-LLSM), **VFC-iLIFE** (Volume Flow Cytometry-integrated Light-Sheet Flow based Enquiry). **And Companies – SPOT** (Single Plane Optical Tomography), **Leica DLS** (Digital Light Sheet), **MuviSPIM-LS** (Live Sample), **MuviSPIM-CS** (Cleared Sample), **MuviSPIM-PM** (PhotoManipulation), **MegaSPIM**, **LCS-SPIM** (Large Cleared Sample), **LightSheet 7**, **QLS scope** (Quantitative Light Sheet), **Smart-SPIM**…

1.4 The Building of a Community

The diversity of available light sheet set-ups led to the broadening of the portfolio of applications of light sheet microscopy in biological research. Developmental biology remains the main driver of light sheet technology development [21]. Animal embryos are large, typically spherical objects rather impenetrable to light. The multi-view imaging implemented by light sheet microscopes gives biologists an equivalent of the access to the dark side of the moon – the part of the embryo away from the detection lens of the microscope. Imaging embryos more or less completely allows biologists to ask questions about long-range interaction between tissues that are difficult to realize with other imaging modalities. Next came applications in neurobiology [22]. Here the speed of light sheet set-ups gives not only access to imaging neuronal reporters in freely behaving animals [23], but has also become the method of choice for volumetric imaging of large, fixed brains [24]. Although resolution is typically not mentioned as the strength of light sheet microscopes, thinner light sheets and higher numerical aperture (NA) objectives have brought the benefits of speed and low phototoxicity also to cell biological investigations of subcellular structures [25]. Light sheet microscopes were always geared toward imaging biological objects in 3D and thus became natural choice for the booming field of organoid research [26]. Affordability of the imaging systems made them also an attractive

choice for researchers in evolutionary biology where light sheets are now routinely used to capture the biodiversity of embryonic and adult forms [27]. In fact, one would have a hard time to find a research field that was not impacted by light sheet microscopy. From plants to beating hearts [28, 29], from single molecule biophysics to histology [30, 31], everywhere, the speed, versatility, and low photodamage of light sheet has opened new avenues. Another purpose of this book is to give the glimpse of the impact that light sheet technology has had on biological research.

Light sheet microscopy is an imaging technique developed to help answer biological questions, yet its impact goes beyond biology. Light sheet microscopes are notorious for producing vast amounts of image data. Regardless, whether a biological object is imaged fast, for a very long time or a single vast object is scanned at high-resolution, the results are Terabytes of imagery. Engineering challenges associated with storing such data, moving them around and opening them for inspection required definition of new ways for representing image data in computers [32]. Moreover, the light sheet data are not only big, but they are also often not directly usable in their raw form. Microscopy image post-processing kept researchers in applied computer science busy long before introduction of light sheets, but with their arrival the challenges multiplied due to the sheer scale of the image data [33]. Finally, the diversity of microscopes combined with diversity of biological applications inevitably resulted in a tremendous diversity of image analysis tasks that need to be solved for the big image data. Together, these challenges have led to a much closer collaboration between biology and computer science communities on light sheet datasets. The beneficial side effect of this interdisciplinary collaboration has been the development of powerful open-source platforms designed to deal with image analysis at scale [34]. Several chapters in this book highlight how light sheet fueled new concepts in biological image data analysis, making the algorithms resilient to whatever volume of data microscopists ultimately reach.

Over the past two decades, an active interdisciplinary research community formed around light sheet microscopy. It unites physicist, engineers, biologists, and computer scientists in a quest to build, disseminate, and apply ever more sophisticated light sheet microscopes to frontier biological questions. This community was initially small and met for intense invitation-only workshops [35]. As it grew, light sheet microscopy became increasingly featured at major microscopy conference venues and the light sheet dedicated workshops grew to full scale conferences. The experimental light sheet systems developed in academic labs, typically for specific research purposes, have become increasingly complemented by general purpose microscopy products developed at companies. An important role in the growth of the light sheet community was played by the EMBO practical courses where the academic labs interacted with the commercial sector [36]. The level of trust and information exchange among the two worlds are rather unprecedented in the light sheet field and supports sustainable development of the technology. It is well documented by the productive co-existence of several open-access light sheet microscopy projects alongside similar but more advanced commercial products [37]. This book reflects the partnership between academia and industry through several chapters dedicated to the open access and commercially available systems.

In 2008 a typical light sheet microscope was a complex-looking arrangement of Thorlabs components on an optical table curiously distinguished by the absence of an eye piece. Nowadays, it is more likely to be a shiny box with a company logo and still no eye piece. Decade ago, light sheet microscopists could be seen running around with a bouquet of capillaries loaded with samples embedded in agarose. These days, light sheet microscopes have been adapted to almost all possible sample-mounting paradigms including the dreaded coverslips. While early on light sheet image processing and analysis was a dark magic accessible only to computer geeks, now it is a matter of clicking a button (almost). Fifteen years ago, we would be impressed with volumetric imaging that lasted for a day or with capturing a slice through a beating heart. Today, organoids are happily growing for weeks under constant surveillance of light sheet microscopes, and light sheets are slicing through tissues expanded to almost macroscopic dimensions. The progress has been spectacular, and it is difficult to predict what will come next. It took ten years to put together this book. Let us hope that in another ten years we and/or our AI assistants will look with nostalgia at the simple light sheet microscopes described in this book, dealing with such ridiculously small data, and achieving only supra-atomic resolution in imaging of natural as opposed to synthetic biological systems.

References

1 Cahan, D. (1996). The Zeiss Werke and the ultramicroscope: the creation of a scientific instrument in context. In: *Scientific Credibility and Technical Standards in 19th and early 20th century Germany and Britain*. Archimedes, vol. 1 (ed. J.Z. Buchwald). Dordrecht: Springer https://doi.org/10.1007/978-94-009-1784-2_3.

2 Siedentopf, H. and Zsigmondy, R. (1903). Über Sichtbarmachung und Groessenbestimmung ultra mikroskopischer Teilchen, mit besonderer Anwendung auf Goldrubinglaesern. *Annals of Physics* 10: 1–39.

3 Thirkill, H. (1909). Ultramicroscopy and ultramicroscopic particles. *Science Progress in the Twentieth Century (1906–1916)* 4 (13): 55–89.

4 Zsigmondy, R. (1916). Nobel Lecture. NobelPrize.org. Nobel Prize Outreach AB 2023. https://www.nobelprize.org/prizes/chemistry/1925/zsigmondy/lecture/ (accessed 13 February 2023).

5 Mappes, T., Jahr, N., Csaki, A. et al. (2012). The invention of immersion ultramicroscopy in 1912-The Birth of Nanotechnology? *Angewandte Chemie International Edition* 51 (45): 11208–11212.

6 Jeffreys S. T. and Petty H. J. (1939). Photosculpture. US patent No. 2163124A. https://patents.google.com/patent/US2163124A/en (accessed 13 February 2023).

7 Huber, D., Keller, M., and Robert, D. (2001). 3D light scanning macrography. *Journal of Microscopy* 203: 208–213. https://doi.org/10.1046/j.1365-2818.2001.00892.x.

8 Barker, D.B. and Fourney, M.E. (1977). Measuring fluid velocities with speckle patterns. *Optics Letters* 1: 135–137.

9 Dudderar, T.D. and Simpkins, P.G. (1977). Laser speckle photography in a fluid medium. *Nature* 270: 45–47.

10 Grousson, R. and Mallick, S. (1977). Study of flow pattern in a fluid by scattered laser light. *Applied Optics* 16: 2334–2336.

11 Karaköylü, E.M., Franks, P.J.S., Tanaka, Y. et al. (2009). Copepod feeding quantified by planar laser imaging of gut fluorescence. *Limnology and Oceanography: Methods* 7: https://doi.org/10.4319/lom.2009.7.33.

12 Voie, A.H., Burns, D.H., and Spelman, F.A. (1993). Orthogonal-plane fluorescence optical sectioning: three-dimensional imaging of macroscopic biological specimens. *Journal of Microscopy* 170 (3): 229–236.

13 Lindek, S., Pick, R., and Stelzer, E.H.K. (1994). Confocal theta microscope with three objective lenses. *The Review of Scientific Instruments* 65: 3367–3372.

14 Stelzer, E.H.K., Lindek, S., Albrecht, S. et al. (1995). A new tool for the observation of embryos and other large specimens: confocal theta fluorescence microscopy. *Journal of Microscopy* 179: 1–10.

15 Fuchs, E., Jaffe, J., Long, R., and Azam, F. (2002). Thin laser light sheet microscope for microbial oceanography. *Optics Express* 10 (2): 145–154.

16 Huisken, J., Swoger, J., Del Bene, F. et al. (2004). Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* 305 (5686): 1007–1009. https://doi.org/10.1126/science.1100035. PMID: 15310904.

17 Keller, P.J., Schmidt, A.D., Wittbrodt, J., and Stelzer, E.H.K. (2008). Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science* 322 (5904): 1065–1069.

18 Power, R.M. and Huisken, J. (2017). A guide to light-sheet fluorescence microscopy for multiscale imaging. *Nature Methods* 14 (4): 360–373.

19 Bouchard, M.B., Voleti, V., Mendes, C.S. et al. (2015). Swept confocally-aligned planar excitation (SCAPE) microscopy for high-speed volumetric imaging of behaving organisms. *Nature Photonics* 9 (2): 113–119.

20 Dunsby, C. (2008). Optically sectioned imaging by oblique plane microscopy. *Optics Express* 16 (25): 20306–20316.

21 Weber, M. and Huisken, J. (2011). Light sheet microscopy for real-time developmental biology. *Current Opinion in Genetics & Development* 21 (5): 566–572.

22 Keller, P.J., Ahrens, M.B., and Freeman, J. (2014). Light-sheet imaging for systems neuroscience. *Nature Methods* 12 (1): 27–29.

23 Ahrens, M.B., Orger, M.B., Robson, D.N. et al. (2013). Whole-brain functional imaging at cellular resolution using light-sheet microscopy. *Nature Methods* 10 (5): 413–420.

24 Ueda, H.R., Ertürk, A., Chung, K. et al. (2020). Tissue clearing and its applications in neuroscience. *Nature Reviews Neuroscience* 21 (2): 61–79.

25 Chen, B., Legant, W.R., Wang, K. et al. (2014). Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution. *Science* 346 (6208): 1257998.

26 Serra, D., Mayr, U., Boni, A. et al. (2019). Self-organization and symmetry breaking in intestinal organoid development. *Nature* 569 (7754): 66–72.

27 Wolff, C., Tinevez, J., Pietzsch, T. et al. (2018). Multi-view light-sheet imaging and tracking with the MaMuT software reveals the cell lineage of a direct developing arthropod limb. *eLife* 7: e34410.

28 Mickoleit, M., Schmid, B., Weber, M. et al. (2014). High-resolution reconstruction of the beating zebrafish heart. *Nature Methods* 11 (9): 919–922.

29 Valuchova, S., Mikulkova, P., Pecinkova, J. et al. (2020). Imaging plant germline differentiation within Arabidopsis flowers by light sheet microscopy. *eLife* 9: e52546.

30 Ritter, J.G., Veith, R., Veenendaal, A. et al. (2010). Light sheet microscopy for single molecule tracking in living tissue. *PLoS One* 5 (7): e11639.

31 Patel, K.B., Liang, W., Casper, M.J. et al. (2022). High-speed light-sheet microscopy for the in-situ acquisition of volumetric histological images of living tissue. *Nature Biomedical Engineering* 6 (5): 569–583.

32 Pietzsch, T., Saalfeld, S., Preibisch, S., and Tomancak, P. (2015). BigDataViewer: visualization and processing for large image data sets. *Nature Methods* 12 (6): 481–483.

33 Preibisch, S., Saalfeld, S., Schindelin, J., and Tomancak, P. (2010). Software for bead-based registration of selective plane illumination microscopy data. *Nature Methods* 7 (6): 418–419.

34 Eliceiri, K.W., Berthold, M.R., Goldberg, I.G. et al. (2012). Biological imaging software tools. *Nature Methods* 9 (7): 697–710.

35 Reynaud, E.G. and Tomancak, P. (2010). Meeting report: first light sheet based fluorescence microscopy workshop. *Biotechnology Journal* 5 (8): 798–804.

36 Reynaud, E.G., Peychl, J., Huisken, J., and Tomancak, P. (2015). Guide to light-sheet microscopy for adventurous biologists. *Nature Methods* 12 (1): 30–34.

37 Pitrone, P.G., Schindelin, J., Stuyvenberg, L. et al. (2013). OpenSPIM: an open-access light-sheet microscopy platform. *Nature Methods* 10 (7): 598–599.

2

Illumination in Light Sheet Fluorescence Microscopy

Rory M. Power[1,2] *and Jan Huisken*[2,3]

[1] *EMBL Imaging Centre, EMBL Heidelberg, Meyerhofstr. 1, 69117 Heidelberg, Germany*
[2] *Morgridge Institute for Research, 330 N Orchard St, Madison, WI 53715, USA*
[3] *Multiscale Biology, Department of Biology and Psychology, Georg-August-University Göttingen, Friedrich-Hund-Platz 1, 37077 Göttingen, Germany*

2.1 Introduction

The light sheet provides the single feature differentiating light sheet fluorescence microscopy (LSFM) from other technologies, and to understand its crucial role, it is essential to appreciate the basis of the microscope itself: the widefield epi-fluorescence microscope. These systems are robust, simple, and commonly used for biological imaging, particularly for imaging thin samples, where they remain a powerful tool in the light microscopist's arsenal. Widefield epi-illumination of the field of view leads to excitation of fluorophores throughout the entire volume (Figure 2.1a) and is most often achieved using a broadband lamp and filters to select the appropriate excitation band. The associated emission is collected by the same objective lens, spectrally filtered to remove stray illumination, and imaged onto a camera in a widefield manner. However, biological systems are inherently three-dimensional (3D), and for thicker samples, this scheme presents several issues. Since the detection system can only maintain a sharp focus over a limited axial range (typically a few micrometers), image contrast is degraded by the diffuse contributions from out-of-focus emitters. Furthermore, to produce volumetric images, the entire volume is repeatedly exposed, resulting in photodamage. The epi-fluorescence microscope does, however, benefit from an efficient detection system capable of imaging the full field of view simultaneously.

The primary counterpart to the epi-fluorescence microscope in modern fluorescence microscopy is the laser-scanning confocal microscope [1]. These systems excite fluorophores within an approximately bi-conic volume produced by a tightly focused (high numerical aperture [NA]) laser beam. The emitted fluorescence is spatially filtered through a confocally situated pinhole onto a single-element detector (photomultiplier tube or avalanche photodiode), which detects only the signal emitted in the vicinity of the beam focus (Figure 2.1b). The confocal microscope possesses the quality of optical sectioning, whereby thick samples not

Light Sheet Fluorescence Microscopy, First Edition. Edited by Emmanuel G. Reynaud and Pavel Tomančák.

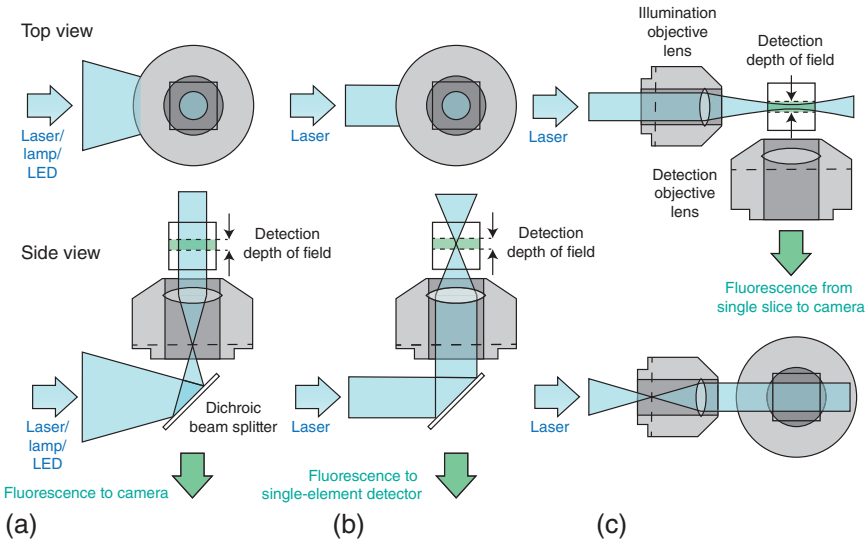


Figure 2.1 Illumination strategies in fluorescence microscopy. (a) Schematic of an inverted widefield epi-fluorescence microscope. The sample volume is illuminated in a widefield manner through the single objective lens. The signal throughout the illuminated volume is collected by the same objective lens (colinear illumination/detection) and imaged onto a camera. A thin slice remains in focus, as governed by the objective lens depth of field. (b) Schematic of an inverted laser-scanning confocal microscope. A biconic region of the sample is illuminated by a tightly focused (high NA) laser beam. Fluorescence is again collected through the same objective lens and a pinhole rejects all signal other than that from the focal region (not shown). (c) Schematic of a light sheet fluorescence microscope (static light sheet as described in Section 2.4.1). In the top view the laser beam is collimated at the back focal plane of a second objective lens used for illumination and focused to a thin sheet. In the side view the beam is focused at the back focal plane of the illumination objective and thus the sheet is collimated at the sample. Fluorescence is collected by a dedicated detection objective lens. Note that the top and side views may be switched in different microscope configurations, but the convention of denoting the focusing dimension as the top view is used throughout. Note that the objective lens schematics throughout are illustrative only and do not reflect the large number of optical elements typically present.

suitable for imaging by widefield epi-fluorescence may be imaged in 3D without contrast-degrading blur. The caveat is that the point-wise illumination and detection scheme is slow and highly inefficient, requiring that the sample be serially scanned to construct a 3D image, exacerbating the issue of overexposure shared with the widefield epi-fluorescence microscope.

The light sheet microscope leverages the benefits of the widefield epi-fluorescence microscope by employing the same efficient detection module as its basis and the optical sectioning ability of the laser-scanning confocal microscope. Rather than illuminating indiscriminately, the light sheet microscope confines excitation to the focal plane of the objective used for detection, thus circumventing the related issues of overexposure and contrast degradation [2]. Furthermore, the peak intensity (power per unit volume) experienced by the sample, which largely governs the rate of photodamage, may be greatly reduced relative to the confocal microscope (for equivalent fluorescence yield). For example, consider the imaging of a field of view

of $1000 \times 1000\,\mu m^2$. In the light sheet case, the entire field of view is illuminated simultaneously. However, in the confocal case, the area probed at any instant is perhaps just c. $0.2\,\mu m^2$ and so to maintain the signal rate, the peak intensity must increase by c. 5×10^6. In LSFM, light confinement is achieved by adding a second optical pathway aligned orthogonally to the first to deliver plane-wise illumination in the form of a light sheet, as shown in Figure 2.1 (iii).

The remarkable simplicity of this scheme belies its power: the light sheet fluorescence microscope is capable of imaging at high speed with a high signal-to-noise ratio and crucially over long periods of time, owing to its low photodamage potential [2]. Other aspects such as robustness, typically large working distances, tunability to a given application, and affordability are no less important. Taken together, these aspects have revolutionized modern biological microscopy, yet it is the light sheet itself that has enabled this success, and it is to its understanding and design that our attention turns.

2.2 Axial Resolution and Optical Sectioning in Light Sheet Microscopy

As much as any other criteria, spatial resolution and optical sectioning ability define the performance of any light microscope. Naturally, other factors are critically important, more so in some cases, but it is in this classical domain that we are most able to make a connection with and draw comparisons with other fluorescence microscopes. Furthermore, an examination of these factors is necessary to comprehend the significance of the light sheet dimensions (discussed in Section 2.3).

While illumination provides the focus of this chapter, understanding the performance of a light sheet microscope requires a more all-encompassing approach. Without taking a holistic view, it is not possible to make any quantitative predictions describing how a light sheet microscope forms an image, and so at this juncture, it is necessary to include the detection path in the discussion. The reader is referred to Chapter 4 for more details in this regard.

2.2.1 The Point Spread Function in Fluorescence Microscopy

Fluorescence provides the dominant contrast mechanism used in light sheet microscopy and so we consider only incoherent imaging. First, we consider the imaging of a generalized object. We can define some object function $O(x,y,z)$, which describes the features of our sample fully; for fluorescent imaging, this may be the position of each emitter in space. Naturally, our desire is to fully reproduce its features when capturing an image, $I(x,y,z)$. In practice, this is not possible since a microscope acts as a low-pass filter, removing high spatial frequency information (diffracted at larger angles from the optical axis), with a cut-off frequency determined by the wavelength of light and NA of the objective lens. Perhaps more intuitively, the microscope blurs the image, masking fine detail and thereby limiting resolution. This blurring or filtering can be quantified by two interrelated

functions: the point-spread function (PSF) and its frequency-space partner, the optical transfer function (OTF). The two are linked by the Fourier transform properties of a lens, and one may consider the image formed in either domain.

$$I(x,y,z) = O(x,y,z) \otimes PSF(x,y,z) \tag{2.1}$$

$$\mathcal{F}[I(x,y,z)] = \mathcal{F}[O(x,y,z)] \cdot OTF(kx,ky,kz) \tag{2.2}$$

where $\otimes$ denotes a convolution. The PSF is a more intuitive description of the resolving power of a microscope, and so we use this description throughout. To illustrate, a point emitter (mathematically equivalent to a δ-function) will produce an image of the PSF. Correspondingly, a more confined PSF delivers a superior, sharper image, and defines the ability of a microscope to discriminate between two neighboring points. This constitutes the most commonly defined description of spatial resolving power, and so the formulation of the PSF is crucial. It is worth noting that this description assumes that the PSF/OTF are invariant across space. This assumption is reasonable for a field of view determined by the specific design of the various optical components of a microscope (most notably the objective lenses). However, in the cases of poorly corrected optical elements or in heavily aberrating/scattering environments, this assumption is invalid, and the PSF/OTF will be spatially variant.

Typically, the PSF of a microscope comprises individual contributions from illumination and detection. Herein lies the justification as to why we cannot quantify spatial resolution by considering of illumination alone. For clarity, we refer to the microscope PSF as the system PSF or PSF_{sys}, and the illumination and detection PSFs as PSF_{ill} and PSF_{det}, respectively:

$$PSF_{sys} = PSF_{ill} \cdot PSF_{det} \tag{2.3}$$

2.2.2 The Point Spread Function in Light Sheet Fluorescence Microscopy

On our way to a formulation of the PSF of a light sheet microscope, it is worth briefly discussing how the widefield epi-fluorescence microscope PSF arises. Since this provides the basis for the light sheet microscope, it is instructive to illustrate how the illumination component affects PSF_{sys}. Since the widefield epi-illumination is delivered homogeneously throughout the volume, it may be stated that PSF_{ill} has unit intensity throughout all space. Correspondingly, PSF_{sys} is equal to PSF_{det}. The formulation of this quantity is beyond the scope of this chapter, and in the remaining discussion, we follow Born and Wolf [3] in calculating the scalar approximation of PSF_{det} (Figure 2.2). The resulting PSF_{sys} is more extended axially than laterally, leading to a lower axial resolution. Furthermore, the PSF features a gradually diminishing intensity away from the focus, yet when integrating over any plane orthogonal to the detection axis, the result is constant, and so an out of focus object contributes as much to the background when out of focus as it does to the signal when in focus. It follows that thick samples cannot be imaged with high contrast with epi-fluorescence microscopy.

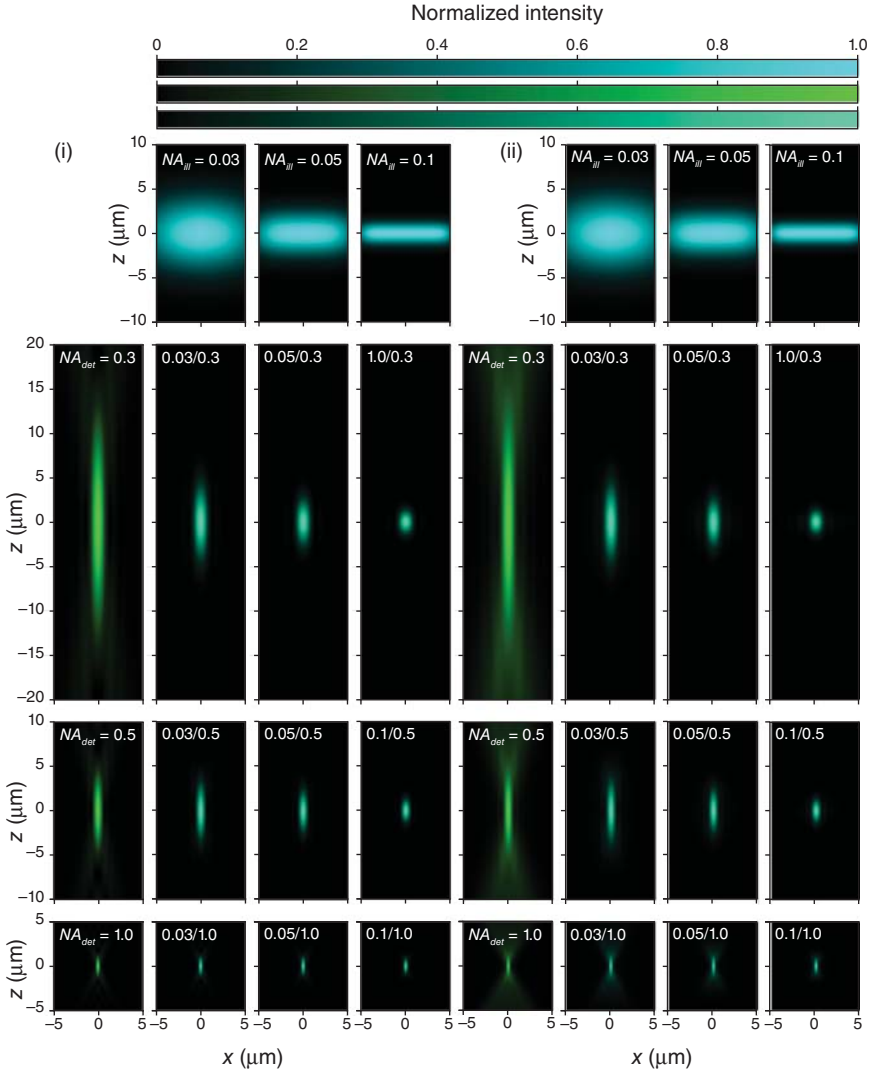


Figure 2.2 Origin of the point spread function (PSF) in light sheet fluorescence microscopy. (i) PSFs displayed as a cross section through their *xz (lateral-axial)* center and illustrating the lateral and axial resolving power. (ii) PSFs displayed as a summed projection along *y* (arbitrarily defined for now but orthogonal to the optical axis *z*) and illustrating the quantity of diffuse out-of-focus background produced when imaging a single incoherent point emitter. Top (cyan): the illumination PSF for different focusing NA and corresponding to a scanned light sheet as described in Section 2.4.2. Left (green): the widefield epi-fluorescence detection PSF for different detection NA. The system PSF arising from the grid-wise combination of individual illumination and detection components (turquoise). All scale bars = 5 μm. Illumination and detection parameters: $\lambda_{0,ill} = 488$ nm $\lambda_{0,det} = 510$ nm, immersion media refractive index, $n_{imm} = 1.33$.

In light sheet microscopy, samples are most commonly illuminated with a Gaussian beam (Section 2.3), and the PSF_{ill} describes the light sheet intensity throughout space (Figure 2.2). Since the illumination and detection PSFs are orthogonally aligned, the rapid decay of intensity away from the focal plane allows the contrast degrading tails of the detection PSF to be suppressed in the resulting PSF_{sys}. It follows that the lateral resolution is unchanged relative to the widefield system, but clearly the form of PSF_{ill} is crucial in determining the improvement of axial resolution and optical sectioning.

It is worth clarifying the terminology here. Axial resolution denotes the ability of a microscope to resolve two point emitters spaced some distance apart along the optical axis. Since the illumination PSF has some finite thickness and a typically Gaussian envelope, the PSF decreases more rapidly away from the detection focal plane, providing superior axial resolving power than epi-fluorescence microscopy. Practicalities relating to the light sheet often dictate that the improvement in axial resolution is marginal and that the optical sectioning power is where the true benefits of the orthogonal illumination–detection scheme lie. Optical sectioning may be viewed as the suppression of out-of-focus fluorescence during the collection of the in-focus signal. This is accomplished in light sheet microscopy by restricting the light sheet to an axial range that is less than the detecting depth of focus. The thicker the light sheet, the worse the optical sectioning will be, converging to the epi-fluorescence case as the sheet thickness increases. Optical sectioning may then be considered to place a practical limit on the achievable resolution. In a poorly sectioned system, out-of-focus light degrades contrast and the ability to resolve two laterally or axially separated points. The axial resolution and optical sectioning ability of a light sheet microscope may be assessed from the associated PSFs. The effect of changing NA_{ill} on these two quantities for different NA_{det} is shown in Figure 2.3. Clearly, a higher NA_{ill} results in superior axial resolution and sectioning, which become particularly apparent as NA_{det} increases (leading to a decrease in the detection depth of field). Why then would one choose not to employ high NA_{ill} to attain high axial resolution and sectioning simultaneously? To fully understand the consequences of such a decision, one must first appreciate the paramount status of the light sheet dimensions and their influence on the performance of the microscope holistically.

2.3 Light Sheet Dimensions

The formulation of the PSF shows the importance of the light sheet thickness in determining the axial resolving and optical sectioning abilities of the microscope. We have shown that thinner light sheets lead to superior axial resolution and sectioning, yet the discussion has intentionally avoided considering how the light sheet dimensions arise. The term “light sheet” suggests a quasi-2D plane of light with uniform thickness. For the sake of discussion, this allows us to define three dimensions for the light sheet. Figure 2.4 illustrates how these axes are defined. Briefly, we denote the light sheet propagation axis, x corresponding to the light sheet length, the

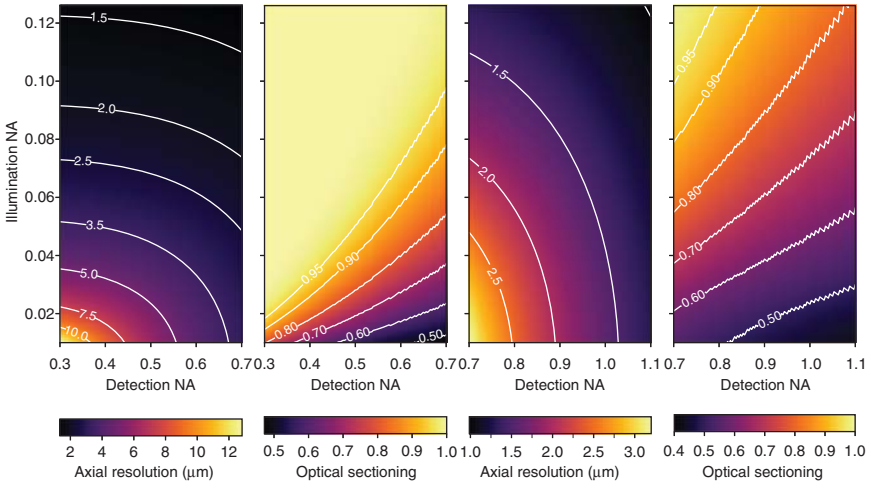


Figure 2.3 Axial resolution and optical sectioning ability of light sheet fluorescent microscopes with different combinations of illumination and detection NA (at the center of the field of view). Low values of axial resolution (high resolving power) and high values of optical sectioning (low out-of-focus contribution to signal) are desirable (a) low-moderate NA_{det}, (b) moderate-high NA_{det}. (i), Axial resolution is calculated as the full-width at half maximum (FWHM) of the system PSF (as in Figure 2.2). (ii) Optical sectioning ability here defined by the integrated intensity of the light sheet contained within the detection system depth of focus (calculated as $\pm\lambda_{0,det}\, n_{imm}/NA_{det}^{\,2}$) relative to the entire simulation volume: (a) $x,y,z = 10 \times 10 \times 30$ μm (b) $x,y,z = 10 \times 10 \times 10$ μm. Illumination and detection parameters: $\lambda_{0,ill} = 488$ nm $\lambda_{0,det} = 510$ nm, immersion media refractive index, $n_{imm} = 1.33$. Gaussian beam simulations have been performed following Section 2.3.

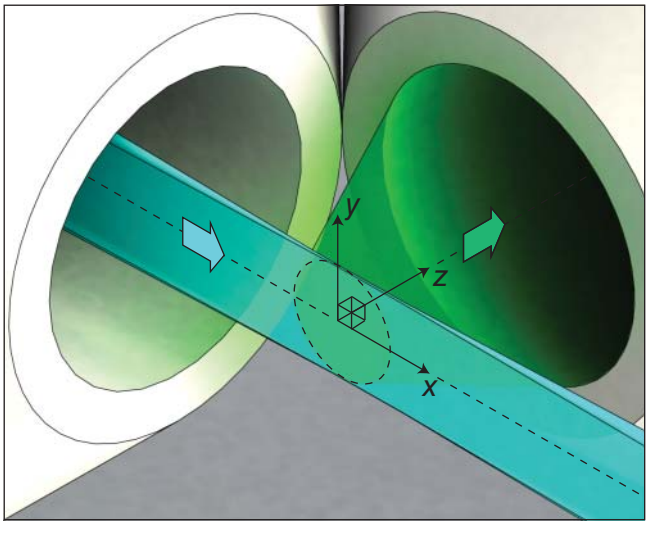


Figure 2.4 Definition of light sheet axes. The light sheet (cyan) propagates in the *x* direction. It is collimated in *y* and is focused along *z*. The light sheet extent in these three axes are denoted the length, height, and thickness, respectively. The thinnest part of the light sheet (its waist) is aligned with the center of the field of view defined by the detection optics. Fluorescent emission collected by the detection objective is shown in green and is rotationally symmetric about the *z*-axis.

detection axis z corresponding to the light sheet thickness, and the third mutually perpendicular axis as y, which most commonly corresponds to the light sheet height. We use this nomenclature regardless of the microscope geometry for consistency throughout (see Chapter 5 for more details on microscope geometry). It should be noted that most optical texts will define z as the propagation axis. However, in our configuration of two optical axes (illumination and detection), we conform to the light sheet microscopy convention of defining the coordinate system relative to the detection axis.

Before describing how the light sheet may be shaped in each of these dimensions, we first note the impact of each on image formation. The light sheet thickness (z-axis) has already been covered with regard to the system PSF, but the importance of the light sheet lateral extent (x,y axes) remains to be addressed. These quantities set an upper limit on the size of the field of view and should be matched to the corresponding detection configuration. We can conclude that the light sheet should be thin (along z) and as large as required (along x and y). The first of these requirements provides a useful starting point: a collimated beam, appropriately focused, may produce a sub-μm thick light sheet; however, the beam will spread rapidly away from its waist (along x), and our description of the illumination source as a light sheet suggests a pseudo-uniform thickness throughout (for simplicity, we can dispense with the y-axis for now, which need not be coupled to the light sheet thickness). Recalling our description of image formation via the system PSF, we see that the condition of spatial invariance is not met for a diverging beam since PSF_{ill} is itself spatially variant. For example, a tightly focused yet highly divergent beam would lead to a highly spatially variant PSF_{sys} (and hence axial resolution and optical sectioning ability) converging to the widefield epi-fluorescence case toward the periphery of the field of view. Correspondingly, the assertion that a thinner light sheet will deliver superior axial resolution and optical sectioning is reasonable at the mutual illumination–detection focus, but divergence of the illumination away from there means the benefits of sheet illumination only apply over a limited distance. Although PSF_{ill} is spatially variant, we may approximate spatial invariance where the divergence is small over the field of view. Under such conditions, Eqs. (2.1)–(2.3) provide excellent approximations. Consequently, the divergence of the light sheet will ultimately limit the usable field of view. To explore the interplay between light sheet thickness and divergence, we must consider in detail how these dimensions arise from beam focusing.

2.3.1 Gaussian Optics Description of Beam Focusing (*x*,*z* Axes)

It should be noted at this juncture that accurately modeling light sheet propagation requires full treatment of diffraction at the lens aperture. One would have to fully define the complex-valued pupil function describing the phase and amplitude of the incident field, which itself may be dependent on aberrations introduced upstream of the lens used for illumination. These treatments fall within the domain of Fourier optics and optical system design, respectively. Several texts exist for readers that wish to explore these topics in detail [4, 5]. In all cases, we will pursue a simple analytic