LIGHT FOR BIO-IMAGING

Joerg Bewersdorf – DOI: 10.1051/epn/2015502
Department of Cell Biology – Yale University School of Medicine – New Haven, CT, USA

The biological sciences have seen tremendous progress over the last decades - sequencing the human genome is just one example - and biology has been proclaimed to be the scientific discipline of the 21st century [1]. These advances have been enabled through tools developed by physicists. Light and light-based technologies, in particular, have been of utmost importance to this progress.

Light technology and biology: a long-standing partnership

Already the discovery of the first cells and the emergence of the field of microbiology in the 17th century were closely linked to progress in optics at the time. It was their pioneering work in the construction of microscopes that allowed Robert Hooke and Antonie van Leeuwenhoek to identify cells in cork sections and find bacteria and other microorganisms, respectively. Towards the end of the 19th century, the mathematical description of optics by Ernst Abbe and others allowed to produce higher quality optical instruments, a development which coincided with the discovery of sub-cellular structures such as chromosomes and the Golgi apparatus. In the 20th century, the invention of phase contrast microscopy by Zernike and the development of laser scanning microscopy, enabled by the invention of the laser, provided new contrast mechanism and imaging modalities in microscopy. Photon detectors sensitive enough to detect single fluorescent molecules and optical tweezers which can pull at single molecules with piconewton forces by means of highly focused laser beams have led to the thriving field of single-molecule biophysics. Even more recently, breaking the diffraction barrier of light in far-field optical microscopy, which has been awarded by the 2014 Nobel Prize in Chemistry (fig. 1), has increased the resolution of light microscopes by another order of magnitude and now allows for the first time to visualize nanoscale dynamics in living cells.

Similarly, progress in optical technology has provided new tools for medical diagnostics and treatment. Examples include the development of endoscopy beginning in the early 1800's and range to more recent innovations such as optical coherence tomography, the optical equivalent of ultrasound imaging which has been enabled by the emergence of bright, low-coherence light sources such as ultrashort laser pulses. Laser scalpels and photodynamic therapy in which a photosensitive drug is applied that, upon application of light, becomes toxic to targeted cells, for example cancer cells, are examples for medical treatments enabled by light.

Light Technology in Biology: More than Just Optics

Over the last century, the nature of the technological breakthroughs that have contributed to new biology has changed. The initial milestones were primarily in classical

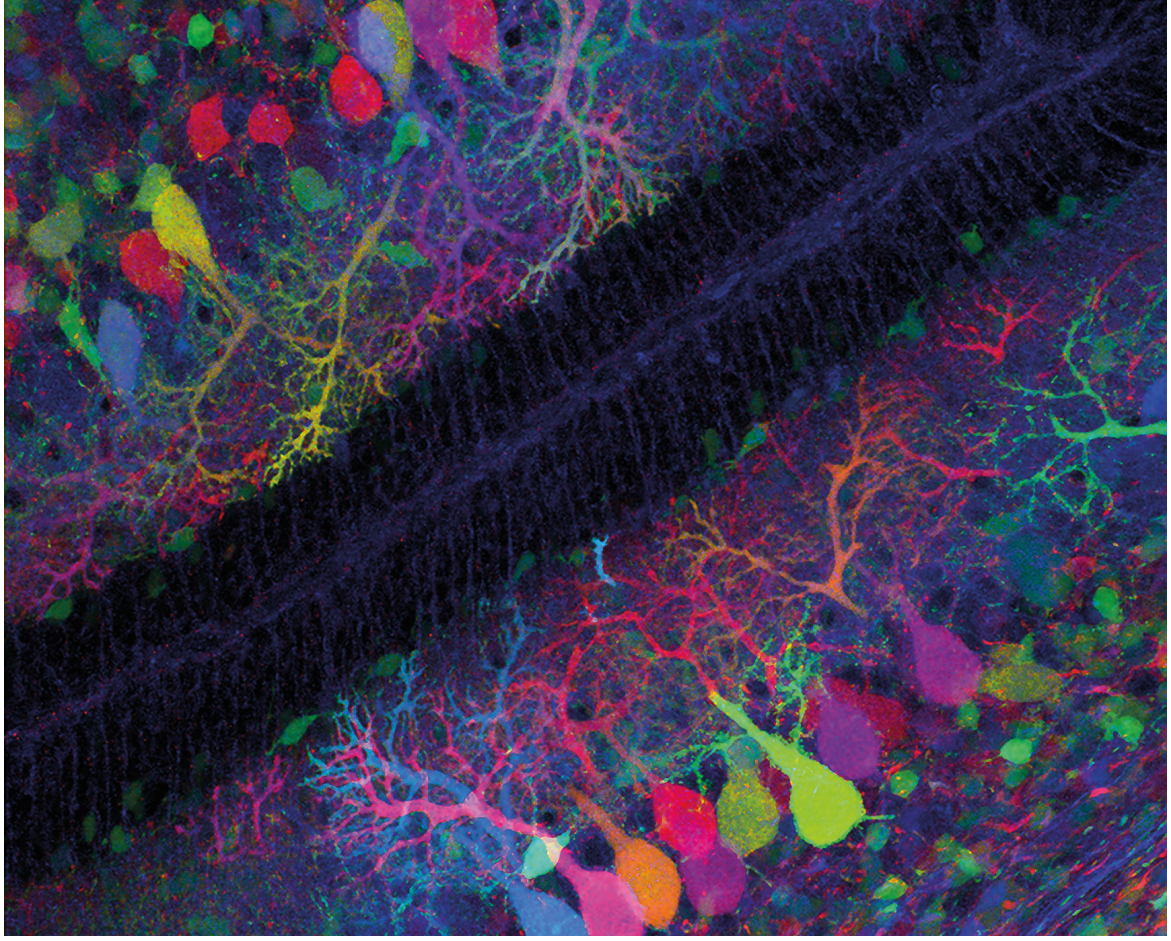
BOX: OPTICAL NANOSCOPY

For more than 100 years, diffraction was considered to be the ultimate barrier in far-field optical microscopy limiting its resolution to about half the wavelength of light [2]. From a pure optics point of view, this still holds true: it is impossible to create a focus of light in the far field of a lens where the majority of the energy is concentrated in a spot significantly smaller than this limit.

Around 1990, Stefan Hell realized that a possible solution to this problem must lie in the interaction of light with the sample, or more concretely, in exploiting the quantum mechanical states a molecule can be in [3]. By getting a molecule into a different, measurable state, it can be distinguished from all other molecules, even if they are closer than the diffraction limit. Using stimulated emission to switch molecules between a fluorescent excited state and the non-fluorescent ground state (fig. 3a), and applying it in a ring around the excitation focus of a laser scanning microscope (fig. 3b,e), Hell and co-workers could demonstrate that, even in far-field microscopy, fluorescence emission can be restricted to a spot much smaller than the diffraction limit (fig. 3c,f) [3]. Modern Stimulated Emission Depletion (STED) 'nanoscopes' achieve about 25 nm resolution in biological samples, about one order of magnitude below that of the best conventional far-field optical microscopes. In fact, the resolution in STED microscopy and related techniques is limited only by how well one can switch molecules between states and eventually by the size of the molecules.

Instead of switching molecules in a spatially targeted manner at the edges of a laser focus, one can alternatively take advantage of stochastic switching, or 'blinking', of individual molecules to overcome the diffraction limit. Based on technological advances pioneered by W.E. Moerner [4], Michel Orrit [5] and others to detect single fluorescent molecules, and the discovery of photoswitchable fluorescent molecules, this concept was realized in 2006 by Eric Betzig, Sam Hess, Xiaowei Zhuang and colleagues [6-8]. Individual molecules are recorded with a camera as they randomly emit bursts of photons (fig. 3h,i). In contrast to STED microscopy, where the location of the emitters is known *a priori* through the position of the laser focus, in this latter group of techniques the molecule positions have to be determined from the recorded data by fitting a model function to each diffraction-limited molecule image (fig. 3h-j).

Current research in both families of techniques, for example by our research group (fig. 4) [9], aims at optimizing optical nanoscopy for imaging of living samples with multiple stainings at different colors in three dimensions.



◂ **FIG. 2:** Fluorescence microscopy image of a mouse brain section. Using a sophisticated genetic approach, each Purkinje nerve cell produces a different ratio of cyan, yellow and red-coloured fluorescent proteins which provides a unique identifier for each cell and allows to trace neuronal networks in the mouse brain [10]. Figure reproduced with kind permission by J. Lichtman.

optics: lenses were improved to the theoretical limits and phase contrast methods that exploit interference phenomena of light were invented. With new devices derived from quantum electronics, in particular the laser and electronic photon detectors becoming readily available, the focus changed to implementing these devices into biological instrumentation, reflecting the progress in the physics and physical technology of the time and forming the field of biophotonics.

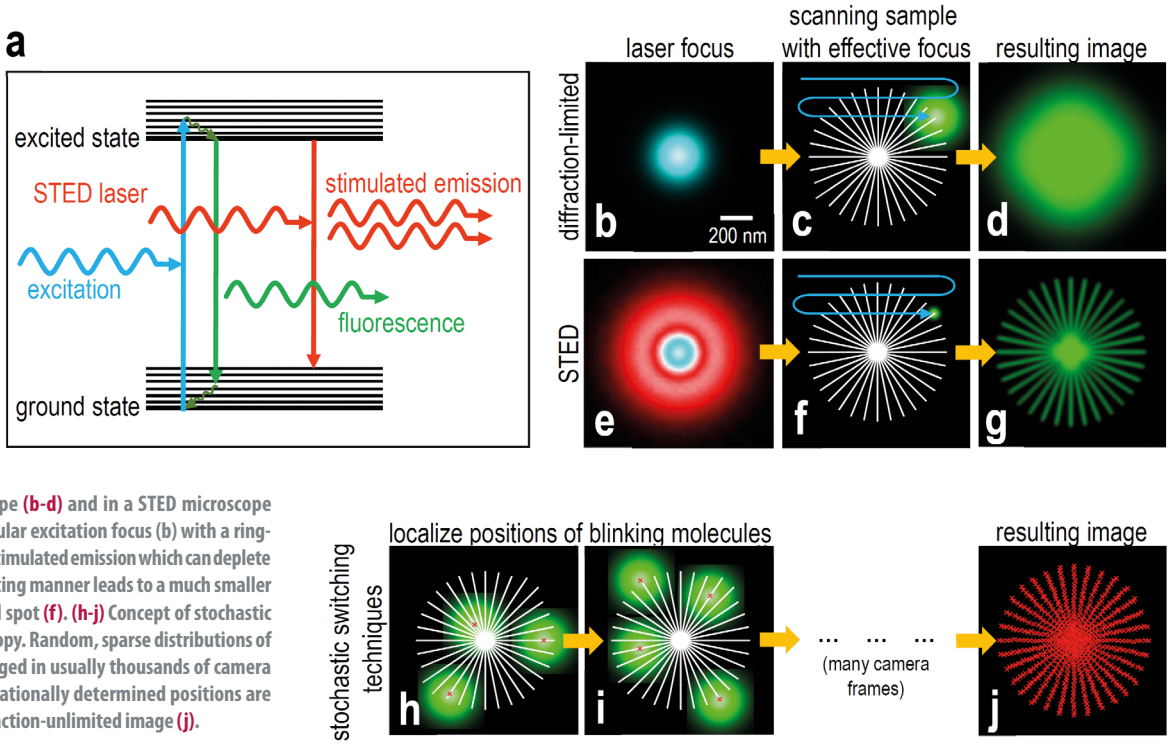
More recently, starting at the end of the 20th century, major breakthroughs emerged primarily at the interface between light and biological samples. The development of antibody-based labelling techniques allowed to specifically highlight nearly every biological structure or protein of interest in a cell with fluorescent molecules. The discovery of green fluorescent proteins (GFPs) and their blue, yellow and red cousins which can be genetically linked to virtually any cellular target of interest boosted live-cell fluorescence microscopy dramatically. The versatility of this new labelling technology, complemented by new detectors sensitive enough to measure the weak fluorescent signal and technology to keep cells in a culture dish alive on a microscope stage, has led to the dominance of fluorescence microscopy as the primary imaging tool in biological imaging (fig. 2).

Moreover, the entrance of versatile molecular probes as major actors on the biological imaging stage significantly expanded the number of parameters to play with and led to inventions previously unimaginable: the diffraction barrier in optical nanoscopes could be broken by taking advantage of the photophysical switching properties of fluorescent molecules (see Box), bioluminescence based on the enzyme luciferase is used to study tumor growth in mice, the new field of optogenetics utilizes photoactuator molecules such as channelrhodopsin to manipulate brain circuits of laboratory animals and essentially 'control their mind' by light. Even genomes can be sequenced by detecting single fluorescent molecules in zero-mode waveguides. Biophotonics has become a truly interdisciplinary venture.

The Future of Light in Biological Research is Bright

Living in a world flooded by photons, organisms are well adapted to light in many aspects. Evolution has found means to harvest the energy of light (chlorophyll), detect light to sense the surrounding (photoreceptors and eyes), actively communicate through light and color (bird feathers and bioluminescence in fireflies), and even deal with the negative effects of photodamage (pigmentation and DNA repair mechanisms). These multifaceted interactions outfit a large molecular toolbox that is continuously utilized by

▾ **FIG. 1:** Eric Betzig, Stefan W. Hell and W.E. Moerner shared the Nobel Prize in Chemistry 2014 "for the development of super-resolved fluorescence microscopy". © Nobel Media AB. Photos: Alexander Mahmoud



▸ **FIG. 3:** Concepts of Optical Nanoscopy. **(a)** Jablonski diagram of a typical fluorescent molecule showing the transitions used in STED microscopy. **(b-g)** Image formation in a conventional laser scanning microscope **(b-d)** and in a STED microscope **(e-g)**. Overlaying the regular excitation focus (b) with a ring-shaped STED focus **(e)** for stimulated emission which can deplete excited states in a saturating manner leads to a much smaller remaining effective focal spot **(f)**. **(h-j)** Concept of stochastic switching optical nanoscopy. Random, sparse distributions of single molecules are imaged in usually thousands of camera frames **(h,i)**. The computationally determined positions are combined to form a diffraction-unlimited image **(j)**.

the research community to inspire and create novel technologies for biophotonics. An end to this development is not in sight with many of the most exciting developments, for example optogenetics, being just a few years old.

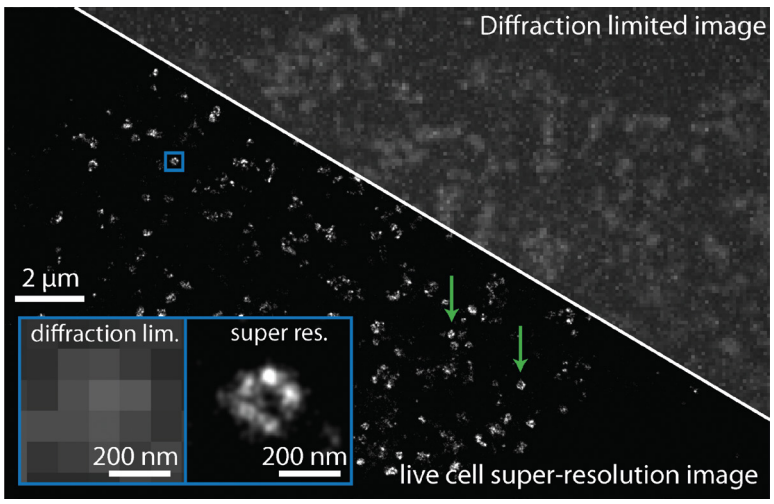
At the same time, optical technology itself is making fast progress which has a positive impact on the development of biological applications: billions of smartphones have given most humans access to high-quality cameras which can be adapted to biodiagnostic usage with little effort. Lasers, LEDs, cameras and other detectors become more powerful every day. Data storage and analysis get cheaper, faster and more accessible, for example through cloud services. All these get implemented into the next generation of optical instrumentation for biological applications making them better, faster and more reliable.

With these developments already in the pipeline, it is safe to predict that light technologies will continue to have a fundamental impact on the advancement of biological research and improving our health. ■

About the author

Joerg Bewersdorf earned his doctoral degree in physics in 2002 under the mentorship of Dr. Stefan Hell. He is now an Associate Professor at Yale University where he works on the development of optical nanoscopy and its biomedical application. Joerg Bewersdorf discloses financial interest in Bruker Corp.

▾ **FIG. 4:** Example of a state-of-the-art optical nanoscopy picture showing clathrin-coated pits, scaffolding structures involved in internalizing extracellular cargo into a cell, in a live HeLa cell. Figure: Fang Huang.



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