#### **BMS 524 - "Introduction to Confocal Microscopy and Image Analysis"**



# **Beyond confocal microscopy Modern 3-D imaging techniques**

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#### **3-D methods based on nonlinear optical phenomena**

# Nonlinear optical phenomena are not part of our everyday experience!

- In "classical" optics the optical properties of materials are independent of the intensity of illumination
- If the illumination is <u>sufficiently intense</u>, the optical properties may depend on the characteristics of light!
  - Several novel 3-D microscopy techniques rely on non-linear optical phenomena
  - 2-p and multiphoton microscopy
  - Higher harmonics microscopy (SGH, TTH)
  - Coherent Anti-Stokes Raman scattering microscopy (CARS)

#### Linear polarization





Position of electron the electric field *E*(*t*)

Harmonic terms

Anharmonic terms

$$P = \frac{1}{2} \varepsilon_0 \chi E_0 \exp(-i\omega t) + c.c., \text{ where}$$

 $\chi = \frac{Ne^2}{\varepsilon_0 m} \frac{1}{\Omega^2 - 2i\Gamma\omega - \omega^2}$ 

P - macroscopic polarization. This is a measure of the response of the electron density distribution to a static electric field.

#### **Origins of optical nonlinearity**

$$m\left[\frac{d^{2}x}{dt^{2}} + 2\Gamma\frac{dx}{dt} + \Omega^{2}x - (\xi^{(2)}x^{2} + \xi^{(3)}x^{3} + \cdots)\right] = -eE(t)$$

- When the anharmonic terms are included there is no longer an exact solution for the equation of motion.
- We can approximate the solution by expressing x as a power series in *E*. Equivalently we can expand *P*:

$$P = \varepsilon_0 (\chi^{(1)}E + \chi^{(2)}E^2 + \chi^{(3)}E^3 + \chi^{(4)}E^4 \cdots)$$

#### **Some examples of nonlinear phenomena**

- 1<sup>st</sup> order (linear) process: absorption and reflection
- 2<sup>nd</sup> order process: SHG, Pockels effect
- 3<sup>rd</sup> order process: 2-photon absorption, Kerr effect, CARS
- 2m-1 order: m-photon absorption

## What is multiphoton (two photon) excitation?

- MPE of molecules is a nonlinear process involving the absorption of multiple photons whose combined energy is sufficient to induce a molecular transition to an excited electronic state. It is a process unknown in nature except in stars
- Quantum mechanically, a single photon excites the molecule to a virtual intermediate state, and the molecule is eventually brought to the final excited state by the absorption of the second photon (for two-photon excitation).

## **History of 2-photon microscopy**

VOLUME 7, NUMBER 6

PHYSICAL REVIEW LETTERS

September 15, 1961

#### TWO-PHOTON EXCITATION IN CaF<sub>2</sub>:Eu<sup>2+</sup>

W. Kaiser and C. G. B. Garrett Bell Telephone Laboratories, Murray Hill, New Jersey (Received August 28, 1961)

With the development of optical masers,<sup>1-3</sup> it is now possible to study two-photon processes which necessitate intense sources of monochromatic radiation. We have investigated the generation of blue fluorescent light around  $\lambda_b = 4250$  A by illuminating CaF<sub>2</sub>:Eu<sup>2+</sup> crystals with red light,  $\lambda_{\gamma} = 6943$  A, of a ruby optical maser.<sup>2</sup> Our experiments differ from the recent investigations by Franken et al.<sup>4</sup> who observed the generation of responding to the diameter of the incident light beam. When pure  $CaF_{2}$  was illuminated by the optical maser in the same way, no light with  $\lambda < \lambda_{\gamma}$  was observed on the photographic plate. This observation is expected from the high symmetry of the  $CaF_{2}$  lattice.

In a second experiment, the light beam leaving the quartz spectrometer was intersected by two mirrors in such a way that the red and blue parts

 The technology of 2-p spectroscopy, developed in '60 by W. Kaiser and C.G.B. Garret was based on a well known quantum mechanical concept presented for the first time by M. Göppert-Mayer in 1931 (Göppert-Mayer M: Über Elementarakte mit zwei Quantensprüngen. Ann Phys 1931, 9:273-295.)

Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence

#### Two-Photon Laser Scanning Fluorescence Microscopy

#### WINFRIED DENK,\* JAMES H. STRICKLER, WATT W. WEBB

Molecular excitation by the simultaneous absorption of two photons provides intrinsic three-dimensional resolution in laser scanning fluorescence microscopy. The excitation of fluorophores having single-photon absorption in the ultraviolet with a stream of strongly focused subpicosecond pulses of red laser light has made possible fluorescence images of living cells and other microscopic objects. The fluorescence emission increased quadratically with the excitation intensity so that fluorescence and photobleaching were confined to the vicinity of the focal plane as expected for cooperative two-photon excitation. This technique also provides unprecedented capabilities for three-dimensional, spatially resolved photochemistry, particularly photolytic release of caged effector molecules.

**E REPORT THE APPLICATION OF** two-photon excitation of fluorescence (1) to laser scanning microscopy (LSM) (2). In LSM, the laser is focused to a diffraction-limited beam waist <1 µm in diameter and is raster-scanned absorption and emission wavelengths (4, 5). Anticipated photodamage to living cells and the limitations of UV lasers have also inhibited this approach.

Two-photon molecular excitation (1, 6) is made possible by the very high local instan-

- 1978: C.J.R. Sheppard and T. Wilson postulated that 2-p phenomenon can be used in scanning microscopy
- 1990: W. Denk, J. Stricker and W.W. Webb demonstrated 2-p laser scanning fluorescencnt microscope. The technology was patented by the Cornell group in 1991

# **2-photon excitation**



- Two-photon excitation occurs through the absorption of two lower energy photons via short-lived intermediate states.
- After either excitation process, the fluorophore relaxes to the lowest energy level of the first excited electronic states via vibrational processes.
- The subsequent fluorescence emission processes for both relaxation modes are the same.

One-photon excitation

Two-photon excitation

#### From 2-photon to multiphoton...



#### Demonstration of the difference between singleand two-photon excitation



Image source: Current Protocols in Cytometry Online Copyright © 1999 John Wiley & Sons, Inc. All rights reserved.

The cuvette is filled with a solution of a dye, safranin O, which normally requires green light for excitation. **Green light (543 nm)** from a continuous-wave helium-neon laser is focused into the cuvette by the lens at upper right. It shows the expected pattern of a continuous cone, brightest near the focus and attenuated to the left. The lens at the lower left focuses an invisible **1046-nm infrared beam** from a mode-locked Nd-doped yttrium lanthanum fluoride laser into the cuvette. Because of the two-photon absorption, excitation is confined to a tiny bright spot in the middle of the cuvette.



Slide credit: Brad Amos, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

#### **Radiance 2100MP at PUCL**



#### Wide-field vs. confocal vs. 2-photon



Drawing by P. D. Andrews, I. S. Harper and J. R. Swedlow

## **Probability of 2-photon excitation**

- For the same average laser power and repetition frequency, the excitation probability is increased by increasing the NA of the focusing lens and by reducing the pulse width of the laser.
- Increasing NA corresponds to spatially confining the excitation power to a smaller focal volume.

$$n_a \propto rac{\delta_2 P_{av}^2}{ au_p f_p^2} \left(rac{\mathbf{N}\mathbf{A}^2}{2\hbar c\lambda}
ight)^2$$

where :

- $n_a$  probability
- $\tau_p$  -pulse duration
- $f_p$  repetition rate
- $\delta_2$  2p cross section
- $P_{av}$  average power of the beam
- $\lambda$  wavelength

## **Resolution of 2-photon systems**

Using high NA pseudoparaxial approximations<sup>1</sup> to estimate the illumination, the intensity profile in a 2-photon system, the lateral (r) and axial (z) full widths at half-maximum of the two-photon excitation spot can be approximated by<sup>2</sup>:

$$r_{0} = \begin{cases} \frac{0.32 \cdot \lambda}{\sqrt{2} \cdot \text{NA}} & \text{NA} \le 0.7 \\ \frac{0.325 \cdot \lambda}{\sqrt{2} \cdot \text{NA}^{0.91}} & \text{NA} > 0.7 \end{cases} \qquad z_{0} = \frac{0.532\lambda}{\sqrt{2}} \left[ \frac{1}{n - \sqrt{n^{2} - \text{NA}^{2}}} \right]$$

Two-photon excitation exhibits localized excitation, the inherent advantage which accounts for the improved resolution available with this method. In 2-p case, equal fluorescence intensity is observed in all planes and there is no depth discrimination. In the two-photon case, the integrated intensity decreases rapidly away from the focal plane.

1

$$V_{2hv} = \pi^{3/2} r_0^2 z_0$$

C. J. R. Sheppard and H. J. Matthews, "Imaging in a high-aperture optical systems," J. Opt. Soc. Am. A 4, 1354- (1987) 1)

W.R. Zipfel, R.M. Williams, and W.W. Webb "Nonlinear magic: multiphoton microscopy in the biosciences," Nat. Biotech. 21(11), 1369-1377 (2003) 2)

#### **Practical resolution**



Centonze VE, White JG. Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging. Biophys J. 1998 Oct;75(4):2015-24.

Effect of increased incident power on generation of signal. Samples of acidfucsin-stained monkey kidney were imaged at a depth of 60 µm into the sample by confocal (550 µW of 532-nm light) and by multiphoton (12 mW of 1047-nm light) microscopy. Laser intensities were adjusted to produce the same mean number of photons per pixel. The confocal image exhibits a significantly narrower spread of pixel intensities compared to the multiphoton image indicating a lower signal to background ratio. Multiphoton imaging therefore provides a high-contrast image even at significant depths within a light-scattering sample. Images were collected at a pixel resolution of 0.27 µm with a Kalman 3 collection filter. Scale bar, 20 µm.

#### **Penetration depth**



Comparison of imaging penetration depth between confocal and multiphoton microscopy. Optical sections through a glomerulus from an acid-fucsinstained monkey kidney pathology sample imaged by confocal microscopy with 2  $\mu$ W of 532-nm light (left, columns 1 and 2) and multiphoton microscopy with 4.3 mW of 1047-nm light (descanned; right, columns 3 and 4) were compared. At the surface, the image quality and signal intensity are similar; however, at increasing depth into the sample, signal intensity and quality of the confocal image falls off more rapidly than the multiphoton image. Images were collected at a pixel resolution of 0.27 µm with a Kalman 3 collection filter. Scale bar, 20 µm.

Centonze VE, White JG. Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging. Biophys J. 1998 Oct;75(4):2015-24.

## We need pulsed lasers for MPE

- The average laser power of 100 mW is focused at the specimen on a diffractionlimited spot of 0.5 µm in diameter. The area of the spot is 2 × 10<sup>-9</sup> cm<sup>2</sup>
- Laser power at the spot = 0.1 W × 1/(2 × 10<sup>-9</sup> cm<sup>2</sup>) = 5 × 10<sup>7</sup> W cm<sup>-2</sup>
- A "femtosecond" laser is on for ~100 femtoseconds every 10 nanoseconds. The pulse duration to gap duration ratio 10<sup>-5</sup>
- The instantaneous power when laser is on equals 5 × 10<sup>12</sup> W cm<sup>-2</sup>



Laser Material	Company; Model	Wavele- length	Pulse Length	Repetition Rate	Power
Ti:Sapphire	Coherent; Mira	700-980	<200 fs	76 MHz	0.7 W,1.3 W
	Spectra Physics; Tsunami	700-1000	<100 fs (or 2ps as option)	80 MHz	0.8 W, 1.4 W
	Coherent; Chameleon - XR	705-980	<140 fs	90 MHz	1.7 W
	Spectra Physics; Mai Tai	710-990	120 fs	80 MHz	1.5 W
	Time Bandwidth; Pallas	780-860	<100 fs	75 MHz	500 mW
	Time Bandwidth; Tiger	780-860	<100 fs	100 MHz	400 mW
	Femtosource	750-850	<12 fs	75 MHz	400 mW 600 mW
Nd:YLF	MicroLase/Coherent Scotland; BioLite	1047	200 fs	120 MHz	500 mW
Nd:Glass	Time Bandwidth; GLX200	1058	<250 fs	100 MHz	>400 mW
Ytterbium	Amplitude Systems	1030	<200 fs	50 MHz	1 W
Cr:LiSAF	Highqlasers	850	100 fs	50 MHz	>1mW
OPO	Coherent and Spectra Physics	350-1200	100 fs		~200 mW 

# **Advantages of 2-p microscopy**

- The tissue above and below the plane of focus is merely subjected to infrared light and multiphoton excitation is restricted to a small focal volume (because fluorescence from the two-photon effect depends on the square of the incident light intensity, which in turn decreases approximately as the square of the distance from the focus.
- 2-p microscopy can image turbid specimens with submicrometer resolution down to a depth of a few hundred microns.
- 2-p microscopy separates excitation and emission light more effectively
- All the emitted photons from multi-photon excitation can be used for imaging (in principle) therefore no confocal blocking apertures have to be used.

## **Second Harmonic Generation**

- An intense laser field induces a nonlinear polarization in a molecule or assembly of molecules, resulting in the production of a coherent wave at exactly twice the incident frequency.
- The magnitude of the SHG wave can be resonance-enhanced when the energy of the second harmonic signal overlaps with an electronic absorption band
- A major constraint of SHG is the requirement of a noncentrosymmetric environment. Why?

$$\begin{split} P_1 &= \mathcal{E}_0 \left( \chi^{(1)} E_1 + \chi^{(2)} E_1^2 + \chi^{(3)} E_1^3 + \cdots \right) \\ E_2 &= -E_1 \qquad P_2 \qquad = \mathcal{E}_0 \left( \chi^{(1)} E_2 + \chi^{(2)} E_2^2 + \chi^{(3)} E_2^3 + \cdots \right) \\ &= \mathcal{E}_0 \left( -\chi^{(1)} E_1 + \chi^{(2)} E_1^2 - \chi^{(3)} E_1^3 + \cdots \right) \\ P_2 &= P_1 \qquad \Rightarrow \qquad \chi^{(2)} = 0 \end{split}$$

In an isotropic medium, reversal of the electric field will produce the same electric polarisation but in the opposite direction.

#### SHG and 2-p combined



2-photon image of liver tissue from an adult mouse. The hepatocytes are visualized by blue autofluorescence (greyscale) from NAD(P)H and lipid soluble vitamins, such as retinol. The collagenous capsule (green) is visualized by SHG.

image from Watt Webb lab at Cornell University

Multiphoton image of a mammary gland from mouse. Blue autofluorescence (green pseudocolor) deliniates cellular structures and lipid droplets. Collagen is visualized by SHG.

image from Watt Webb lab at Cornell University. It was acquired in collaboration with Alexander Nikitin, Dept. of Biomedical Sciences, Cornell.

#### **Higher harmonic microscopy**



Time series showing mitosis processes inside a live zebrafish embryo *in vivo* monitored with SHG, and THG. The imaging depth is 400-µm from the chorion surface. THG (purple) picks up all interfaces including external yolk syncytial layers, cell membranes, and nuclear membranes while SHG (green) shows the microtubule-formed spindle biconical arrays.

![](_page_21_Picture_3.jpeg)

(A)-(G) An in vivo sectioning series of a zebrafish larva at 5 days after fertilization. (H) The enlarged view inside a somite showing distribution of muscle fibers. (I) An optical section at the center of the larva showing the segments inside the vacuolated notochord and the distribution of somites alongside the notochord. Image size: (A)-(G) and (I):  $235 \times 235$ -µm<sup>2</sup>; (H): 40 × 40-µm<sup>2</sup>.

from "Higher harmonic generation microscopy for developmental biology" by Chi-Kuang Sun et al., *Journal of Structural Biology*, 147(1), 2004, Pages 19-30

## 4π confocal microscopy

- 4π confocal microscopy was proposed as a means to increase the aperture angle and therefore improve the axial resolution of a confocal microscope.
- Since in a confocal arrangement the PSF is given by the product of the illumination and the detection PSF's, three types of  $4\pi$  confocal microscope have been described:
  - in a type-A  $4\pi$  confocal microscope the illumination aperture is enlarged
  - in a type-B  $4\pi$  confocal microscope the detection aperture is increased.
  - A type-C  $4\pi$  confocal microscope combines both types A and B, leading to further resolution enhancement along the optical axis.

## 4π PSF

$$PSF_{exc} = h_{exc}(r, z) = \left| \vec{E}(r, z) \right|^{2}$$

$$PSF_{conf} = PSF_{conf} \cdot PSF_{det} \qquad h_{conf}(r, z) = h_{exc}(r, z) \cdot h_{det}(r, z)$$

Type A - the two illumination wave fronts interfere at sample:

$$h_{4\pi}^{A}(r,z) = \left|\vec{E}_{1,exc}(r,z) + \vec{E}_{2,exc}(r,z)\right|^{2} \cdot \left|\vec{E}_{1,det}(r,z)\right|^{2}$$

Type B - the two detection wave fronts interfere in the detector:

$$h_{4\pi}^{B}(r,z) = \left|\vec{E}_{1,exc}(r,z)\right|^{2} \cdot \left|\vec{E}_{1,det}(r,z) + \vec{E}_{2,det}(r,z)\right|^{2}$$

Type C - both illumination and detection wave fronts interfere:

$$h_{4\pi}^{C}(r,z) = \left|\vec{E}_{1,exc}(r,z) + \vec{E}_{2,exc}(r,z)\right|^{2} \cdot \left|\vec{E}_{1,det}(r,z) + \vec{E}_{2,det}(r,z)\right|^{2}$$

# History of 4π microscopy

![](_page_24_Figure_1.jpeg)

- Exploiting counter propagating interfering beams for axial resolution improvement was first attempted by placing a mirror beneath the sample in an epifluorescence microscope. The interference between the reflected and the incoming beam creates a flat standing wave of fluorescence excitation.
- The concept of 4π microscopy was developed by Prof.
  Stefan Hell at the Max Planck Institute for Biophysical Chemistry in Goettingen, Germany and was refined and turned into a commercial system by Leica Microsystems.

#### Sketch of the $4\pi$ microscope of type C

Excitation light originating from the microscope stand is divided by the beam splitter BS and focused onto the same spot by the opposing objective lenses O1 and O2. The lenses L1, L2, and L3 and the mirrors M1, M2, and M3 form the intermediate optical system, ensuring that the two scanning pivotal points coincide with the entrance pupils of the two objective lenses. Fluorescence is collected by both lenses, recombined at BS, and directed toward the microscope stand. The pathlength difference between the two interferometric arms is smaller than the coherence length of the fluorescence light, so that fluorescence interferes at the detector as well. Dispersion compensation over a large wavelength range is ensured by movable optical wedges in the lower interferometric arm whose thickness is compensated by a glass window in the upper arm.

Gugel H, Bewersdorf J, Jakobs S, Engelhardt J, Storz R, Hell SW. Biophys J. 2004 Dec;87(6):4146-52.

![](_page_25_Figure_3.jpeg)

![](_page_26_Picture_0.jpeg)

#### Intensity along z axis

The two waves can be written as:

$$E_1 = E_0 \exp(i[kz - \omega t]) \qquad E_2 = E_0 \exp(i[-kz - \omega t])$$

The total electric field along the z axis is therefore the sum:

The intensity along *z* will be:

$$E = E_{1} + E_{2} = E_{0}e^{-i\omega t} [\exp(ikz) + \exp(-ikz)]$$

$$I = EE^{*} = E_{0}^{2} [\exp(ikz) + \exp(-ikz)] \cdot [\exp(-ikz) + \exp(ikz)]$$

$$= E_{0}^{2} [2 + \exp(2ikz) + \exp(-2ikz)]$$

$$= E_{0}^{2} [2 + 2\cos(2kz)]$$

$$= 2E_{0}^{2} [2\cos^{2}(kz)]$$
maximum at  $kz = nn$   
and minima at  $kz = n/2 + nn$ 

The actual intensity distribution of a microscope along z is the sinc function:

And therefore the total intensity for a 4p system is the multiplication of these two:

$$I = \left[\frac{\sin(k \cdot \mathrm{NA}^{2}z/4)}{k \cdot \mathrm{NA}^{2}z/4}\right]^{2}$$
$$I = \left[\frac{\sin(k \cdot \mathrm{NA}^{2}z/4)}{k \cdot \mathrm{NA}^{2}z/4}\right]^{2} \times \cos^{2}(kz)$$

Slide credit: Dmytry Podolsky & Yuval Garini

## Intensity along z axis

![](_page_28_Figure_1.jpeg)

![](_page_28_Figure_3.jpeg)

- Intensity surface plots of the PSF of the (a) 4πconfocal, (b) confocal, and the (d) pointdeconvolved 4π-confocal microscope as determined with light scattering beads.
- The plot in (c) represents the lobe function, *I*(*z*). The arrangement of the figures symbolizes the fact that the 4π-PSF can be approximated by the convolution of the peak function of (d) with the lobe function of (c).
- The comparison between (b) and (d) reveals a 4.5fold improvement of the axial resolution in 4πconfocal microscopy over regular confocal microscopy.

## Axial response of $4\pi$ system

![](_page_29_Figure_1.jpeg)

Axial resolution of the  $4\pi$  microscope of type C using two-photon excitation for water immersion (*left*) and glycerol immersion (*right*).

#### Signal-to-noise ratio and resolution

![](_page_30_Figure_1.jpeg)

![](_page_30_Figure_2.jpeg)

The influence of Poisson noise on two intensity distributions separated spatially according to the Rayleigh criterion.

# Aliasing

![](_page_31_Figure_1.jpeg)

- It is believed that the Nyquist theorem states that a signal must be sampled at least twice as fast as the bandwidth of the signal to accurately reconstruct the waveform
- Otherwise the high-frequency content will *alias* at a frequency inside the spectrum of interest. An alias is a false lower frequency component that appears in sampled data acquired at too low a sampling rate.
- The figure shows a sine wave sampled at 10 samples/ $\pi$ , 60 samples/ $\pi$  and 20 samples/ $\pi$ .

#### **Sampling – what is the minimal rate?**

![](_page_32_Figure_1.jpeg)

#### Sampling – cont.

![](_page_33_Figure_1.jpeg)

The Nyquist criterion states that, in order to prevent undesired aliasing, one must sample a signal at a rate equal to at least twice its bandwidth.

![](_page_33_Figure_3.jpeg)

#### **Structured illumination – or "breaking" the Nyquist criterion**

- Structured illumination methods use sampling rates below Nyquist!
- Yes, you can use aliasing to your advantage with undersampling (super-Nyquist sampling)!
- When a signal is sampled at less than the Nyquist rate, the aliased signal appears at  $f_s f_a$ , where  $f_s$  is the sampling frequency and  $f_a$  is the frequency of the input signal. Because you know ahead of time that the signal is aliasing, you can reverse the  $f_s - f_a$  relationship to recover the actual frequency.
- You still are not really breaking the Nyquist criterion because Nyquist actually said the sampling rate must be at least double the signal's bandwidth, not the signal's highest frequency component.

#### **Resolution extension through the moiré effect**

If the illumination contains a spatial frequency  $\mathbf{k}_1$ , then each sample frequency  $\mathbf{k}$  gives rise to moiré fringes at the difference frequency  $\mathbf{k} - \mathbf{k}_1$ . Those fringes will be observable in the microscope if  $|\mathbf{k} - \mathbf{k}_1| < k_0$ 

![](_page_35_Figure_2.jpeg)

If an unknown sample structure (*a*) is multiplied by a known regular illumination pattern (*b*), moiré fringes will appear (*c*). The Moiré fringes occur at the spatial difference frequencies between the pattern frequency and each spatial frequency component of the sample structure and can be coarse enough to observe through the microscope even if the original unknown pattern is unresolvable. Otherwise-unobservable sample information can be deduced from the fringes and computationally restored.

Gustafsson, M.G.L. (2005) Proc. Natl. Acad. Sci. USA 102, 13081-13086

## **Optigrid and Apotome systems**

A diffraction grating can be imaged in the sample plane. The resulting intensity:

$$I = I_0 + I_c \cos \varphi + I_s \sin \varphi$$

where  $I_0$  describes the contribution of a conventional wide-field image, and  $\varphi$  characterizes a spatial phase due to the grating.

Let's record three images with  $\varphi_1=0$ ,  $\varphi_2=2\pi/3$ , and  $\varphi_3=4\pi/3$  by slightly shifting the grating. We will obtain an optically sectioned image, where  $I_0$  as well as  $\varphi$  are eliminated:

$$I_p = \left[ (I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2 \right]^{1/2}$$

The conventional image can be also recovered:

$$I_0 = \frac{I_1 + I_2 + I_3}{3}$$

# **Sectioning capability**

#### OptiGrid

![](_page_37_Picture_2.jpeg)

A single 6  $\mu$ m FocalCheck<sup>TM</sup> microsphere optically sectioned with each instrument. Shown is a view though the center of the sphere in the XY plane, and in the XZ plane through the image stack.

![](_page_37_Figure_4.jpeg)

Axial response of a system built by Neil, Juskaitis, and Wilson

Slide credit: Adam Puche, University of Maryland

#### **Saturated structured illumination**

![](_page_38_Figure_1.jpeg)

Huang, Bo, Mark Bates, and Xiaowei Zhuang. 2009. "Super-Resolution Fluorescence Microscopy." *Annual Review of Biochemistry* 78 (1): 993-1016. doi:10.1146/annurev.biochem.77.061906.092014.

(a) A diffractive grating in the excitation path splits the light into two beams. Their interference after emerging from the objective and reaching the sample creates a sinusoidal illumination pattern with alternating peaks and zero points. Strong excitation light saturates the fluorescence emission at the peaks without exciting fluorophores at the zero points, leading to sharp dark regions in the excitation pattern. (b) When a sinusoidal illumination pattern is applied to a sample, a moiré pattern at a significantly lower spatial frequency than that of the sample can be generated and imaged by the microscope (SIM panel, *lower part*). Multiple images that resulted from scanning and rotating the excitation pattern are then used to reconstruct the sample structure. SSIM introduces a high-frequency component into the excitation pattern, allowing features far below the diffraction limit to be resolved

#### **Structured illumination – history**

#### Optischen Abbildung unter Überschreitung der beugungsbedingten Auflösungsgrenze

von W. LUKOSZ und M. MARCHAND Physikalisches Institut, Technische Hochschule, Braunschweig, Germany

(Received 5 February 1963, and in revised form 1 July 1963)

Bekanntlich setzt die Beugung dem mit einem optischen System erreichbaren Auflösungsvermögen (präziser formuliert : der Bandbreite des vom System durchgelassenen Orts-Frequenzbandes) eine prinzipielle Grenze.

In der vorliegenden Arbeit wird ein neues Verfahren zur optischen Abbildung mit einem über die beugungsbedingten Grenzen hinausgehenden Auflüsungsvermögen erläutert : Das optische System selbst wird unverändert benutzt. In (bzw. in der Nähe) der Objektebene wird aber eine Maske mit örtlich variabler Transmission (z.B. ein Gitter) angebracht oder

- Lukosz and Marchand suggested in 1963 that lateral light patterns could be used to enhance resolution
- Practical implementation was reported by T. Wilson et al. in 1997. (Neil, M. A. A., Wilson, T. & Juskaitis, R. (1997) Opt. Lett. 22, 1905-1907.)

#### The principle of stimulated emission depletion (STED) microscopy

![](_page_40_Figure_1.jpeg)

(a) The process of stimulated emission. A ground state  $(S_0)$  fluorophore can absorb a photon from the excitation light and jump to the excited state  $(S_1)$ . Spontaneous fluorescence emission brings the fluorophore back to the ground state. Stimulated emission happens when the excited-state fluorophore encounters another photon with a wavelength comparable to the energy difference between the ground and excited state. (*b*) The excitation laser and STED laser are combined and focused into the sample through the objective. A phase mask is placed in the light path of the STED laser to create a specific pattern at the objective focal point. (*c*) In the *xy* mode, a donut-shaped STED laser is applied with the zero point overlapped with the maximum of the excitation laser focus. With saturated depletion, fluorescence from regions near the zero point is suppressed, leading to a decreased size of the effective point spread function (PSF).

#### **Excitation and deexcitation beams for 3D STED**

ST

Ζ

![](_page_41_Figure_1.jpeg)

Klar T A et al. PNAS 2000;97:8206-8210

#### **Resolution improvement in STED**

![](_page_42_Figure_1.jpeg)

Klar T A et al. PNAS 2000;97:8206-8210

# Example: Subdiffraction resolution fluorescence imaging of microtubules

![](_page_43_Figure_1.jpeg)

Hein B et al. PNAS 2008;105:14271-14276

# Example: Subdiffraction-resolution imaging of the ER in a living mammalian cell.

![](_page_44_Figure_1.jpeg)

Hein B et al. PNAS 2008;105:14271-14276

#### Stochastic optical reconstruction microscopy (STORM) or (fluorescence) photoactivation localization microscopy ((F)PALM)

Zhuang, Xiaowei. 2009. "Nano-imaging with Storm." *Nature photonics* 3 (7): 365-367. doi:10.1038/nphoton.2009.101.

 Target structure
 Localizing activated subset of probes
 STORM image

 Image
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Different fluorescent probes marking the sample structure are activated at different time points, allowing subsets of fluorophores to be imaged without spatial overlap and to be localized to high precision. Iterating the activation and imaging process allows the position of many fluorescent probes to be determined and a super-resolution image is then reconstructed from the positions of a large number of localized probe molecules.

![](_page_45_Figure_4.jpeg)

#### **Super-resolution imaging principles.**

![](_page_46_Figure_1.jpeg)

Schermelleh L et al. J Cell Biol 2010;190:165-175

# Resolvable volumes obtained with current commercial super-resolution microscopes.

![](_page_47_Figure_1.jpeg)

Schermelleh, Lothar, Rainer Heintzmann, and Heinrich Leonhardt. 2010. "A guide to super-resolution fluorescence microscopy." *The Journal of Cell Biology* 190 (2) (July 26): 165 -175. doi:10.1083/jcb.201002018.

#### **Selective Plane Illumination Microscopy (SPIM)**

![](_page_48_Figure_1.jpeg)

## **Super-resolution microscopy of biological samples.**

![](_page_49_Figure_1.jpeg)

175. doi:10.1083/jcb.201002018.