Introduction of laser fluorescence microscope
(Lecture in English)

Leica Microsystems

This lecture introduces the fundamentals of fluorescence confocal laser scanning microscopy. Topics include the principles of fluorescence, confocal optical design, image formation, optical sectioning, three-dimensional imaging, and advanced functional imaging options such as fluorescence lifetime imaging (FLIM). The advantages and limitations of confocal microscopy, together with common biological applications, will be discussed. The course is intended for students and researchers seeking a basic understanding of fluorescence imaging techniques.

Introduction of laser fluorescence microscope

2026 Intensive Course in Technical Seminar
/ Central Research Laboratory Special Seminar
Shiga University of Medical Science

September 9th (Wed), 2026
Leica Microsystems K.K.

From Eye to Insight

Agenda

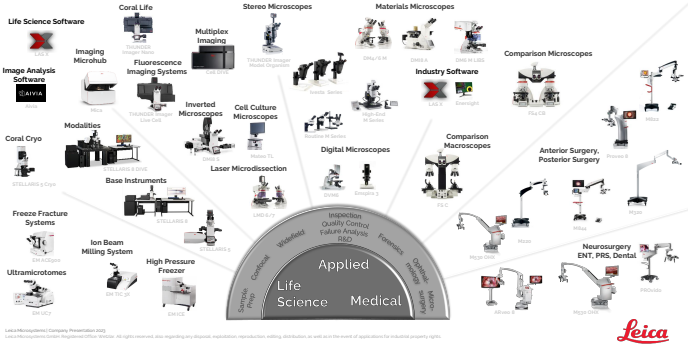
1. Light Microscope
2. Confocal Laser Scanning Microscope
3. Advanced application: fluorescent lifetime imaging
4. Advanced application: super resolution imaging

Who we are - Leica Microsystems

Who we are - Leica Microsystems

- 1849 - Carl Kellner establishes the “Optical Institute” in Wetzlar — 177th anniversary
- 1869 – Ernst Leitz takes over the “Optical Institute” and renames the company Ernst Leitz — 157th anniversary
- 1914 – Oskar Barnack invents the first 35mm film camera, Ur-Leica — 112th anniversary

Leica Microsystems Product Portfolio



Agenda

1. Light Microscope
2. Confocal Laser Scanning Microscope
3. Advanced application: fluorescent lifetime imaging
4. Advanced application: super resolution imaging

Light Microscope

Types for purpose

1. For Life science research
2. For Industrial, material science microscopes/polarization microscopes
3. Stereomicroscopes/Digital microscopes (viewing without eyepiece)

Advanced microscope systems:
- Confocal laser scanning microscopes
- Super-resolution microscopes
- Laser Microdissection

and so on

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Light Microscopes

Biological microscopes and industrial microscopes come in two types:
upright and **inverted**, in which the objective lens is positioned below the specimen.

Intelligent Upright Microscope
Leica DM6B

Intelligent Inverted Microscope
Leica DMi8

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Beam Pathway

Upright Microscope

Inverted Microscope

Figure 5: An upright microscope features the objective above and the condenser below the specimen (left). On an inverted microscope this set-up is turned around (right).

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Industrial/Material microscopes

Samples

Upright Microscope

Inverted Microscope

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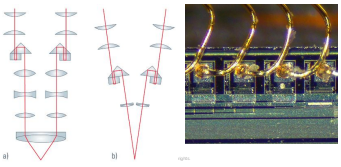
Stereo Microscopes

Independent left and right optical beam path systems
Large working distance (WD) and field of view

Used for
dissection/observing small animals, embryos, plants, and insects
inspecting materials and electronic components, assembly work



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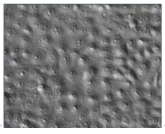
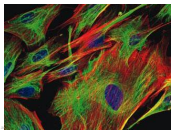
Various Microscopic Methods – how to observe?

Transmitted Light Microscopy
Observing light that has passed through samples such as cells or tissue sections

Brightfield microscopy (BF)
Phase contrast microscopy (PH)
Differential interference contrast microscopy (DIC)

Fluorescence Microscopy
labeling specific substances using dyes to observe their localization, behavior and dynamics





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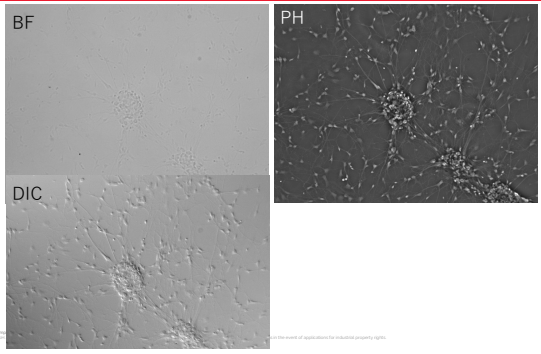
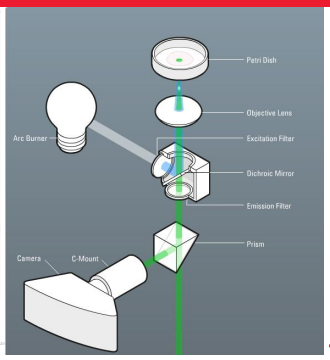
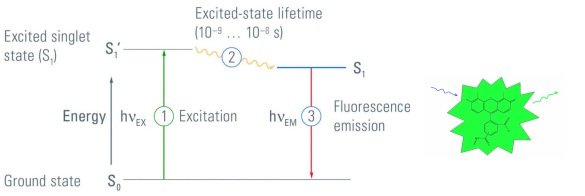
Transmitted Light Microscopy



Fluorescence Microscopy



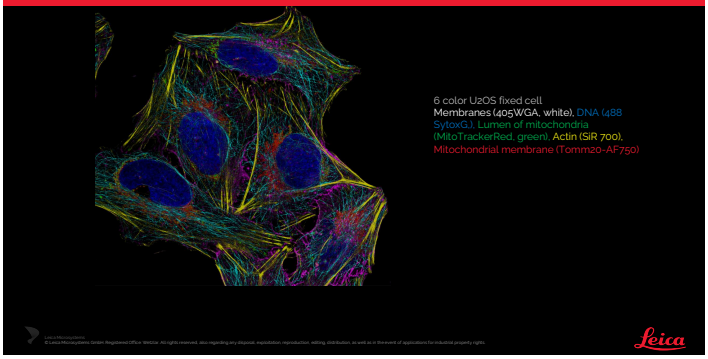
What is fluorescence?



fluorescence Jablonski diagram

A. Jablonski, Efficiency of Anti-Stokes Fluorescence in Dyes, Nature (1933) vol. 131, pp. 839-840, DOI: 10.1038/131839b0.

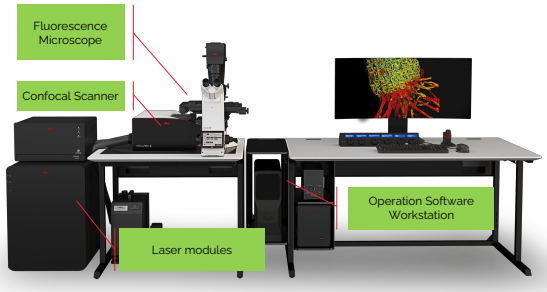
Fluorescence imaging



Agenda

1. Light Microscope
2. **Confocal Laser Scanning Microscope**
3. Advanced application: fluorescent lifetime imaging
4. Advanced application: super resolution imaging

Confocal Laser Scanning Microscope



Confocal Laser Scanning Microscope – Purpose of use

Used in a wide range of research fields,
including **life sciences** and **materials science**

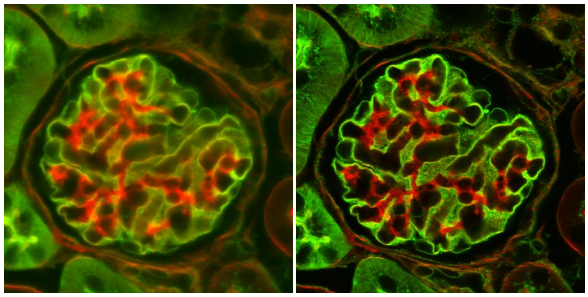
Research institutions at universities and government agencies
(in various fields such as medicine, science, pharmacy, and agriculture)
Pharmaceutical companies engaged in drug development
as well as manufacturers of cosmetics and consumer goods
Industrial/Material applications
(foreign object inspection, semiconductors, paper manufacturing, etc.)

Specimens to be Observed

Cells (animal and plant), bacteria, virus
Whole animal and plant specimens (mice, rats, zebrafish, C. elegans, etc.)
Ink, multilayer films, paper, wafers, and various material-based samples

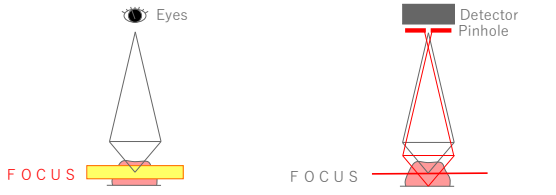
What is confocal laser scanning microscope (CLSM)?

Comparison with an image taken by fluorescence microscope

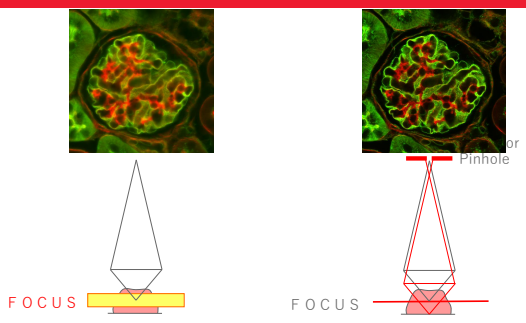


Fluorescence microscope Confocal laser scanning microscope

Principle of Confocal laser scanning microscope



Principle of Confocal laser scanning microscope

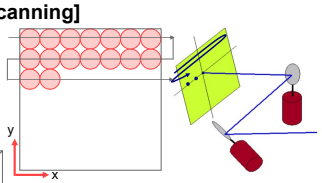


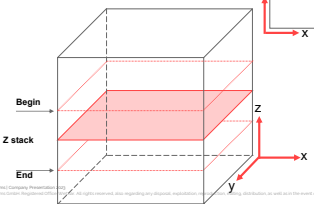
Principle of Confocal laser scanning microscope

Laser scanning [Point Scanning]

Laser is a point illumination
Detectors (PMT,HyD) are a point detection

plane detection (CCD camera)





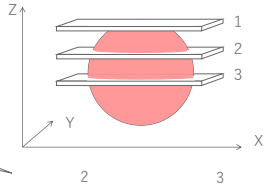
Using two mirrors called galvanometer mirrors, images are acquired as the excitation laser scans across the sample in the XY directions.

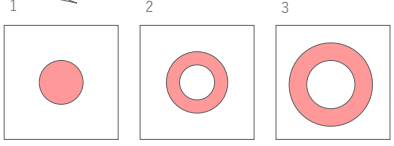
Furthermore, by shifting the focus, the sample can be observed in the Z direction.

Principle of Confocal laser scanning microscope

Optical Sectioning

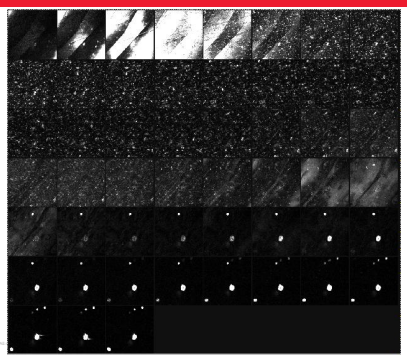
increase resolution
in Z-direction





Optical sectioning – polymer film z-stack series

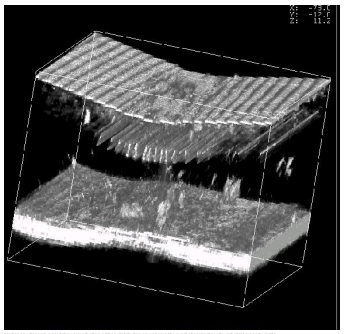
Surface



Inner area

Optical sectioning – polymer film z-stack 3D volume rendering

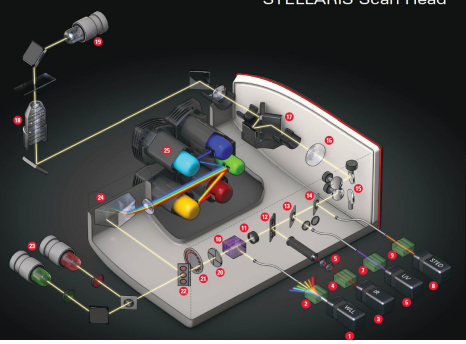
Surface



Inner area

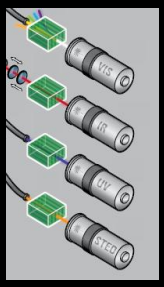
Optical Beam Pathway

STELLARIS Scan Head



1. White light laser and up to six visible line lasers
2. Acousto-optical tunable filter (AOTF)
3. Up to three infrared (IR) laser lines *
4. Acousto-optical modulation (AOM)
5. Vario Beam Expander (VBE)
6. Ultraviolet (UV 405 nm) lasers *
7. AOTF or direct modulation (DMOD)
8. STED laser *
9. Acousto-optical tunable filter (AOTF)
10. Acousto-optical beam splitter (AOBS), other options available
11. FRAP Booster *
12. Multi-function port: IR or special visible laser incoupling *
13. Ultraviolet (UV 405 nm) laser incoupling with CS2 UV optics
14. STED laser incoupling
15. FOV Scanner with Tandem Scanner option
16. Scan optics with alternative HIVISR or VISIR
17. Scan field rotation *
18. Objective lens (different options available)
19. Transmitted light detection *
20. Square confocal pinhole
21. Fluorifier disc *
22. Outcoupling with X1 port *
23. External detection *
24. Prism-based dispersion
25. SP Detection with up to five detectors (Power HyD S, Power HyD X or Power HyD R)

Optical Beam Pathway - Lasers

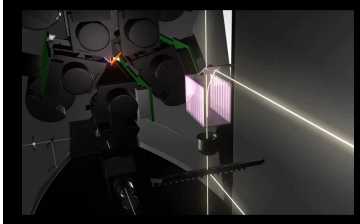


Laser Excitation

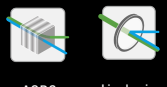
Diode Lasers

WLL

Optical Beam Pathway – Beam Splitter



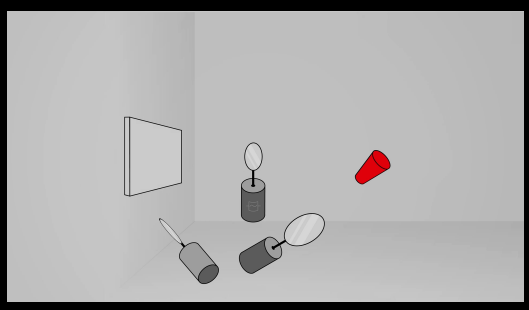
Beam Splitter



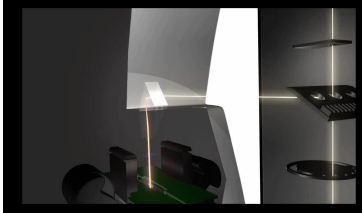
AOBS

Liachroic

Optical Beam Pathway – Scanner



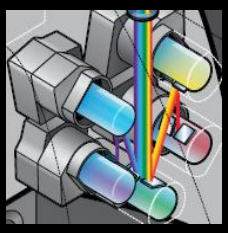
Optical Beam Pathway – Detector system



Prism

Photon-preserving dispersion

Optical Beam Pathway – Detector system

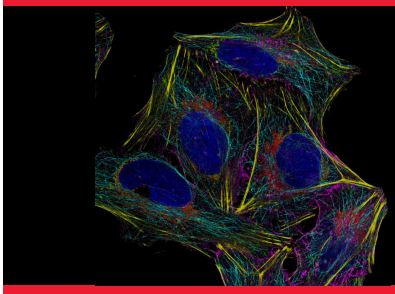


Spectral Detection

Agenda

1. Light Microscope
2. Confocal Laser Scanning Microscope
3. **Advanced application: fluorescent lifetime imaging**
4. Advanced application: super resolution imaging

Fluorescence imaging focus on spectral contrast….



6 color U2OS fixed cell
Membranes (405WGA, white), DNA (488 SytoxO), Lumen of mitochondria (MitoTrackerRed, green), Actin (SiR 700), Mitochondrial membrane (Tomm20-AF750)

..fluorescence contains much more information

What is Fluorescent Lifetime ?

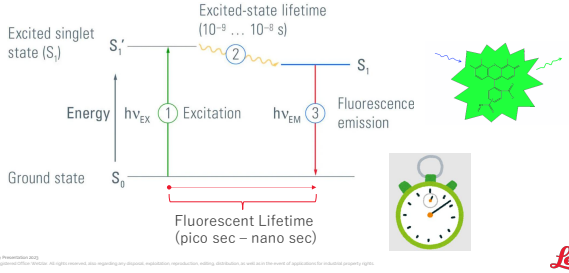
Q: What is Fluorescent Lifetime ?

A: The average time a fluorescent molecule spends in the excited state after being excited and before returning to the ground state

The time between when illuminate on a fluorescent substance and when fluorescence is emitted

What is Fluorescent Lifetime ?

The time between when illuminate on a fluorescent substance and when fluorescence is emitted



FLIM: Fluorescent Lifetime Imaging Microscopy

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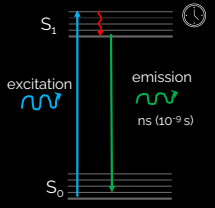
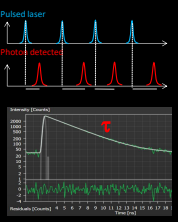
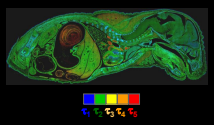
Fluorophore behavior…



..measured as lifetime…



..generating a FLIM image



FLIM creates images with lifetime information

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FLIM: Fluorescent Lifetime Imaging Microscopy

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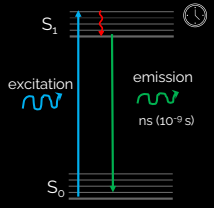
Fluorophore behavior…



Depends on
- Micro-environment (pH, temperature, ion concentration)
- Binding

Independent from
- Fluorophore concentration
- Bleaching
- Laser intensity

Fluorescence Lifetime

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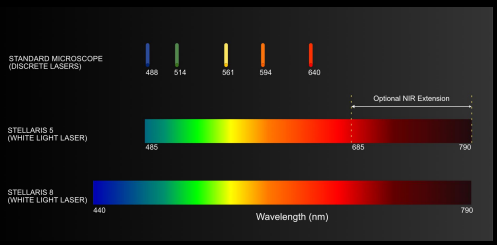
Key Components: The Next Generation White Light Lasers



- Complete spectral freedom with excitation perfectly matched to the fluorochrome
- Less complexity, more flexibility: a single laser to do the work of many. Up to 8 single excitation lines can be used simultaneously

#CONFOCALREIMAGINED

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Key components: fast electronics and FLIM components

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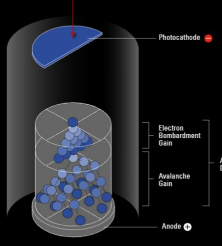
Fast electronics and FLIM components, fast HyD X/HyD R

- Up to 4 channels simultaneous and combinable
- Full system dead time: 1.5 ns
- Time resolution: 97 ps



The perfect detectors for FLIM

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STELLARIS FALCON for key applications

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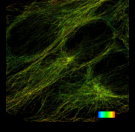
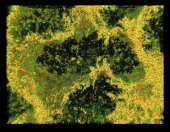
Molecular interaction
FLIM-FRET



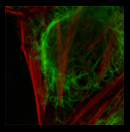
FLIM-FRET with AF488Tubulin/AF555Tubulin LUT 0-35% FRET efficiency

Biosensing
Changes in lifetime



Membrane tension change during osmotic pressure using FLIPPER TR

Species Separation
Component analysis



Phasor separation of AF647 phalloidin (red) and Atto647N vimentin (green)

FLIM is a great tool for functional imaging

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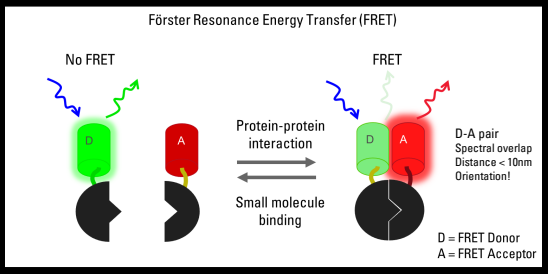
Why FRET?



FRET is THE tool to address molecular interaction

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STELLARIS FALCON applications

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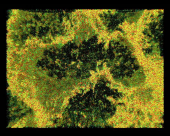
Biosensing

Changes in lifetime



Membrane tension change during osmotic pressure using FLIPPER TR

STELLARIS FALCON: Membrane tension visualization with FlipperTR®

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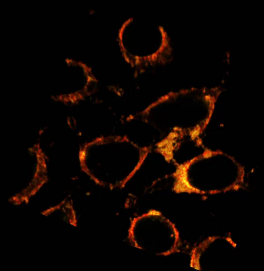

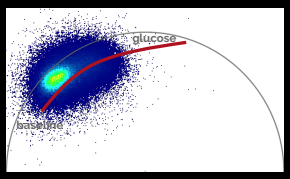

HeLa cells labeled with FLIPPER TR and treated with glucose to induce shrinking (reduce membrane tension)

STELLARIS FALCON: Membrane tension visualization with FlipperTR®

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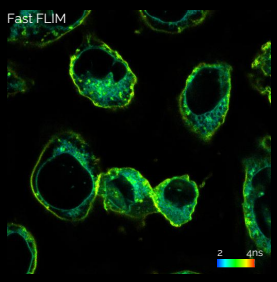
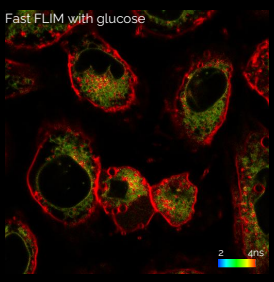
Fast FLIM



Fast FLIM with glucose



Fast FLIM image and FLIM phasor plot of U2OS cells labeled with Flipper TR, left cell in cell culture medium, right cell treated with 5% concentration glucose

STELLARIS FALCON applications

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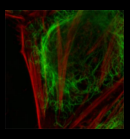
Species Separation

Component analysis



Phasor separation of AF647 phalloidin (red) and Atto647N vimentin (green)

STELLARIS FALCON: Fluorophores with overlapping spectra

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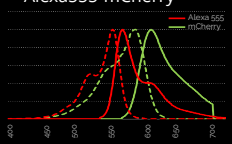
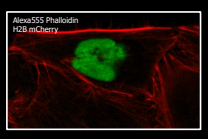
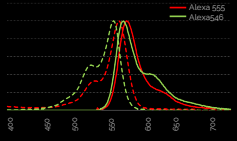
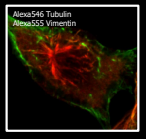
- Alexa555-mCherry





- Alexa555-Alexa546





Agenda

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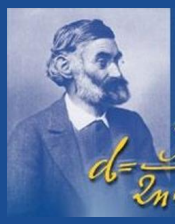
1. Light Microscope
2. Confocal Laser Scanning Microscope
3. Advanced application: fluorescent lifetime imaging
4. Advanced application: super resolution imaging

Theoretical limitation of the resolution



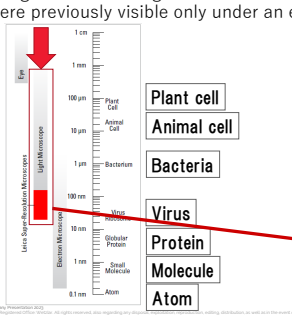
Ernst Abbe
1840-1905

As long as an optical microscope uses light, which is a wave, it cannot observe objects that are much smaller than the wavelength. (Abbe's diffraction limit)

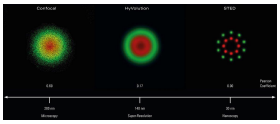
$d = \frac{\lambda}{2 n \sin\alpha} \fallingdotseq$ **200nm**

Super resolution microscope

Observing intracellular organelles and structures by using optical microscope that were previously visible only under an electron microscope



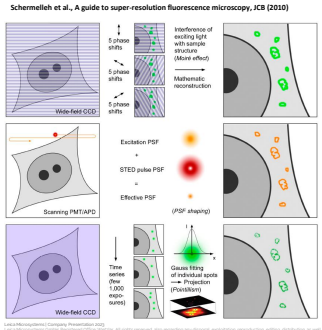
Plant cell
Animal cell
Bacteria
Virus
Protein
Molecule
Atom



Confocal 200nm | Confocal Super resolution 120nm | Nanoscopy <50nm

20～120nm

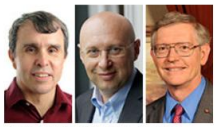
Super resolution microscope



1. Structured Illumination
   - SIM
   - 3D SIM
   - SSIM
2. Stimulation Emission Depletion
   - STED
3. Localization
   - PALM / FPALM
   - STORM / dSTORM
   - PALMIRA
   - GSDIM

2014 Nobel Prize in Chemistry

Awarded 2014 Nobel Prize in Chemistry
for Development of super-resolved fluorescent microscopy
STED：Stefan W. Hell
PALM: Eric Betzig, William E. Moener



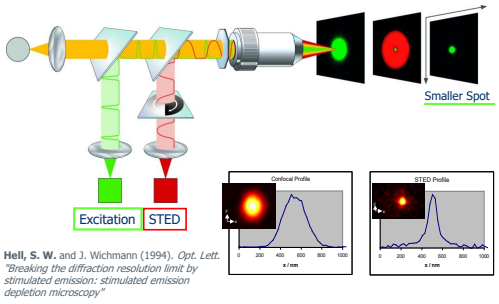
Photos: M. Staley/HHMI, B. Schuller, Max-Planck-Institut, Wikimedia Commons, CC-BY-SA-3.0

2014 Nobel Prize in Chemistry

The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

http://www.nobelprize.org/nobel_prizes/chemistry/

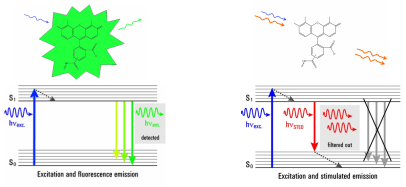
STED Microscopy: The Principle



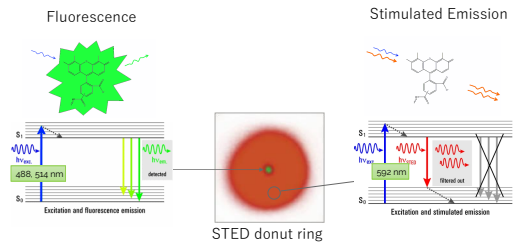
Hell, S. W. and J. Wichmann (1994). *Opt. Lett.* *"Breaking the diffraction resolution limit by stimulated emission: stimulated emission depletion microscopy"*

How to achieve super-resolution in STED?

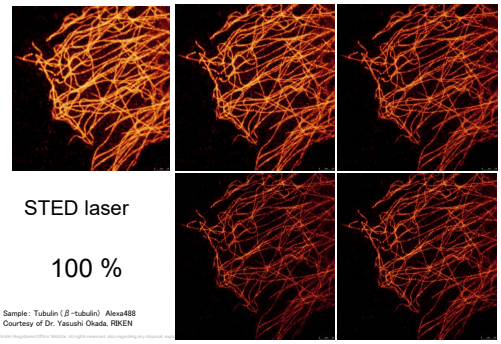
Fluorescence and Stimulated Emission



How to achieve super-resolution in STED?



How to achieve super-resolution in STED?

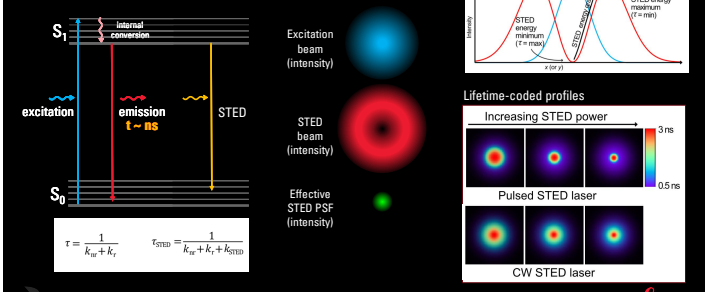


STED laser

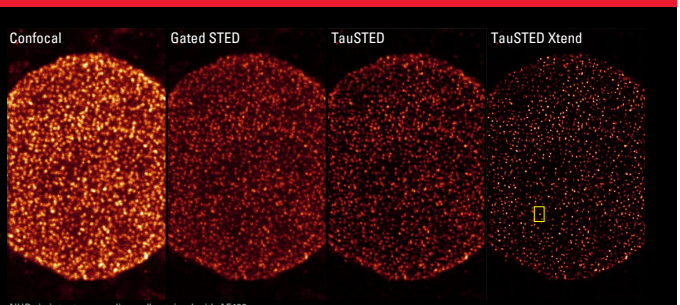
100 %

Sample: Tubulin (β-tubulin) Alexa488
Courtesy of Dr. Yasushi Okada, RIKEN

Fluorescence Lifetime and STED

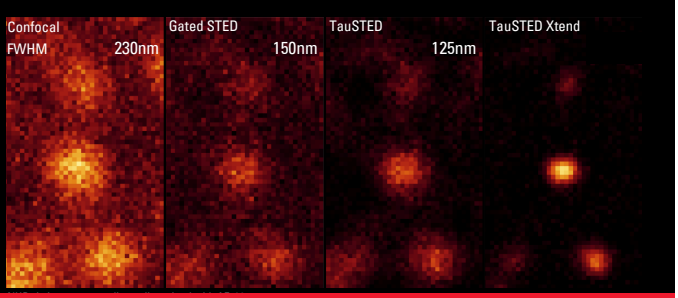


Xtend on green fluorophores



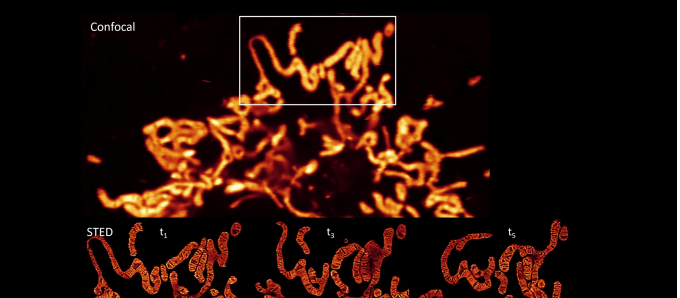
NUPs in intact mammalian cells stained with AF488.

Xtend on green fluorophores

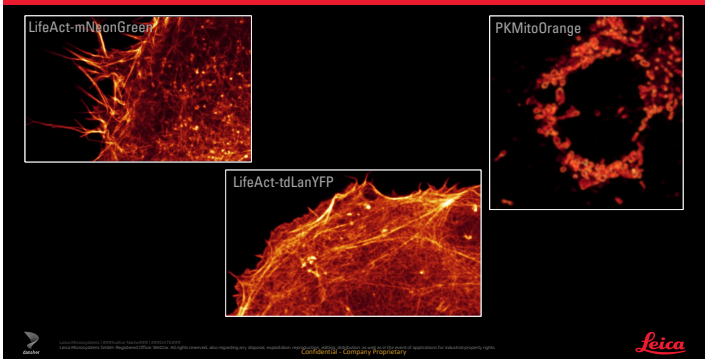


TauSTED Xtend pushes resolution in (even more) gentle imaging conditions

Gentle Live Cell Imaging - PKmitoOrange



What Do These Have in Common?



TauSTED Xtend: Gentle live cell imaging at nanoscale

TauSTED Xtend: Sharp. Simple Stunning.

Agenda

1. Light Microscope
2. Confocal Laser Scanning Microscope
3. Advanced application: fluorescent lifetime imaging
4. Advanced application: super resolution imaging

References

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