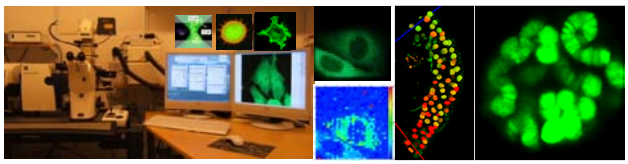



Fluorescentna mikroskopija. Tehnike oslikavanja, poboljšanje rezolucije zaobilaženjem difrakcione barijere i izučavanje dinamičkih procesa

Dr Vladana Vukojević, Associate Professor

Karolinska Institutet, Department of Clinical Neuroscience (CNS)
Center for Molecular Medicine (CMM), Stockholm, Sweden

Fakultet za fizičku hemiju, Univerzitet u Beogradu
27. juni, Beograd, Srbija

Karolinska Institutet, a medical university with a mission to contribute to the improvement of human health through research, education and information



Research goals
The goal for our research is to achieve scientific breakthroughs that change the view of human health and disease, as well as normal vital processes. Research results should lead to innovations and practical applications that can be implemented within the health service sector.

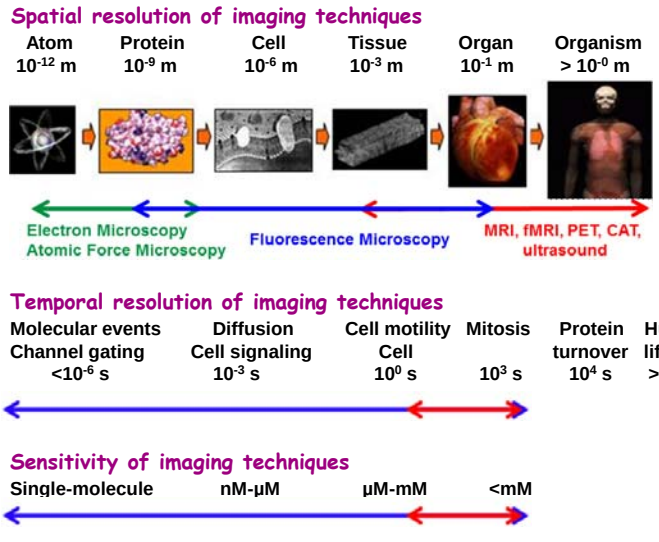
Educational goals
The goal for our educational programs is to strengthen their link to research, and to prepare the students for engagement in research. They should provide the best possible conditions to work in, lead and continue to develop activities in collaboration with other professions.

http://search.ki.se/search.do;jsessionid=1C744137F1321DCBDC6866C5BF4207C7?q=facts+about+karolinska+ppt&site=internwebben

Outline

- **Brief recapitulation of basic concepts: light-matter interaction**
  - **What is light?**
  - **What is fluorescence?**
  - **What are the most important properties of fluorophores and why is this important for you to know them?**
  - **What is fluorescence quenching and how to avoid it?**
- **Fluorescence Imaging - Confocal Laser Scanning Microscope (CLSM)**
  - **The confocal principle**
  - **Upright vs. inverted microscopes**
  - **Epifluorescence microscopes**
  - **The light path in epifluorescence microscopes**
  - **Magnification vs. resolution**
  - **Limits of resolution in epifluorescence microscopy**
  - **Evading the resolution limit imposed by the diffraction of light**
  - **Super-resolution fluorescence microscopy imaging techniques**

Imaging techniques in biomedical research

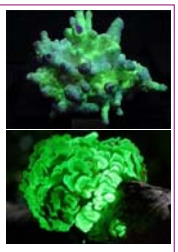


fFMI

DSLM
FCS
FCCS
FIONA
FLIM
FLIP
FRAP
FRET
LSFM
mSPIM
PALM
2PEM
RICS
SIM
SPIM
SHREK
STED
STORM
TIRF
TREXM

(Image modified from http://www.hinz.org.nz/uploads/journal/fig1_041205.jpg)

What is fluorescence?



Fluorescence is a naturally occurring, two-step dissipative process that may arise when the UV-VIS-IR electromagnetic field interacts with matter. The first step in this interaction is absorption of light, *i.e.* absorption of a photon by a molecule, which is quickly followed by the emission of a photon of lower energy, *i.e.* the emission of light of longer wavelength.

1500-1000 B.C. – first written recordings describing the phenomenon of luminescence

1646 – Athanasius Kircher wrote a book "Ars Magna Lucis et Umbrae"

1845 – Sir John Frederick William Bart Herschel described quinine fluorescence Phil.Trans.Roy.Soc. London 1845, **135**, 143-145

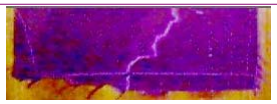
1843 - Sir George Gabriel Stokes coined the word fluorescence to describe luminescence calcium fluoride (fluorite). Phil.Trans.Roy.Soc. London 1852, **143**, 463-562

1875 – Eugen Cornelius Joseph von Lommel suggested that a substance must absorb light before it can emit it back as fluorescence

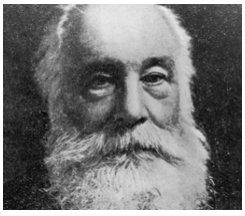
1930 – Alexander Jablonski "On the influence of the change of wavelengths of excitation light on the fluorescence spectra"

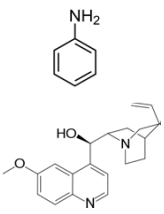
Goldberg and Weiner. The Science of Luminescence. http://pubs.acs.org/doi/pdf/10.1021/bk-1989-0383.ch001
Newton Harvey. A History of Luminescence. From the earliest time until 1900
https://archive.org/stream/historyoflumines00harv#page/n7/mode/2up

What are fluorophores?



Fluorophores are naturally existing or synthetic molecules which have the capacity to absorb light, *i.e.* electromagnetic radiation in the UV-VIS-IR part of the spectrum, and emit the absorbed energy in the form of light of longer wavelength. In this way, the excited molecule returns to its ground state, some energy is lost as heat and the electromagnetic radiation is converted from one wavelength to another.





Sir William Henry Perkin (1838 –1907) discovered the first aniline dye, mauveine, while trying to synthesize quinine.

1850-1900 Advances in fluorescent dyes development preceded and were crucial for the development of fluorescence microscopy.

1857 – Synthetic dye industry developed by William Perkin facilitated the synthesis, chemical and spectral characterization of many new fluorescent dyes, such as fluorescein (1871, Adolf von Baeyer), Eosin B (1875, Caro), rhodamine B and G (1887 and 1891, Ceresole), thioflavine (1888, Rosenheck), acridine orange (1889, Bender).

Theories of light

1665 – Micrographia
Robert Hooke

1690 – The wave model
Christiaan Huygens - birefringence

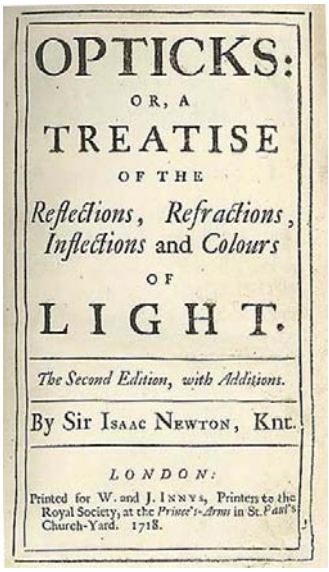
1704 – "Enforced" the corpuscular model
Sir Isaac Newton
Opticks, or, A treatise of the reflections, refractions, inflections and colors of light

1801-1849 - Coexistence
Thomas Young - interference of light (1801)

1849 – Disproved the corpuscular theory
Léon Foucault and Hippolyte Fizeau

1865 - Light is an electomagnetic wave
James Clerk Maxwell

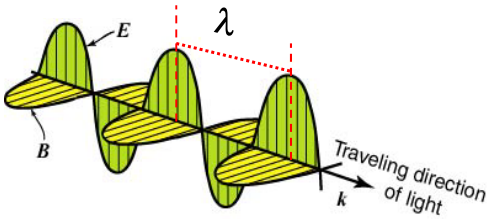
1905 – Reinstated the corpuscular model
Albert Einstein


OPTICKS:
OR, A
TREATISE
OF THE
Reflections, Refractions, Inflections and Colours
OF
LIGHT.
The Second Edition, with Additions.
By Sir Isaac Newton, Knt.
LONDON:
Printed for W. and J. Innys, Printers to the Royal Society, at the *Prince's-Arms* in St. *Paul's* Church-Yard. 1718.

http://galileo.phys.virginia.edu/classes/609.ral5q.fall04/LecturePDF/L20-LIGHTII.pdf
http://www.gutenberg.org/files/15491/15491-h/15491-h.htm
http://io9.com/5877660/was-robert-hooke-really-sciences-greatest-asshole

What is light?

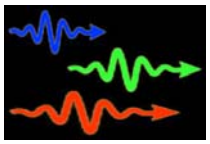
Light is a propagating electromagnetic wave, with the electric and magnetic field components being mutually perpendicular, and also perpendicular to the direction of light propagation.



- Amplitude of the electromagnetic wave defines the brightness of the light beam
- Wavelength defines its color
- Angle of vibration defines polarization

http://micro.magnet.fsu.edu/primer/java/wavebasics/index.html

According to the modern interpretation of the corpuscular model, light is a stream of photons whose energy is $E = h\nu = hc/\lambda$.



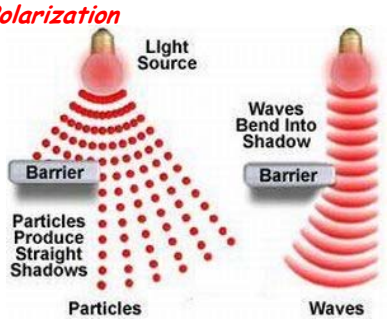
- Photon is an elementary particle with integer spin and no electric charge or rest mass.
- These bosons are carriers of the electromagnetic field.

http://www.windows2universe.org/physical_science/magnetism/photon.html

Dual nature of light and limitations of the individual models

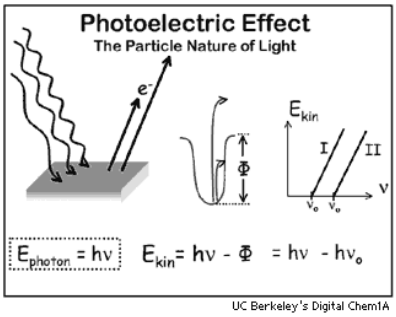
The corpuscular model cannot explain

Diffraction
Interference
Polarization



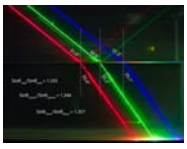
The wave model cannot explain

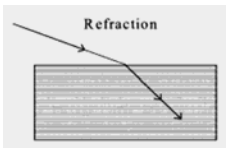
Photoelectric effect

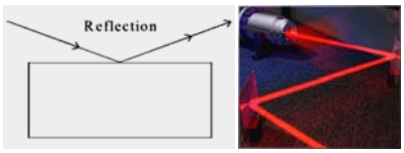


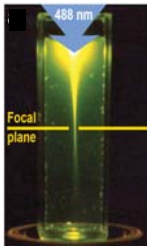
UC Berkeley's Digital ChemLA

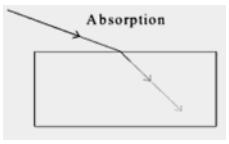
Light interaction with matter

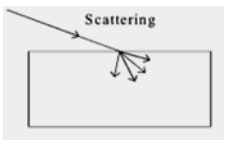






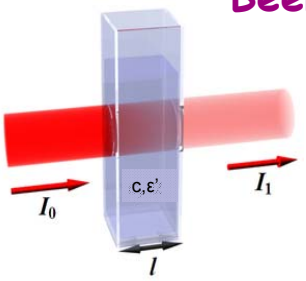






http://4.bp.blogspot.com/-nq51398Pg0Q/TuFrhlaK7cI/AAAAAAAABWk/Wb0F3SXpYpk/s1600/Snells_7380label.jpg
http://www.warren-wilson.edu/~physics/PhysPhotOfWeek/20050401LaserRefraction/LaserRefractn.jpg
http://www.nature.com.proxy.kib.ki.se/nbt/journal/v21/n11/pdf/nbt899.pdf
http://www.omegafilters.com/Capabilities/Specialized_Testing_/Light_Scattering_Measurements

Beer-Lambert law



$$-\ln\frac{I}{I_0} = \varepsilon'\cdot c\cdot l$$

$$\log_b(x) = \frac{\log_a(x)}{\log_a(b)} \Rightarrow \log_{10}\left(\frac{I}{I_0}\right) = \frac{\ln\left(\frac{I}{I_0}\right)}{2.303}$$

$$dI = -I_0\cdot\varepsilon'\cdot c\cdot dl$$

$$\frac{dI}{I_0} = -\varepsilon'\cdot c\cdot dl$$

$$\ln I - \ln I_0 = -\varepsilon'\cdot c\cdot(l-0)$$

$$\ln\frac{I}{I_0} = -\varepsilon'\cdot c\cdot l$$

$$-\log\frac{I}{I_0} = A = \frac{\varepsilon'}{2.303}\cdot c\cdot l = \boldsymbol{\varepsilon}\cdot c\cdot l$$

I - intensity of transmitted light
I_0 - intensity of incident light
ε- *molar extinction coefficient ($M^{-1}cm^{-1}$)*
c - concentration (M = mol dm^{-3})
l - optical path length (cm)

http://sciencefair.math.iit.edu/techniques/spectrophotometer/
http://bouman.chem.georgetown.edu/S98/spectrometer.htm

Electronic Configuration of Elements

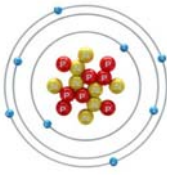
Pauli exclusion principle - two identical fermions (particles with half-integer spin) may not occupy the same quantum state simultaneously.

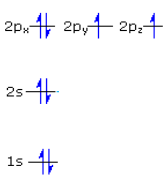
When applied to the electronic structure of an atom, the Pauli principle asserts that only two electrons with opposite spins can occupy the same atomic orbital.

The **"Aufbau principle"** – a hypothetical process of atom "building" formulated by Niels Bohr and Wolfgang Pauli stating that: The orbitals of lower energy are filled in first with electrons and only then the orbitals of high energy are filled.

Hund's rule of maximum multiplicity - unoccupied orbitals will be filled before occupied orbitals are reused by electrons having different spins.

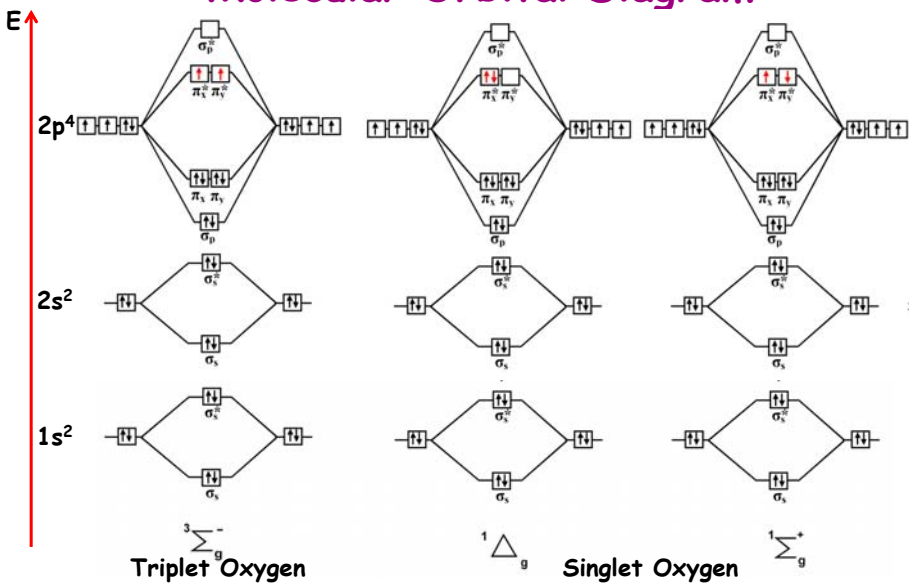
$^{16}_{8}O$





http://www.livescience.com/28738-oxygen.html
http://footage.shutterstock.com/clip-4385954-stock-footage-oxygen-atom-with-element-s-symbol-number-mass-and-element-type-color.html

Molecular Orbital Diagram

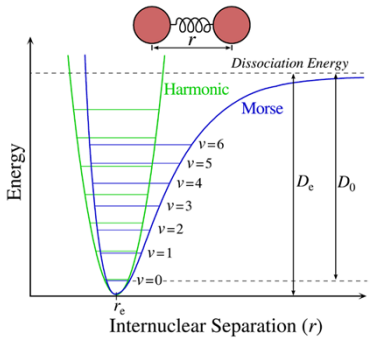


http://en.wikipedia.org/wiki/File:Molek%C3%BClorbital-Sauerstoff.png

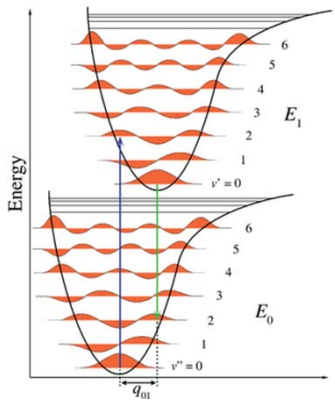
Coupling between vibrational and electronic levels. Potential well diagram

Potential energy of a diatomic molecule

$$V(r) = D_e(1 - e^{-a(r-r_e)})^2$$



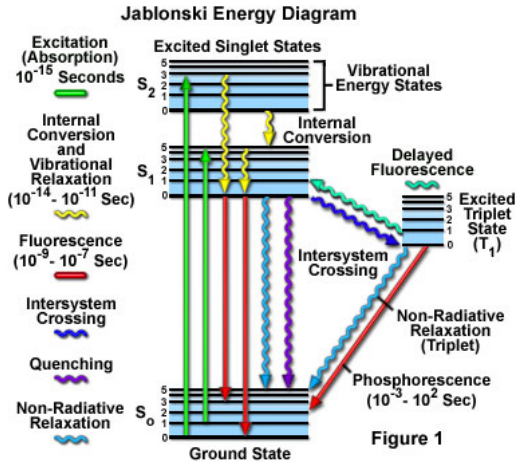
Morse Potential



Franck-Condon principle

http://en.wikipedia.org/wiki/File:Morse-potential.png
http://en.wikipedia.org/wiki/File:Franck-Condon-diagram.png

Jablonski diagram

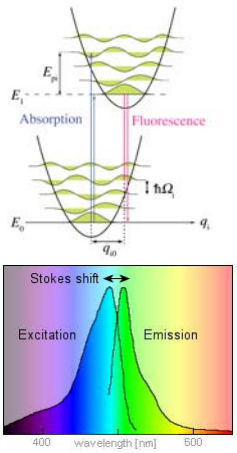


- **Electronic states** are arranged vertically by energy and grouped horizontally by spin multiplicity.
- **Radiative transitions** are indicated by straight arrows and non-radiative transitions by wavy arrows.
- **The vibrational ground states** of each electronic state are indicated with thick lines, the higher vibrational states with thinner lines.

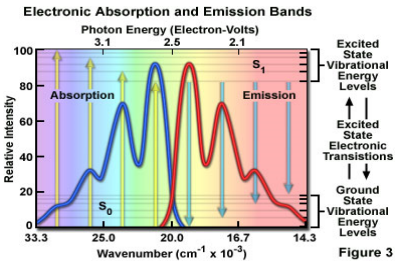
http://www.olympusmicro.com/primer/java/jablonski/jabintro/index.html

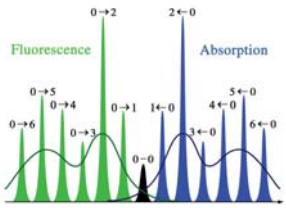
Characteristics of fluorescence emission

Stokes shift



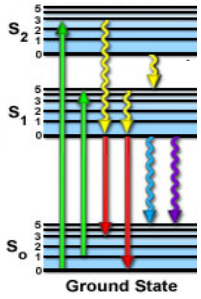
The mirror-image rule

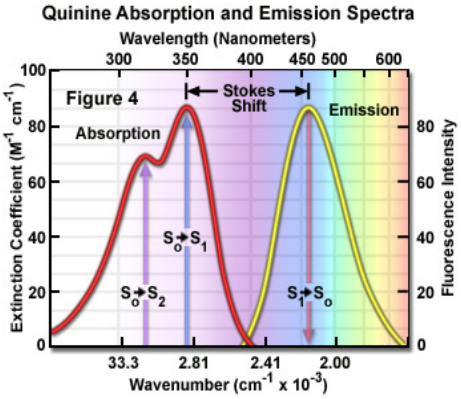




Lakowicz JR. Principles of Fluorescence Spectroscopy

Exceptions from the mirror-image rule





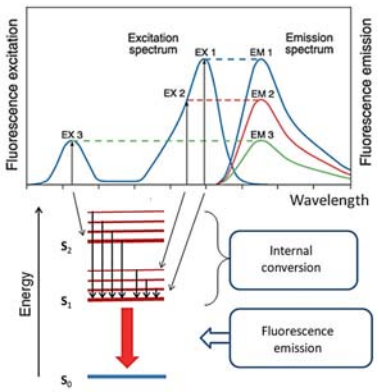
http://www.olympusconfocal.com/theory/fluoroexciteemit.html

Emission spectra and Kasha's rule

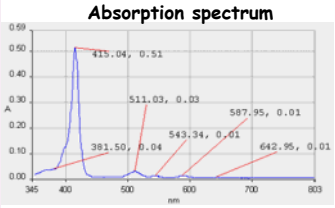
cross-excitation and spectral bleed through (crossover, crosstalk)

Photon emission (fluorescence or phosphorescence) occurs in an appreciable yield only from the lowest excited state of a given multiplicity.

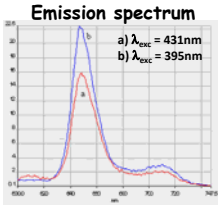
The same fluorescence emission spectrum is generally observed irrespective of the excitation wavelength.

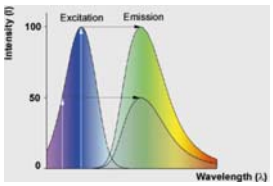


Absorption spectrum



Emission spectrum

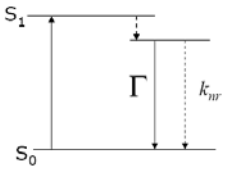




Demchenko et al. Angew. Chem. Int. Ed. 2014, 53, 2 – 11
Fagadar-Cosma et al. Digest Journal of Nanomaterials and Biostructures, 2007 **2(1):** 175 - 183

Fluorescence lifetime

The lifetime (τ) of the excited state is the average amount of time that the molecule spends in that state before returning to the ground state.
Γ and k_{nr} are rate constants for the radiative and nonradiative decay, respectively.



$$\tau = \frac{1}{\Gamma + k_{nr}}$$

$$\tau_{nat} = \frac{1}{\Gamma} \quad \textit{natural lifetime (in the absence of nonradiative processes)}$$

$$\Gamma \cong 2.88\times10^{-9} N^2 \frac{\int_{\bar{\nu}} F(\bar{\nu}) d\bar{\nu}}{\int_{\bar{\nu}} F(\bar{\nu}) d\bar{\nu} / \bar{\nu}^3} \int_{\bar{\nu}} \frac{\varepsilon(\bar{\nu})}{\bar{\nu}} d\bar{\nu}$$

$$\Gamma = 2.88\times10^{-9} N^2 \left\langle \bar{\nu}^{-3} \right\rangle^{-1} \int_{\bar{\nu}} \frac{\varepsilon(\bar{\nu})}{\bar{\nu}} d\bar{\nu}$$

$$\tau = \tau_{nat} \cdot Q$$

Typically fluorescence lifetimes are < 10 ns.
Vibrational: internal relaxation occurs on much shorter timescales (10^{-12} s).

The fluorescence process is random. The population of the excited state is governed by Boltzmann statistics. The decay to the ground state is exponential in time – hence the lifetime is an average value: at t=τ, about 63% of the molecules have decayed and the remaining 37% will decay t>τ.

Quantum yield

Quantum yield is among the most important properties of a fluorescent dye. It is defined as the number of photons emitted relative to the number of photons absorbed.

Molecules such as rhodamine have a quantum yield near 1.

Quantum yield is determined by the rate constants for radiative and non-radiative emission:

$$\Phi_F = \frac{k_r}{k_r + k_{chem} + k_{dec} + k_{ET} + k_{et} + k_{pt} + k_{tict} + k_{ic} + k_{isc}}$$

$$\Phi_F = \frac{k_r}{k_r + k_{nr}}$$

Rate constants:

k_r	= radiative
k_{chem}	= photochemistry
k_{dec}	= decomposition
k_{ET}	= energy transfer
k_{et}	= electron transfer
k_{tict}	= proton transfer
k_{tict}	= twisted-intramolecular charge transfer
k_{ic}	= internal conversion
k_{isc}	= intersystem crossing

Lakowicz JR. Principles of Fluorescence Spectroscopy;
http://pubs.rsc.org.proxy.kib.ki.se/en/content/articlepdf/2009/pp/b903357m

Brightness

Brightness is equal to the product of the extinction coefficient and the quantum yield, divided by 1000 (convention).

$$BR = \frac{\varepsilon \times \Phi_F}{1000}$$

ε - molar extinction coefficient ($M^{-1}cm^{-1}$)
Φ_F - quantum yield

eGFP:

Φ_F = 0.6

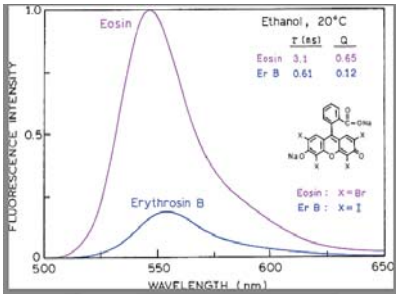
ε = 56000 $M^{-1}cm^{-1}$

BR = 33.6 $M^{-1}cm^{-1}$

Relative brightness:

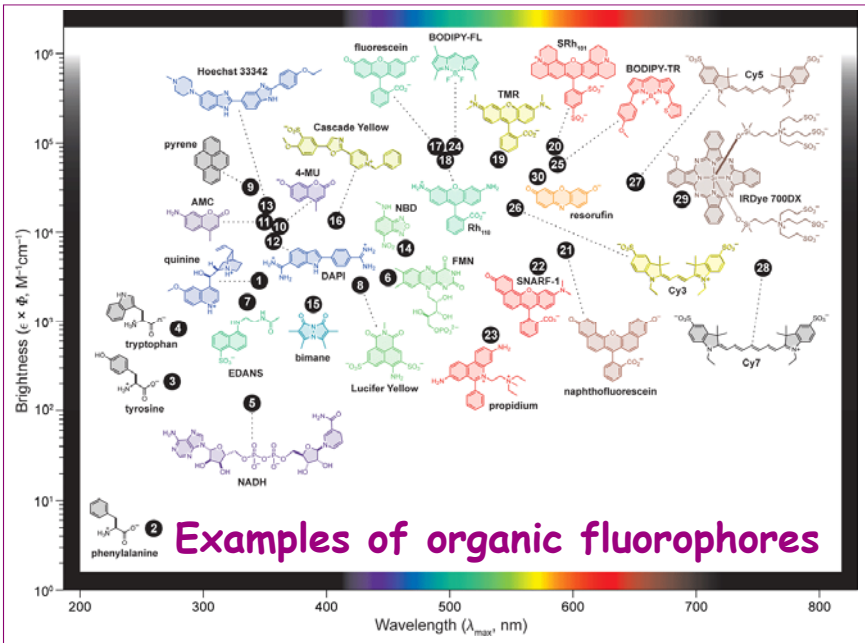
$$BR_{relative} = \frac{BR}{BR_{eGFP}}$$

Molecular Brightness



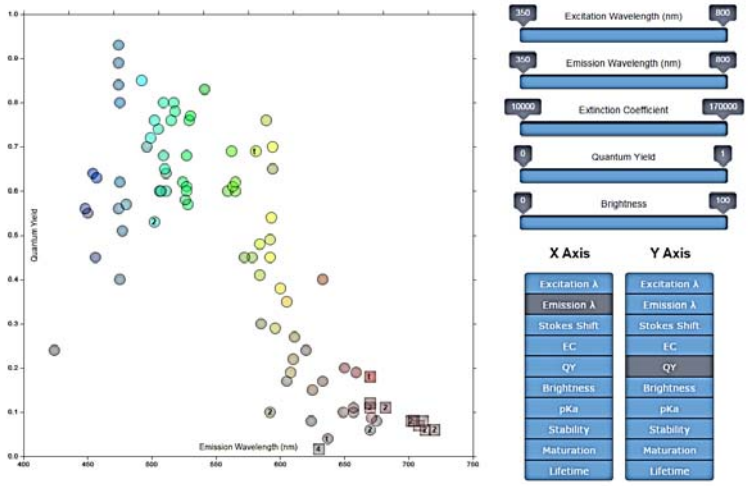
Protein	Excitation peak, nm	Emission peak, nm	EC, $M^{-1}cm^{-1}$	QY	Relative brightness	pKa
Sirius	355	424	15,000	0.24	0.11	<3.0
Azurite	383	447	26,000	0.55	0.43	5.0
EBFP2	383	448	32,000	0.56	0.54	4.5
TagBFP	402	457	52,000	0.63	0.99	2.7
mTurquoise	434	474	30,000	0.84	0.76	4.5
ECFP	434	477	32,500	0.40	0.39	4.7
Cerulean	433	475	36,000	0.57	0.62	4.7
TagCFP	458	480	37,000	0.57	0.64	4.7
mTFP1	462	492	64,000	0.85	1.65	4.3
mUkG1	483	499	60,000	0.72	1.31	5.2
mAG1	492	505	55,000	0.74	1.23	5.8
AcGFP1	475	505	50,000	0.55	0.83	-
TagGFP2	483	506	56,500	0.61	1.05	5.0
EGFP	489	509	55,000	0.60	1.00	5.9
mWasabi	493	509	70,000	0.80	1.70	6.5
EmGFP	487	509	57,500	0.68	1.19	6.0
TagYFP	508	524	64,000	0.62	1.20	5.5
EYFP	514	527	84,000	0.61	1.55	6.5
Topaz	514	527	94,500	0.60	1.72	-
SYFP2	515	527	101,000	0.68	2.08	6.0
Venus	515	528	92,200	0.57	1.59	6.0
Citrine	516	529	77,000	0.76	1.77	5.7
mKO	548	559	51,600	0.60	0.94	5.0
mKO2	551	565	63,800	0.57	1.10	5.5
mOrange	548	562	71,000	0.69	1.49	6.5
mOrange2	549	565	58,000	0.60	1.06	6.5
TagRFP	555	584	100,000	0.48	1.42	<4.0
TagRFP-T	555	584	81,000	0.41	0.99	4.6
mStrawberry	574	596	90,000	0.29	0.79	<4.5
mRuby	558	605	~90,000	0.35	1.06	4.4
mCherry	587	610	72,000	0.22	0.48	<4.5
mRaspberry	598	625	86,000	0.15	0.39	-
mKate2	588	633	62,500	0.40	0.76	5.4
mPlum	590	649	41,000	0.10	0.12	<4.5
mNeptune	600	650	67,000	0.20	0.41	5.4
T-Sapphire	399	511	44,000	0.60	0.79	4.9
mAmetrine	406	526	45,000	0.58	0.78	6.0
mKeima	440	620	14,400	0.24	0.10	6.5

Lakowicz JR. Principles of Fluorescence Spectroscopy
Chudakov et al. Physiological Reviews 2010 **90**:1103-1163. (http://physrev.physiology.org/content/physrev/90/3/1103.full.pdf)



http://www.hhmi.org/research/tailoring-fluorescent-molecules-biological-applications

Fluorescent protein properties



http://nic.ucsf.edu/FPvisualization/
http://nic.ucsf.edu/dokuwiki/doku.php?id=fluorescent_proteins
Chudakov et al. Physiological Reviews 2010 **90**:1103-1163. (http://physrev.physiology.org/content/physrev/90/3/1103.full.pdf)

Physical and optical data

Protein	Ref.[a]	λ_{ex}[b]	λ_{em}[c]	ε[d]	φ[e]	Brightness[f]	Photostability[g]	pKa[h]	Maturation[i]
LanYFP	‡	513	524	150	0.95	424	ND	3.5	ND
dLanYFP	‡	513	524	125	0.90	335	ND	ND	ND
mNeonGreen	‡	506	517	116 ± 4	0.80 ± 0.016	276	158 ± 13	5.7	< 10
Clover	7	505	515	111	0.76	251	50	6.2	30
YPet	2	517	530	104	0.77	238	49	5.6	ND
mCitrine	2	516	529	77	0.76	174	49	5.7	ND
mVenus	2	515	528	92	0.57	156	15	6.0	ND
EYFP	2	514	527	83	0.61	151	60	6.9	ND
mEmerald	2	487	509	57	0.68	116	101[j]	6.0	ND
sfGFP	2	485	510	83	0.65	161	157[j]	5.5	ND
mWasabi	15	493	509	70	0.80	167	93	6.5	ND
mAG	16	492	505	42	0.81	100	ND	6.2	ND
mEGFP	2	488	507	56	0.60	100	150[j]	6.0	25[k]

[a]Source of data unless otherwise noted. [b]Excitation maximum in nm. [c]Emission maximum in nm. [d]Extinction coefficient in $mM^{-1}cm^{-1}$, determined by alkali denaturation method. [e]Fluorescence quantum yield. [f]Product of ε and (x003C6), expressed as a percentage of mEGFP brightness. [g]Time in s to photobleach from 1000 to 500 photons per s per molecule in live cells under widefield arc-lamp illumination. [h]pH at which fluorescence intensity is 50% of its maximum value. [i]Time in min for fluorescence to reach its half-maximal value after exposure to oxygen at 37 °C. ND = not determined.

Shaner et al. Nat Methods. 2013 May; 10(5): 10.1038/nmeth.2413

Fluorescence quenching

The intensity of fluorescence can be decreased by a wide number of processes.

Collisional quenching is the most typically encountered fluorescence quenching process where the fluorophore in the excited state is deactivated by a collision with another molecule in the sample, but the molecules are not chemically altered in the process.

The decrease in intensity for collisional quenching can be described by the following equation:

$$A^* + Q \rightarrow A + Q^*$$

$$\frac{F_0}{F} = 1 + K \cdot [Q] = 1 + k_q \tau_0 \cdot [Q]$$

K - Stern-Volmer quenching constant

k_q - bimolecular quenching constant

τ_0 - unquenched lifetime

[Q] - quencher concentration

$$\Phi_f^0 = \frac{k_f}{k_f + k_d} = \frac{k_f}{\frac{1}{\tau_0}} = k_f \tau_0 \quad \text{In absence of quencher}$$

$$\Phi_f = \frac{k_f}{k_f + k_d + k_q[Q]} = \frac{k_f}{\frac{1}{\tau_0} + k_q[Q]} \quad \text{In presence of quencher}$$

$$\frac{\Phi_f^0}{\Phi_f} = \frac{\frac{k_f}{\frac{1}{\tau_0}}}{\frac{k_f}{\frac{1}{\tau_0} + k_q[Q]}} = 1 + k_q \tau_0 [Q] \quad \text{Dividing these equations}$$

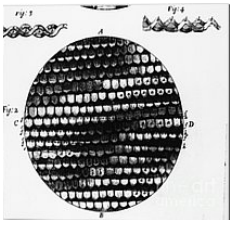
$$\frac{\Phi_f^0}{\Phi_f} = \frac{I_f^0}{I_f} = \frac{\tau_0}{\tau} = 1 + k_q \tau_0 [Q] \quad \textit{Stern-Volmer Equation}$$

The Photochemistry Portal
photochemistry.wordpress.com

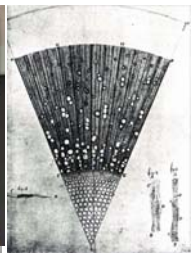
Lakowicz JR. Principles of Fluorescence Spectroscopy
https://photochemistry.wordpress.com/category/quenching/

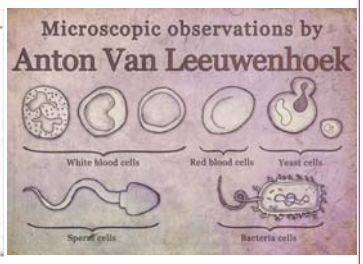
Instrumentation for optical imaging

1665 Robert Hook



1677 Antonie van Leeuwenhoek





Milestones in optical microscopy and the birth of fluorescence microscopy

1874 – Abbe E. A Contribution to the Theory of the Microscope and the nature of Microscopic Vision. Proceedings of the Bristol Naturalists' Society 1874 **1**:200–261.

1883 – Abbe E. The Relation of Aperture and Power in the Microscope (continued). Journal of the Royal Microscopical Society. 1883 **3**:790–812.

Microscope objectives that minimized the effects of spherical and chromatic aberrations could be readily produced, but limitations associated with resolution, contrast, noise, sensitivity and specificity remained.

1850-1900 Advances in fluorescent dyes development preceded and were crucial for the development of the fluorescence microscope – synthetic dye industry developed by William Perkin facilitated the synthesis, chemical and spectral characterization of many new fluorescent dyes, such as fluorescein (1871, Adolf von Baeyer), Eosin B (1875, Caro), rhodamine B and G (1887 and 1891, Ceresole), tioflafine (1888, Rosenheck), acridine orange (1889, Bender)

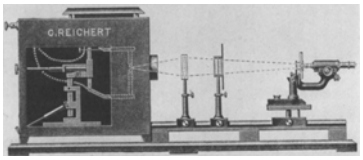
Masters BR. The Development of Fluorescence Microscopy. Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester. doi: 10.1002/9780470015902.a0022093; http://www.physics.bilkent.edu.tr/news/masters/ELS_Hist_Fl_Micro.pdf
Cell Structure and Function by Microspectrofluorometry, Elli Kohen, Academic Press, 1989
http://books.google.se/books?id=iLhQAwAAQBAJ&pg=PA4&lpg=PA4&dq=Acridine+orange+Bender&source=bl&ots=Y6PJS2HyRb&sig=Wr28HhEaR86JO_c7NmWOOjlNvqI&hl=en&sa=X&ei=AENWVNS_JNGradvZgPgM&ved=0CCgQ6AEwAQ#v=onepage&q=Acridine%20orange%20Bender&f=false

The forerunners of fluorescence microscope

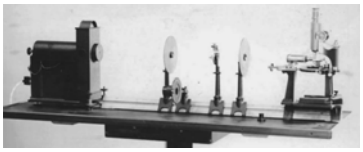
1904 – the UV absorption microscope, Carl Zeiss, Germany

August Köhler
Koehler A. New Method of Illimination for Photomicro-graphical Purposes. Journal of the Royal Microscopical Society 1894 **14:** 261–262.

1911– Heimstädt's fluorescence microscope, Reichert Company, Austro-Hungarian Empire



1912 – Slit ultramicroscope by Siedentopf and Zsigmondy, Zeiss, Germany

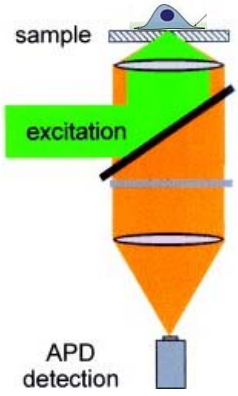


Masters BR. The Development of Fluorescence Microscopy. Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester. doi: 10.1002/9780470015902.a0022093; http://www.physics.bilkent.edu.tr/news/masters/ELS_Hist_Fl_Micro.pdf
Cell Structure and Function by Microspectrofluorometry, Elli Kohen, Academic Press, 1989
http://books.google.se/books?id=iLhQAwAAQBAJ&pg=PA4&lpg=PA4&dq=Acridine+orange+Bender&source=bl&ots=Y6PJS2HyRb&sig=Wr28HhEaR86JO_c7NmWOOjlNvqI&hl=en&sa=X&ei=AENWVNS_JNGradvZgPgM&ved=0CCgQ6AEwAQ#v=onepage&q=Acridine%20orange%20Bender&f=false

Fluorescence microscopy types

Wide-field microscopy

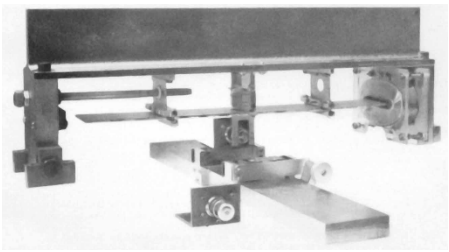
Confocal microscopy



http://www.science.uva.nl/research/molphot/research/sm/peterSMS.html

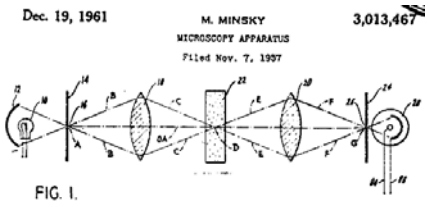
The Confocal Laser Scanning Microscope (CLSM)

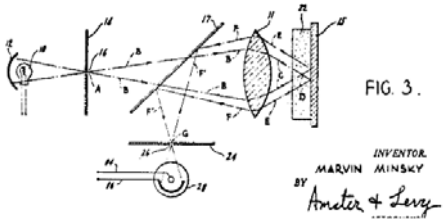
Marvin Minsky



http://global.britannica.com/EBchecked/topic/384568/Marvin-Minsky

The confocal principle



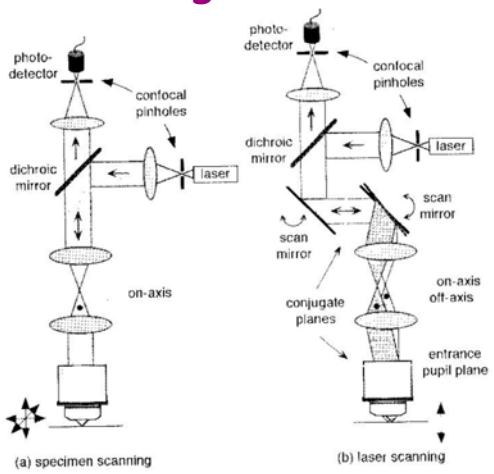


A confocal imaging system achieves out-of-focus light rejection by two strategies:
•by illuminating a single point of the specimen at any one time using a focused beam, so that illumination intensity drops off rapidly above and below the plane of focus
•by blocking the out-of-focus light by a pinhole aperture in a conjugate focal plane to the specimen so that light emitted away from the illuminated point in the specimen is blocked from reaching the detector.

The axial resolution that is obtained can be better 1.4 times than the resolution obtained by wide field illumination.

http://hugroup.cems.umn.edu/Research/imageproc.html; http://phym.unige.ch/cmi/Docs/Arnaudeau-Confocal.pdf
http://www.loci.wisc.edu/optical-sectioning/confocal-imaging; http://www.pdfs.name/handbook-of-biological-confocal-microscopy

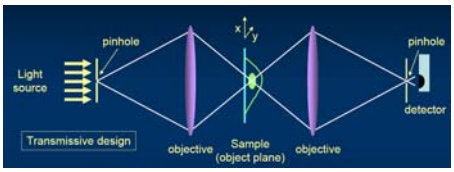
Specimen scanning *versus* laser scanning

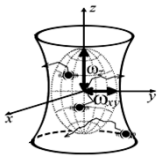


Advantage of specimen scanning is that the field of view can be large and all points in the image are illuminated under the same angle. Dissadvantage, image acquisition is slow.

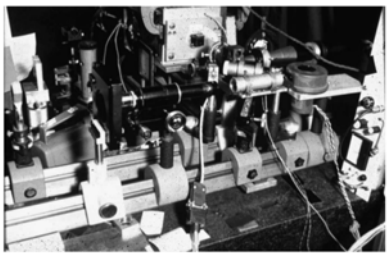
Dissadvantage of single point scanning is that all points are illuminated under different angles, and the maximum field of view is limited in size. Advantage, image acquisition is fast.

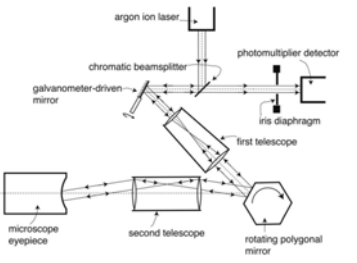
The confocal laser scanning microscope (CLSM)

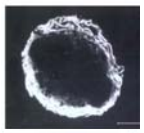


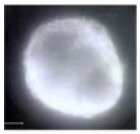


Prototype CLSM at the MRC Laboratory of Molecular Biology in 1986



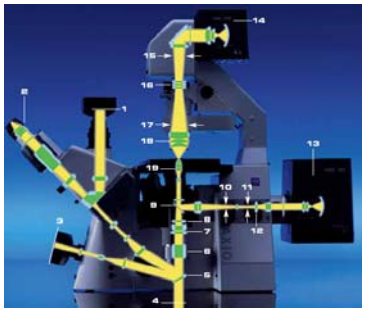


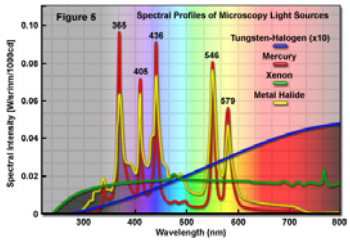


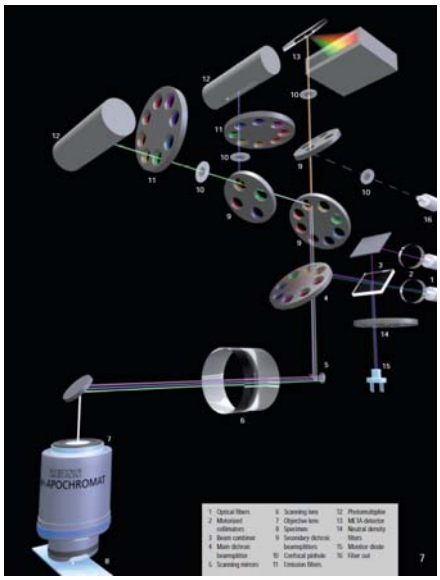


http://web.media.mit.edu/~minsky/papers/ConfocalMemoir.html
Amos WB, White JG. How the confocal laser scanning microscope entered biological research. Biol Cell. 2003 **95**:335-42. http://onlinelibrary.wiley.com.proxy.kib.ki.se/doi/10.1016/S0248-4900(03)00078-9/pdf

Confocal microscope







LSM510-ConfoCor2

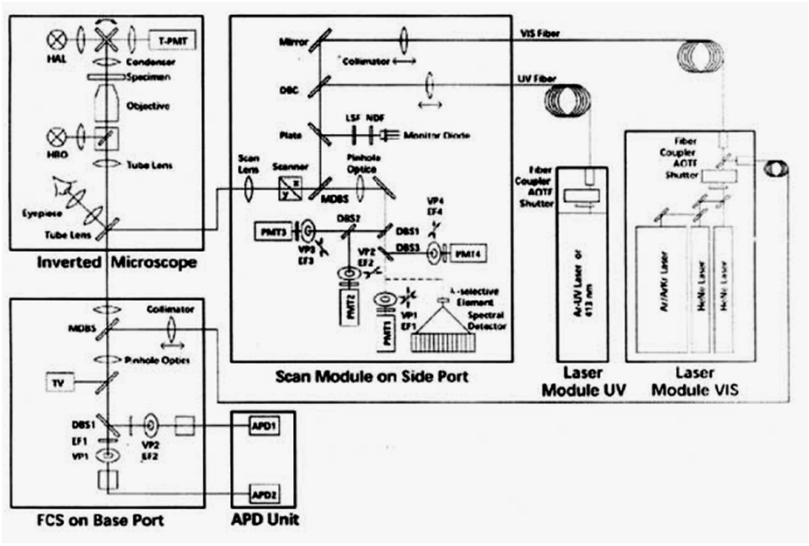
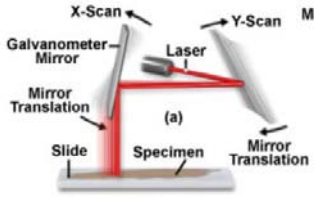
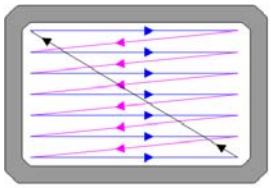


Image acquisition

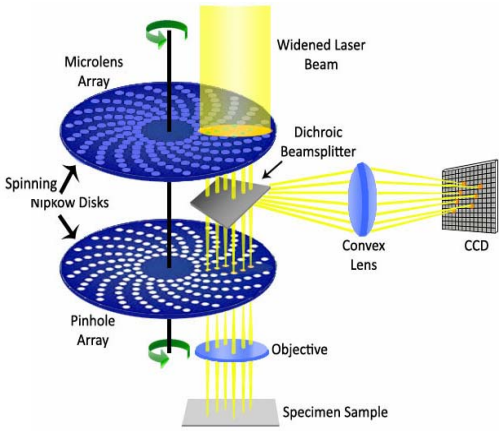
Laser point scanning



Raster scanning



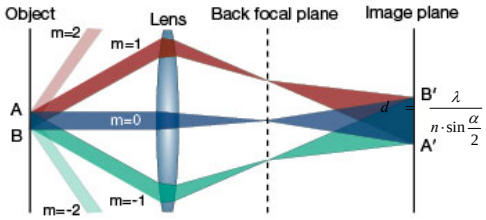
Spinning (Nipkow) disc

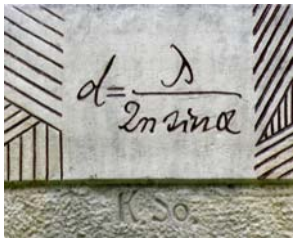


http://micro.magnet.fsu.edu/primer/techniques/confocal/confocalintroduction.html
http://zeiss-campus.magnet.fsu.edu/tutorials/spinningdisk/yokogawa/index.html

Diffraction of light & Abbe's theory of image formation

- The object AB when illuminated with coherent light, diffracts the light.
- The different orders of diffraction collected by the lens are separated in the back focal plane of the objective lens.
- Diffracted orders interfere in the image plane, forming the image B'A'.





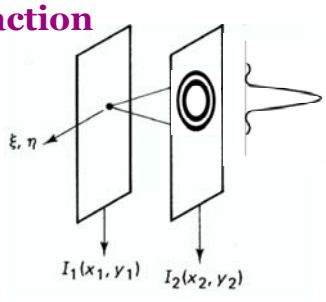
- The smallest separation that can be resolved (d) depends on the wavelength of the illumination light in vacuum (λ), one half of the angular aperture of the microscope objective (α) and the refractive index of the immersion medium (n).

http://www.osa-opn.org/home/articles/volume_18/issue_2/features/ernst_abbe_and_the_foundation_of_scientific_micros/#.Up-biBDVbag
E. Abbe. Beiträge zur Theorie des Mikroskops und der Mikroskopischen Wahrnehmung. Archiv für Mikroskopische Anatomie, IX, 413-68 (1873).

Resolution limit imposed by diffraction

Diffraction effects, aberrations in the optical system and detector noise will spread the image of a point source captured by a confocal microscope over a finite area.

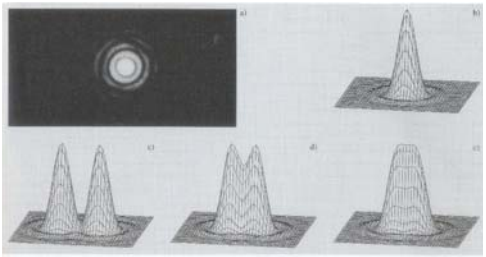
Irradiance distribution in an image of a point source is called the point spread function (PSF).



Resolution of a diffraction-limited optical microscope

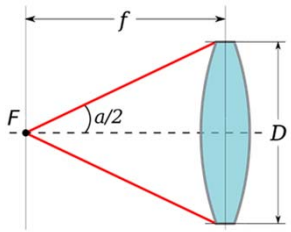
Radial resolution $R = \dfrac{1.22 \cdot \lambda}{2 \cdot NA}$

Axial resolution $Z \approx \dfrac{0.64 \cdot \lambda}{n - \sqrt{n^2 - NA^2}}$



Resolution in confocal microscopy

Angular aperture of a lens is the apparent angle of the lens aperture as seen from the focal point.



$$\frac{\alpha}{2} = arctg\left(\frac{D}{2f}\right)$$

f – focal length

D –diameter of the aperture

Numerical aperture (NA)

$$NA = n \cdot \sin\frac{\alpha}{2}$$

n – refractive index of the immersion medium

Improving the resolution of fluorescence microscopy

Improving the axial resolution

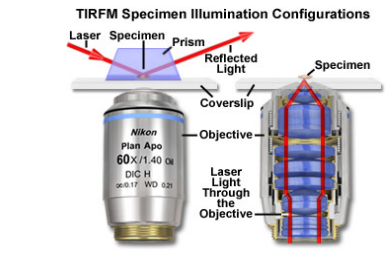
- Total Internal Reflection (TIRF) Microscopy
- Light Sheet Fluorescence Microscopy (LSFM)
- Single Plane Illumination Microscopy (SPIM)
- 2-Photon excitation Microscopy (2-PEM)

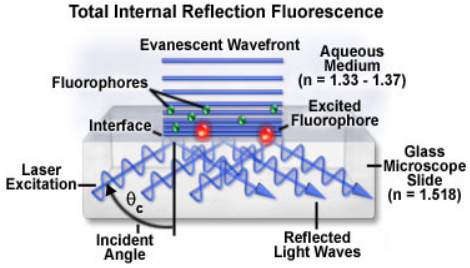
Evading the diffraction limit – Super-resolution fluorescence imaging techniques

- Structured Illumination Microscopy (SIM)
- Fluorescence Imaging with One Nanometer Accuracy (FIONA)
- Stochastic Optical Reconstruction Microscopy (STORM)
- (Fluorescence) Photoactivation Localization Microscopy ((f)PALM)
- Stimulated Emission Depletion (STED) Microscopy

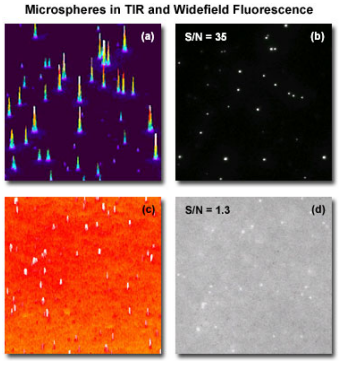
http://www.youtube.com/watch?v=UCJ6oQSdxN0

Total Internal Reflection (TIRF) Microscopy

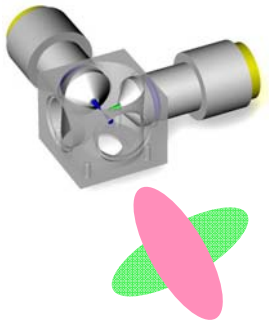


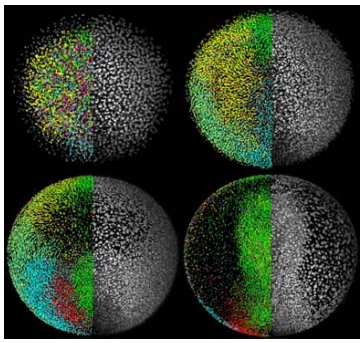


http://www.microscopyu.com/articles/fluorescence/tirf/tirfintro.html



Light Sheet Fluorescence Microscopy (LSFM)

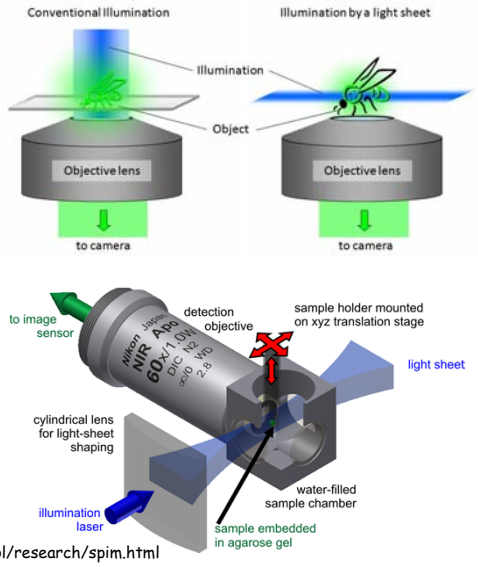




Improved axial resolution

Philipp J. Keller, Annette D. Schmidt, Joachim Wittbrodt, Ernst H. K. Stelzer. Reconstruction of Zebrafish Early Embryonic Development by Scanned Light Sheet Microscopy. Science, 2008, Science. 2008 322(5904):1065-9

Single Plane Illumination Microscopy (SPIM)



http://www.dkfz.de/Macromol/research/spim.html

2-Photon Excitation Microscopy (2-PEM)

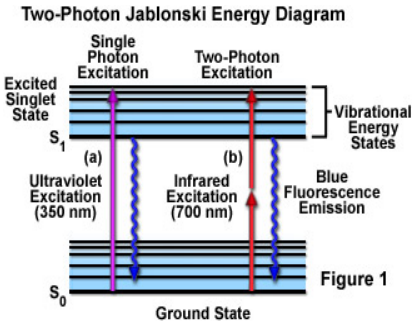
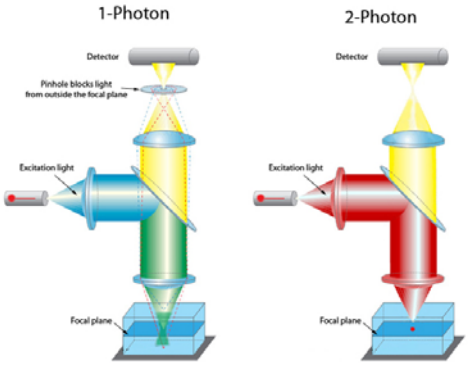
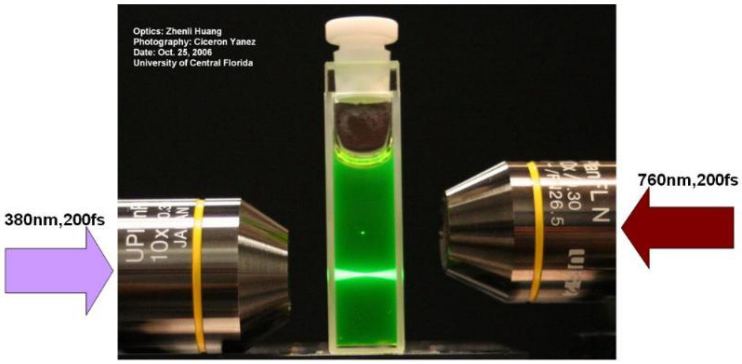


Figure 1



http://www.microscopyu.com/articles/fluorescence/multiphoton/images/multiphotonintrofigure1.jpg

2-Photon vs 1-photon absorption



2-photon excitation requires a pulsed laser to enable excitation via the two step process (the half-way state is very short lived so the second photon needs to act very soon after the first), necessitating femtosecond pulsed laser.
Radial resolution of 2-PEM is somewhat lower than the radial resolution of single-photon excitation because of the longer wavelength of the excitation beam, whereas axial resolution and penetration depth are signifficantly improved.

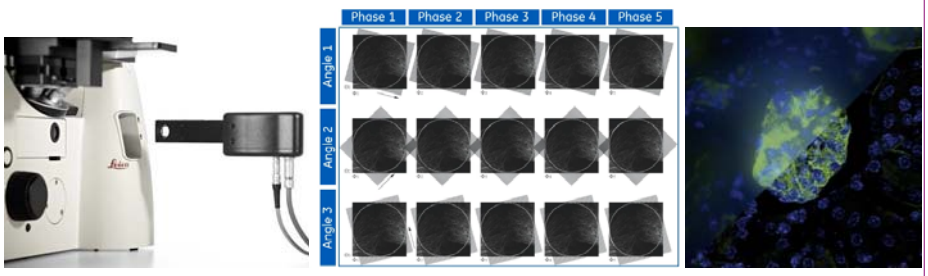
http://belfield.cos.ucf.edu/image/Gallery/on%20vs%20two/fluorescein.jpg
http://microscopy.duke.edu/introtomicroscopy/twophotonex.html

Structured Illumination Microscopy (SIM)

Moiré effect is a visual perception that occurs when viewing patterns that are superimposed on each other, which differ in relative size, angle, or spacing.

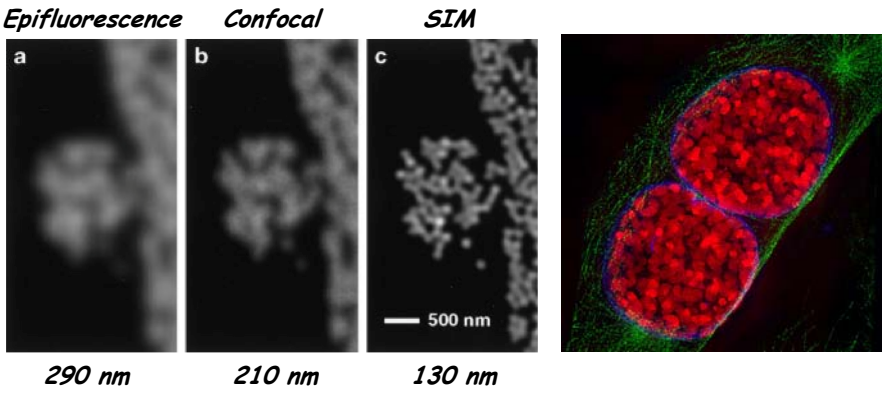
By superimposing the images of a low spatial frequency pattern or a grid and the object, information that exists beyond the resolving power of a fluorescent microscope can be revealed.

This technique can offer at least a doubling of spatial resolution, and in the presence of nonlinear saturation effects, can theoretically offer unlimited resolution.



http://www.mathematik.com/Moire/
Gustaffson MGL. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. Journal of Microscopy 2000, 198:82-87
Gustafsson MGL. Nonlinear structured-illumination microscopy: wide-field fluorescence imaging with theoretically unlimited resolution. Proc Natl Acad Sci U S A 2005, 102:13081-13086.

Spatial resolution of SIM



Gustafsson MGL PNAS 2005;102:13081-13086 http://www.allthebestbits.net/structured-illumination-microscopy/

Nobel prize for chemistry 2014

For making it possible to see features at the scale of billionths of a meter, smashing a theoretical barrier for optical microscopy.

Eric Betzig **Stefan Hell** **William Moerner**

The signal-to-noise ratio (SNR) in a digital image

$$\sigma = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^4 b^2}{a^2 N^2}\right)} \approx \frac{\lambda}{2\sqrt{N}}$$

photon noise — *pixelation effect due to the finite pixel size of the detector* — *background*

s_i – standard deviation of the Gaussian distribution (PSF width/2.2)
a – the effective pixel size of the detector, which is equal to the pixel size divided by magnification
b – background
N – number of collected photons

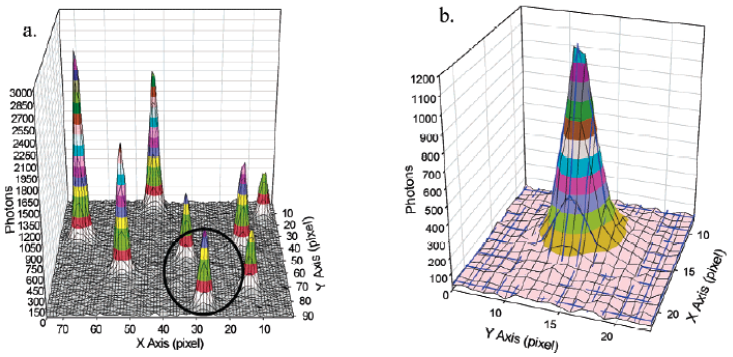
Single molecules can be localized with an arbitrary high precision by increasing the signal-to-noise ratio (SNR)!

Yildiz A, Selvin PR.Fluorescence imaging with one nanometer accuracy: application to molecular motors. Acc Chem Res. 2005 Jul;38(7):574-82.
http://link.springer.com.proxy.kib.ki.se/content/pdf/10.1007%2F978-1-61779-261-8_4

Fluorescence Imaging with One Nanometer Accuracy (FIONA)

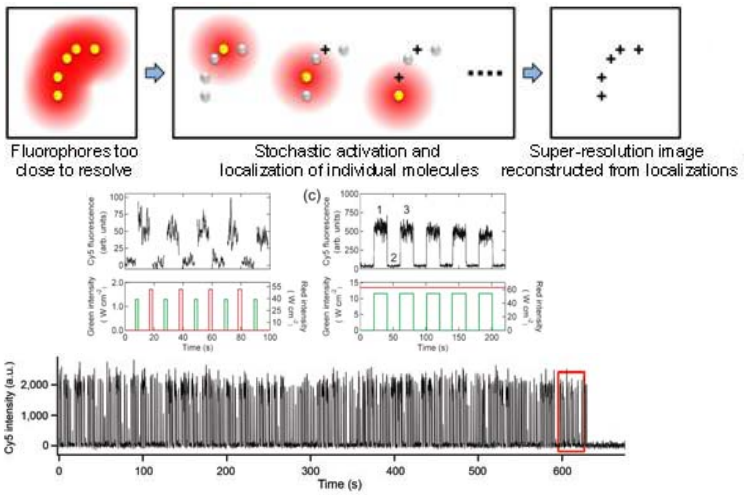
Determining the center, *i.e.* the mean value of the photon distribution ($\mu = x_0, y_0$), and its uncertainty, the standard error of the mean, σ.

If 10,000 photons can be collected in the absence of background before the fluorophore bleaches or is switched off, the center of localization can be determined with an accuracy of approximately 1 to 2 nanometers.!



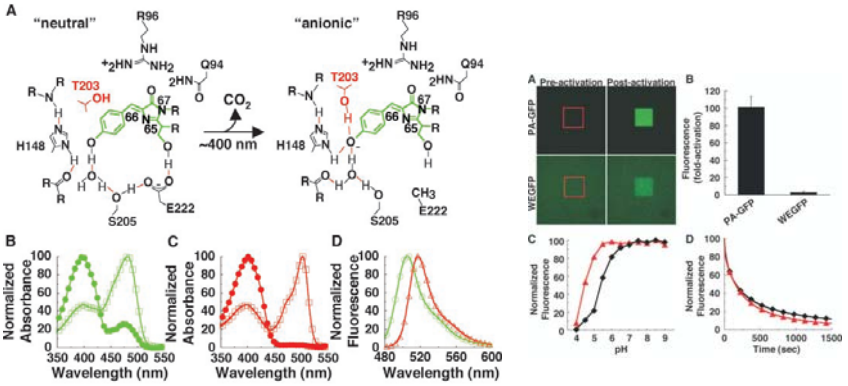
Yildiz A, Selvin PR.Fluorescence imaging with one nanometer accuracy: application to molecular motors. Acc Chem Res. 2005 Jul;38(7):574-82.
http://www.youtube.com/watch?v=Bom9d-Knz0w

Stochastic Optical Reconstruction Microscopy (STORM)



Rust MJ, Bates M, Zhuang X: Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nat Methods 2006, 3:793-796.
B. Huang, M. Bates & X. Zhuang, Annu. Rev. Biochem., 78, 993-1016 (2009). http://huanglab.ucsf.edu/STORM.jpg
Churchman S, Okten Z, Rock RS, Dawson JF, Spudich SA. Single molecule high-resolution colocalization of Cy3 and Cy5 attached to macromolecules measures intramolecular distances through time. PNAS, 2005, **102: 1419–1423.**
https://www.youtube.com/watch?v=w2Qo__sppcI

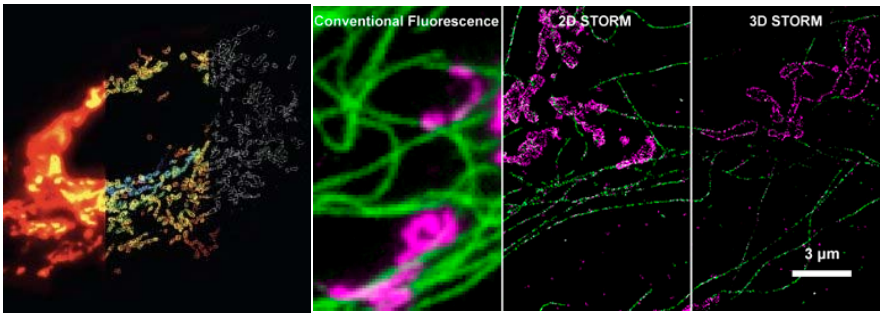
Photoactivatable GFP



Photoconversion in wild-type GFP is thought to involve a shift in the chromophore population from the neutral phenolic form to the anionic phenolate form. Rotation of the T203 and decarboxylation of glutamic acid 222 (E222) *are structural rearrangements that may be key features of GFP* photoconversion. *Native (filled circles) and photoactivated (open squares) absorbance spectra* of **(B) WEGFP (wild-type EGFP) and (C) T203H mutant (PA-GFP) are shown normalized to the** highest absorbance. **(D) Emission spectra were collected under excitation at 475 nm of photoactivated** WEGFP (green circles) and PA-GFP (red triangles).

Patterson GH, Lippincott-Schwartz J. A Photoactivatable GFP for Selective Photolabeling of Proteins and Cells. Science, 2002, 297:1873-1877; http://www.youtube.com/watch?v=Bom9d-Knz0w

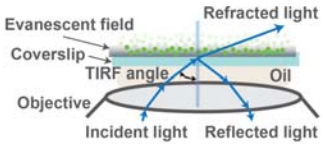
2D and 3D STORM with nanometer-scale resolution

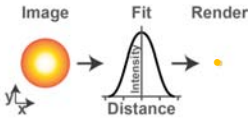


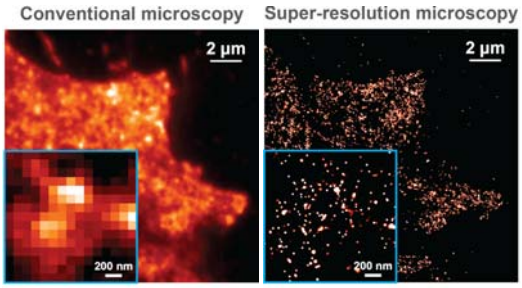
Huang B, Jones SA, Brandenburg B, Zhuang X. Whole cell 3D STORM reveals interactions between cellular structures with nanometer-scale resolution. Nat Methods. 2008 December ; 5(12): 1047-1052.

Opioid receptor lateral organization by pcPALM

Dr. Tijana Jovanović-Talisman
City of Hope
Beckman Research Institute
Duarte, CA 91010, USA

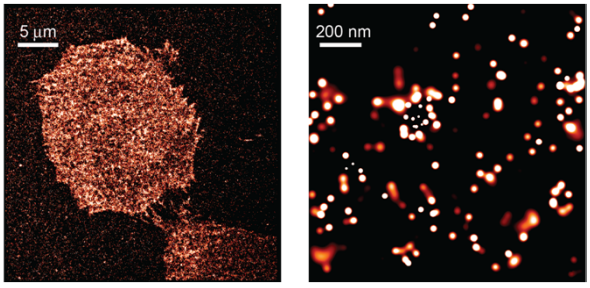






Sengupta P, Jovanovic-Talisman T, Skoko D, Renz M, Veatch SL, Lippincott-Schwartz J. Probing protein heterogeneity in the plasma membrane using PALM and pair correlation analysis. Nat Methods. 2011 **8:**969-975.

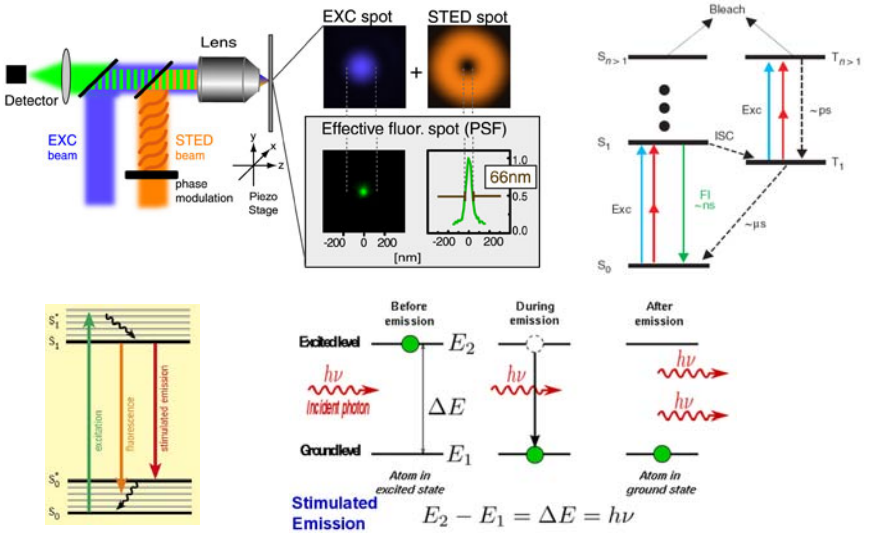
Nanoscopic distribution of endogenous MOP in MDA-MB-468 cells



Nanoscopic distribution of endogenous MOP in MDA-MB-468 cells using anti-MOP antibody (ab64746) labeled with Cage-552 fluorescent dye (Abberior)

Tobin et al. PLoS One 2014 9(2):e87225
V. N. Belov et al., "Rhodamines NN: A Novel Class of Caged Fluorescent Dyes", Angew. Chem. Int. Ed. 2010 **49:**3520–3523

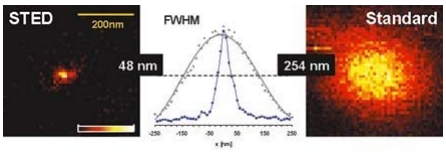
Stimulated Emission Depletion (STED) Microscopy



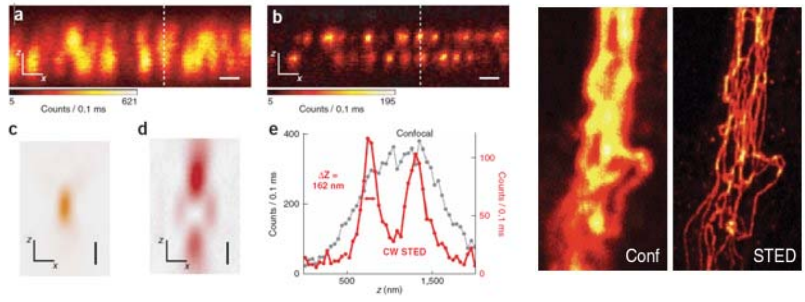
$$E_2 - E_1 = \Delta E = h\nu$$

Donnert G, Eggeling C, Hell S. Major signal increase in fluorescence microscopy through dark-state relaxation. Nature Methods 2007, 40: 81-86;
http://universe-review.ca/R13-11-QuantumComputing.htm

Spatial resolution of STED Microscopy



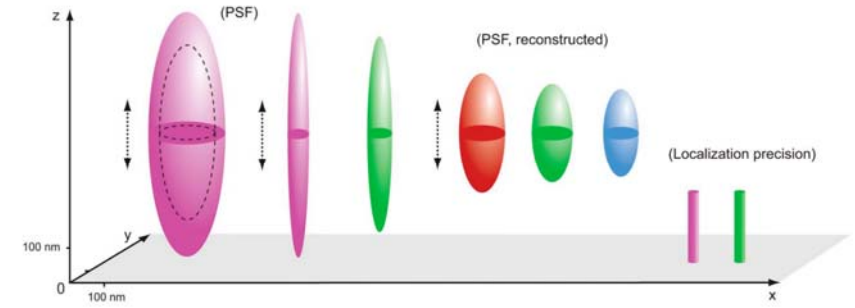
$$d = \frac{\lambda}{2n\sin\alpha\sqrt{1 + I/I_{sat}}}$$



http://www.whatsnextnetwork.com/technology/media/sted_microscopy.jpg
Willig KI, Harke B, Medda R, Hell SW. STED microscopy with continuous wave beams.
Nature Methods, 2007, 4: 915-918

Resolvable volumes obtained with current commercial super-resolution microscopes

	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λ_{em} [nm]	460-670	670	520	620	520	460	670	520



~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

Schermelleh et al. A guide to super-resolution fluorescence microscopy, J. Cell Biol. 2010 **190**:165-175

Limitations of super-resolution methods

Low temporal resolution of STORM and PALM, several milliseconds at best but most often in the minutes range

High excitation intensity – STED ~ 50 MW cm^{-2},
2-PEM ~ GW cm^{-2}
Single-photon ~ 100 kW cm^{-2}

Perturbation effects - singlet oxygen evolution and local overheating