Advanced Technologies in Biological Research:

Optical Spectroscopy and Biosensors

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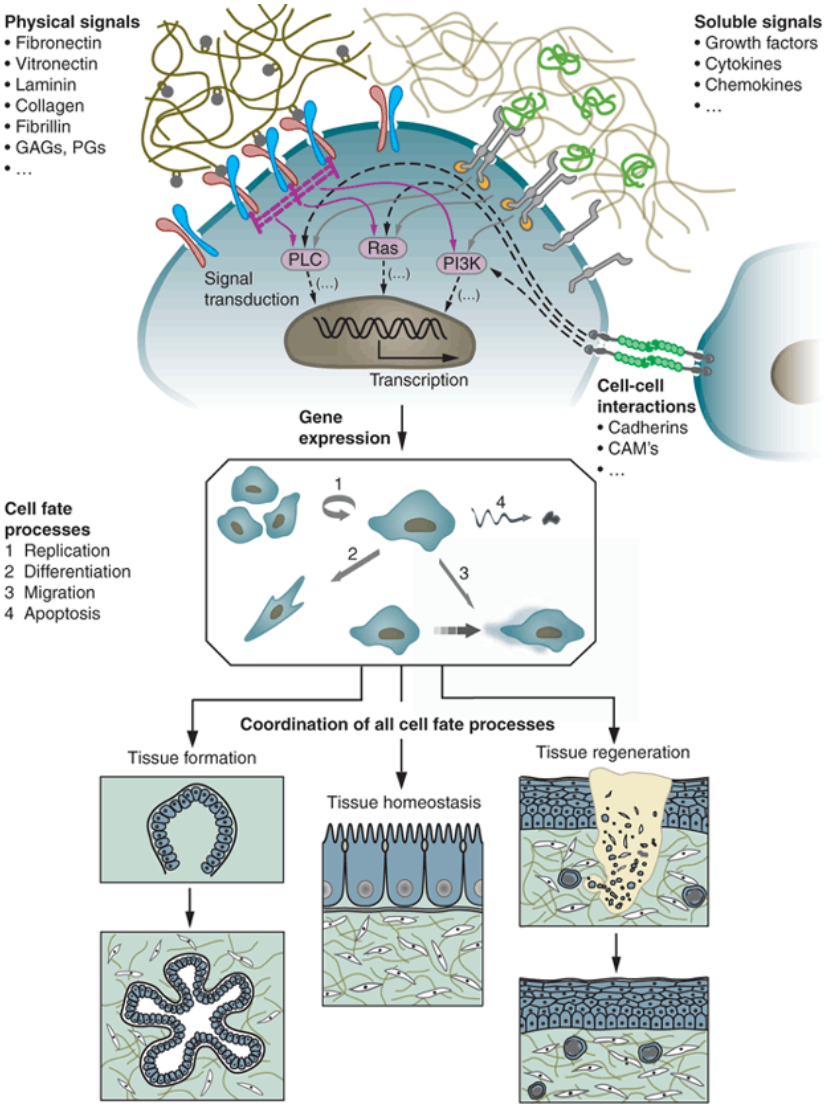
Tissue Engineering

Aims at “ ...tissue growth and applying this to produce functional replacement tissue for clinical use…”

* Langer R, Vacanti JP (1993) *Science* **260** (5110): 920–6

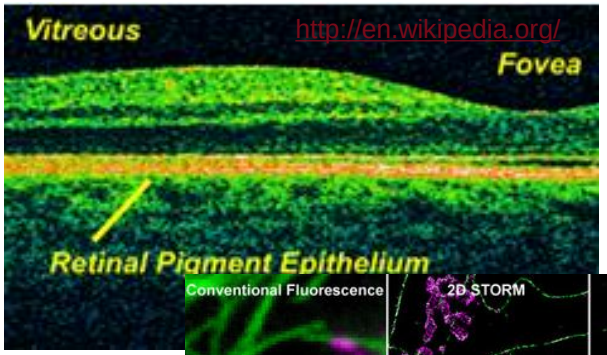


M P Lutolf & J A Hubbell, *Nature Biotechnology* **23**, 47 - 55 (2005)

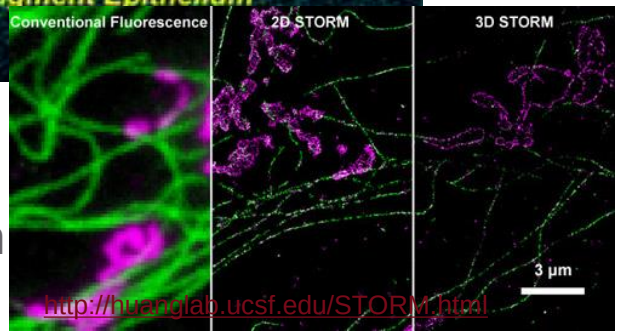
Optical techniques provides tools for imaging cell cultures, individual cells and were deployed for the investigation / detection of biomolecules and observation their interactions at surfaces, biointerfaces, inside cells,…

Optics for Imaging / Spectroscopy in Tissue Engineering

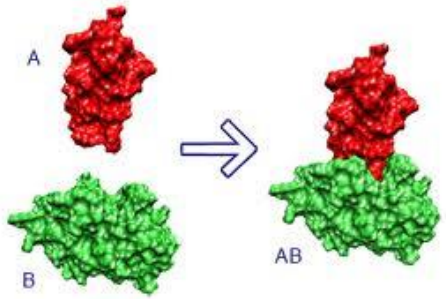
Optical techniques are at the heart of the majority of tools enabling observation of tissues, cells, and biomolecules. Typically nondestructive and rather sensitive.



Optical tomography: optical coherence tomography (OCT)



Microscopy: phase contrast, fluorescence, fluorescence resonant energy transfer (FRET), super-resolution microscopy (stimulated emission depletion - STED, photo activated localization microscopy - PALMS, STORM…)



Spectroscopy: IR Absorption, SERS, Surface plasmon resonance (SPR)…

Outline

A. Introduction to light propagation and confinement: ray, wave, and electromagnetic optics. Total internal reflection, guided wave optics, surface plasmon resonance.

B. Optical micro- and nano-structures: Top down and bottom up fabrications, electron beam lithography, colloidal lithography, nano-imprint lithography.

C. Optical spectroscopy: absorption, fluorescence, FRET, Raman and IR spectroscopy.

D. Super-resolution microscopy: Diffraction limit and approaches to break it, STED, PALMS, STORM.

E. Optical tweezers: manipulation with cells and force measurements.

F. Biosensors: Transducers, key performance characteristics, application areas including medical diagnostics, and drug development.

G. Surface plasmon resonance (SPR) biosensors.

H. Reaction kinetics and evaluation of affinity binding constants for molecular interaction analysis (BIA).

I. Single molecule interaction analysis by using fluorescence, FRET and LSPR monitoring.

J. Optical spectroscopy-based biosensors. Colorimetric, fluorescence, Raman.

A. PROPAGATION AND CONFINEMENT OF LIGHT

Optics / Photonics - Light Propagation / Confinement

Propagation of light and its interaction with matter can be treated at different levels (accuracy):

Less accurate (and simpler to use…)

		Size of an object Δx
Ray optics	- refraction, reflection…	Δx>>λ
Wave optics	- wavelength λ, phase, interference…	Δx<~λ
Electromagnetic optics	- polarization, surface waves…	
Quantum optics	- quantized energies (photons), lasers…	

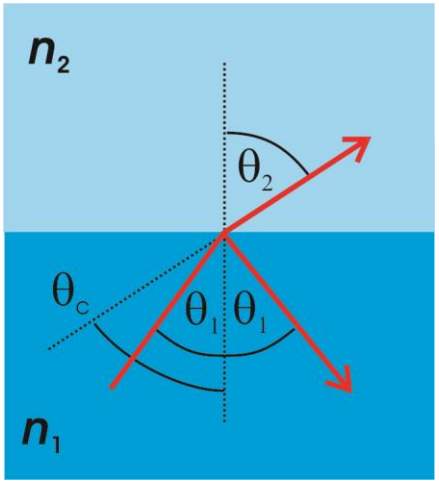
More general (and complicated…)

Ray Optics

Refractive index *n* describes optical density of matter in which a light beam – ray – propagates. At a plane interface between $n_1 > n_2$, reflection and refraction occurs.

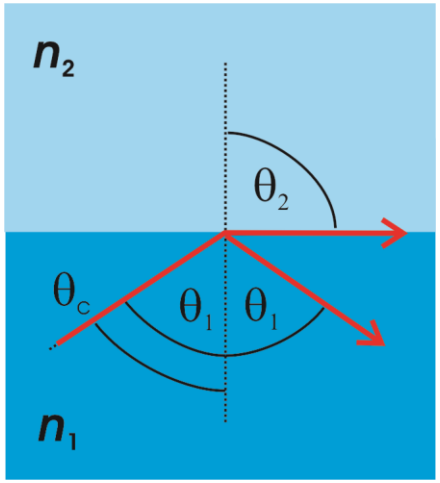
Snell law $n_1 \sin(\theta_1) = n_2 \sin(\theta_2) \rightarrow \theta_c = \arcsin(n_2 / n_1)$

$\theta_1 < \theta_c$



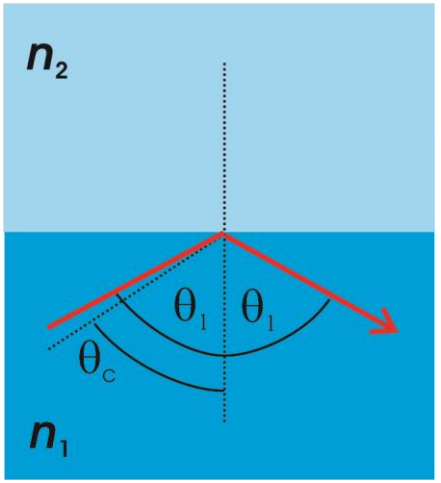
Refracted beam propagates to n_2 medium

$\theta_1 = \theta_c$



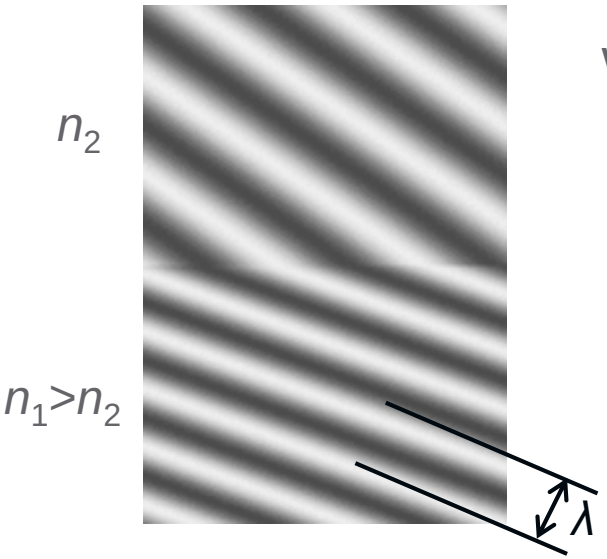
Beam propagates along the interface

$\theta_1 > \theta_c$



Beam undergoes total internal reflection - TIR

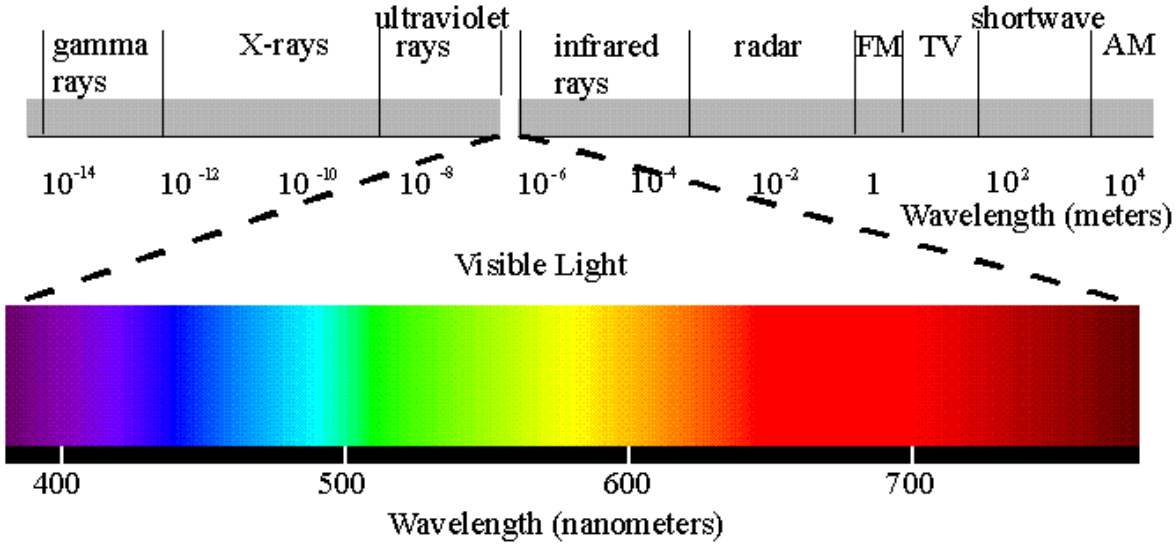
Wave Optics



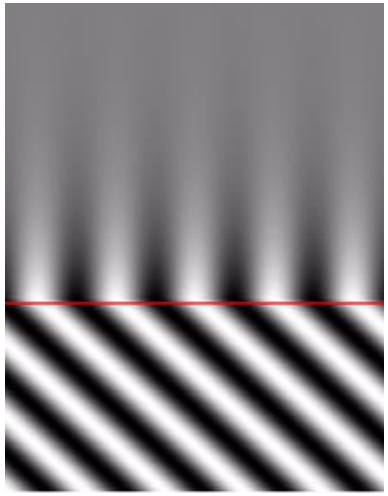
Wave equation $$\left(\frac{d^2}{dx^2}-\frac{1}{v^2}\frac{d^2}{dt^2}\right)u(x,t)=0$$

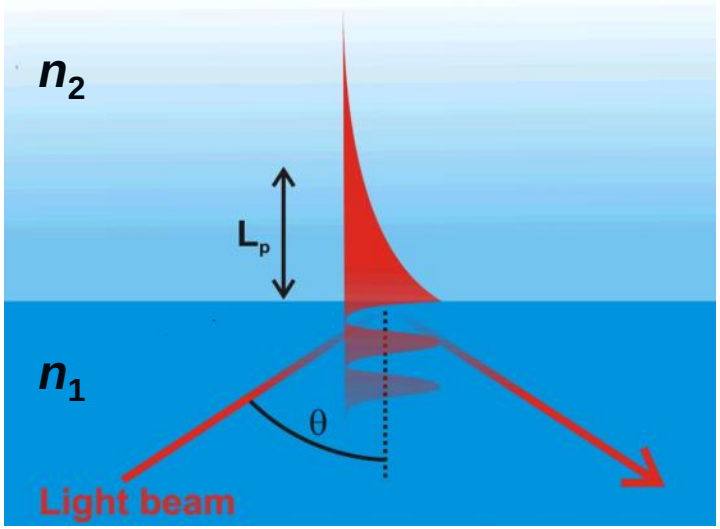
Plane wave (scalar) $$u(x,t)=A\sin(k_0 n x-\omega t)$$

$k_0=\omega/c$	propagation constant in vacuum
ω	angular frequency
$v=c/n$	velocity of light
$\lambda=2\pi c/\omega$	wavelength in vacuum



Total Internal Reflection - TIR





At angles $\theta>\theta_c$ field amplitude exponentially decays to medium n_2

$$u(x) = u_0 e^{-x/L_p}$$

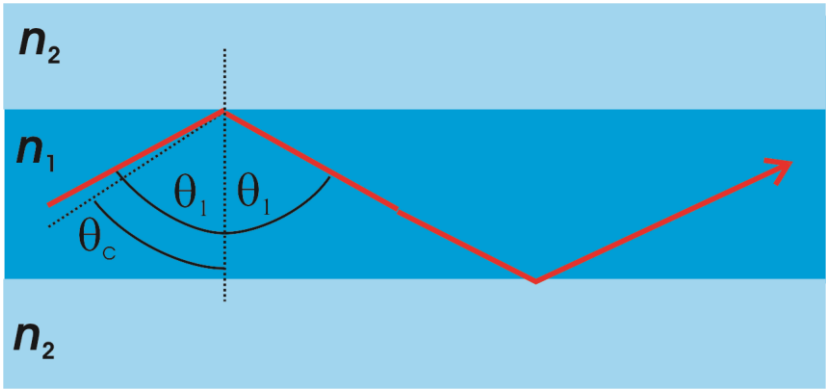
The field probes limited penetration depth from the interface:

$$L_p = \frac{\lambda}{4\pi\sqrt{n_1^2 \sin^2(\theta) - n_2^2}}$$

Slab Optical Waveguide

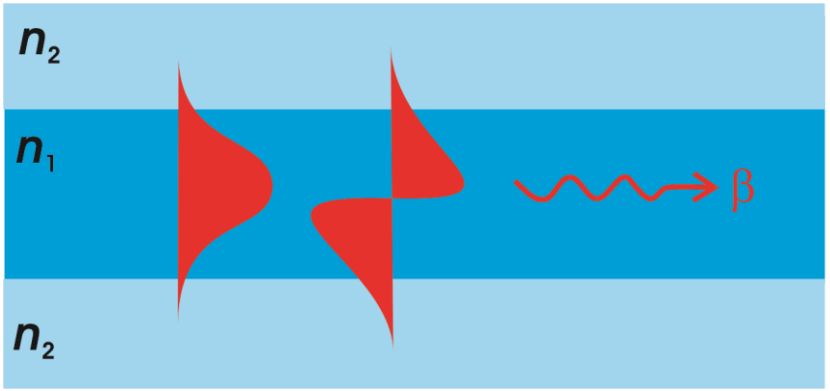
Ray optics point of view:

Light propagation is confined by TIR at opposite interfaces

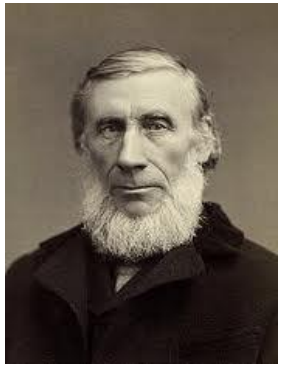


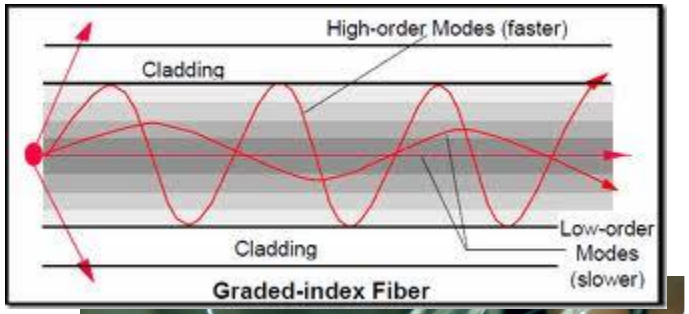
Wave optics point of view:

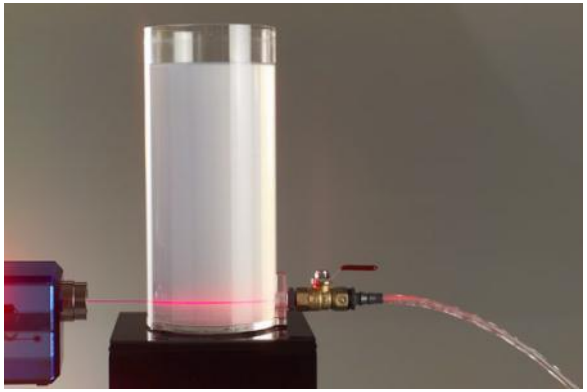
Only discrete modes with certain propagation constants β can travel through the waveguide

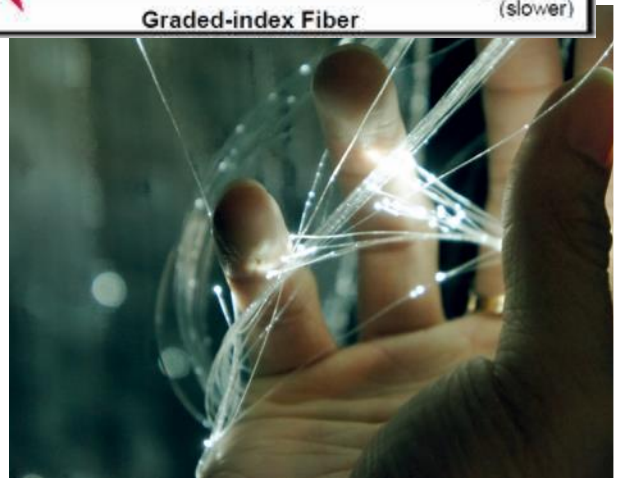


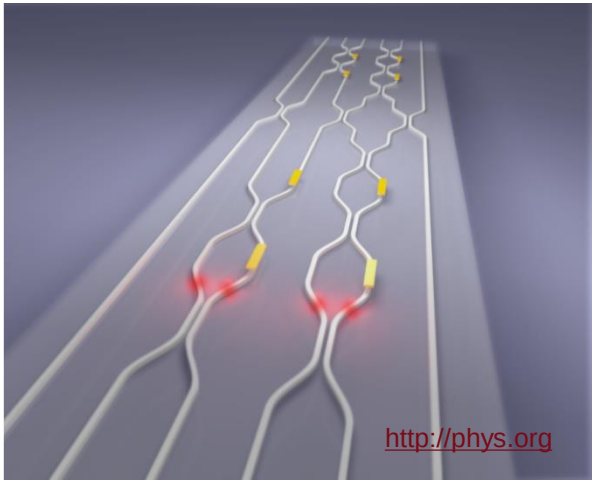
Examples of Dielectric Optical Waveguide











Historical Tyndall experiment at 1870.

Optical fibers

Optical circuits

Examples of Dielectric Optical Waveguide

Optical fiber bundles used for imaging of remote places (right) and catheters (down).

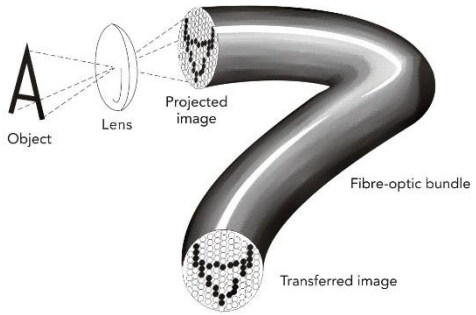
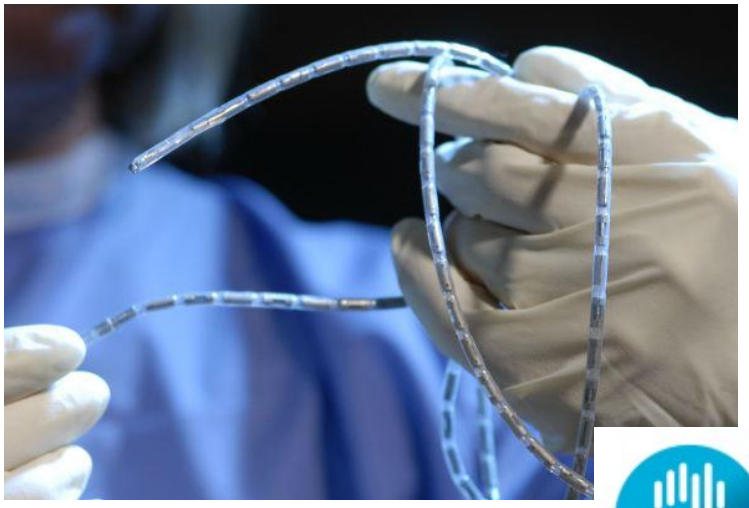
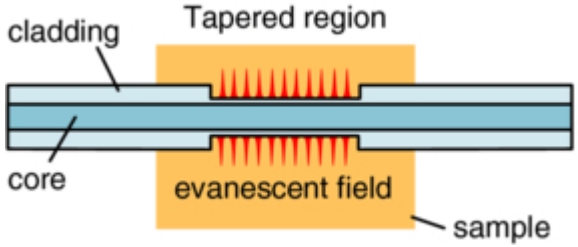


Figure 7-4 Fiber optic endoscopy



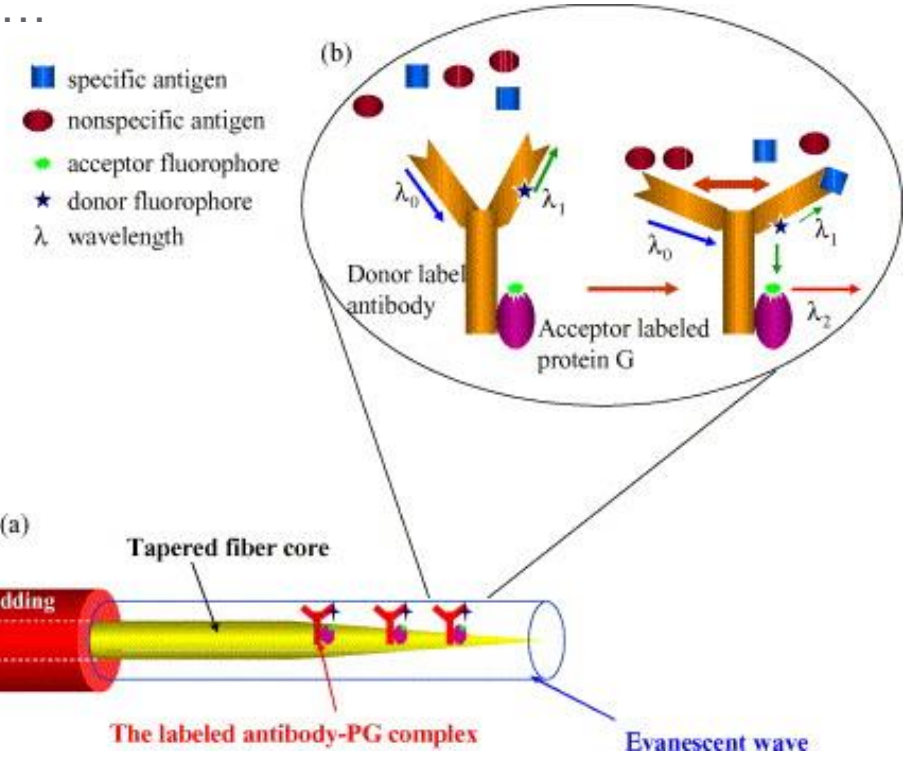
Examples of Dielectric Optical Waveguide

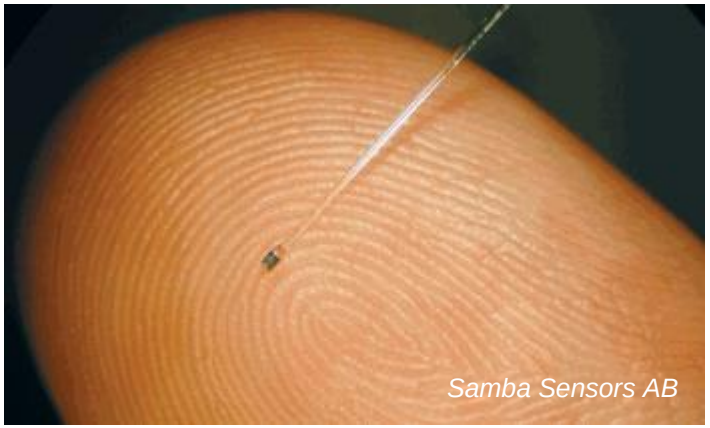
Optical fibers allow for design of miniature sensors relying on probing by evanescent field:



Hodgkinson et al, 2013 *Meas. Sci. Technol.* **24** 012004

Oxygen
Pressure
Fluorescence, FRET
…



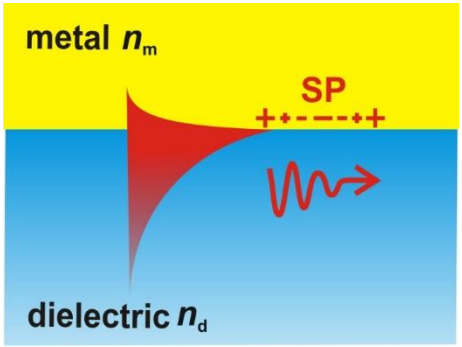


Sungho Biosensors and Bioelectronics
Volume 21, Issue 7, 15 January 2006, Pages 1283–1290

Metallic Waveguides – Surface Plasmons (SP)

Surface plasmons (SPs) or also called surface plasmon polaritons (SPPs) are waves originating from coupled oscillations of electron plasma density and associated electromagnetic field on a metal – dielectric interface.

They travel along single interface which serves a waveguide.



Propagation constant β can be analytically expressed as:

$$\beta = \frac{2\pi}{\lambda}\sqrt{\frac{n_m^2 n_d^2}{n_m^2 + n_d^2}}$$

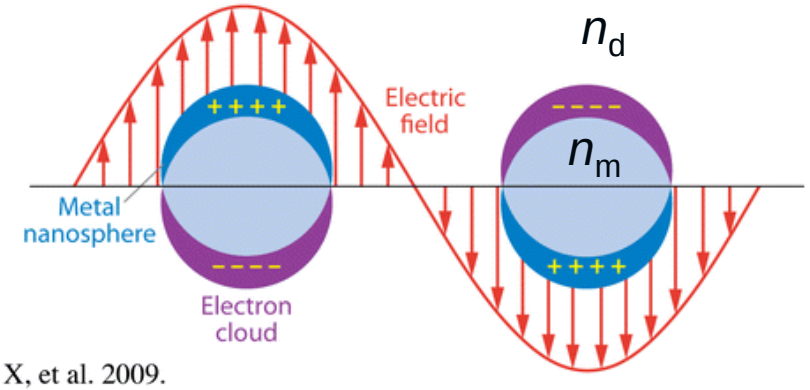
- SPs allows for tight confinement of electromagnetic field at the interface.
- For visible near infrared wavelength typically gold and silver is used where the Re$\{n_m^2\}$<0.
- Majority of the field is probing the dielectric n_d.

Localized Surface Plasmons (LSPs)

Localized surface plasmons (LSPs) are associated with electron plasma density oscillations on metallic nanoparticles. Provides unique optical / plasmonic characteristics.

Resonant effect, e.g. for spherical metallic nanoparticle with $d<<\lambda$ the resonance wavelength λ_{LSPR} obeys:

$$\mathrm{Re}\{n_m^2(\lambda)\} + 2n_d(\lambda) = 0$$



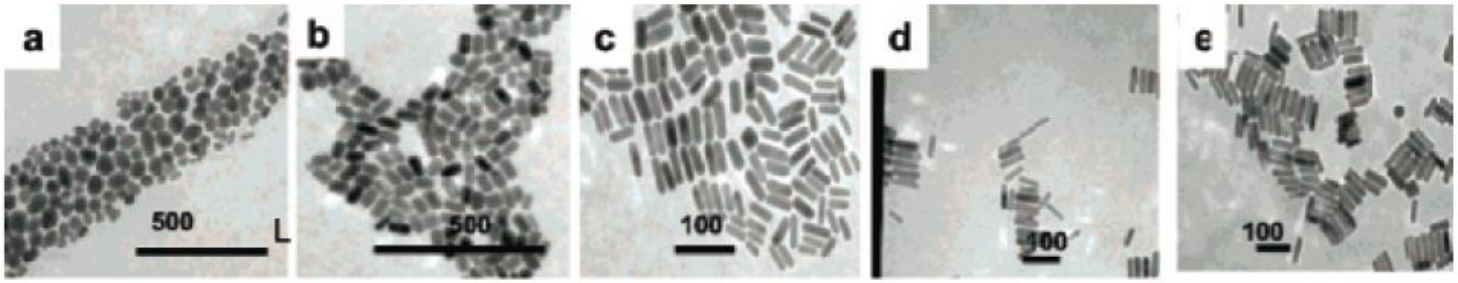
Lu X, et al. 2009.
Annu. Rev. Phys. Chem. 60:167–92

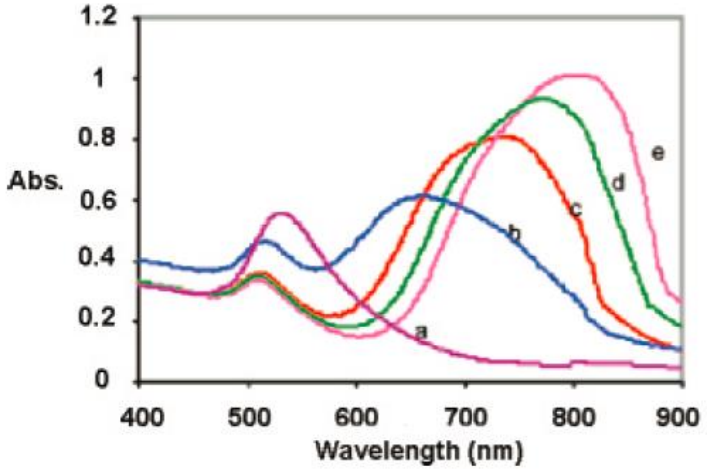
Localized surface plasmon resonance is associated with strong:

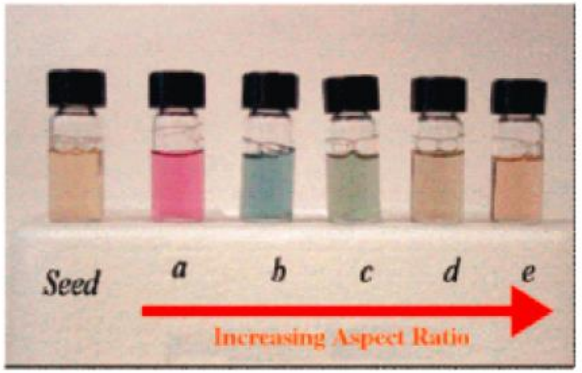
- **Absorption**
- **Scattering**
- **Field confinement and enhancement**

Localized Surface Plasmons (LSPs)

Synthesized metallic nanorods - tuning of λ_{LSPR} by changing the aspect ratio.

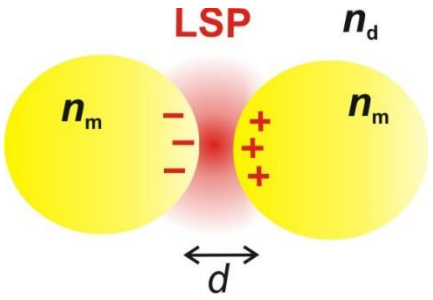


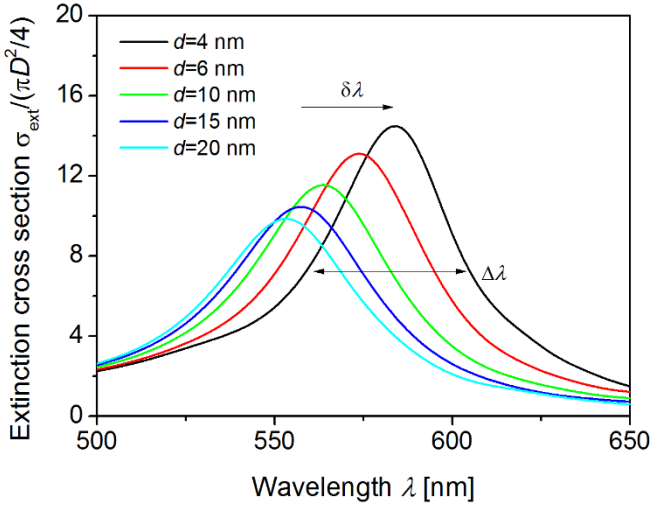




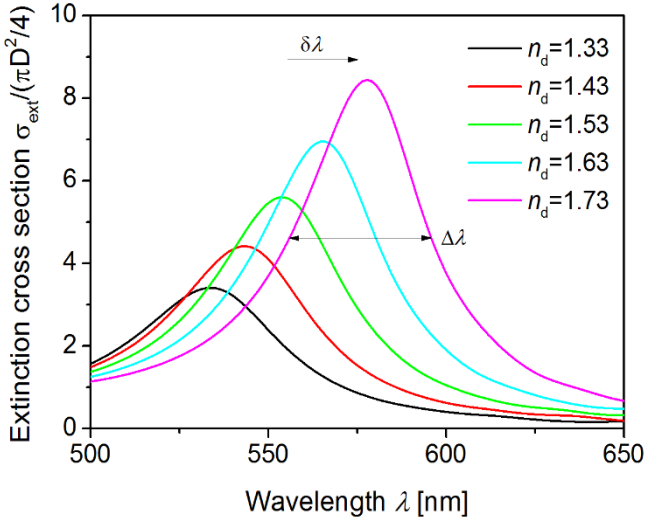
Watt, F.; Bettiol, A. A.; Van Kan, J. A.; Teo, E. J.; Breese, M. B. H.
Int. J. Nanosci. ***2005, 4, 269.***

Spectroscopy of Surface Plasmons





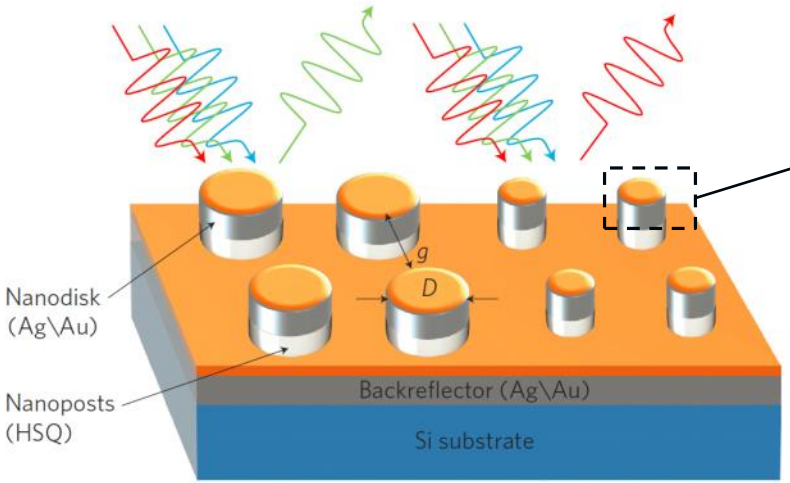
- Changes in the refractive index or geometry of metallic nanoparticles alter λ_{LSPR}
- These variations can be probed by, e.g., transmission spectrometry



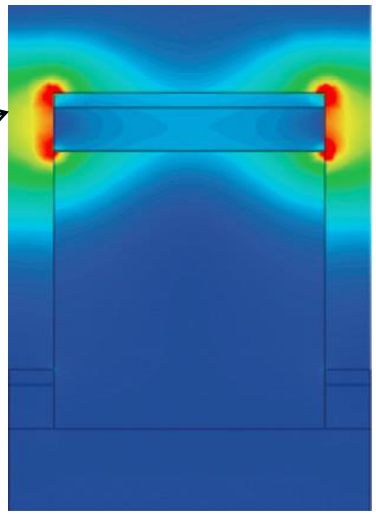
Localized Surface Plasmons (LSPs)

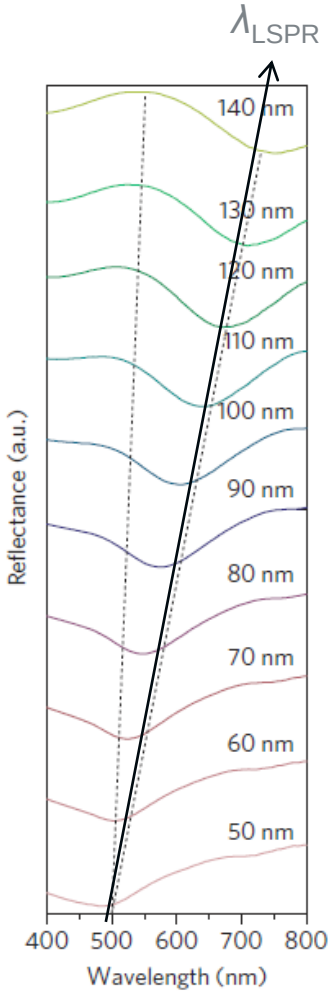
Strong absorption, scattering and field enhancement $|E/E_0|^2$ occurs at resonant wavelength λ_{LSPR} which can be tuned by:

- Choice of metal (e.g., silver, gold)
- Refractive index change of a dielectric
- Shape and inter-NP interaction



$|E/E_0|^2$ field profile at resonance λ_{LSPR}

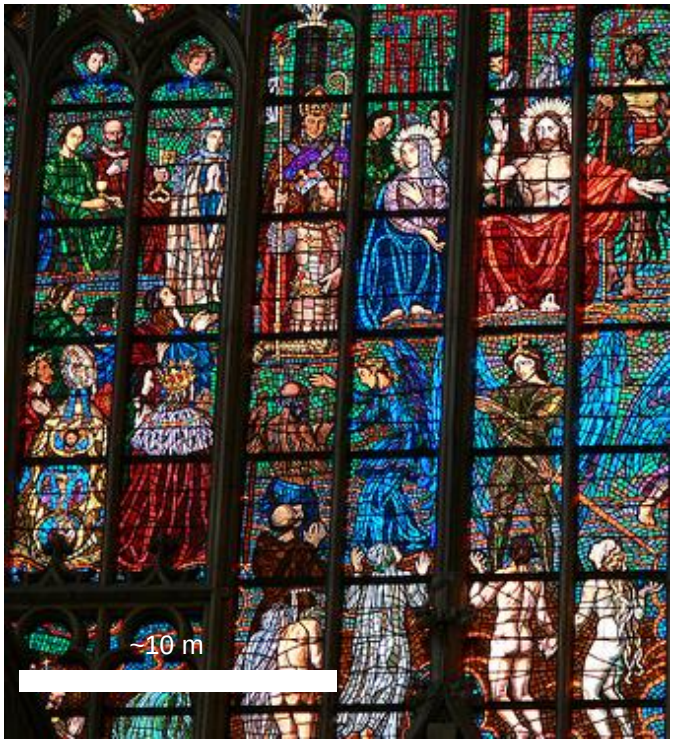




Kumar et al., Nature Nanotechnology (2012) DOI: 10.1038/NNANO.2012.128

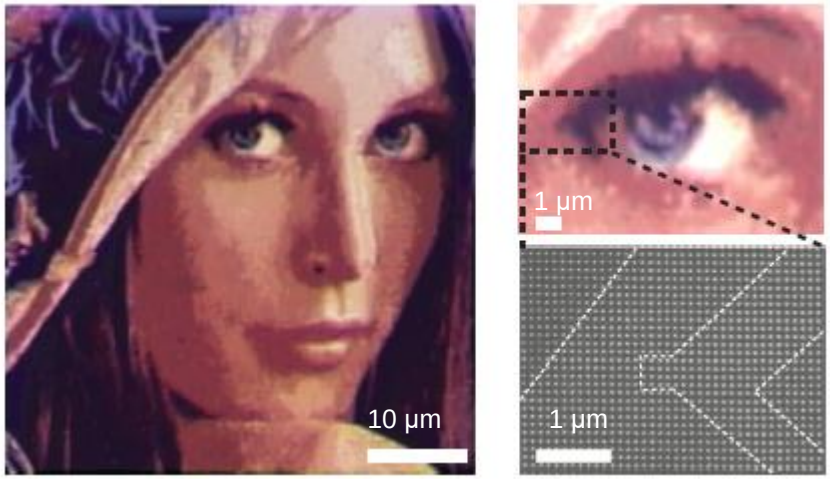
Example of LSPs

Blend of glass with metallic nanoparticles - used for centuries in stained glass.



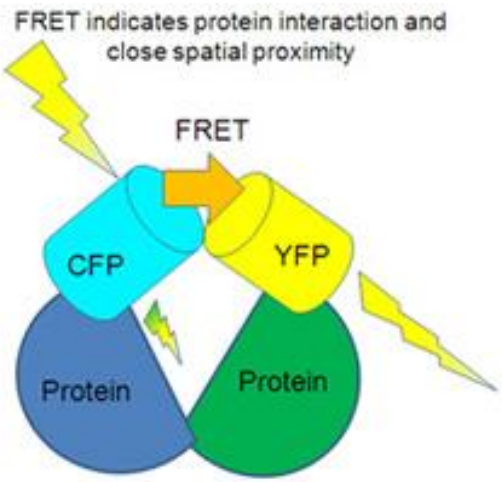
St. Vitus cathedral, Prague

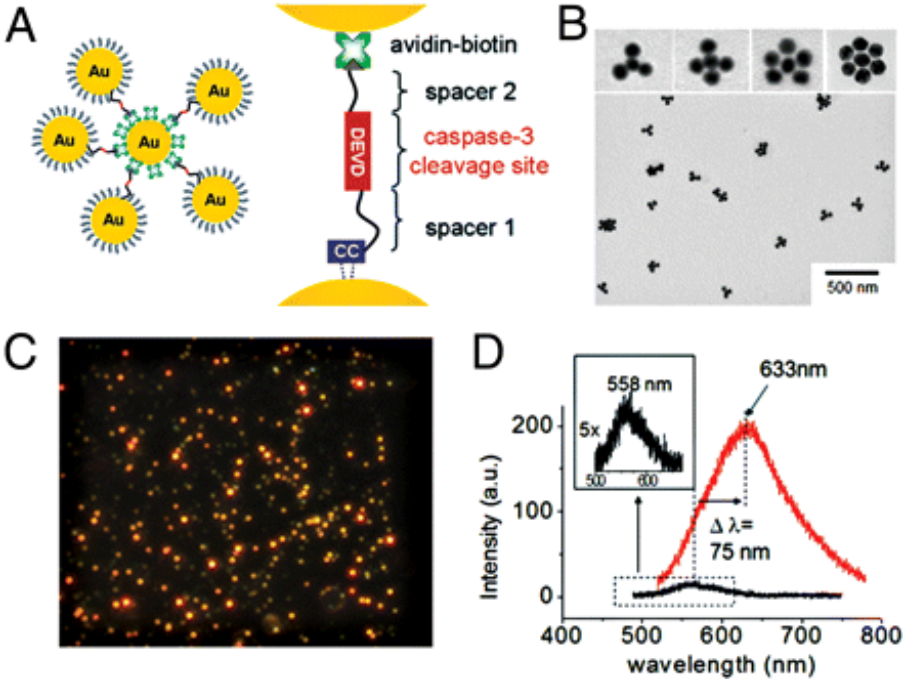
Structures from previous slide employed for printing with diffraction limit accuracy (10^5 dpi).



Kumar et al., Nature Nanotechnology (2012) DOI: 10.1038/NNANO.2012.128

Example of Plasmonics in Biotechnologies



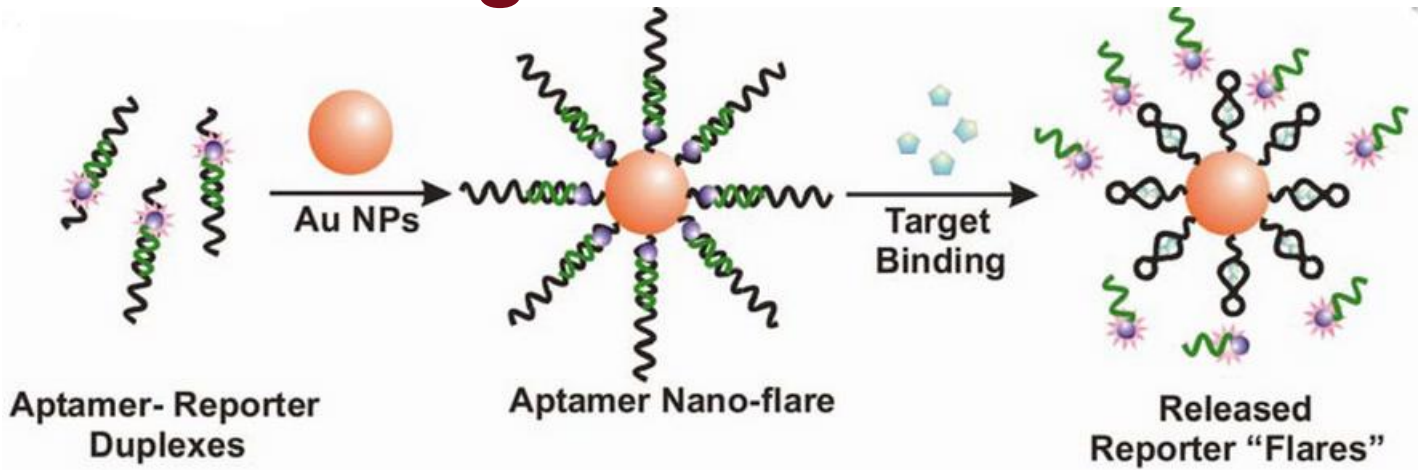


FRET – fluorescence resonant energy transfer. Used for interaction analysis of biomolecules at distances $r < 10$ nm.

Plasmonic ruler – a concept relying on coupling of LSPs enables monitoring of interactions at larger distances $r > 10$ nm

Alivisatos lab: 10.1073/pnas.0907367106

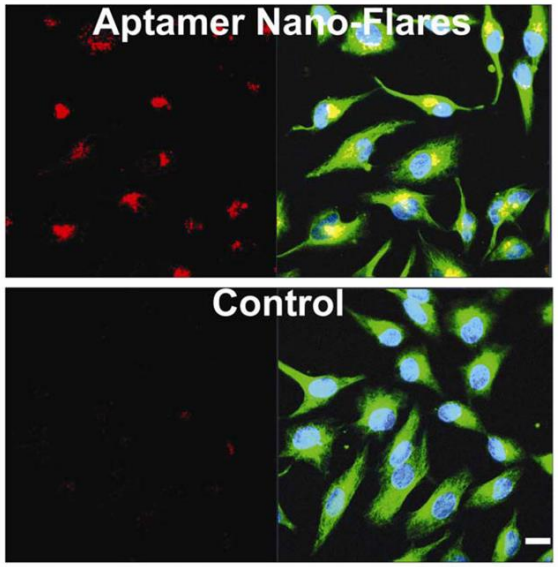
Example of Plasmonics in Biotechnologies - Nanoflare



FRET – metallic NP serves as a donor and quenches the fluorescence

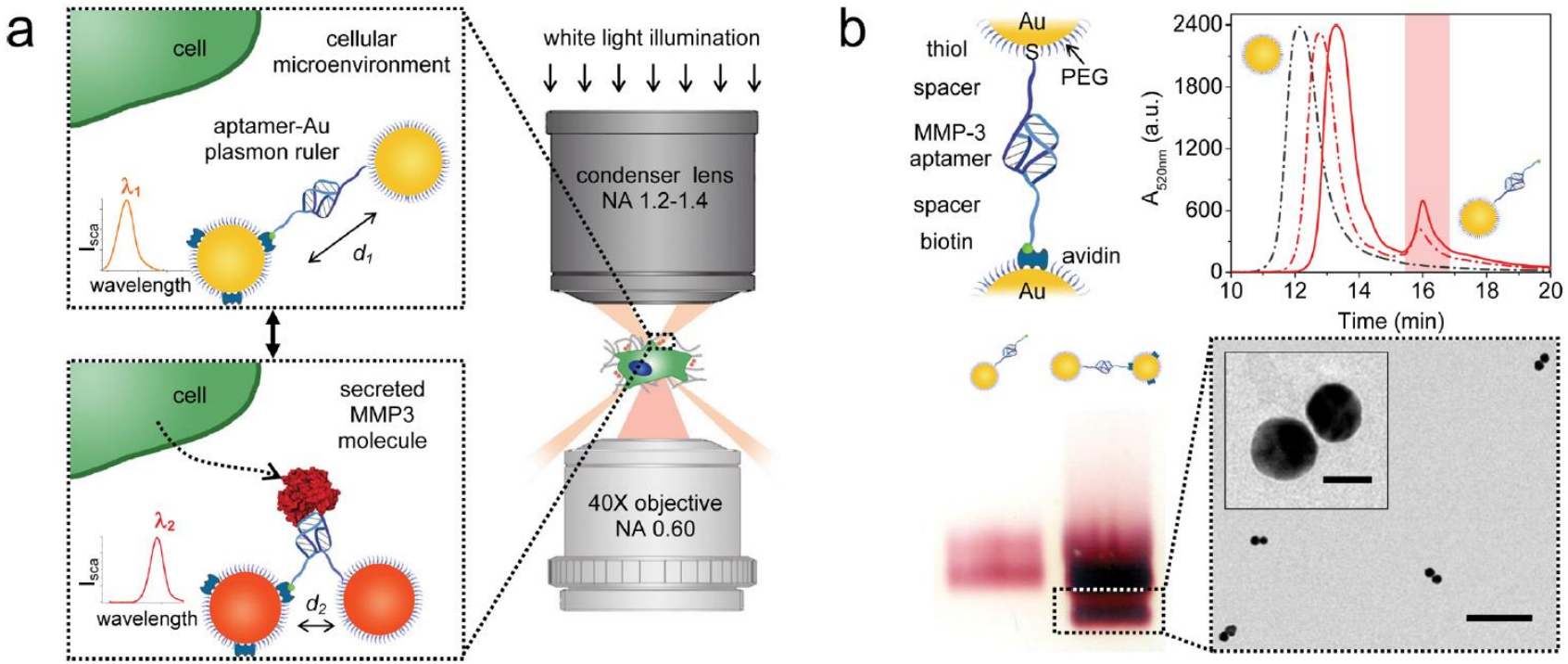
Spherical DNA – allows for simple cellular uptake

Aptamer – oligonucleotide designed for specific capture of molecular analytes (ATP in this case)



Mirkin lab: doi:10.1021/nl901517b

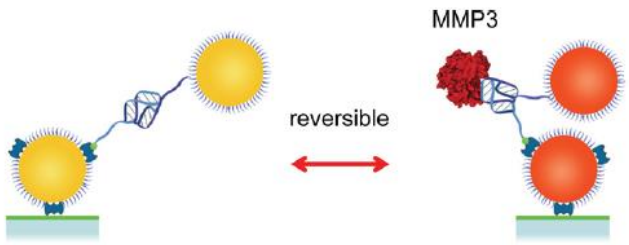
Example of Plasmonics in Biotechnologies – Reversible Live-Cell Measurements

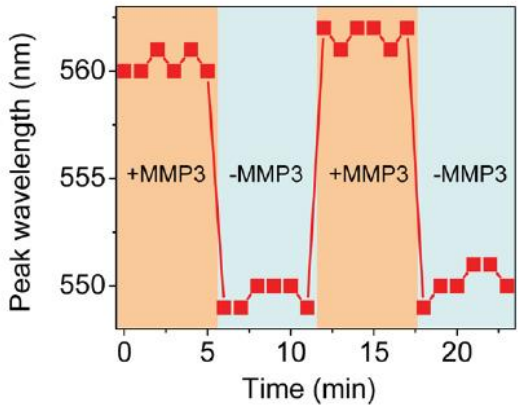


Concept pursued for real-time detection of growth factors, proteases…
Investigation of cellular microenvironment, concentration gradients.

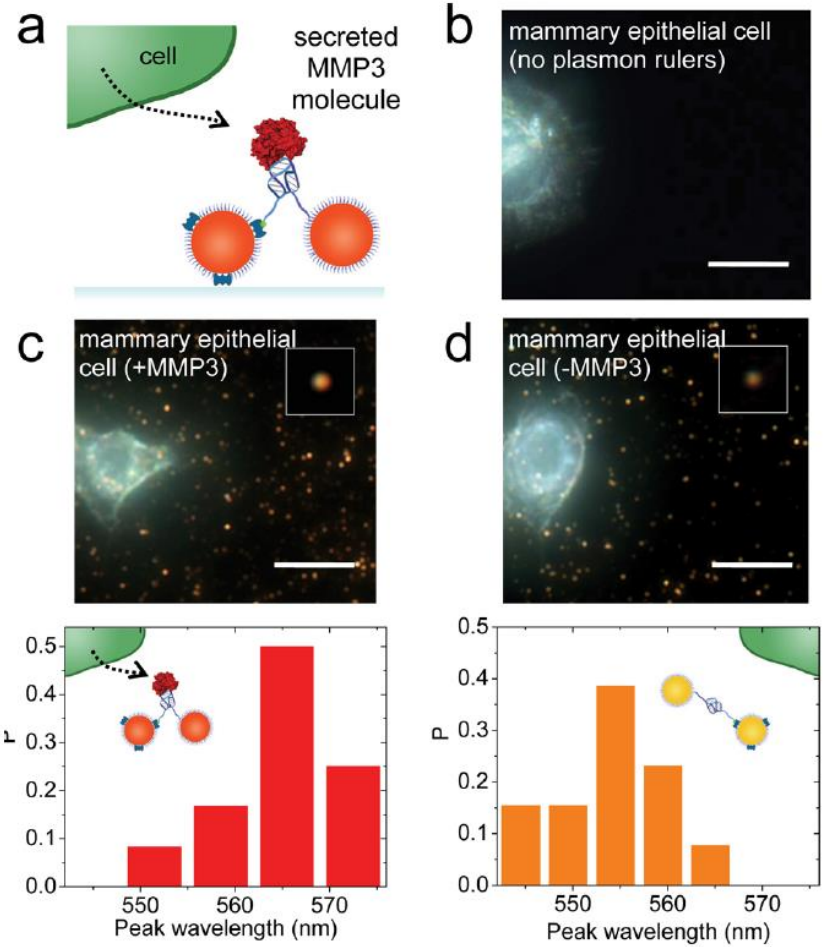
Alivisatos lab: http://pubs.acs.org/doi/abs/10.1021/acs.nanolett.5b01161

Example of Plasmonics in Biotechnologies – Reversible Live-Cell Measurements



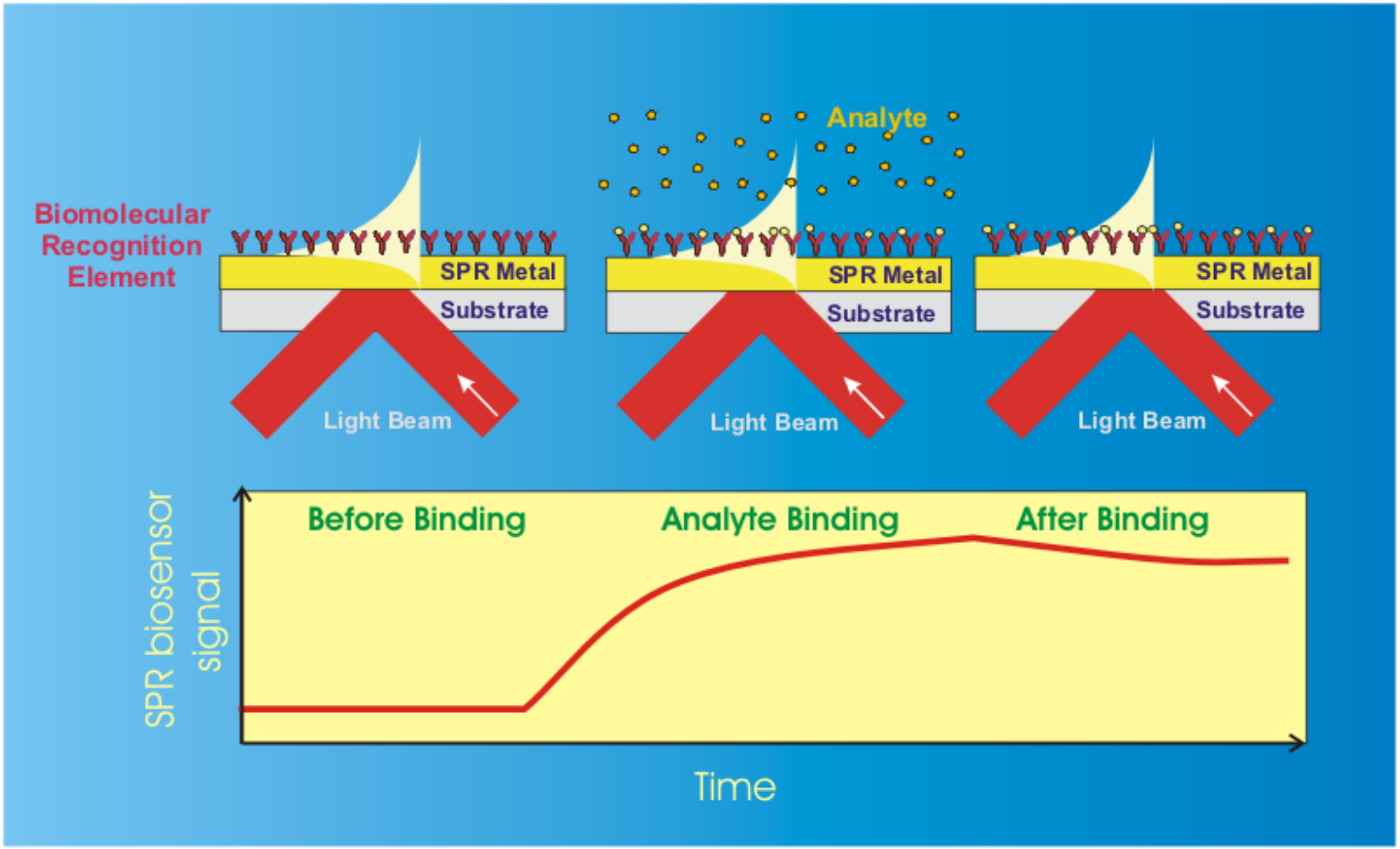


Demonstrated for detection of metalloproteinase (MMP3) secreted by epithelial cells.



Alivisatos lab: http://pubs.acs.org/doi/abs/10.1021/acs.nanolett.5b01161

Example of Plasmonics in Biotechnologies – Biomolecular Interaction Analysis



A - Summary

- Light propagation, refraction and reflection of light beams, total internal reflection - TIR
- Dielectric waveguides – optical fibers.
- Metallic waveguides - propagating surface plasmons
- Metallic nanoparticles - localized surface plasmons LSPs, optical properties of metallic nanoparticles associated with LSPs.
- Confinement and enhancement of light intensity, probing of molecular binding events.
- Metallic surface quenches fluorescence.

B. PREPARATION OF OPTICAL STRUCTURES

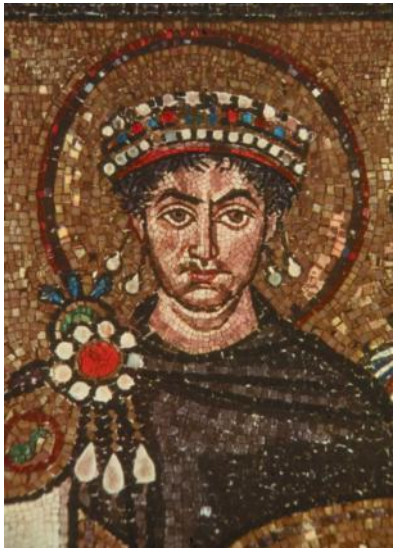
Preparation of Optical Nano/Micro - Structures

"Top down" and "bottom up" approaches can be used for preparation of micro/nano structures.

A subtractive process from bulk starting materials

David (Michelangelo), Florence

An additive process that starts with precursor atoms or molecules

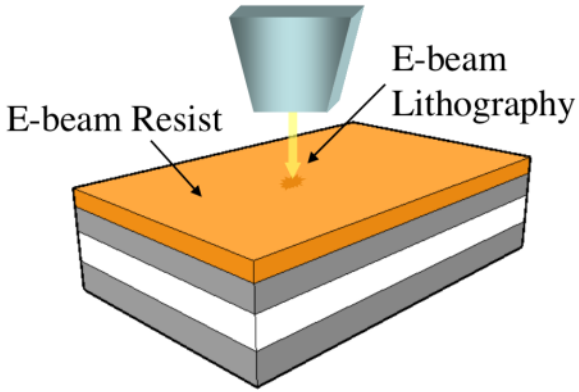


Mosaic of Justinian and Retinue at Apse Entry, San Vitale, Ravenna, 6th century

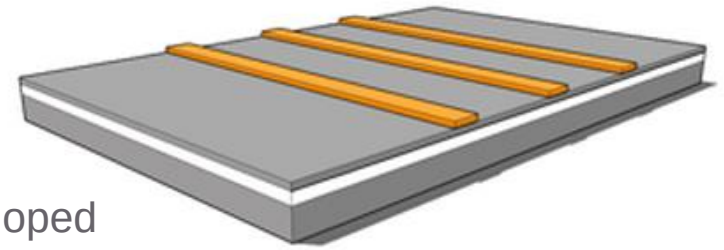
Electron Beam Lithography (EBL)

A method to pattern resist layers (e.g. PMMA) spun onto a substrate. Features of few tens of nanometers or below are possible to prepare. Limitation is the (slow) patterning time and difficult structuring of large areas (>100 × 100 µm).

1) Electrons from a scanning electron microscope are accelerated and passes through the resist and into the silicon. Secondary electrons are produced.
2) These electrons travel through the resist where they break the bonds of the polymer chain.
3) When the sample is developed, the now short chained polymers are dissolved, leaving the written pattern behind.



Scanning over the resist surface

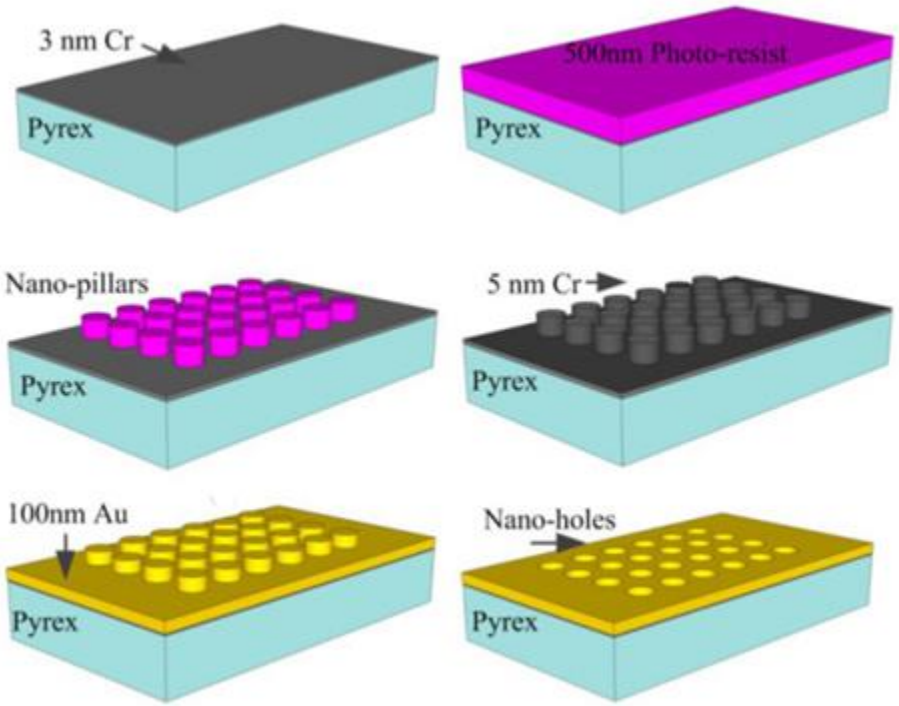


Resist is developed leaving the patterned resist mask

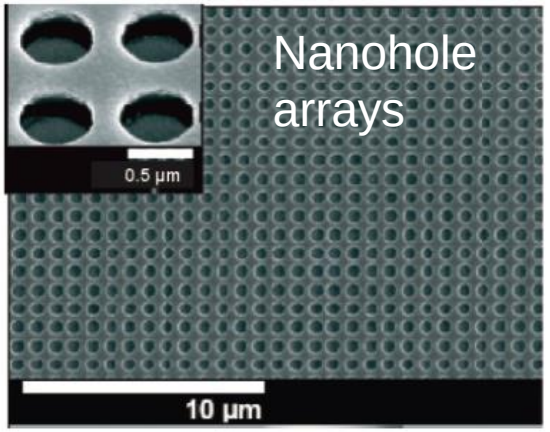
http://people.ece.cornell.edu/lipson/nature/fabrication.htm

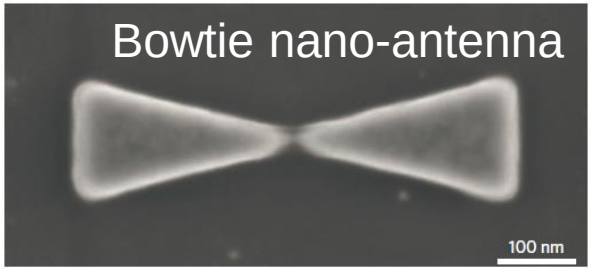
EBL – Examples

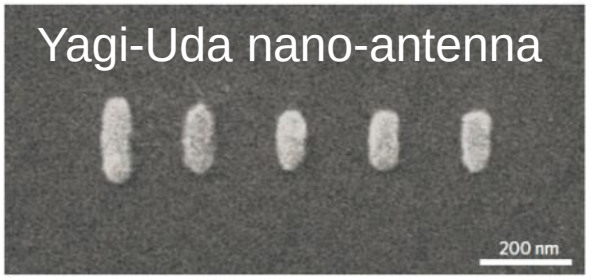
Lift-off of sacrificial resist layer is combined with EBL to prepare target micro/nano-structures.



Optics Express, Vol. 19, Issue 27, pp. 26186-26197 (2011)



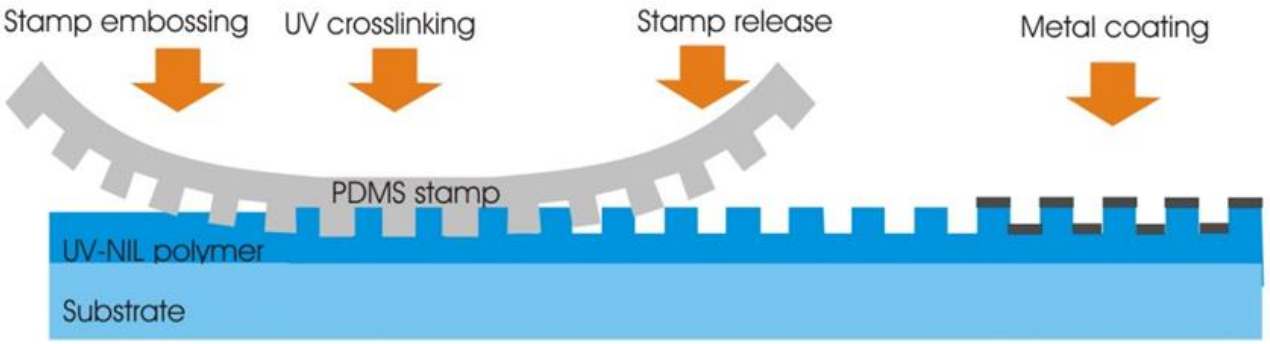




Novotny, van Hults, Nature Photonics, doi: 10.1038/nphoton.2010.237

Nano-Imprint Lithography (NIL)

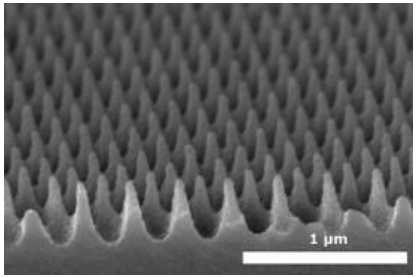
Method aimed at structuring with low cost, high throughput, and high resolution. Patterns are made by a mechanical deformation of an imprint resist. The imprint resist is typically a polymer that is cured by heat or UV light during the imprinting.



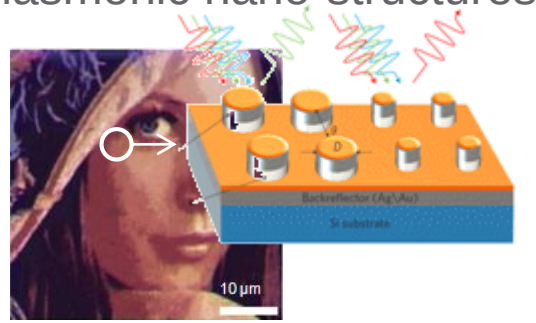
By NIL, wide range of structures can be made:

CDs

Anti-reflection coatings



Plasmonic nano-structures



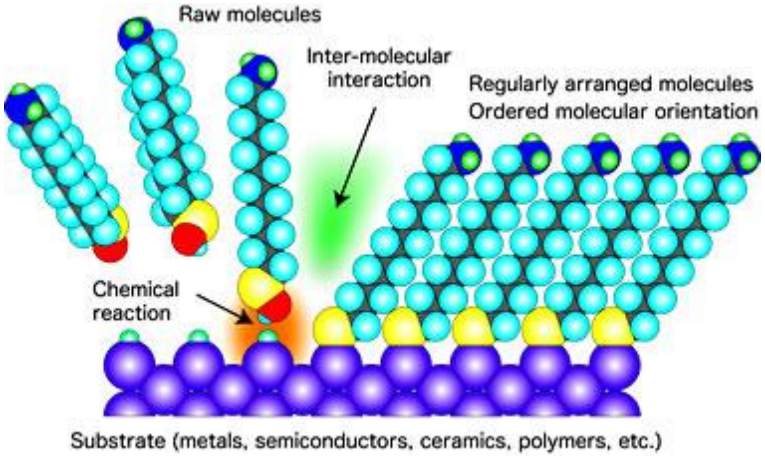
http://www.nilt.com

Self Assembly

Molecules or nanoparticle can self-arrange in well defined structures. Creates a high quality layer of material. Layers are deposited one layer at a time over large areas. Self assembled monolayer (SAM).

Building blocks with different characteristic size:

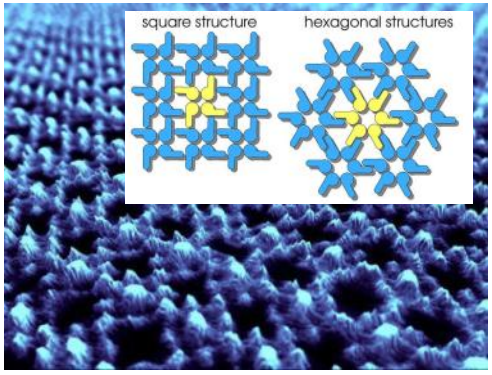
< 1 nm



Thiol SAM

http://www.mtl.kyoto-u.ac.jp/english/laboratory/nanoscopic/nanoscopic.htm

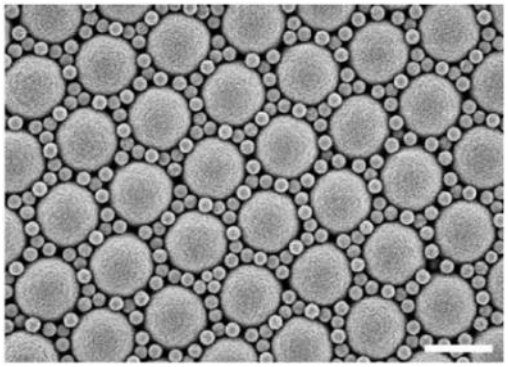
several nm



S-layer

Sleytr, FEMS Microbiology Letters (2007), 267 (2), 131–144

> 100 nm



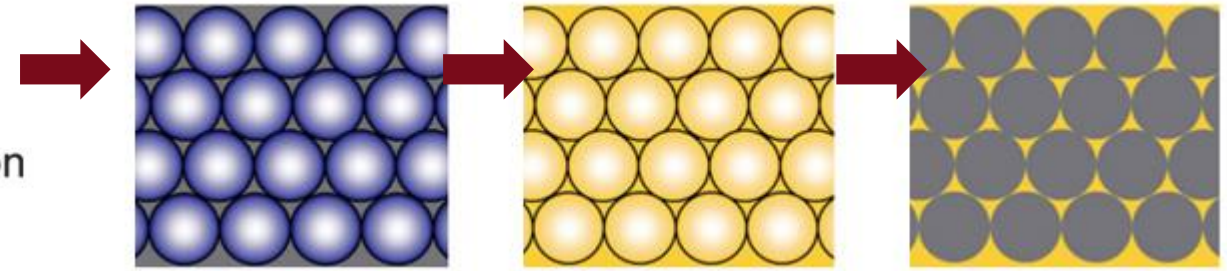
Colloidal crystal

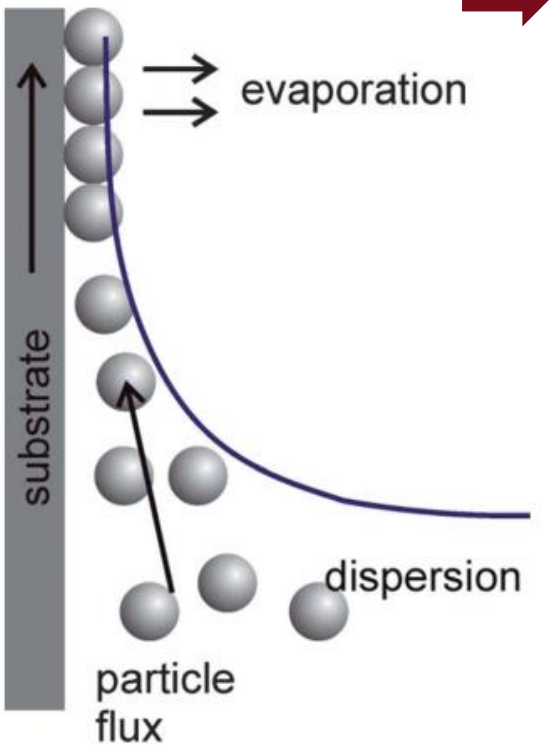
N. Vogel, et al., Adv. Funct. Mater., 2011, 21, 3064–3073.

Colloidal Lithography

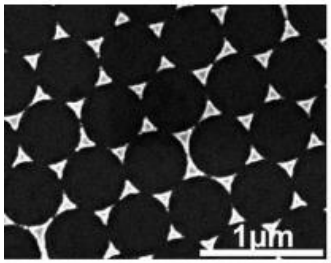
Colloidal crystal monolayer is employed as a mask for further fabrication of desired structures.

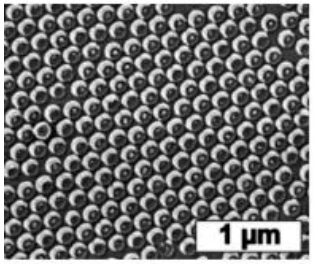
Colloid mask assembly / Metal deposition / Removal of colloid





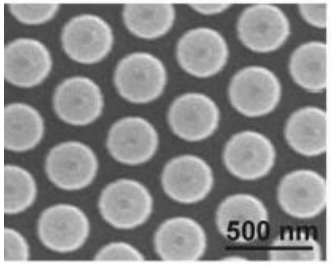
Triangles

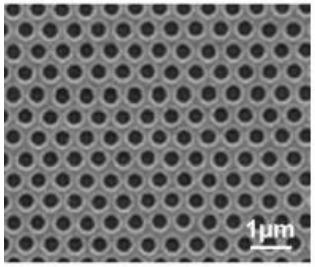




Crescents

Nano disks



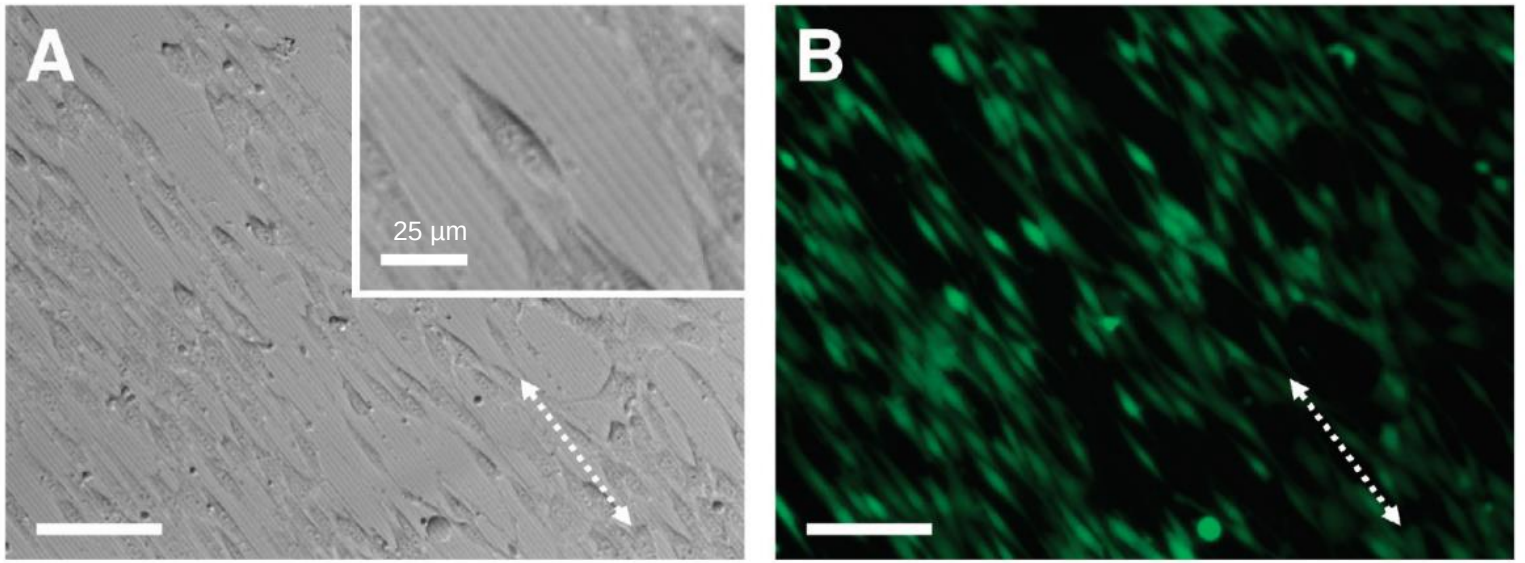


Nano holes

N. Vogel et al ***Soft Matter***, 2012,**8**, 4044-4061
DOI: 10.1039/C1SM06650A

Beyond - Controlled Cell Interaction with Surfaces

It’s not only optics - similar techniques exploited for structuring of polymer surfaces enabling control of cell attachment – e.g. orientation of fibroblasts shown below.



Biomacromolecules 2009, 10, 994–1003

B - Summary

- “Top down” approaches for preparation of structures: e-beam lithography, lift-off, NIL.
- “Bottom up” techniques based on self assembly – SAM, colloidal crystals, colloidal lithography.
- Can be used to control optical characteristics as well as to define morphologies with different chemical characteristics (cell-surface interactions).

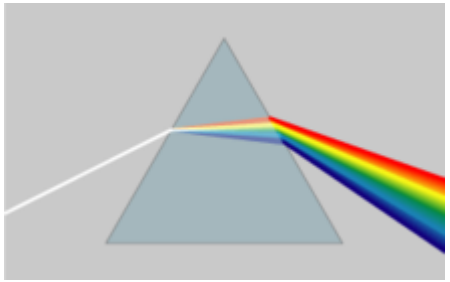
C. OPTICAL SPECTROSCOPIES LIGHT- MATTER INTERACTION

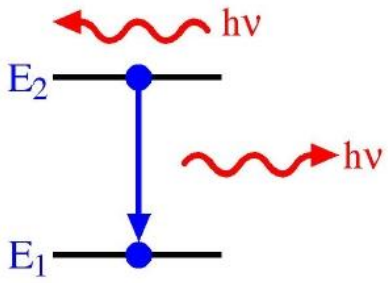
Refractive Index

Parameter that describes interaction of light with matter composed of elements (e.g. atoms) that are << λ and exhibit polarizability. By averaging over many atoms that are be polarized by the oscillating electric field.

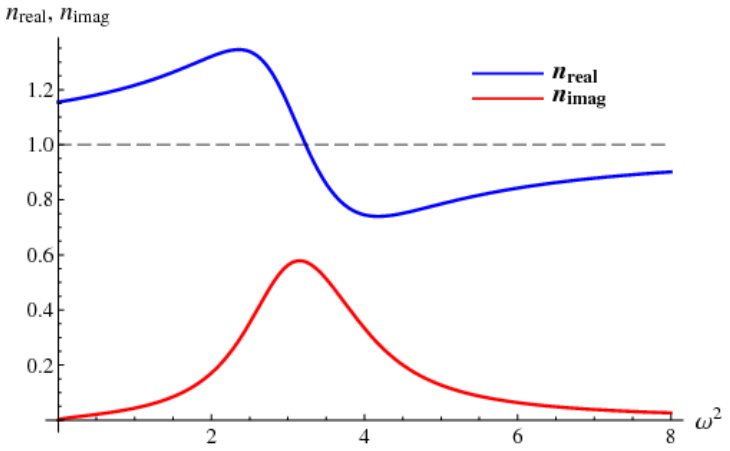
$$\tilde{n} = n + ik$$

Real part – refraction





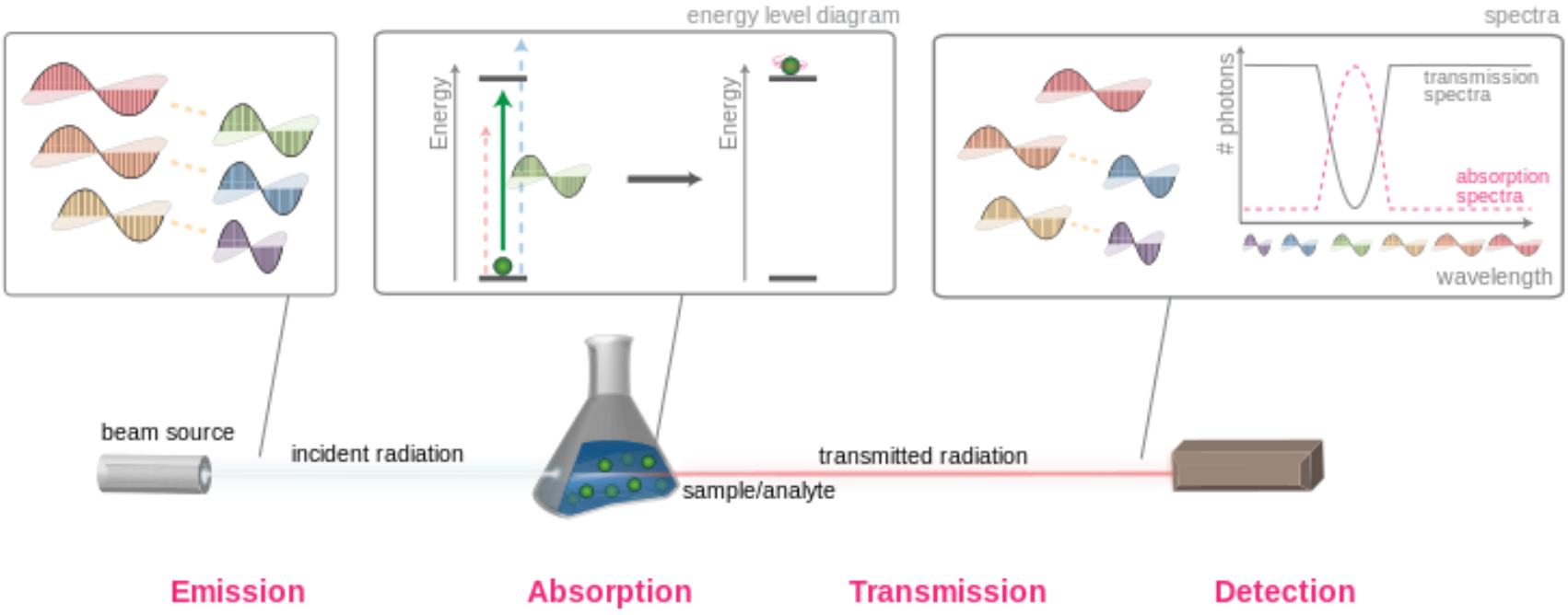
Imaginary part - absorption



For more details, see Lorenz Lorenz or Clausius-Mossotti theories.

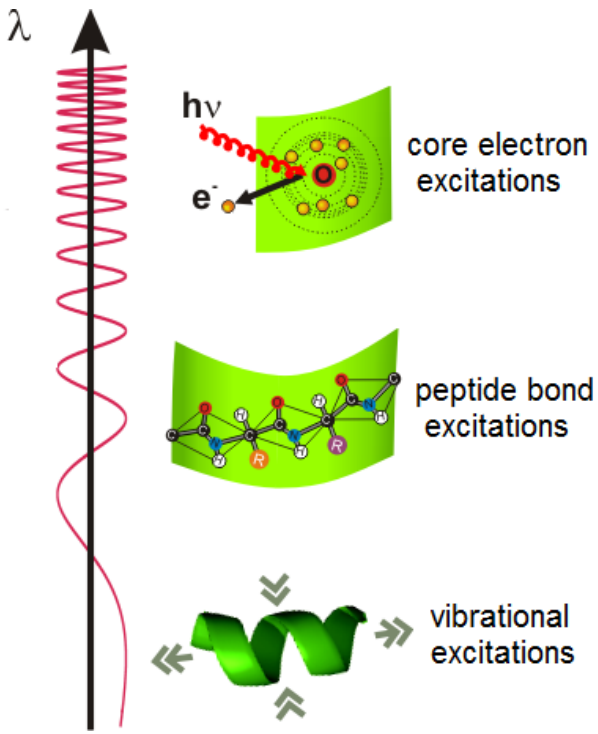
Absorption of Molecules

Absorption of light by molecules is accompanied with their transition from a ground state to excited state (followed by a relaxation). It typically occurs at distinct energies leading to specific bands in absorption spectrum.



E.g. is routinely used for measuring total concentration of proteins – UV absorption spectroscopy.

Absorption of Molecules - Wavelength Spectrum



Electronic lines correspond to a change in the electronic state of an atom or molecule. Typically UV-Vis.

Vibrational lines correspond to changes in the vibrational state of the molecule and are typically found in the infrared region.

Rotational lines, for instance, occur when the rotational state of a molecule is changed. Rotational lines are typically found in the microwave spectral region.

Combination of above can lead to rather complex spectra.

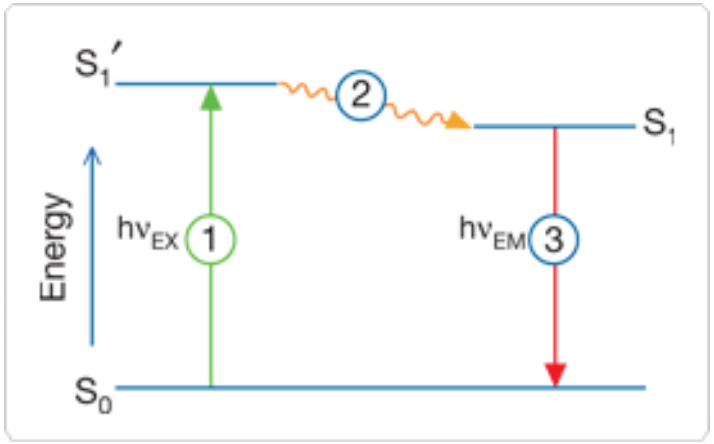
Fluorescence

Process in which a fluorophore (e.g. a dye molecules, quantum dot) absorbs a photon and re-emits it at a higher wavelength. Form of luminescence:

Luminescence

Fluorescence: Lifetime from $<10^{-10}$ to 10^{-7} s (from singlet state).

Phosphorescence: Lifetime from 10^{-5} to $>10^{+3}$ s from (triplet excited state).



Jablonski diagram

(1) Photo-excitation: from the ground electronic state S_0 creates excited states S_1, (S_2, …, S_n)

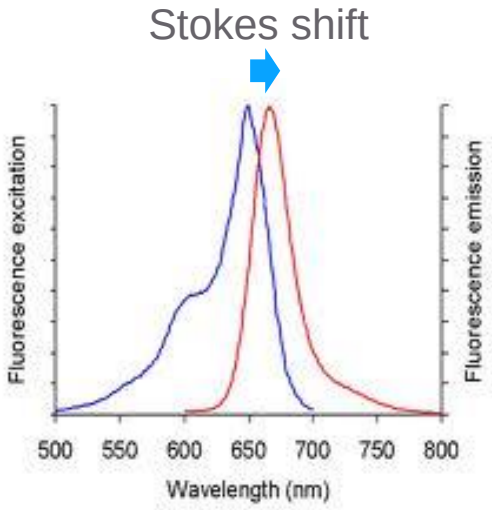
(2) Internal conversion: Molecules rapidly (10^{-14} to 10^{-11} s) relax to the lowest vibrational level of S_1.

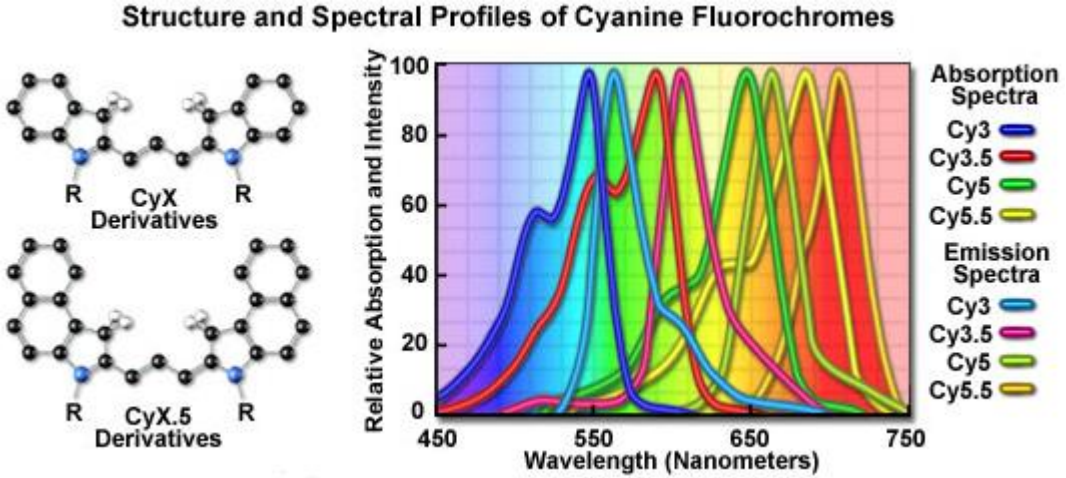
(3) Returning to its ground state S_0.

As the energy hvex is higher than that emitted – Stokes shift occurs.

Fluorophore Characteristics

Quantum yield: Ratio of number of emitted vs. absorbed photons.
Absorption cross-section: Describes the strength of the absorption.
Quenching: Loss of fluorescence signal, interactions between the fluorophore and the local molecular environment (collisions), including other fluorophores (e.g., fluorescence resonant energy transfer FRET).
Photo-bleaching: Only certain number of absorption / emission is possible before destruction of the excited fluorophores occurs.





Abs. and Emission spectrum of Cy5 dye

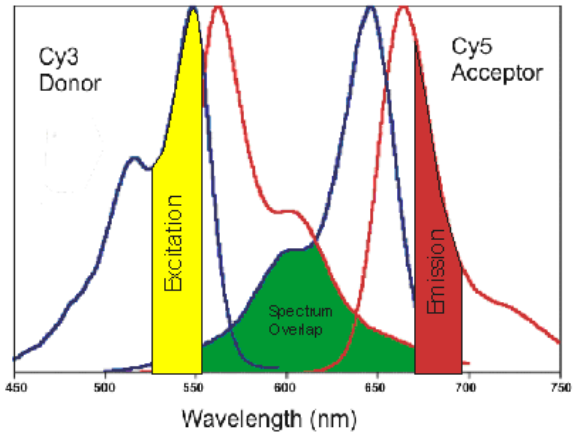
http://www.olympusmicro.com

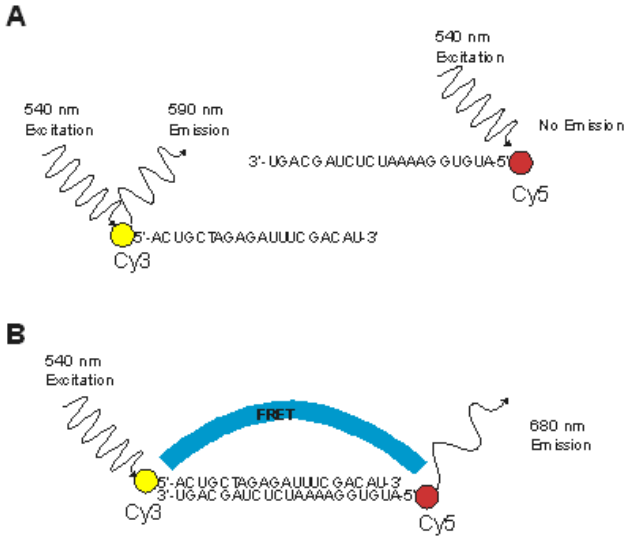
Fluorescence Resonant Energy Transfer - FRET

Förster / fluorescence resonant energy transfer: dipole-dipole coupling of two fluorophores which changes the emission spectrum. Efficient at small distances, typically $r < 10$ nm.

$$E = \frac{1}{1 + (r/R_0)^6}$$

R_0 is Förster distance at which E=50%





Spectral overlap of absoprtion and emission bands of donor and acceptor chromophores

Applications: conformation changes studies, immunoassays, DNA hybridization.

Raman @ IR Spectroscopy

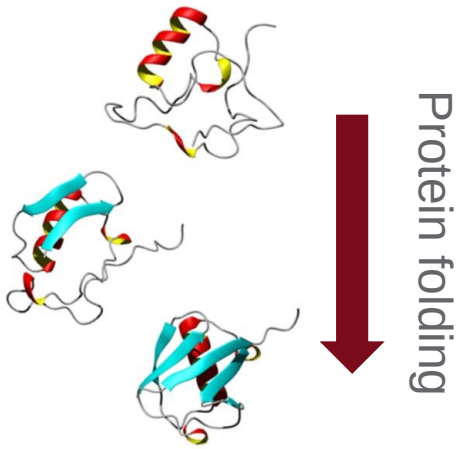
Vibrational spectroscopies - IR and Raman are the most common vibrational spectroscopies for assessing molecular motion and fingerprinting species.

IR and Raman obeys complementary selection rules

- Selection rules dictate which molecular vibrations are probed.
- Some vibrational modes are both IR and Raman active.

Applications

- Commonly used in chemistry, since vibrational information is specific to the chemical bonds and symmetry of molecules. Therefore, it provides a fingerprint by which the molecule can be identified.
- For larger molecules – information on conformation changes can be obtained rather than identification of a protein itself.



http://web.mit.edu/~tokmakofflab/ResearchProtein.htm

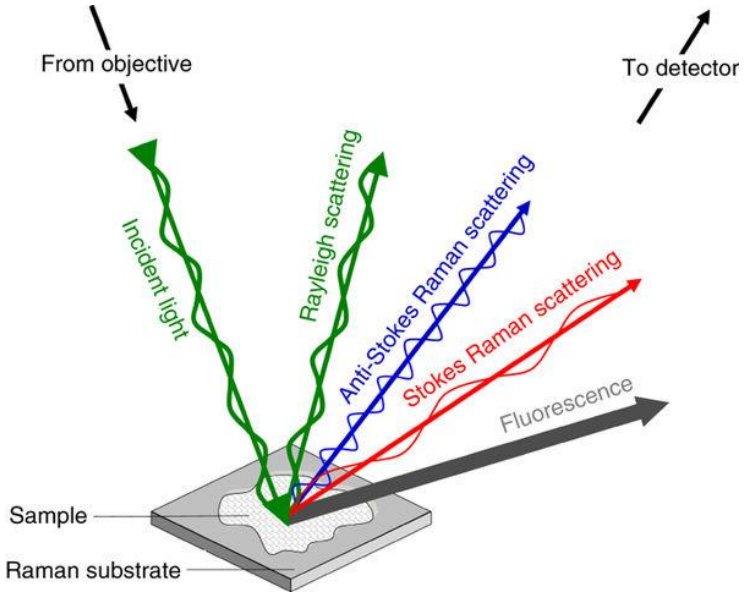
Raman Spectroscopy

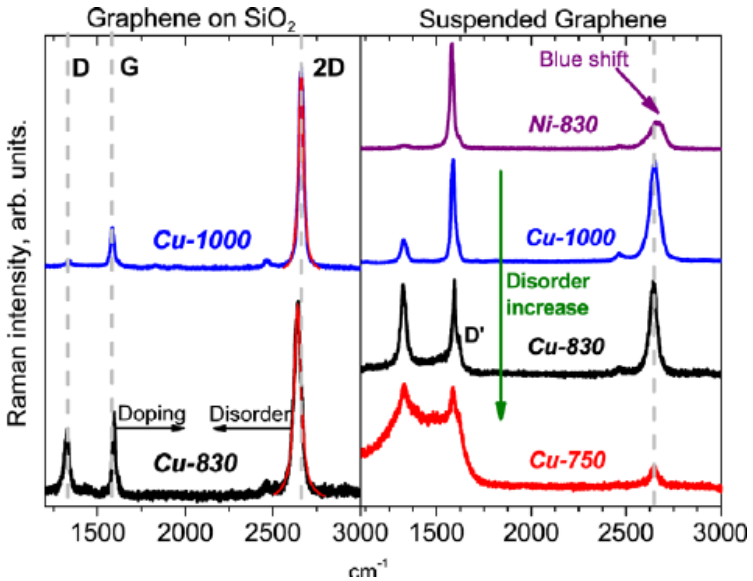
Field intensity

Raman scattering probability ~ E^4 IR absorption rate ~ E^2

Raman is much weaker effect (often masked by fluorescence) compared to IR.

Selection rules related to symmetry
Rule of thumb: symmetric=Raman active, asymmetric=IR active





DOI: 10.1088/0957-4484/22/27/275716

C - Summary

- Absorption occurs at different spectral ranges for different molecular transitions (between electronic, rotational, and vibration states).
- Fluorescence, Stokes shift, lifetime, quantum yield, FRET.
- Raman and IR spectroscopy, fingerprinting for small molecules. Information on vibration and rotation of molecules.
- Raman scattering is weak and provides a practical tool in connection with amplification – surface-enhanced Raman scattering (SERS)

Scientific Background on the Nobel Prize in Chemistry 2014

D. “Super-resolved” fluorescence microscopy

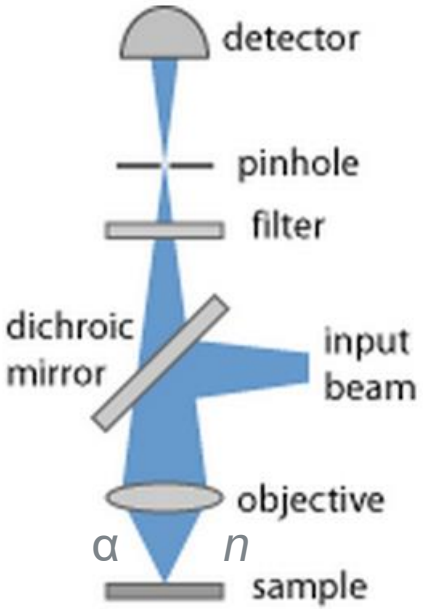
The **Nobel Prize in Chemistry 2014** was awarded jointly to **Eric Betzig**,**Stefan W. Hell** and **William E. Moerner** *"for the development of super-resolved fluorescence microscopy"*.

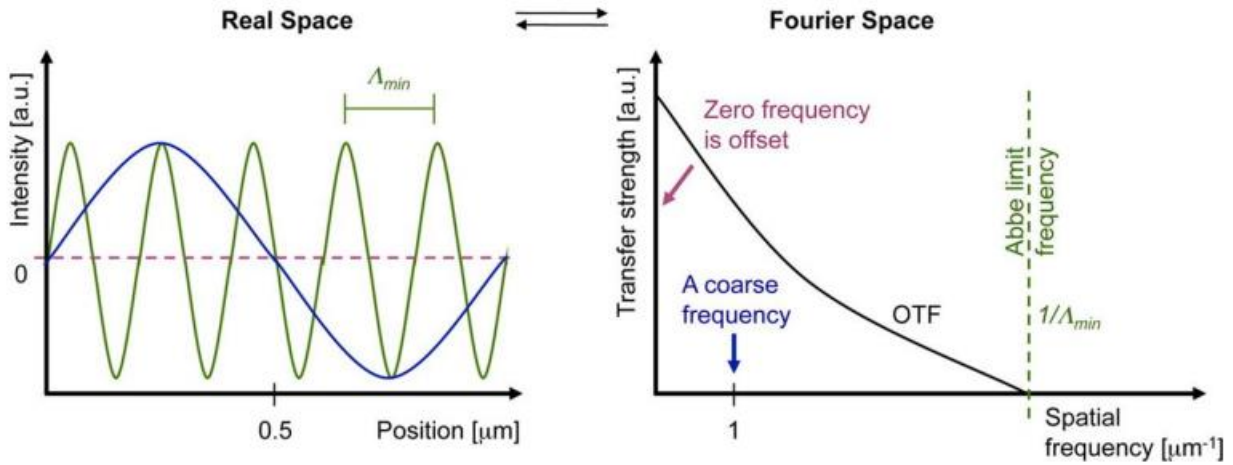
http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/advanced-chemistryprize2014.pdf

Optical Microscopy

Abbe diffraction limit: In the far field, the minimum distance between two points that can be distinguished is:

$$(\delta x_{\min}, \delta y_{\min}) = \Delta_{\min} = \frac{\lambda}{2n \sin \alpha}$$

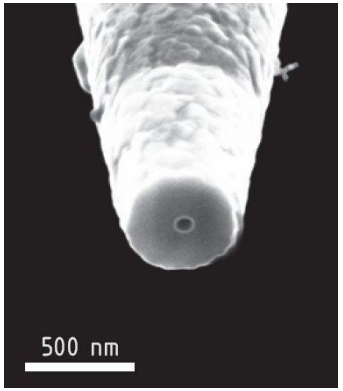




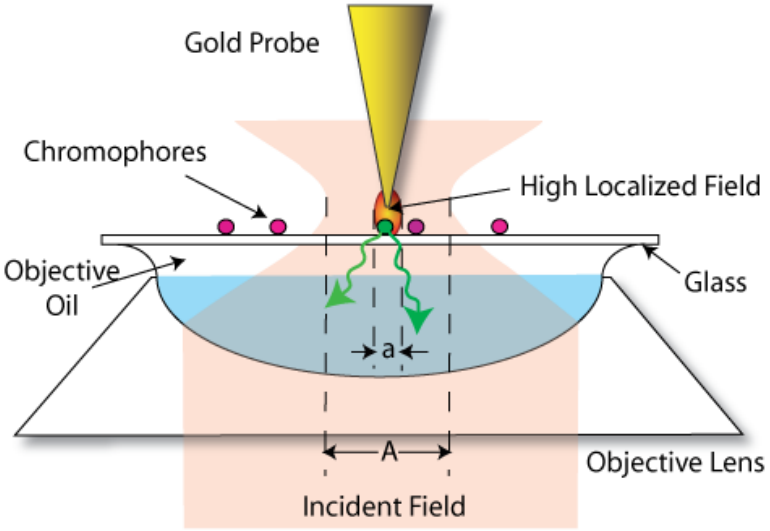
Confocal microscope

How to fight the diffraction limit

Scanning near field optical microscope (SNOM)



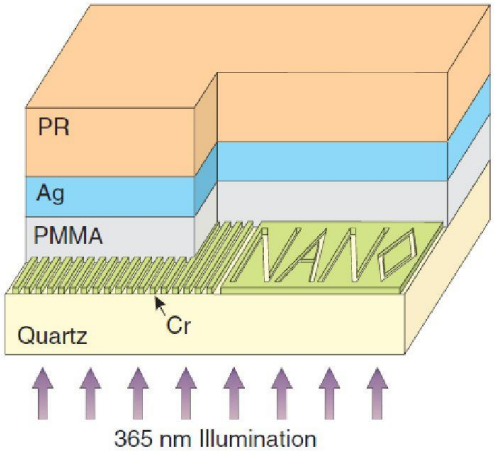
https://gerhardt.ch/science.php

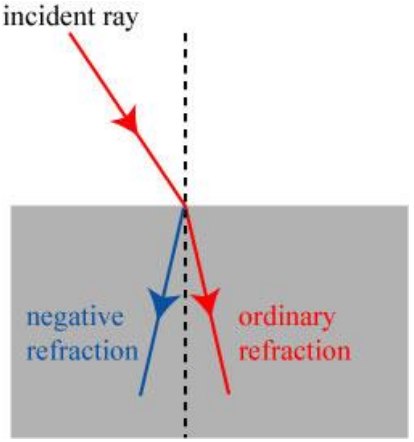


Optical meta-materials:

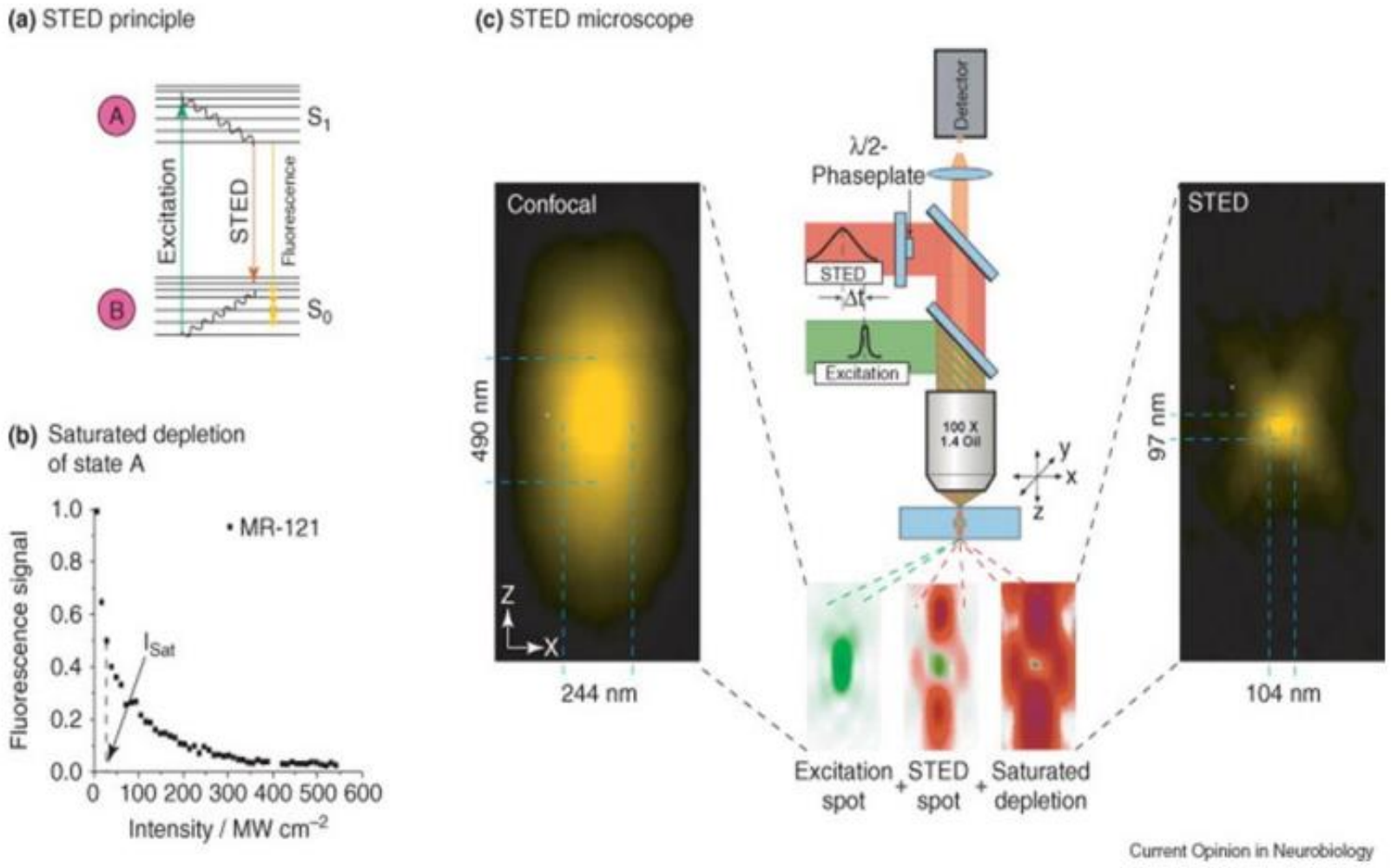
Concept of a lens made of materials with “negative refractive index” allows for perfect imaging.

http://www.intechopen.com/books/plasmonics-principles-and-applications/plasmonic-lenses

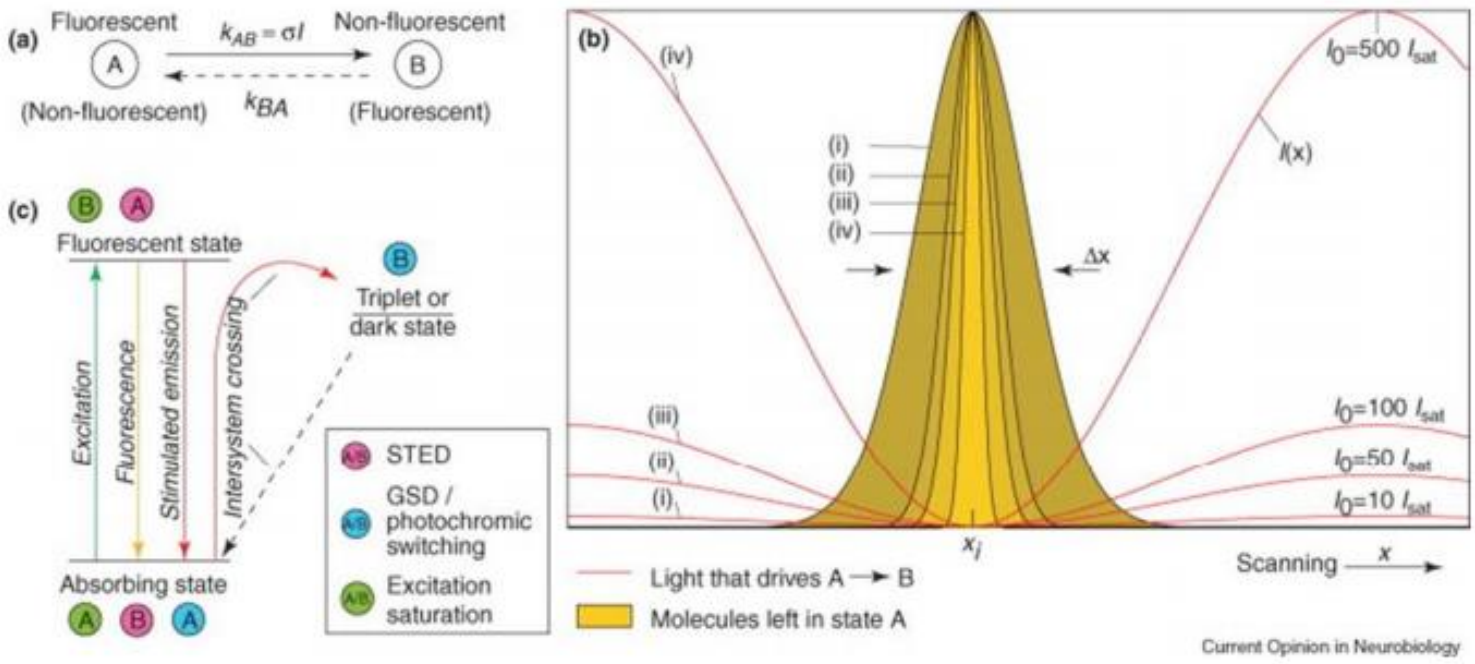




STED – Stimulated Emission Depletion

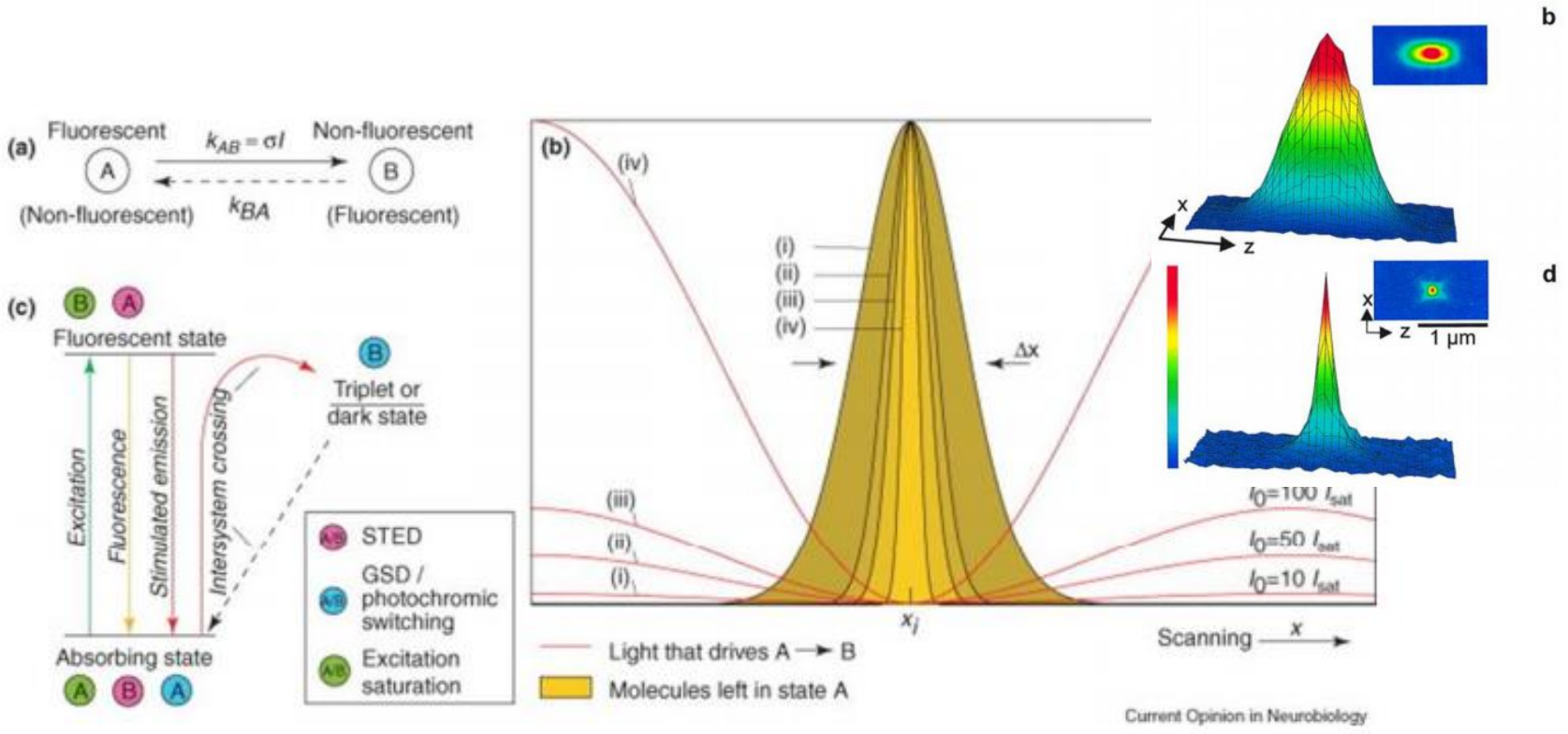


STED – Stimulated Emission Depletion



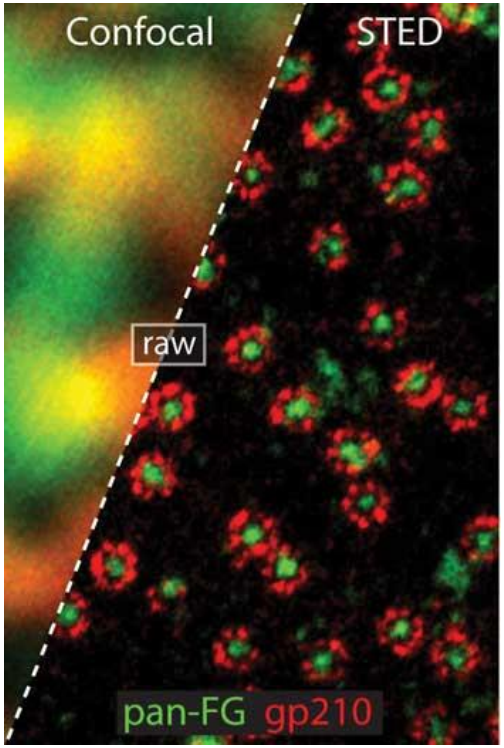
Switching off fluorophores around a narrow zone in the center allows for localization of the fluorescence emission. From the principle point of view - no limit in resolution.

STED – Stimulated Emission Depletion



Switching off fluorophores around a narrow zone in the center allows for localization of the fluorescence emission. From the principle point of view - no limit in resolution.

Other approaches



STED imaging showing two color co-localized recordings of nuclear pore complexes in amphibian cells at resolution of 20 nm (red) and ~30 nm (green channel). The imaging is described in Biophys J 105, L01 - L03, (July 2013). Göttfert et al.

Other approaches followed based on other implementations of the concept that is based on ability of selectively “switch on and off” fluorescence.

STORM – stochastic optical reconstruction microscopy

PALMS – photo-activated localization microscopy

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D - Summary

- Regular optical microscopes can resolve features down the size of several hundreds of nanometers.
- Resolution hindered by wave-nature of light. Abbe diffraction limit.
- STED, PALMS, STROM – fluorescence-based techniques that allow breaking the diffraction limit and advance the spatial resolution to sub 100 nm distances when probing with visible light.