ZF Nanoscale Molecular Imaging - FS22 JZ

Topic 1 - Fluorescence microscopy (L1-3) (Light)

- Super-resolution imaging using patterned illumination: STED, RESOLFT, SSIM
- Super-resolution imaging using Single Fluorophore Localization: PALM, STORM, FPALM

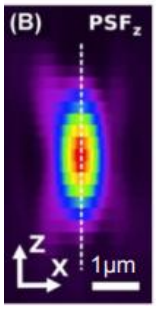
Fluorescence microscopy
Advantages (over e.g. EM):

- Live sample real time
- Molecule-specific binding
- High sensitivity (single molecule)

Disadvantage:

- Limited spatial resolution (lateral ~200 nm, axial ~500 nm)

Point spread function PSF: Point is imaged as diffraction pattern (Airy disc, Gaussian shape)
Axial PSF is 2.5x the size of the lateral (490 nm vs 190 nm)



Abbe's diffraction / Rayleigh resolution limit:

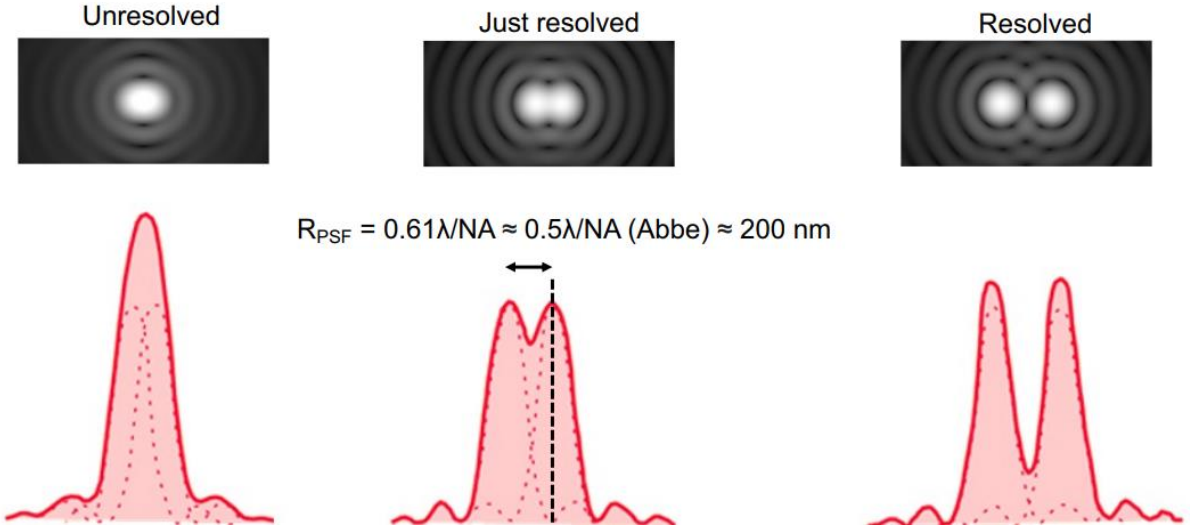
$$R_{Lateral} = \frac{\lambda}{2nsin\alpha}$$ (Abbe's limit for the lateral resolution of an optical microscope)

$$R_{Axial} = \frac{2\lambda}{n(sin\alpha)^2}$$ (Rayleigh's limit for the axial resolution of an optical microscope)

(NA = n * sin α)

Blurry images are caused by overlapping PSF's

Rayleigh resolution criterion: Resolved when the maximum of the second fluorophore falls on the first minimum of the first fluorophore's PSF



How to break the diffraction limit of ~200 nm? => Two approaches:

- Far-field (collection of emitted fluorescence far from fluorophore >100 µm):
  1. Confocal microscopy
  2. Multiphoton microscopy
  3. 4Pi & I^{5}M microscopy
  4. Structured illumination microscopy (SIM)
- Near-field (collection close to fluorophore <100 µm):
  1. Total internal reflection fluorescence (TIRF)
  2. Scanning near-field optical microscopy (SNOM)

Far-field

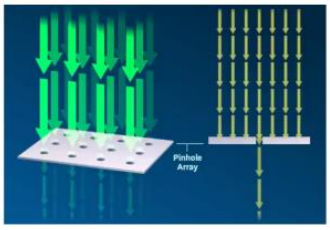
1. Confocal fluorescence microscopy

Concept by Marvin Minsky in 1957: Focused laser for excitation and pinhole for detection (filter non-focused rays)
20-30 % improvement in lateral and axial resolution
Reconstruction of 3D images possible (optical sectioning)

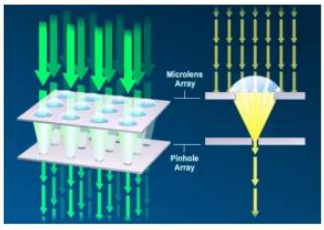
Flavors: Laser-scanning or spinning disc

Laser-scanning: Laser instead of white light, much stronger excitation, but raster scanning (slow). High quality and very flexible. Spectral unmixing using a diffraction grating → accurate recording of one particular wavelength possible

Spinning disc: 1000 pinholes (50 µm, 2.5 µm spacing), single frame each 30° rotation, 12 frames per rotation. Up to 5000 rotations per minute => speed must match camera frame rate, ideal for live cell imaging.
Uses microlens array for increased illumination



Light transmission: 4%



Light transmission: 56%

Spatial resolution could be improved by confocal microscopy

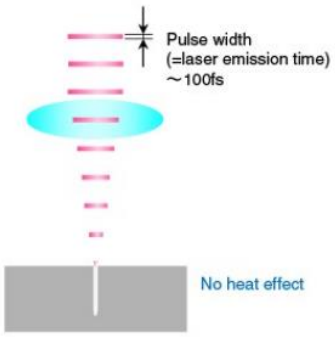
Widefield **Confocal**

$$R_{Lateral} = \frac{0.61\lambda}{NA} \rightarrow \frac{0.4\lambda}{NA}$$

$$R_{Axial} = \frac{2n\lambda}{NA^2} \rightarrow \frac{1.4n\lambda}{NA^2}$$

2. Two-photon microscopy

Simultaneous excitation of one fluorophore by two photons (longer wavelength than emitted light, requires femtosecond Ti-sapphire pulsed laser). Lower axial PSF, allows optical sectioning. Two photons are absorbed → probability for fluorescent emission increases quadratically.
Very low probability of two-photon absorption happening due to short time of fluorophore in intermediate virtual state and being a very small target.
Density of photons needs to be increased (a million times more photons than in single photon fluorescence)



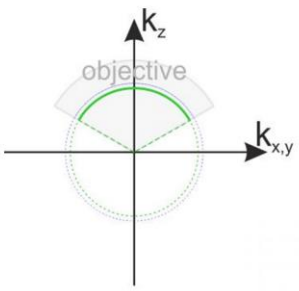
Femtosecond laser

Usage of femtosecond laser, which pulses 50-100 fs wide at high peak power

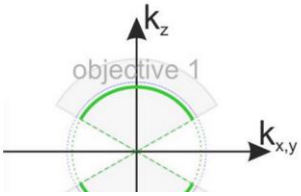
Advantages: up to 1 mm deep penetration, less damaging
Limitations:
Focal spot is diffraction limited
Requires fs lasers: very expensive

3. 4Pi microscopy

Two opposing microscope objectives with a common focus to increase microscopy aperture for laser excitation and signal collection



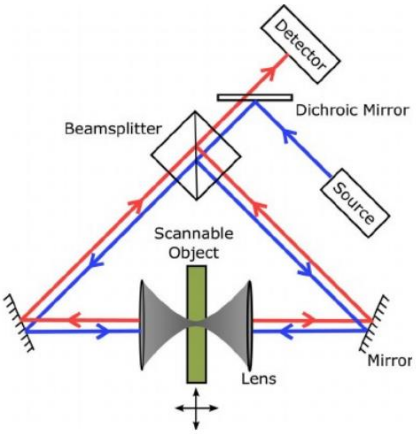
Normal microscope



Difference in optical path length through the two objective lenses is aligned to be minimal. Objectives are illuminated with coherent wave fronts that are:

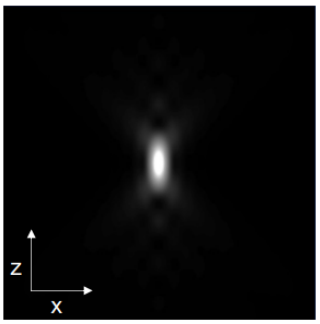
- Interfering constructively in common focus (type A)
- Fluorescent light that is collected from both sides interferes constructively in the common point detector (type B)
- Both (type C, highest increase in axial resolution)

Max aperture angle of 140° is possible (1.3 π). 4 π to indicate the basic idea (aims to reduce axial resolution by adding second objective below sample)

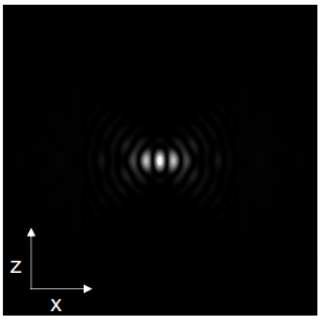


Optical set-up of a 4Pi microscope

Confocal PSF

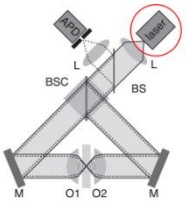


4Pi PSF



3. I^5 microscopy (I^5M)

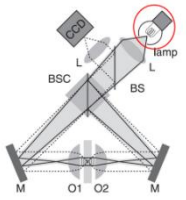
$I^5 = I^2$ (image interference) + I^3 (incoherent interference illumination)
Same principle as 4Pi - spatially incoherent light source (standard arc lamp) instead of laser is used
Axial resolution: ~100 nm



4Pi

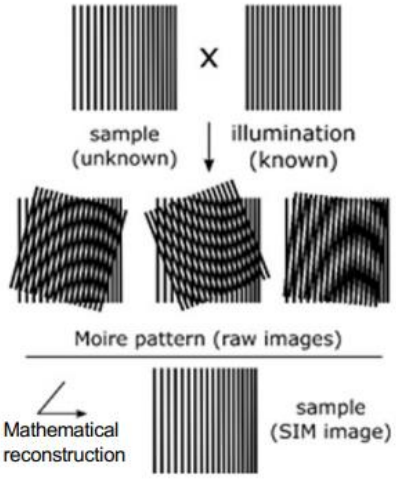
4. Structured illumination microscopy (SIM)

Principle: Diffraction grating is inserted in excitation beam path, laser beams interfere at focal plane and create sinusoidal illumination pattern, which superimposes with the high spatial frequencies present in the sample and generates a “Moiré pattern”



I^5M

Homogeneous illumination: small distances not resolved due to diffraction limit
Structured illumination: high spatial frequencies overlap with the high frequency of the illumination stripes, creating a Moiré pattern of lower frequency which can be well resolved
Final image is mathematically reconstructed: Several raw images at different orientation (moving of the diffraction grating)



2D-SIM image: 9 raw images required (3 translations x 3 rotations)
3D-SIM image: 15 raw images required (5 translations x 3 rotations)

SIM advantages:
2x increase in spatial resolution over widefield (lat: 100 nm, ax: 250 nm)
4D imaging at fast frame rate
Labelling using conventional fluorophores

Summary

- **Light microscopy**
  - Still the most popular tool in life sciences because of two fundamental reasons:
    1) Only way to look inside live cells
    2) Proteins and molecules of interest can be visualized using fluorophore tagging
- **Widefield microscopy**
  - Spatial resolution is diffraction-limited to approx. 200 nm in lateral direction and 500 nm in axial direction
  - Not nanoscale - internal structure of cell organelles can not be visualized
- **Breaking the diffraction barrier**
  - Spatial resolution of widefield microscopy can be improved using far-field approaches: confocal microscopy, 2-photon microscopy, 4Pi/I^5 microscopy and SIM
  - Best case scenario: 100 nm resolution in all three dimensions, by combining 4Pi/I^{5}M and SIM
  - Not limited by fluorophore photophysics or photochemistry, applicable to all fluorophores
  - However, still essentially diffraction-limited

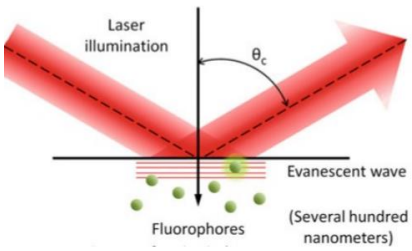
Near-field
1. Total internal reflection fluorescence (TIRF) microscopy
Total internal reflection: Waves do not transmit into medium 2, completely reflected back into medium 1

- Snell's law: $n_1 \sin\theta_1 = n_2 \sin\theta_2$

$$\theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right)$$

- Conditions for TIR:
  - $n_1 > n_2$
  - $\theta > \theta_c$

Evanescent field:
(penetration depth: approx. 100 nm, waves decay exponentially, excitation of fluorophores a few hundred nm in are excited)

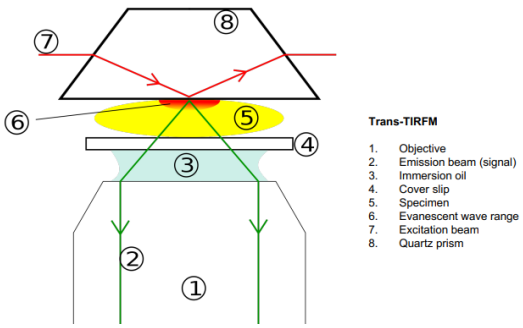


Advantages:
Substantially decreased
No out-of-focus fluorescence
Limited phototoxicity

background

Two flavors: Prism and objective based
Prism based:
Prism/solution interface required
Excitation light path and emission channel are separated
Clean evanescent wave with decay close to theoretically predicted function
Advantages:

- Much cheaper
- Easy integration
- Large range of incidence angles



Disadvantages:

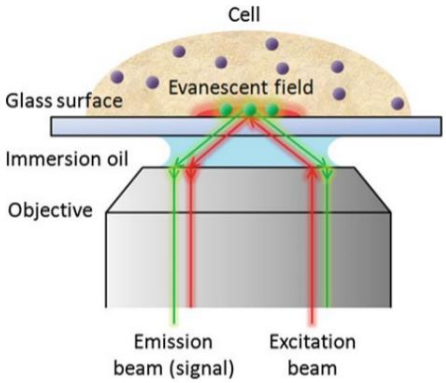
- Illumination introduced on specimen side opposite of objective → imaging through the bulk of the specimen
- Complex design
- Not used by many biologists (rather by physicists)

Objective based:
Excitation light and emission channel share objective
Objective lens has high NA
Advantages:

- Stable, easy to set up and align
- Works with free collimated laser, optical fiber or conventional arc sources
- Easy switch between widefield and TIRF
- Easier for cellular microscopy

Disadvantages:

- Evanescent wave is contaminated with intense stray light

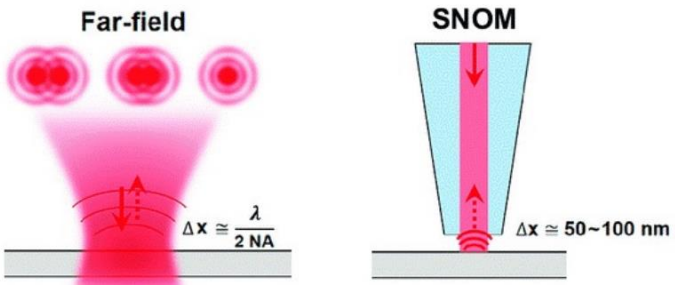


TIRFM applications:

- Ligand binding and receptor movement
- Vesicles undergoing exocytosis
- Proteins in exo/endocytosis
- size, movement and distance apart of regions of contact between a cell and solid substrate

2. Scanning near-field optical microscopy (SNOM) (near-field scanning optical microscopy NSOM)

Laser is focused through an aperture with a diameter smaller than the excitation wavelength: evanescent field on far side of the aperture
Non-propagating light near fluorophore containing high-frequency spatial information is collected
Detector must be placed very close to the sample (few nanometers)



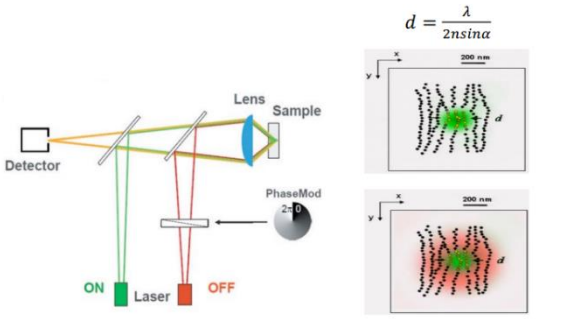
Applications: Single-molecule spectroscopy, study of quantum dots, excitonic photoluminescence in TMDCs
Limitations: Zero working distance and extremely small depth of field. Extremely long scan times, low transmissivity of aperture, only features at the surface, problematic optic probes for imaging soft materials (due to spring constants)

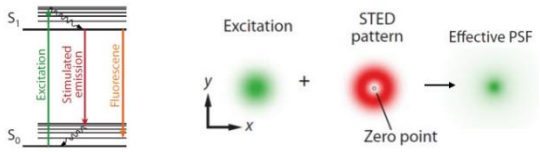
Super Resolution Fluorescence microscopy

- Patterned illumination: STED, GSD, RESOLFT, SSIM
  - introducing sub-diffraction limit features in the excitation pattern
  - non-linear effects to sharpen the PSF of the microscope
- Localization of individual fluorophores: STORM, PALM, FPALM

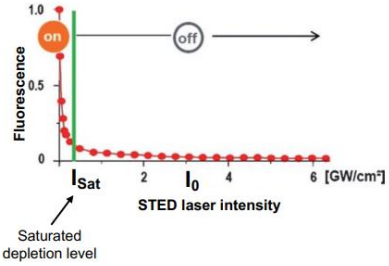
Stimulated emission depletion (STED) microscopy

- Two lasers for excitation and stimulated emission are used
- Stimulated emission beam is donut shaped, extinction of fluorescence via non-radiative relacation from the off-center fluorophores
- Key physical condition: STED light should be red-shifted compared to excitation laser (prevent excitation of molecules again)
- STED pattern: restricted by diffraction limit



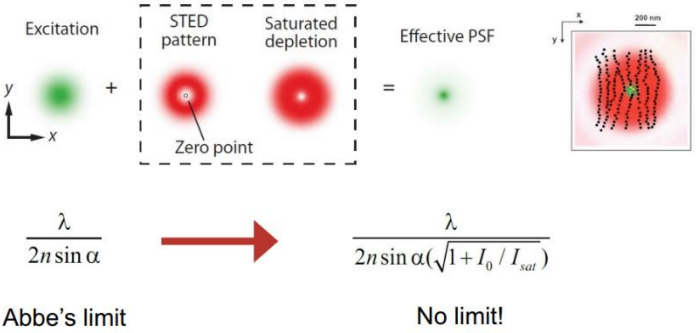


Key to super-resolution: Nonlinear dependence of the stimulated emission
STED laser intensity (I_{sat}) quenches all fluorescence
Increasing STED intensity leads to expansion of saturation depletion region
The size of the reduced PSF is restricted by the practical power level of STED



STED depletes essentially all adjacent fluorophores to the region of interest, leading to the removal of spatial resolution limit.

Lateral: ~104 nm
Axial: ~97 nm
FWHM down to 2.4 nm possible (lat)

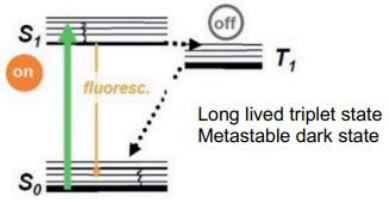


Other types of on/off mechanisms:

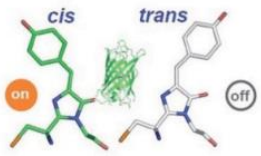
- GSD (using triplet state)
- RESOLFT (switchable fluorescent proteins using cis-trans isomerization)

Ground state depletion (GSD) microscopy

Usage of triplet state T1 (possible for all fluorophores, metastable), optical suppression due to a spin flip taking place. T1 population by repetitively exciting the dye to first singlet state S1, until the nonradiative intersystem crossing to T1 occurs.
Same wavelength for excitation and depletion, ordinary continuous wave lasers and standard fluorophores can be used.

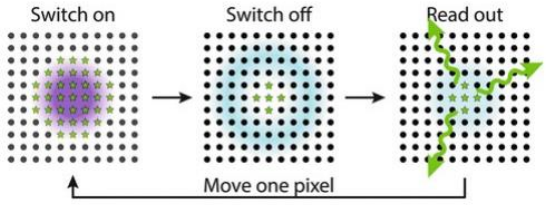


Reversible saturable optically linear fluorescence transition (RESOLFT)
Using cis-trans isomerism, fluorophores reversibly switch between on and off state
<40 nm spatial resolution possible
Superfast imaging possible (100'000 donuts in 2 seconds)



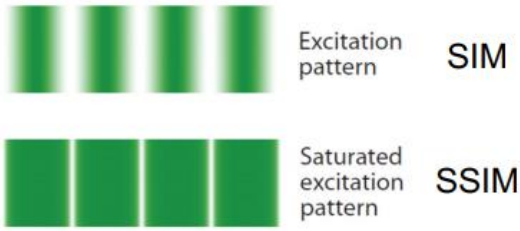
Switchable fluorescent proteins
cis–trans isomerization

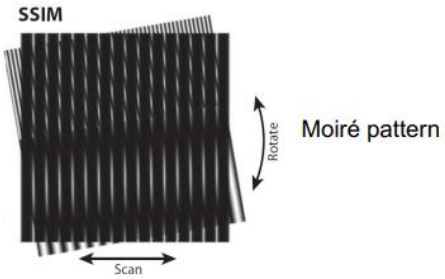
Combinatory approaches:
3D 4Pi-RESOLFT nanoscopy (3D actin microfilaments in live HeLa cell)



Same idea as STED - a donut is created and scanned over a sample

Saturated structured illumination microscopy (SSIM)
SIM but using strong excitation light to saturate the fluorescence emission at the peaks, w/o exciting fluorophores at the zero points





Single Fluorophore Localization: PALM, STORM, FPALM
Detection and imaging of single molecules in dense media

- Stochastic optical reconstruction microscopy (STORM)
- Fluorescence photoactivation localization microscopy (FPALM)
- Photoactivation localization microscopy (PALM)
- Points accumulation for imaging in nanoscale topography (PAINT)

Requirements for super-resolution imaging:

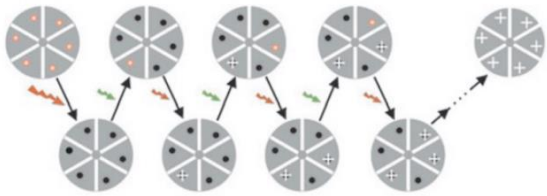
- Super-localization
- Active control of emitter concentration and sequential imaging

Shown by using

- Difference in excitation resonance frequency
- Difference in fluorescence lifetime

Photo-switchable fluorophores required for STORM, FPALM and PALM

Stochastic optical reconstruction microscopy (STORM)
Photoexcitation with red laser, longer exposure => photobleaching
Photo-recovery to fluorescent state with green laser
20 nm spatial resolution possible



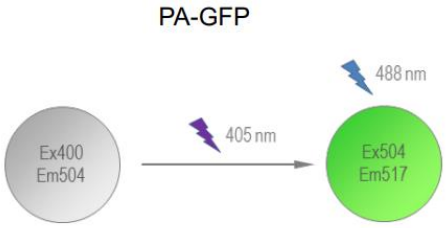
STORM principle

Cy5 excitation with red laser, gives green signal → too long, photobleaching
Bring back to live with green laser → several cycles possible

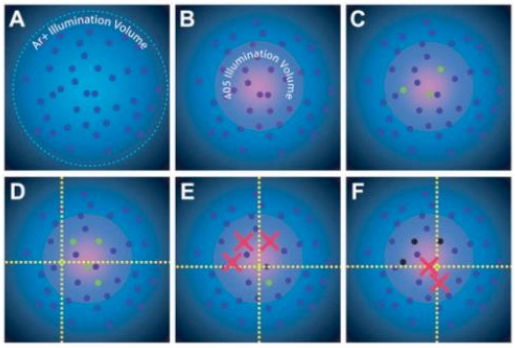
1. Photobleach all
2. Weak pulse green laser to excite only few
3. repeat

Fluorescence photoactivation localization microscopy (FPALM)

- Proof of principle study



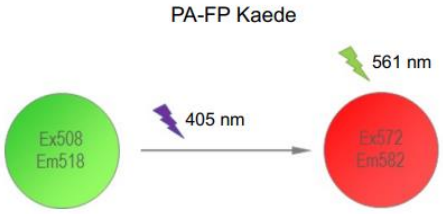
FPALM principle



Ar⁺ laser: 496.5 nm

(Purple laser to activate, then blue to get signal. Photobleaching with longer exposure)

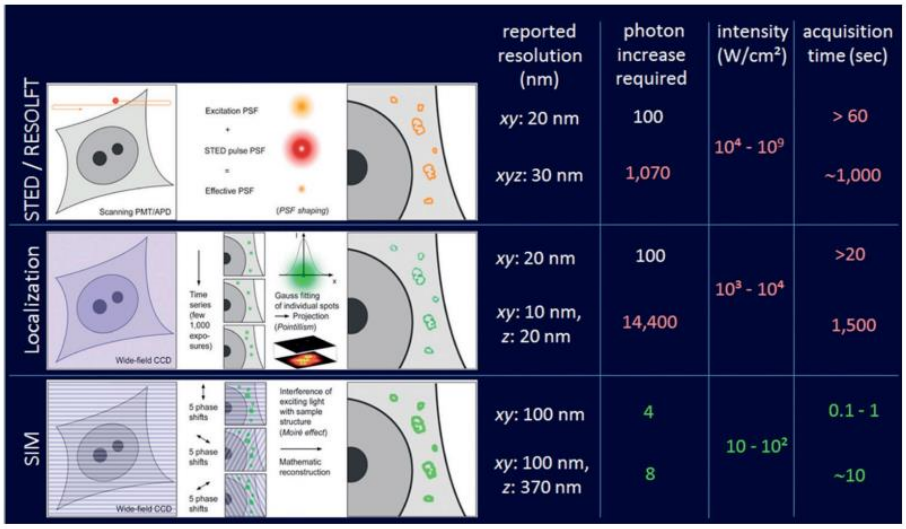
Photoactivated localization microscopy (PALM)
PA-FP (photoactivated fluorophore)
subset activation with 405 nm radiation, imaging with 561 nm until bleached, fluorophore localization in one frame, reiteration for many cycles
Resolution is determined by uncertainty of localization and labelling density
Dual color super-resolution imaging possible by using Eos/Dronpa pair (Eos: photoconversion, irreversible, Dronpa: Photoswitching, reversible)



iPALM: 3D cellular ultrastructure using interferometric PALM
fluorescence emitted light is made to interfere, increases z-resolution to sub-20 nm and provides correlative height information.

SRFM limitations:

- Overexpression of target protein can alter physiological state of the cell
- Exogenous dyes could have limited affinity for the desired target
- High residual background
- Chemical fixation alters the ultrastructure
- High intensity required



	reported resolution (nm)	photon increase required	intensity (W/cm²)	acquisition time (sec)
STED / RESOLFT	*xy*: 20 nm	100	10^4 - 10^9	> 60
	xyz: 30 nm	1,070		~1,000
Localization	*xy*: 20 nm	100	10^3 - 10^4	>20
	xy: 10 nm, *z*: 20 nm	14,400		1,500
SIM	*xy*: 100 nm	4	10 - 10^2	0.1 - 1
	xy: 100 nm, *z*: 370 nm	8		~10

Average sunlight intensity:

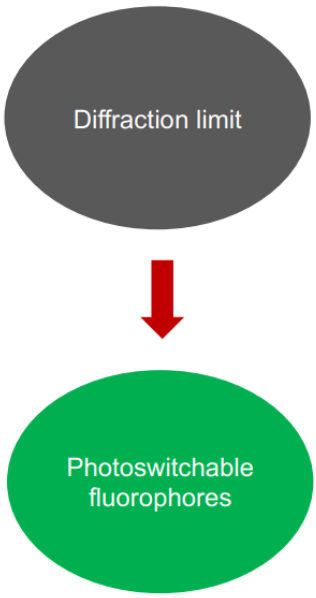
0.14 W/cm²

Holy grail: **Molecular resolution imaging of a live cell at 0.1 W/cm²**

Summary:

Optical microscopy: (Long) journey to the nanoscale

- **Simple magnifying glasses (13th century)**
- **Fluorescence microscopy**
- **Breaking the diffraction barrier: Extending resolution of optical microscopy**
  - Using far-field
    - Confocal microscopy
    - Multiphoton microscopy
    - 4Pi and I5M microscopy
    - Structured illumination microscopy (SIM)
  - Using near-field
    - Total internal reflection fluorescence (TIRF) microscopy
    - Scanning near-field optical microscopy (SNOM)
- **Super-resolution fluorescence nanoscopy**
  - Using patterned illumination (2000)
    - Stimulated emission depletion (STED) microscopy
    - Ground state depletion (GSD) microscopy
    - Reversible saturable optical fluorescence transitions (RESOLFT) microscopy
    - Saturated structured illumination microscopy (SSIM)
  - Using single molecule imaging (2006)
    - Photoactivated light microscopy (PALM)
    - Stochastic optical reconstruction microscopy (STORM)
    - Florescence photoactivated light microscopy (FPALM)



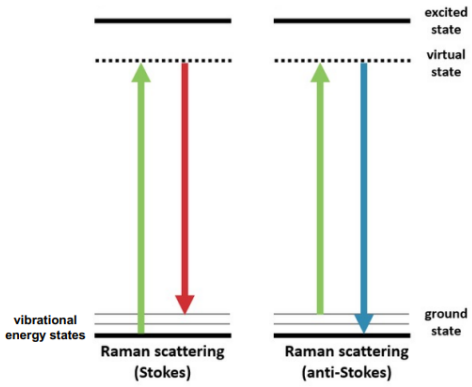
Topic 2 - Raman spectroscopy (L4-6) (Light)

- Scanning near-field optical microscopy (SNOM): with and without aperture
- Tip-enhanced Raman spectroscopy (TERS)

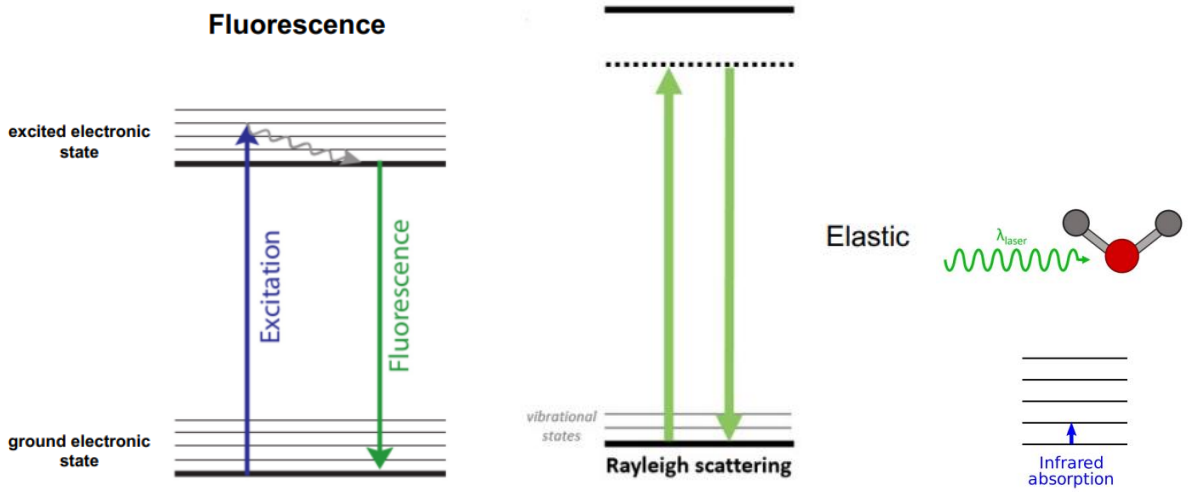
Rayleigh: Sky is blue because the molecules are smaller than most wavelengths except the smallest, which then scatters more. Clouds are white because the particles are larger and not only one color, but all are scattered (Mie scattering, same analogy to milk, sugar and salt). Why is sunset/sunrise red then? Longer distance travelled, blue is scattered away.

Why is the sea blue? Rayleigh: Sea reflects the light from the sky. Raman: Water is blue because it also scatters light, which is proportional to the inverse of the wavelength to the power of four (blue is scattered the most, Raman could prove this experimentally).

Raman effect: Change in the color of light when it interacts with molecules. Photons interact with molecules and can either lose energy to excite vibration in the molecule, leading to lower energy of the scattered photon and thus longer wavelength (red shift, Stokes Raman scattering). Or the excited vibrational molecule can transfer the energy to the photon, leading to shorter wavelength (blue shift, Anti-Stokes Raman scattering).
Ground state molecules are much more likely than excited molecules; Stokes Raman scattering therefore is more prevalent. Intensity determined by temperature - ratio can be used to measure temp. Raman signal is much weaker than fluorescence! The virtual state does not exist, the process is instantaneous.

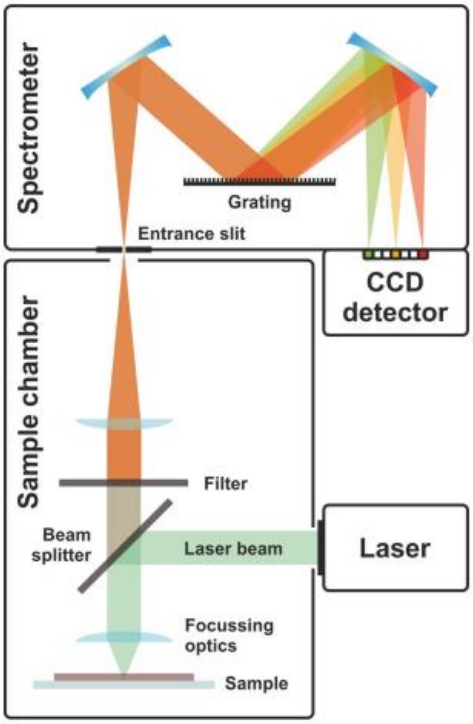


Raman vs Fluorescence vs Rayleigh vs infrared



Raman microscope build

1. Excitation laser
   - UV, Blue, green, red, near-IR, IR
2. Microscope: upright or inverted
   - Sample stage: Piezo-scanner controlled or stepper motor-controlled
3. Dichroic mirror or filter
4. Spectrometer with a diffraction grating
   - 300, 600, 1800, 2400 lines/mm
5. CCD detector



Important: Blue light causes shift to even higher energy, possibly destroying the sample
Denser grating causes the light to spread more, enabling the detection of wavelengths close together. Spectroscope resolution vs. Raman signal

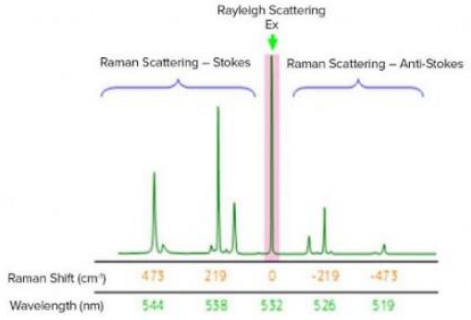
Molecules are detected by Raman shift

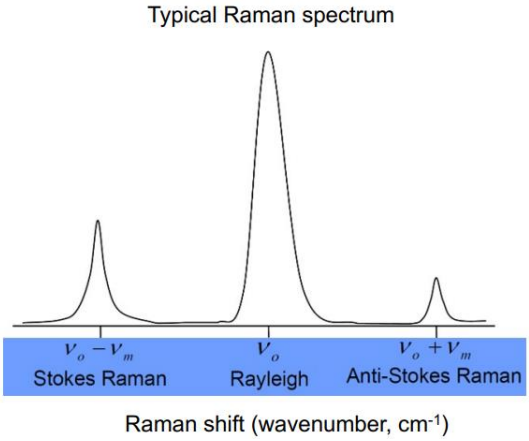
$$Raman\ \ shift[cm^{-1}] = \frac{10^7}{\lambda_{ex}[nm]} - \frac{10^7}{\lambda[nm]}$$

Excitation laser = 532 nm, Raman wavelength = 550 nm:
Raman shift = 615.2 cm^{-1}

Key condition is the change in polarizability of a chemical bond, requiring electron-rich groups like triple or double bonds.

S

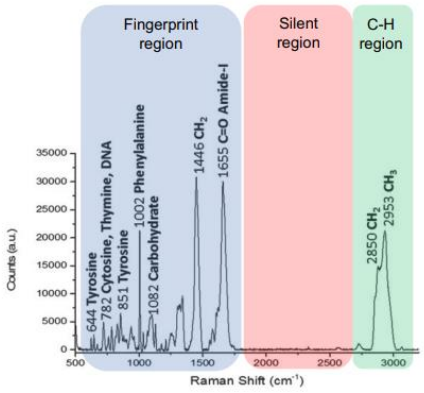


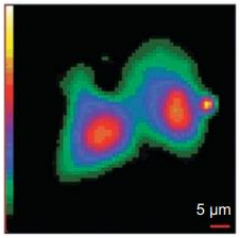


Confocal Raman spectroscopy

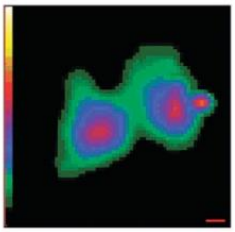
Imaging of biological cells

SKBr3: Fixed human breast cancer cells

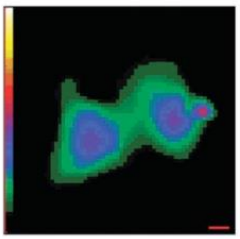




1033 cm^{-1}, phenylalanine, protein marker



1057 cm^{-1}, amide 1, protein marker



2880 cm^{-1}, CH_2, Lipid marker

Chemometrics: Principal component analysis, 2100 – 3100 cm^{-1}

Label-free, non-destructive, ambient, but not nanoscale
Diffraction limited spatial resolution and low sensitivity (1 in 10 mio photons)

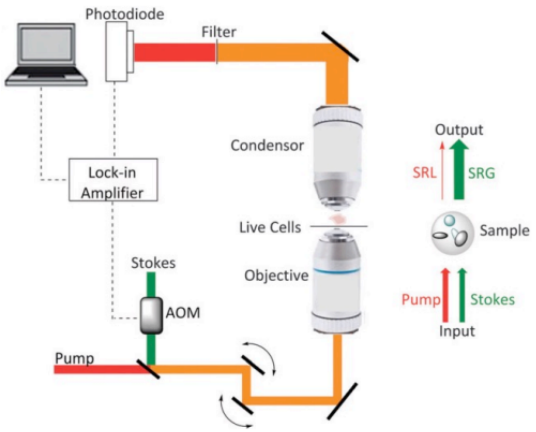
Stimulated Raman spectroscopy (SRS)
Overcomes low signal and resolution limitation. Two lasers are used: Probe beam to excite the molecule and Stokes beam to deexcite the molecule to a chosen vibration mode, resulting in the signal. Vibrational mode is resonantly excited in the molecule with a very large amplitude. The SRS signal is the loss in the probe/pump beam, which is orders of magnitudes higher than spontaneous Raman scattering. No signal if no vibrational mode has frequency => no background!

Advantages:

- Faster (single channel detection, no spectrometer, photodiode detector)
- Higher resolution (2 photon principle)
- Higher sensitivity (Resonant excitation of a vibrational mode)

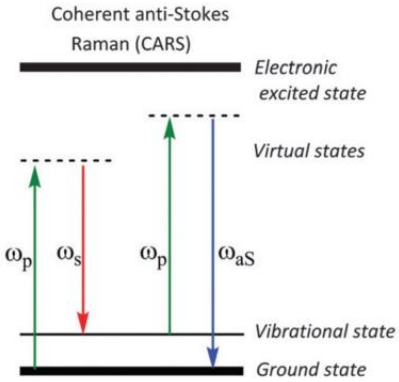
SRS microscope

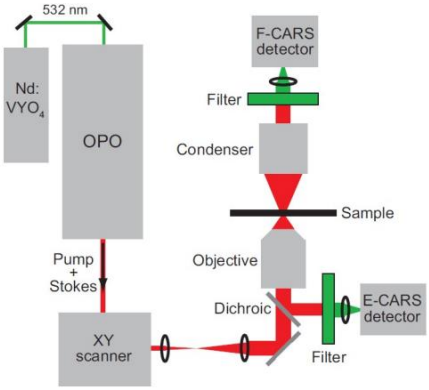
- Synchronized pairing (spatially and temporally) of the picosecond or femtosecond pulsed pump and Stokes beams
- An objective lens focuses the beams onto a common focal spot within the target sample which generates a stimulated Raman process at its focus.
- A high numerical aperture condenser collects the transmitted beams
- Filter blocking the Stokes beam and subsequent detection of the pump beam is achieved via a photo-diode.
- A lock-in amplifier is used to extract the SRL signal from the laser intensity, providing a Raman intensity value at each pixel
- 3D imaging is achieved by raster scanning the laser focus across the sample and moving the focus depth into the sample
- Lateral resolution: **130 nm**



Multiple cells in large area with high contrast very fast (within minutes).
Imaging of specific components via alkyne tagging (e.g. EdU), multicolor possible via isotopic labelling.

Coherent anti-stokes Raman spectroscopy (CARS)
Molecule absorbs photon and is excited to virtual state. Stokes beam photon causes de-excitation to vibrational state. Third photon of the probe beam excites the system to another virtual state with instantaneous de-excitation, which leads to the emission of a photon at anti-stokes frequency. Absorption of all three photons happens rarely, pulsed lasers with very high powers are required. No energy transfer to three molecules, CARS can occur even in the absence of background → Non-resonant background.

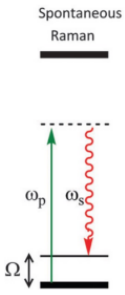


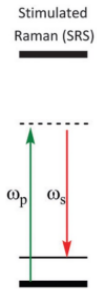


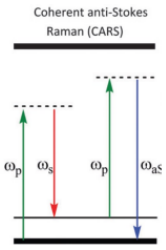
Summary

Hyperspectral molecular imaging:

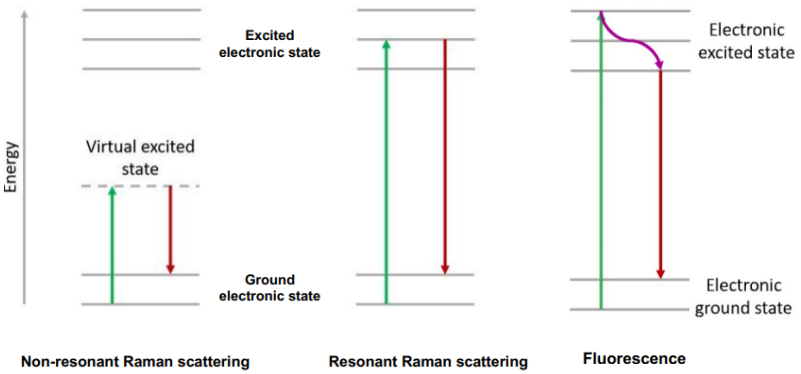
- Confocal Raman spectroscopy
  - Label-free, non-destructive, ambient imaging
  - Low sensitivity and spatial resolution
- Stimulated Raman spectroscopy (SRS)
  - 2-photon process
  - Significantly improves sensitivity and spatial resolution
  - Much faster compared to spontaneous Raman
- Coherent anti-tokes Raman spectroscopy (CARS)
  - 3-photon process
  - Significantly improves sensitivity and spatial resolution
  - Much faster compared to spontaneous Raman
  - Less photo damage







Resonance Raman scattering (RRS)
Raman scattering signal can be enhanced when excitation laser wavelength is close to an electronic absorption band: The smaller the frequency difference between laser and electronic transition, the stronger the RRS intensity. Resonance effects are only expected if laser line coincides with electronic absorption => sensitive and selective.



RRS limitations:

- Only special molecules with resonance Raman scattering
- Tunable laser required (expensive)
- Fluorescence background can be significant
- Analyte photodecomposition under resonant excitation conditions
- Spatial resolution still diffraction-limited

Confocal Raman microscopy key limitations:

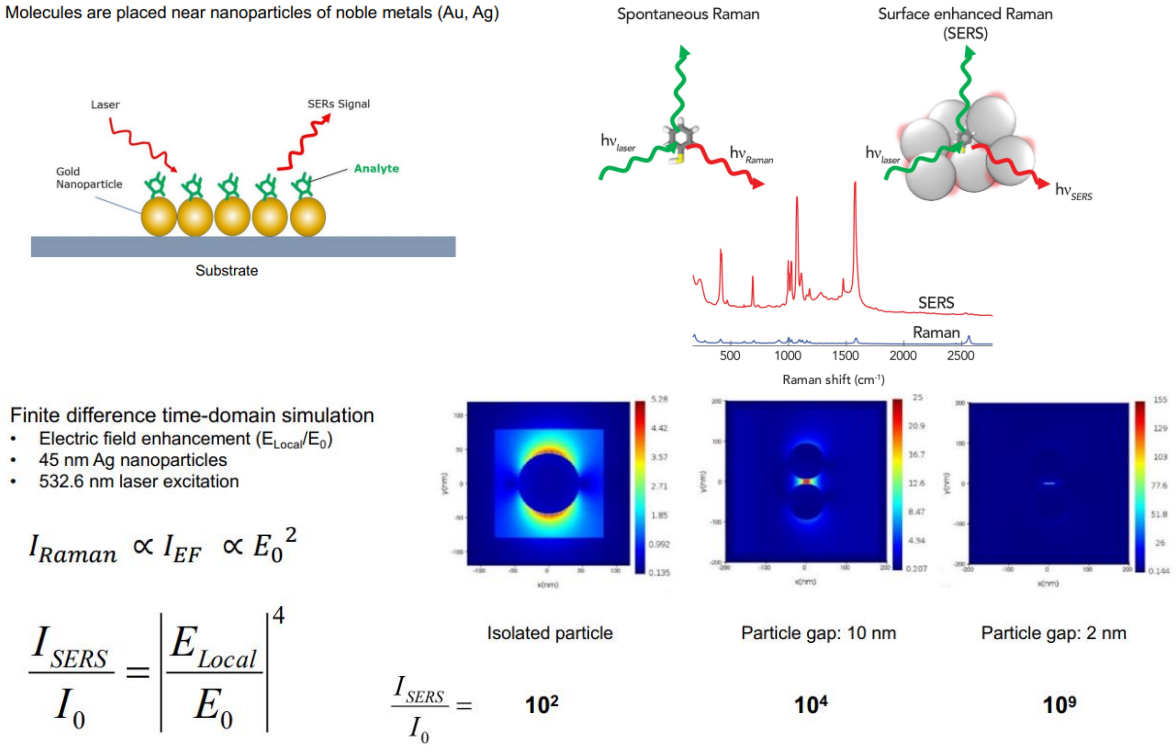
- Low scattering efficiency (low sensitivity)
  enhanced by either RRS, SRS or CARS
- Diffraction-limited spatial resolution (large laser spot - issue for nanoscale)

(Aperture SNOM does not work for Raman: Weak signal created - 50 nm hole allows for only low intensity laser spot - fluorescence is much stronger effect)

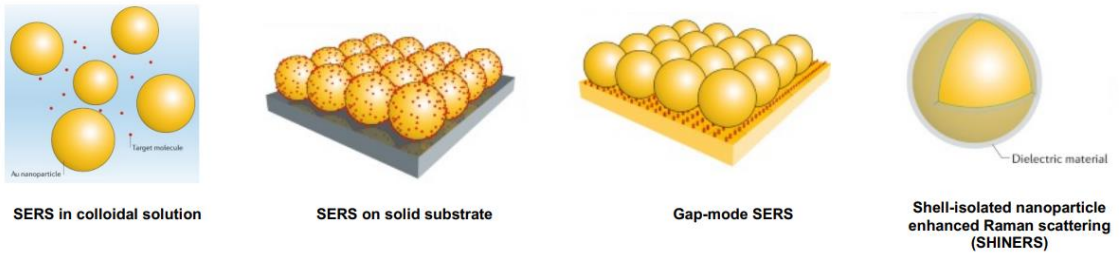
Localized surface plasmon resonance (LSPR): Resonance created in phase with the excitation laser. Localized because the particles have no where to go and start resonating, creating a very high intensity amplitude (5x).

Surface-enhanced Raman spectroscopy (SERS)

Molecules placed close to plasmon resonance particles → Extremely intense electric field is generated, creating the resonance in phase with the Raman signal, thus increasing the signal. Raman wavelength differs not much from excitation laser wavelength.



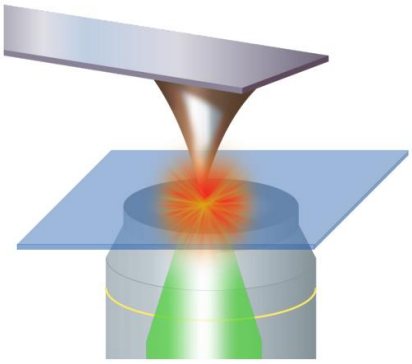
Experimental design:

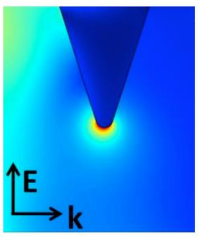


SERS in colloidal solution | **SERS on solid substrate** | **Gap-mode SERS** | **Shell-isolated nanoparticle enhanced Raman scattering (SHINERS)**

Colloidal solution - Concentration can be low (single molecule possible), Molecules on particles or vice versa (Gap-mode), SHINERS: Prevents catalytic activity (no direct interaction with molecules). Most intense signal for Gap-mode SERS and least for SHINERS.

Tip-enhanced Raman spectroscopy (TERS)

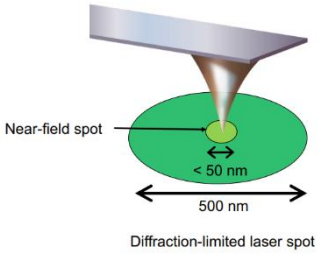




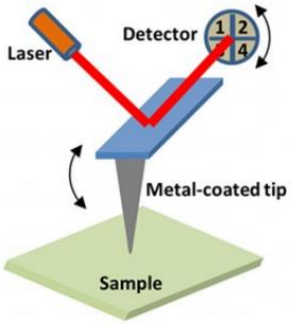
$$\frac{I_{TERS}}{I_0} = \left|\frac{E_{Local}}{E_0}\right|^4$$

SERS variant combining scanning probe microscopy and Raman spectroscopy. (SERS but sticking tiny tip (silicone or gold) in laser spot - much more intense Raman signal LSPR-concept). Tip is placed close to the sample, which can be moved in 1 nm steps.
Lightening rod effect: Sharpest part of the object has the highest charge => very strongly charged entity at the tip.
Excitation spot is effectively reduced from 500 nm to 50 nm using the tip.

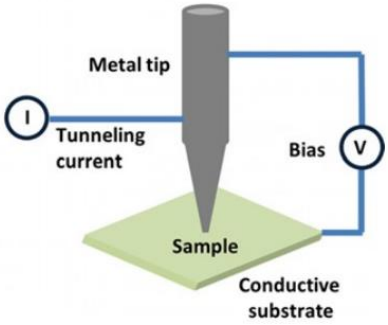
Strongest electrical field when metal tip is parallel polarized: all electrons are pushed to the apex (increases e- density)



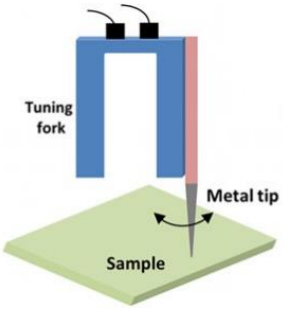
TERS concepts:



AFM feedback



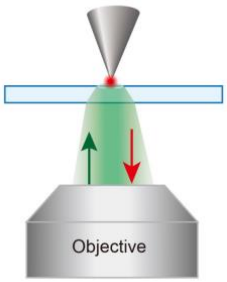
STM feedback



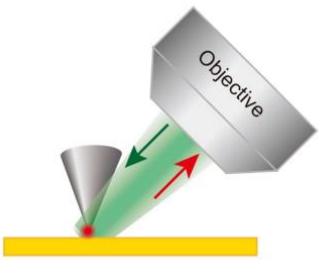
Shear force feedback

AFM - Atomic Force Microscopy (see later)
STM - Scanning tunneling microscopy (see later)

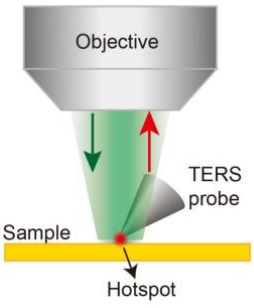
Bottom illumination



Side illumination



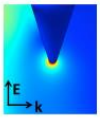
Top illumination



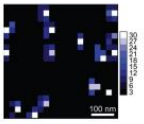
Summary

Hyperspectral molecular imaging:

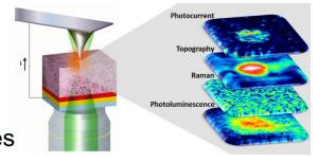
- Tip-enhanced Raman spectroscopy (TERS)
  - High surface sensitivity
  - Nanoscale spatial resolution



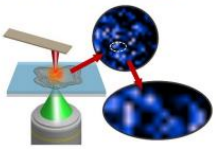
- Imaging structural defects in graphene



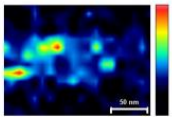
- Structure-chemistry-function in organic photovoltaic devices



- Visualizing small molecules within a biological cell



- Imaging catalytic activity in heterogeneous catalysts



Topic 3 - infra-red spectroscopy (L7+8) (Light)

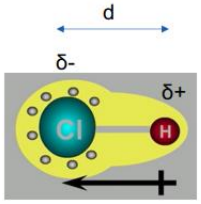
- Nanoscale Fourier-transform Infrared Spectroscopy (Nano-FTIR)
- Photothermal AFM-IR

Infrared absorption: molecular vibrations - unique reflection of a molecule's structure
IR and Raman give complementary vibrational information. Strong Raman modes have weak IR signal and vice versa. IR signal is much stronger than Raman.

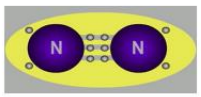
Raman Spectroscopy	IR Spectroscopy
It is due to the scattering of light by the vibrating molecules.	It is the result of absorption of light by vibrating molecules.
The vibration is Raman active if it causes a change in polarisability (distortion of electron cloud, size, shape, orientation) Symmetric bands are active	Vibration is IR active if there is change in dipole moment. Asymmetric bands are active
The molecule need not possess a permanent dipole moment.	The vibration concerned should have a change in dipole moment during vibration.
Water can be used as a solvent as it donot absorb Visible or NIR	Water cannot be used due to its intense absorption of IR, there is no single solvent suitable throughout complete IR range
Sample preparation is not very elaborate, Sample can be in any state. Glass can be used	Sample preparation is elaborate. Glass can not be used. Na, K, Ag, Ca salts are used
Expensive instrumentation	(Relatively) inexpensive instrumentation

IR absorption selection rules:

- Vibration must cause a change in dipole moment within a molecule to absorb infrared light
- The greater the change in dipole moment, the stronger the IR absorption
- Dipole moment = dδ

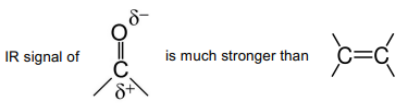


IR active



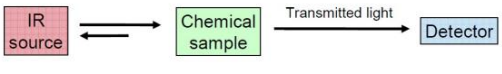
IR inactive

Changing dipole moment creates oscillating electric field, where the incident IR radiation field starts interacting with the molecule
If natural frequency of oscillation = IR frequency, then the energy is resonantly transferred to the molecule => large IR absorption and oscillation amplitude increases

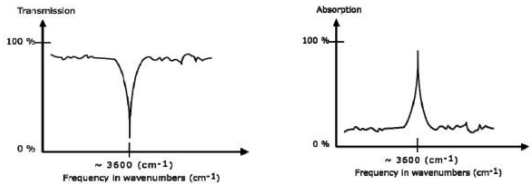


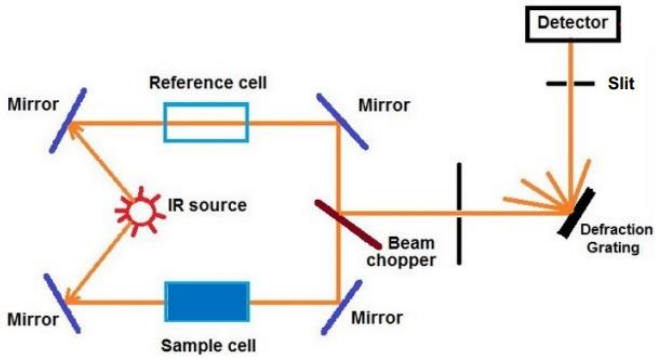
IR spectroscopy
Frequencies detected one by one (slow)
All wavenumbers get detected individually by comparing with reference cell ray.

Spectrum is shown as either transmitted light or absorbed wavelength by the molecule.



IR spectrometer

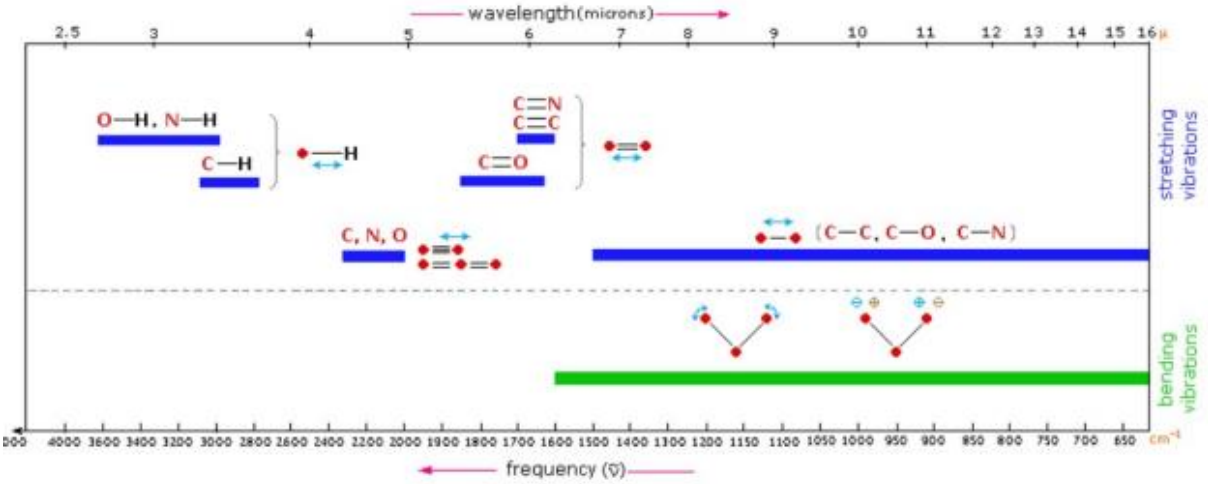




Dispersive spectrometer

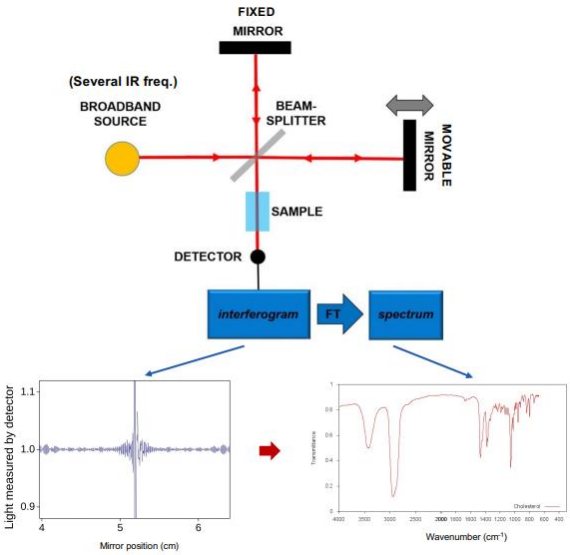
IR-active is only when dipole moment changes => symmetric stretch of linear molecules won't show up (e.g. CO_2).

Number of vibrational modes for n-atoms - 3n degrees of freedom:
6: translations and rotations of the molecule
3n - 6: vibrational (3n - 5 for linear molecules)



Fourier-transform infrared spectroscopy (FTIR)

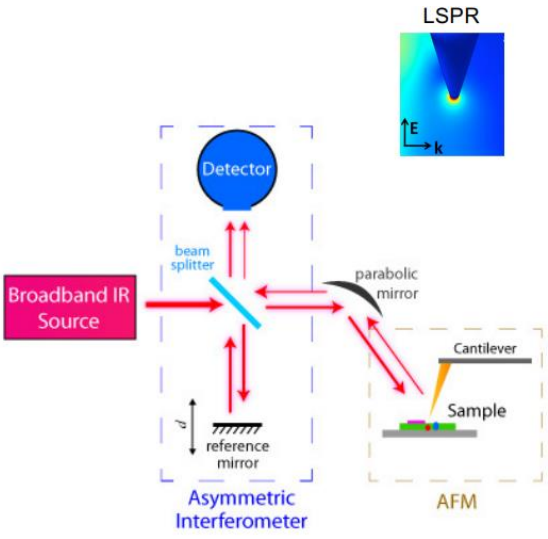
Simultaneous collection of high-res spectral data over wide range.
Based on Michaelson interferometer:
Movement of one mirror creates variable condition for constructive interference, when moving, each wavelength in the beam is periodically blocked and transmitted due to interference. Different wavelengths are then modulated at different rates, at each moment or mirror position, the beam coming out of the interferometer has a different spectrum.
Recorded is an interferogram of light intensity vs mirror position, which is converted to intensity vs IR frequency => IR spectrum.
Higher signal to noise ratio and much faster.



The movement of the mirror leads to the constructive or destructive interference for certain wavelengths at certain mirror positions. How often a certain frequency is absorbed is determined from how high the signal is.

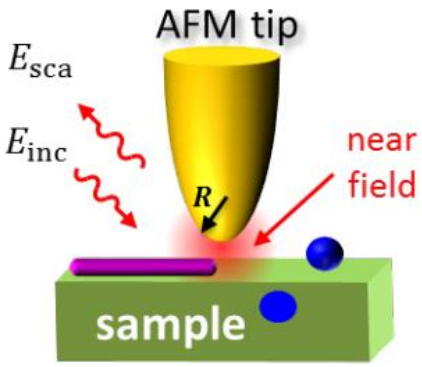
Nano-FTIR spectroscopy

(FTIR but tip-enhanced)
Combination of FTIR and scattering-scanning near-field optical microscopy (s-SNOM).
Sample stage is placed in one of the interferometer arms, allowing recording of amplitude and phase of the detected light.
Using a metallized AFM tip enhances the illuminating IR light in nanoscopic volume. The interferogram is created from the backscattered signal while translating the reference mirror.
Fourier transformation returns the near-field IR spectra. Hyperspectral imaging (complete spectrum at every pixel) is possible due to the tip, with nanoscale spatial resolution determined by tip apex size.



Nano-FTIR principle

- Sample is placed at one arm of the interferometer:
  - Boosts weak near-field signal due to interference with strong reference field
  - eliminates background by parasitic scattering off everything that falls into large diffraction-limited beam focus
  - allows for recording of amplitude and phase
- Nano-FTIR signal generation and extraction:
  - signal requires technical and mathematical manipulation to be extracted
  - near-field and apex of metallic tip interacts with molecular sample → depends on the complex dielectric constant of the sample
  - sample response is scattered by the tip, composing of amplitude and phase
  - scattered response from near-field interferes with reference beam, is demodulated by a lock-in detector and recorded as asymmetric interferogram. Fourier transform yields nano-FTIR amplitude and phase spectra
  - the phase of the scattered radiation is proportional to the imaginary component of the complex dielectric constant of the sample, which in turn is proportional to the IR absorption, so is the phase of the scattered radiation
    => phase spectrum of nano-FTIR matches perfectly with the absorption spectrum of conventional FTIR



FTIR vs nano-FTIR: Identical spectra, better resolution for the later. Organic compounds, polymers, biological and other soft matter, nano-FTIR spectra can be directly compared to the standard FTIR databases.

AFM-IR or photothermal induced resonance (PTIR) spectroscopy

AFM-IR principle: AFM - high spatial resolution, IR - rich chemical information

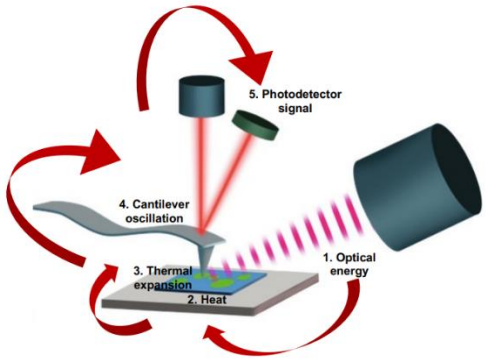
Setup:

- Pulsed, wavelength tunable laser
- AFM
- Optics to focus laser at the sample beneath the tip

Signal detection:

- Conventional spectroscopic methods - light-sensitive detectors in the far-field
- AFM-IR - detection by sharp tip of the AFM cantilever (senses IR absorption via photothermal expansion of the sample underneath)
- Amplitude of induced oscillation is proportional to local absorption of the sample
- AFM-IR absorption spectra: AFM tip stationary and sweeping laser λ
- AFM-IR absorption images: illuminating the sample at fixed λ while scanning the AFM tip over the sample

AFM-IR: Energy transduction



AFM-IR signal

The cantilever contribution ← cantilever modal stiffnesses, frequencies, shapes and deflection sensitivity, tip height and cantilever tilt angle

Mechanical contribution ← sample thermal expansion coefficient

Intensity of the incident laser

$$S_{\text{AFM-IR}} \propto H_{\text{AFM}} H_{\text{m}} H_{\text{th}} H_{\text{opt}}(\lambda) I_{\text{inc}}(\lambda)$$

Thermal contribution ← sample thermal properties

Optical contribution: fraction of incident light absorbed by the sample ← sample refractive index n(λ) + ik(λ)

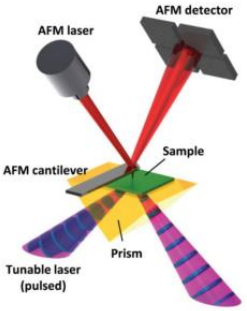
- **AFM-IR signal amplitude:** Depends on local thermo-mechanical properties of the sample and the local tip–sample mechanical interactions *via* scaling factor ($H_{AFM}H_mH_{th}$) → quantification of AFM-IR data challenging
- However, since the scaling factor is wavelength-independent (i.e. constant across a spectrum), peak ratios can provide **semi-quantitative analysis**

$$S_{AFM-IR} \propto k(\lambda)$$

AFM-IR configurations

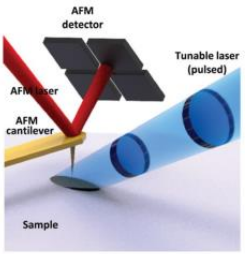
Total internal reflection (TIR) configuration:

- Sample is deposited on top surface of IR-transparent prism
- Limited light penetration depth (sample thickness several µm)
- Complex sample preparation: microtoming, spin coating, drop casting
- Advantages:
  - no propagating light
  - passive uncoated Si or SiN cantilevers (don't enhance field in proximity of the tip) can be used



Top illumination configuration:

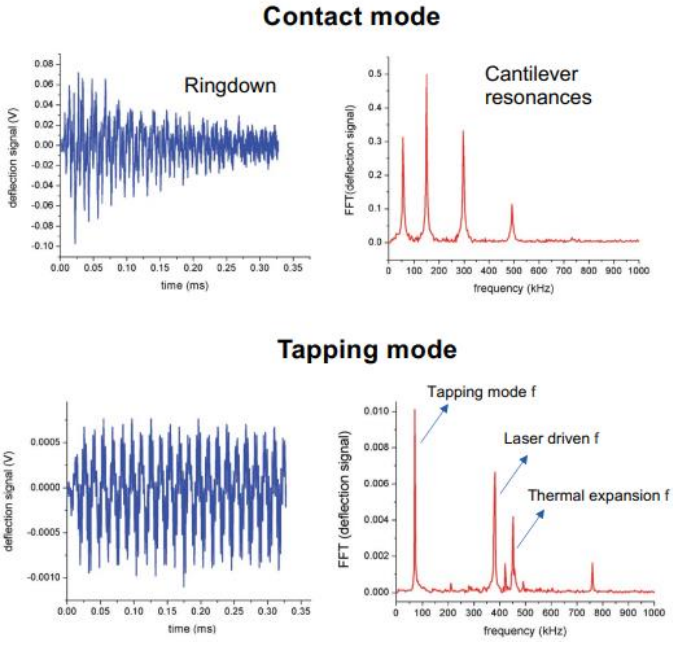
- Simplified sample preparation (can be arbitrarily thick)
- Gold-coated cantilevers to limit self-absorption
- Plasmonic enhancement from gold-coated tips:
  - not so important for thick samples
  - necessary for extremely thin samples



Contact mode resolution: 20 nm (sample pushes tip really hard, causing the ringdown/oscillation)

Tapping mode resolution: 10 nm (higher laser powers needed, sticky/soft samples possible)
Cantilever oscillates with driving frequency, laser drives oscillation as well and finally the sample contributes to the oscillation as well.

AFM-IR vs bulk FTIR: Well matching spectra since cantilever oscillation is linearly dependent on IR absorption coefficient



Topic 4 - Ions (L9)

- Nanoscale secondary ion mass spectrometry (NanoSIMS)

Mass spectrometry

Measures mass-to-charge ratio (m/z) of ions by first ionizing the sample molecule and fragment them, then accelerate or deflect, then detect
Identification is done by correlating known masses to the identified masses and through characteristic fragmentation patterns

Basic instrumentation:
Ionization source: gives the material a positive charge (removal of electron or addition of hydrogen). Gas/vapor: electron ionization or chemical, liquid: electrospray ionization ESI, solid: matrix assisted laser desorption and ionization MALDI, ion bombardment SIMS.
ESI/MALDI: soft ionization by adding hydrogen with little to no fragmentation

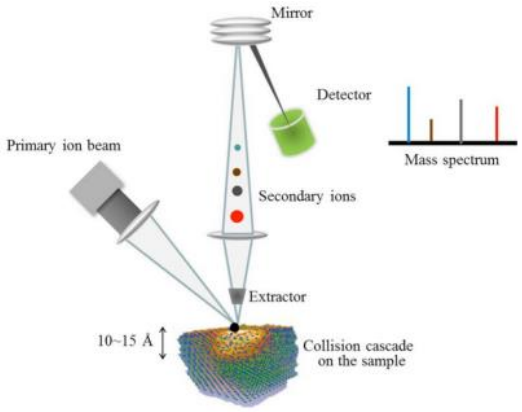
Mass analyzer: Separates ions according to m/z using physical methods. Types:

- Sector: static electric or magnetic sector
- Time of flight (TOF): Static electric field, separation in time (light ions are faster)
- Quadrupole mass filter: oscillating electrical fields selectively stabilize paths of ions, so only a certain range of m/z ratio ions can pass → mass-selective filter
- Orbitrap: electrostatic trapping of ions to orbit around a central, spindle shaped electrode. Ions oscillate back and forth, which generates an image current in the detector plate. Frequencies depend on the m/z ratio and obtained by Fourier transformation

Secondary ion mass spectrometry (SIMS)

Solid surfaces and thin films are sputtered with a focused (primary) ion beam, ejected secondary ions are collected and analyzed. Elemental, isotopic or molecular composition of the surface to a dept of 1-2 nm is determined with a mass spectrometer.
SIMS instrument:

- Primary ion gun (primary ion beam: Ar_{40}^+, Ar_{700}^+, C_{60}^+, Bi_3^+. Au_3^+)
- Primary ion column: accelerates and focuses the beam onto the sample
- High vacuum chamber: holds sample and secondary ion extraction lens
- Mass analyzer: ToF, Sector, quadrupole
- Detector: Faraday cup, electron multiplier, microchannel plate detector



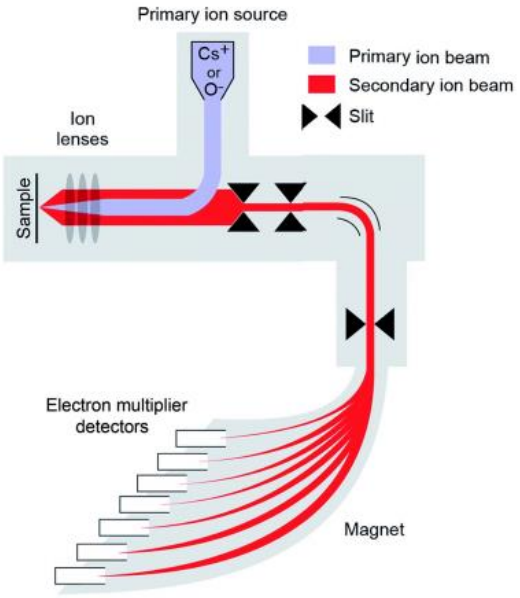
Advantages:

- Extremely high surface sensitivity (down to ppb levels)
- Minimal sample preparation (no matrix like MALDI required)
- Primary ions can be focused to a tight spot (up to few µm spatial resolution possible)
- Depth profiling possible

Limitations:

- No large biomolecules → ESI or MALDI needed
- Expensive (2-3 M$)

Nanoscale secondary ion mass spectrometry (NanoSIMS)
SIMS with spatial resolution of ~50 nm and high mass resolution



Primary ion beam hits the surface vertically using a coaxial lens assembly, secondary ions are collected with the same lens assembly. Primary beam rastered on the surface, beams: Cs+ for -ve analysis, O- for +ve ion analysis.
Primary and secondary beams must be of opposite polarity! 10x better spatial resolution than SIMS

Sample preparation:

- Must be vacuum compatible
- Flat surface (otherwise will cause varying ionization trajectories)
- Surface must be conductive (sputter coating)
- Cells and tissue require chemical fixation followed by resin embedding and microtome sectioning

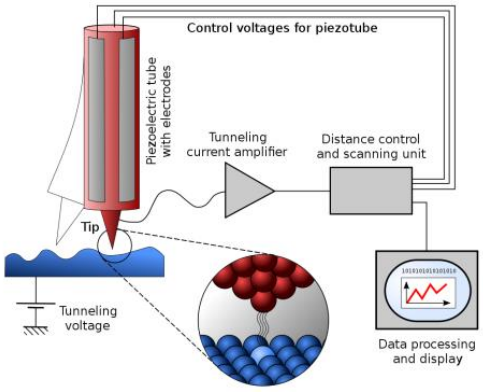
Applied for direct visualization of ions, immunogold labeling, stable isotope labeling

Topic 5 - direct visualization (L10+11)

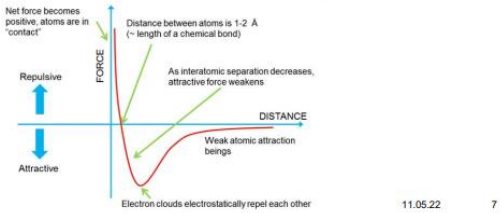
- Ultrahigh vacuum (UHV)-TERS (Light)
- Atomic Force Microscopy (AFM) (Atomic Force)
- Scanning Tunneling Microscopy (STM) (Electrons)
- Cryogenic electron microscopy (Cryo-EM) (Electrons)

Scanning Tunneling Microscopy (STM)

Imaging surfaces at the atomic level using an extremely sharp conducting tip, distinguishing features smaller than 0.1 nm with 0.01 nm depth resolution.
At ambient, ultrahigh vacuum, cryogenic and high temp conditions



Quantum tunneling: Wavefunction can propagate through a potential barrier, transmission is finite and depends exponentially on the barrier height and width. Electron tunneling: thickness of 1-3 nm.



By moving the tip (using a piezoelectric scanner, which can be altered by a control voltage), changes in surface height and population of the electronic states cause changes in the tunneling current.
Two modes:

- Constant current mode: feedback electronics adjusts height by applying voltage to the piezoelectric scanner
- Constant height mode: piezoelectric scanner voltage is kept constant as the tip moves back and forth across the surface in the tunneling current, exponentially dependent on the distance.

STM instrumentation:

- Sharp conductive tips (W, Au or Ag - electrochemical etching, Pt-Ir - mechanical shearing)
- Stepper motors (100 nm step size)
- Piezoelectric scanners (e.g. lead zirconate titanate ceramic at 5 nm/V)
- Vibration isolation by magnetic levitation or active/passive isolation

Application: Direct visualization of 1D polymerization / polymerization in 2D polymers, manipulation of single molecules, molecular arrangement in photocatalytic coupling reactions

UHV-TERS

TERS but in vacuum and cold

Atomic force microscopy (AFM)

Cantilever on sample with photo detector and light beam above.

Main methods to know for the exam (20 min oral)

Nanoscale molecular imaging using fluorescence spectroscopy
-Structured Illumination Microscopy (SIM)
-Stimulated Emission Depletion Microscopy (STED, GSD and RESOLFT)
-Direct stochastic optical reconstruction microscopy (dSTORM)

-Photoactivated localization microscopy (PALM, FPALM)

Nanoscale molecular imaging using Raman spectroscopy
-Scanning near-field optical microscopy (aperture SNOM)
-Tip-enhanced Raman spectroscopy (TERS): Based on both atomic force microscopy (AFM) & scanning tunnelling microscopy (STM)

Nanoscale molecular imaging using infra-red (IR) spectroscopy
-Nanoscale Fourier-transform Infrared Spectroscopy (Nano-FTIR)
-Tapping AFM-IR
-Photothermal AFM-IR

Nanoscale molecular imaging using ions
-Nanoscale secondary ion mass spectrometry (NanoSIMS)

Single molecule imaging techniques
-Scanning probe microscopy: STM & AFM
-Ultrahigh vacuum (UHV)-TERS
-Cryogenic electron microscopy (Cryo-EM)