REVIEW on a few relevant tecniques in super-res micorscopy

7TH NIC@IIT ADVANCED MICROSCOPY PRACTICAL WORKSHOP

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Point Spread Function

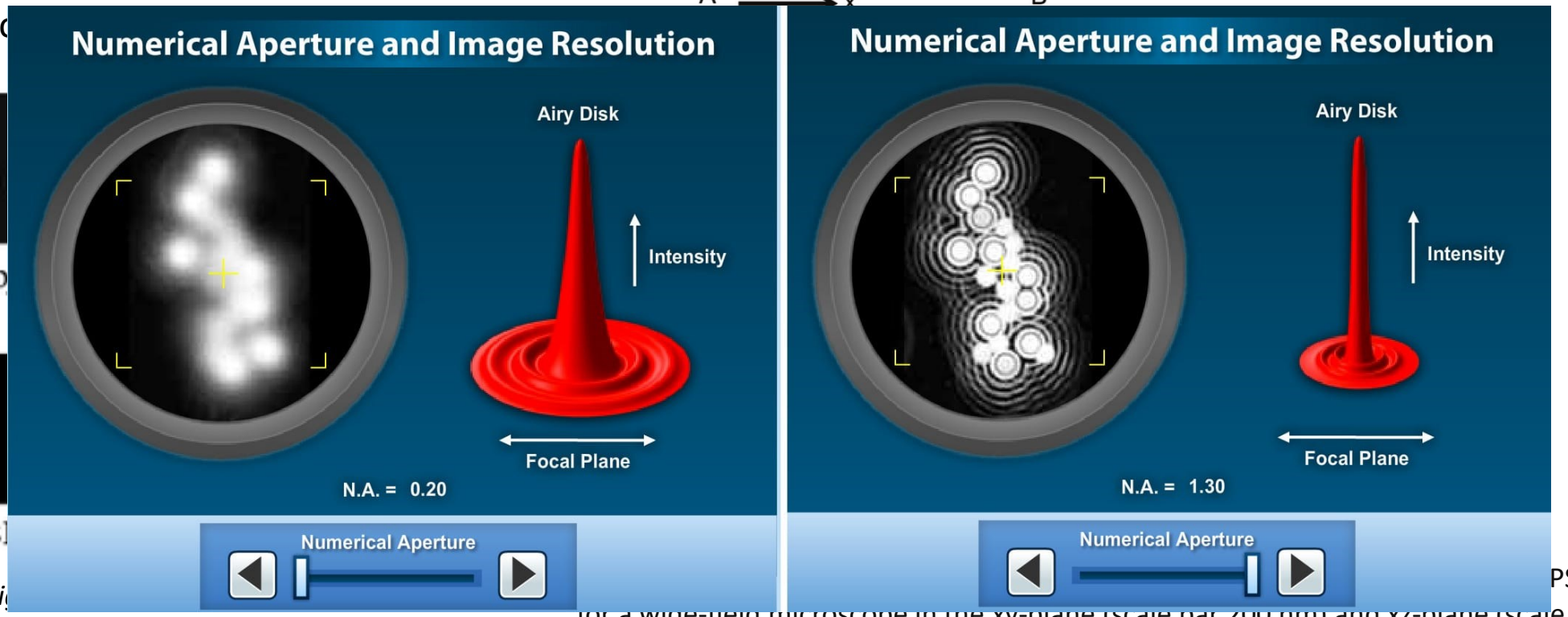
IMAGE FORMATION

In c

Ob

PS

SYSTEM RESOLUTION



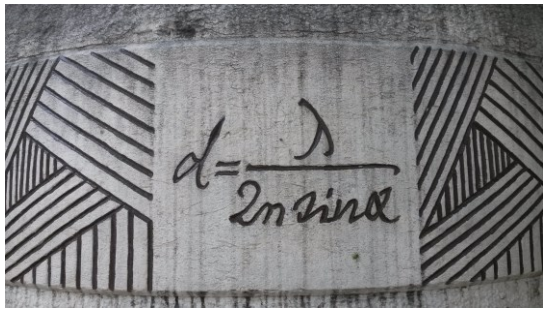
Fi PSF profile for a wide-field microscope in the xy-plane (scale bar 200 nm) and xz-plane (scale bar 600 nm); **B** two particles at resolvable and unresolvable distances as seen in a microscope.

confocal microscope.

SUPER-RESOLUTION MICROSCOPY

Microscopy tecniques that allow to reach a **resolution higer** than the one imposed by the Abbe diffraction limit.

Abbe diffraction limit



"Maximum resolution possible for a theoretically perfect, or ideal, optical system"

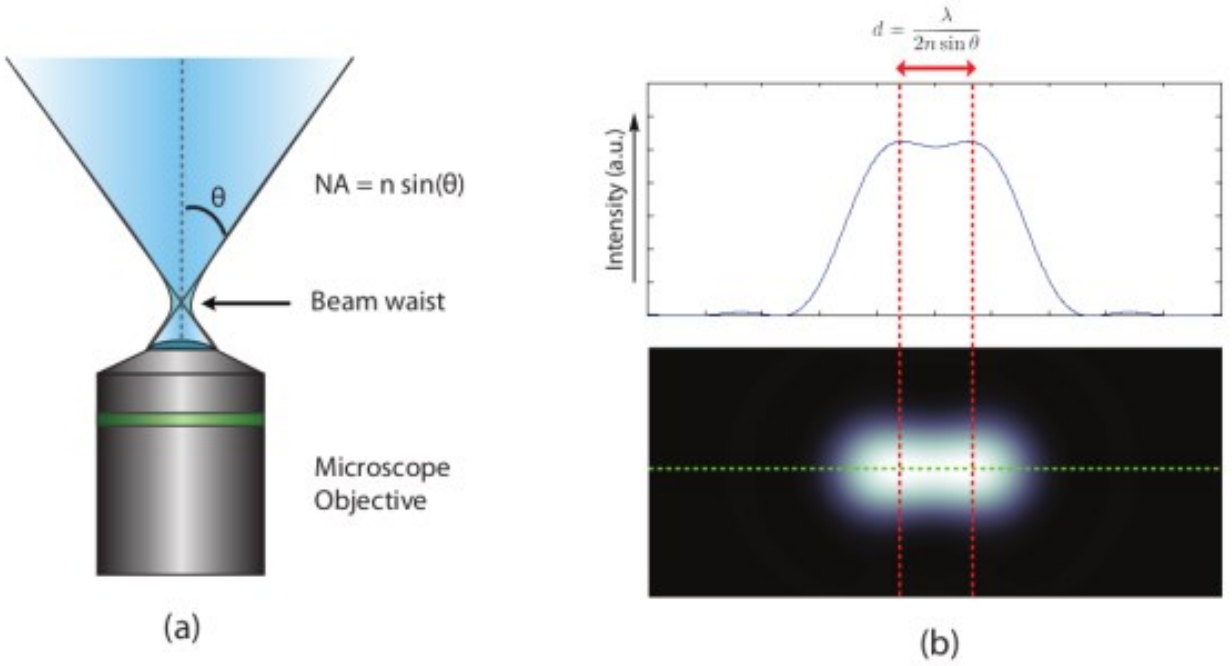


Figure 3 [10] – diffraction limit. d is the smallest resolvable distance between two points

EXAMPLES of SUPER-RES TECHINQUES

- *Structured Illumination Microscopy*
- *Stimulated Emission Depleted Microscopy*
- *Stochastic Optical Reconstruction Microscopy*

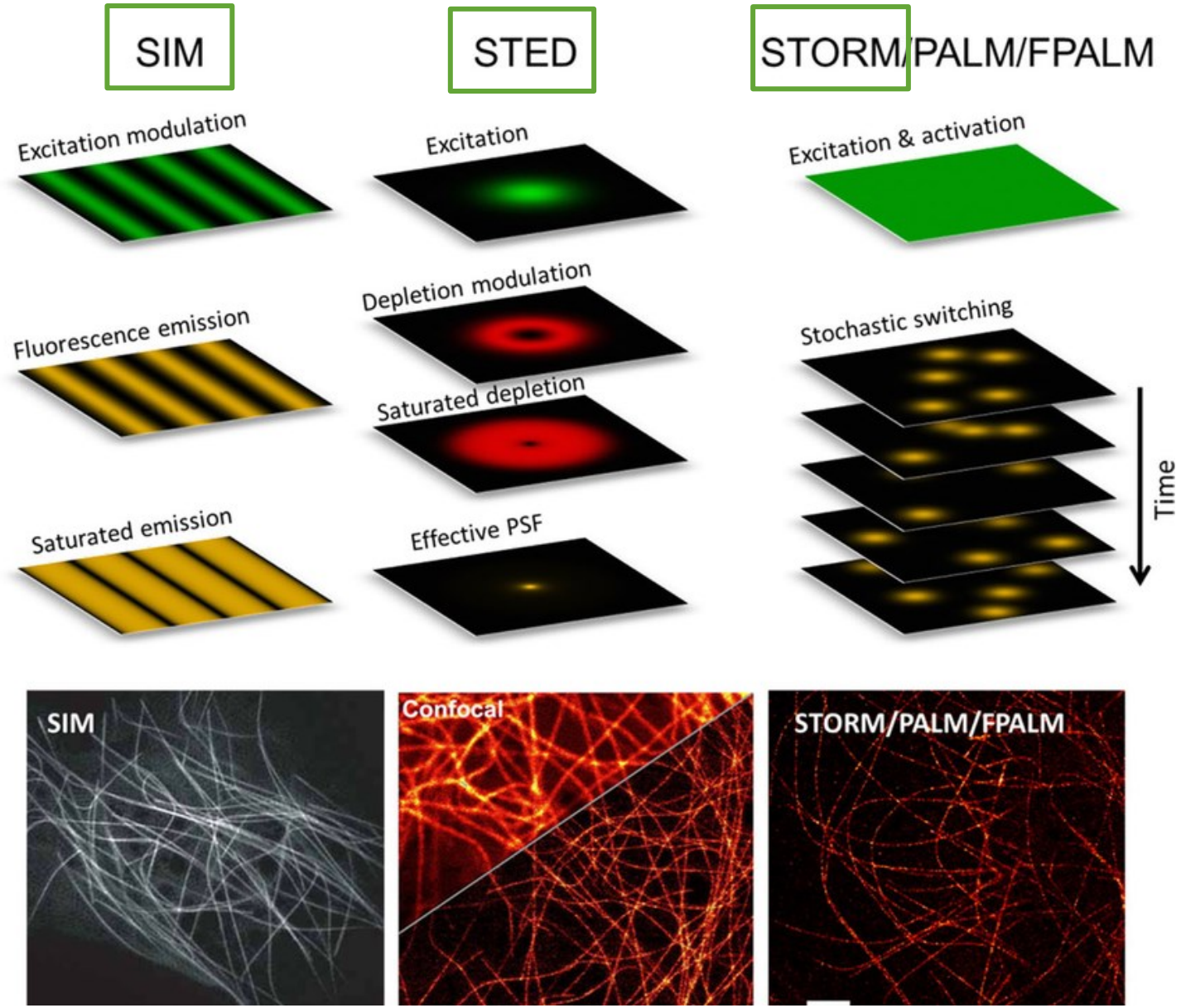


Figure 4 [11] – different super-resolution tecniques

SIM

Structured Illumination Microscopy

Principle: sample excited with a known spatially structured pattern of light, it relies on the generation of **interference** patterns known as Moiré effect

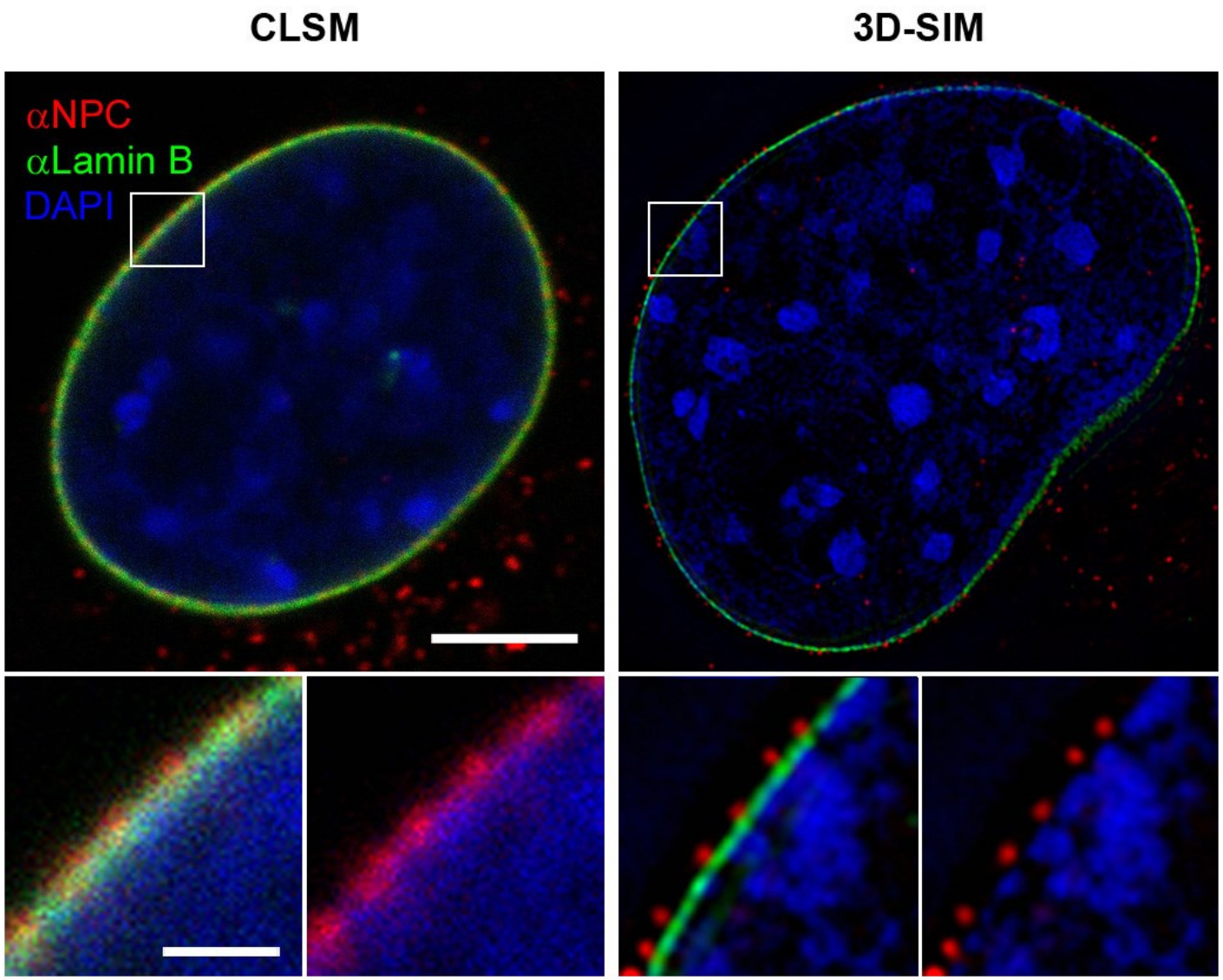


Figure 5 [12] – Simultaneous imaging of DNA, nuclear lamina, and NPC epitopes by 3D-SIM. C2C12 cells are immunostained with antibodies against lamin B (green) and antibodies that recognize different NPC epitopes (red). DNA is counterstained with DAPI (blue).

GENERAL FEATURES

- Laser-based **wide-field** microscopy
- Movable diffraction **grating** into the excitation beam path
- Laser beams (± 1) **interfere** with each other at the focal plane of the objective and create an illumination in stripes
- This **stripe pattern** by its superimposition with the **sample** generates a so-called **Moiré effect**

Overlap between the high frequency organization of the objects and the high frequency of the illumination stripes → pattern of **lower frequency** well collected by the objective

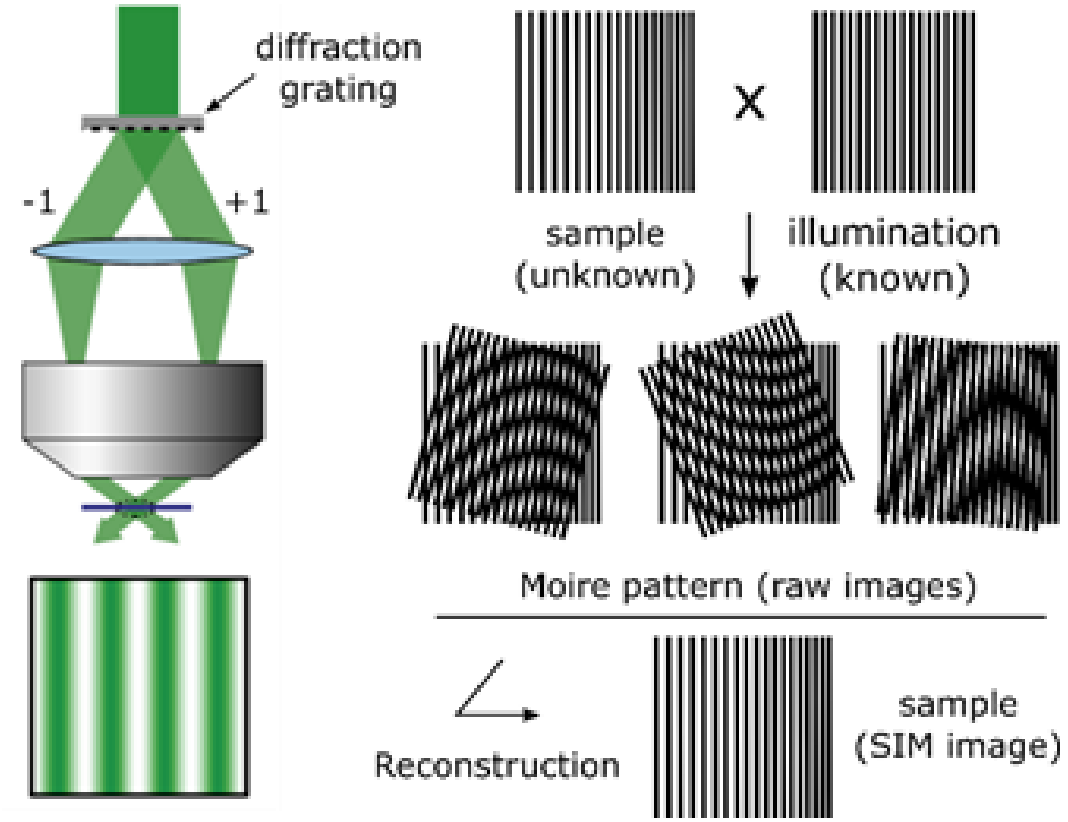


Figure 6 [13] – Moire pattern formation

IMAGE RECONSTRUCTION

- Shift and change of the orientations of the illuminating bands → **image reconstruction**
- Number of shifts and rotations depends if 2D or 3D, in order to cover a certain Fourier Space

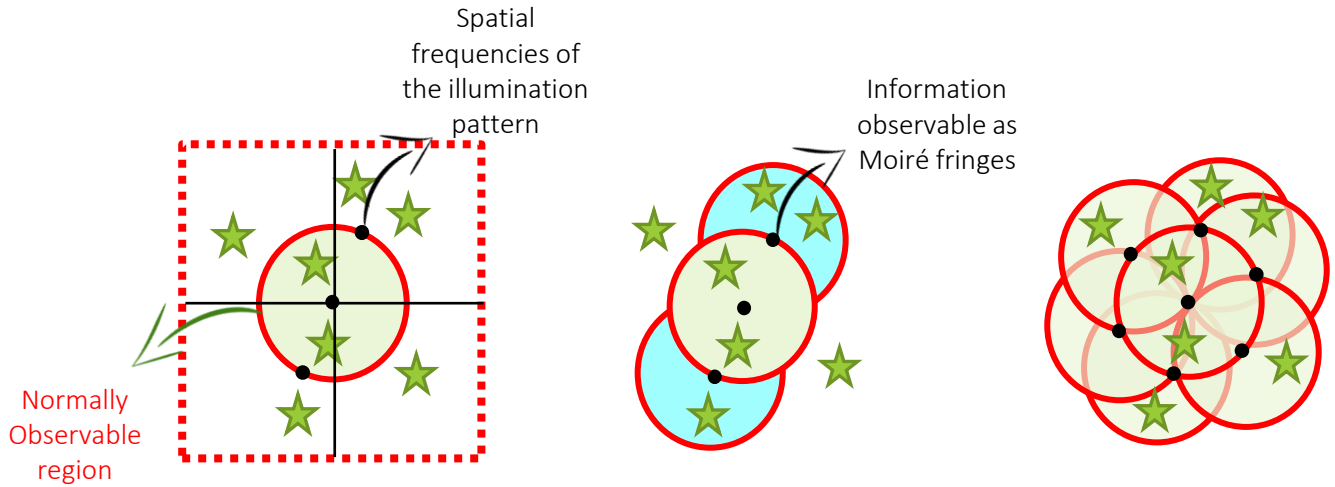
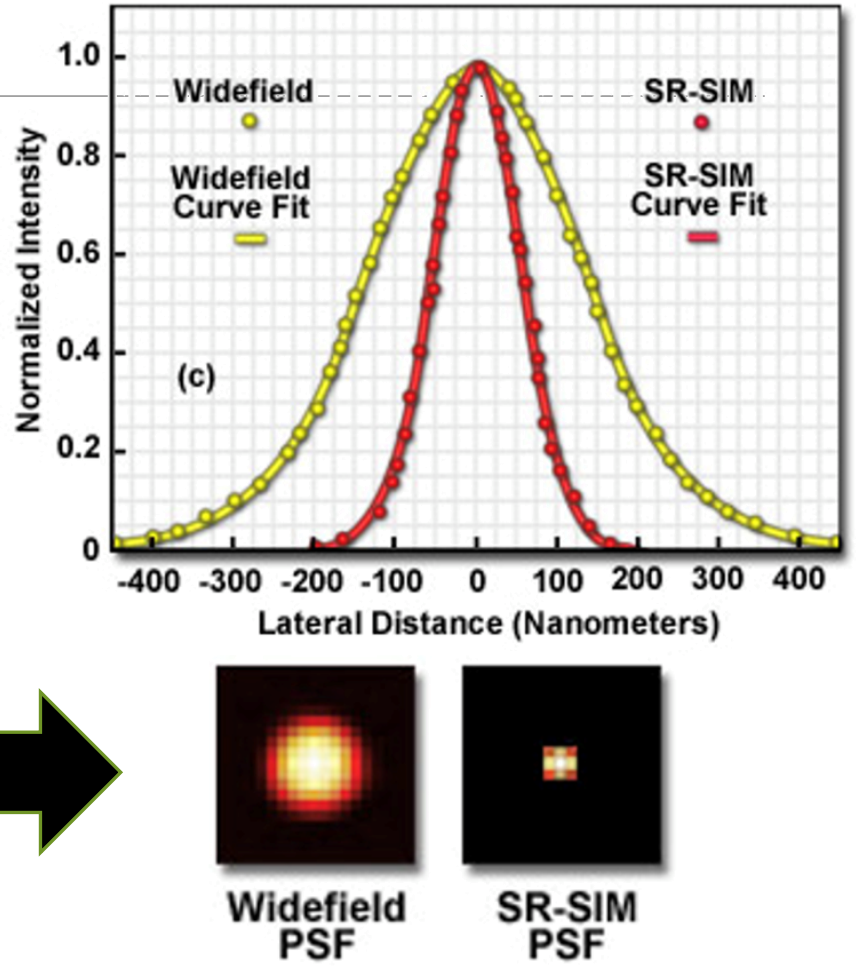


Figure 7 [6] – comparison between widefield and SR – SIM PSFs



OPTICAL SETUP

- **Laser** source directed into the microscope optical train
- The light is directed through a polarizer and a **diffraction grating** and is then projected onto the specimen
- The rays are **collimated** by the objective in the specimen focal plane where they **interfere** and generate a three-dimensional illumination pattern
- Fluorescence emission is captured by a **CCD camera**

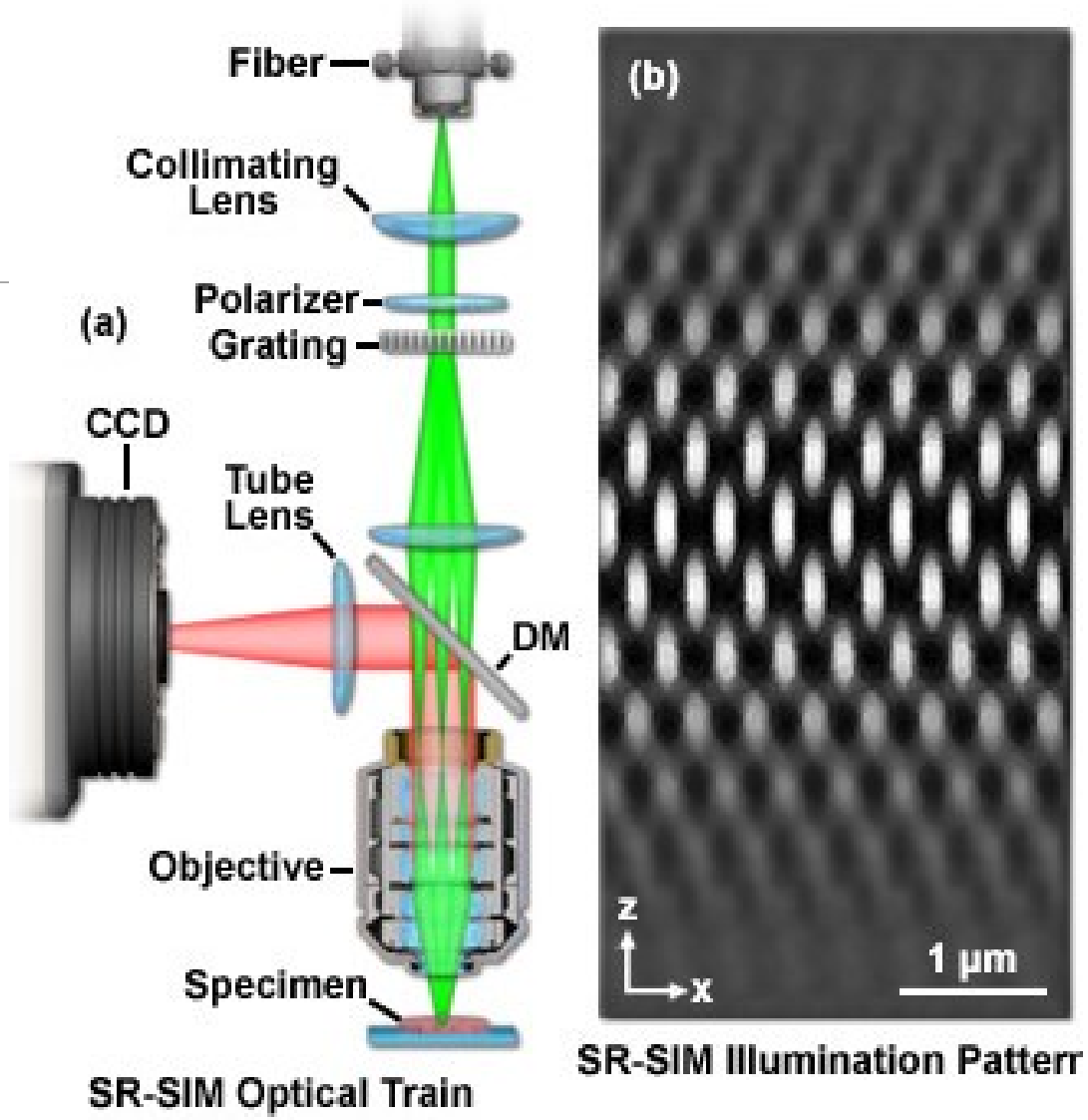


Figure 8 [6] – SR – SIM optical setup

PROS & CONS

- Increased resolution (100nm lateral and 250nm axial)
- Fast frame rate of imaging (high **temporal resolution**), being wide-field
- Use of **conventional** fluorophores

- Obtained resolution not as good as STORM/PALM and STED (~20nm)
- Possible **artefacts** during image reconstruction
- Sensitive to out of focus light → difficult with dense/thick samples

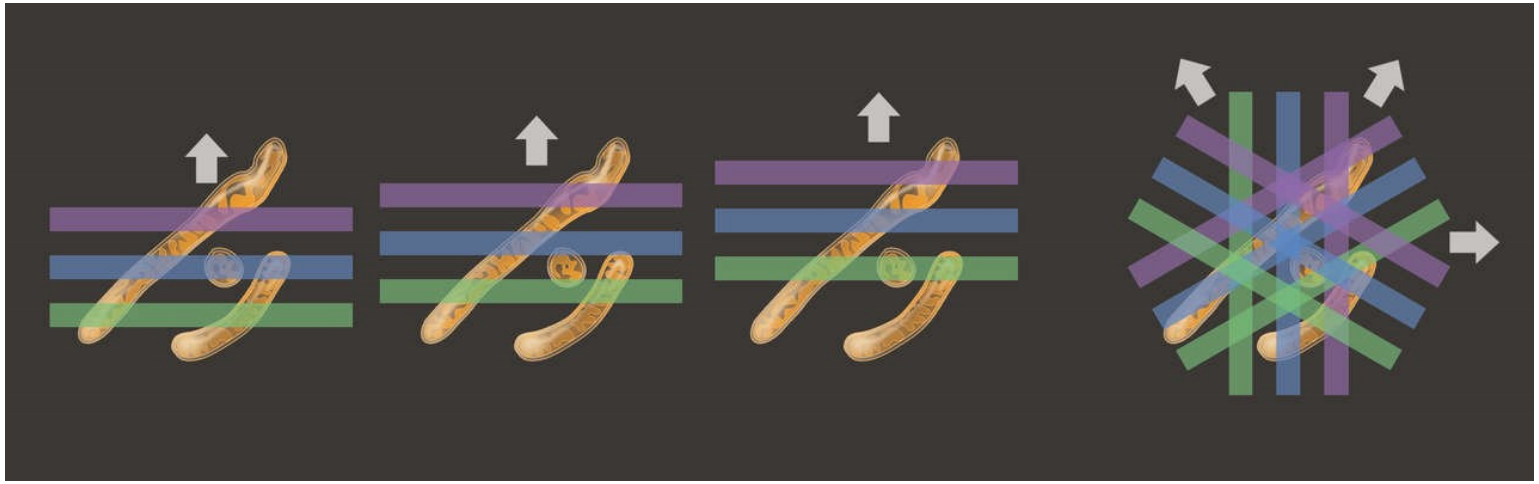


Figure 9 [14] – Capture multiple images with structured illumination that is shifted in phase.
Repeat this process for three different angles. These images are then processed using advanced algorithms to obtain super-resolution images.

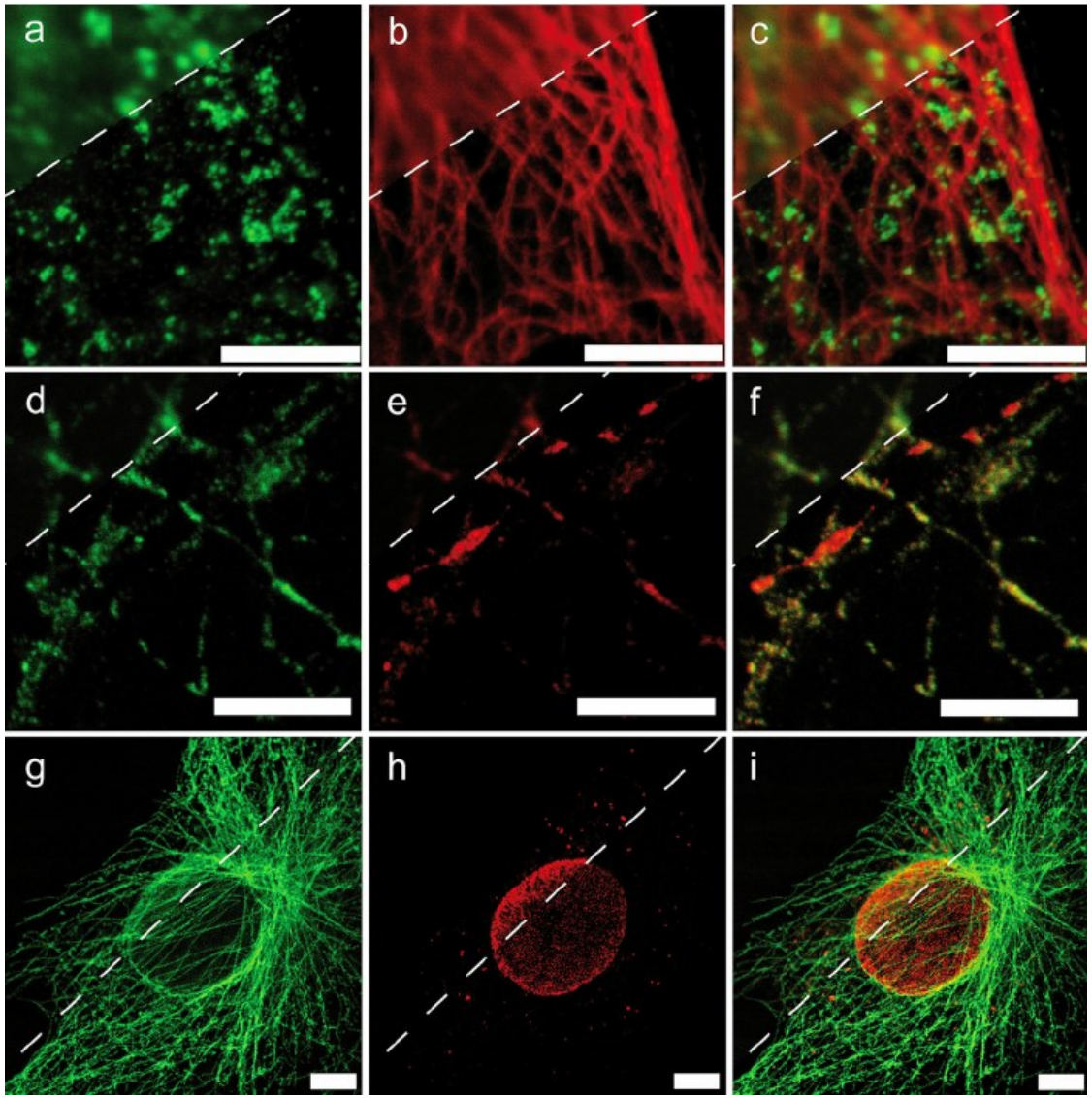


Figure 10 [8] – **Multicolor STED** imaging.
Confocal – STED.
a – c: **Hela cells** in which clathrin is stained by ATTO 590 (green) and tubulin by ATTO647N (red)
d – f: Culture of **hippocampal neurons** in which SynI is labeled with ATTO 594 (green) and vGAT with Abberior STAR635p (red)

Principle: switching off the fluorescence of dye molecules by **stimulated emission** using intense laser light in the outer regions of the diffraction limited excitation focus, and detecting the **fluorescence** from dye in the center

STED

Stimulated Emission Depletion Microscopy

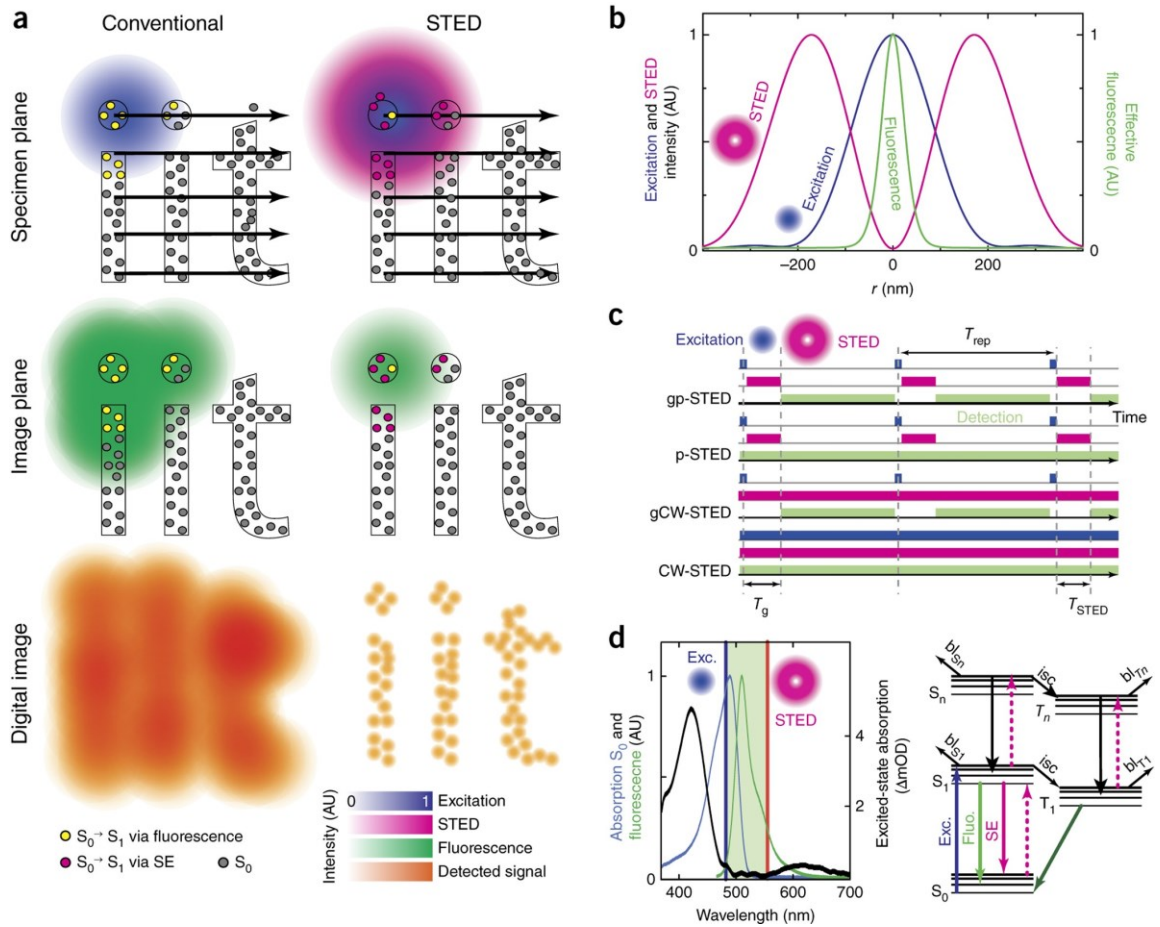


Figure 11 [7] – (**a**) Conventional scanning microscope (left) and a STED microscope (right). (**b**) Spatial conditions. (**c**) Temporal conditions (**d**) Spectral conditions.

GENERAL FEATURES

- Pulsed lasers in relation to the time scales for **molecular relaxation** → radially symmetric depletion zones (**non-linear depletion**)
- **Phase modulator** modifies the STED beam → zero-intensity node at the center of focus with exponentially growing intensity toward the periphery
- Fluorophores within the **zero-node** region → fluorescence
- Fluorophores exposed to the STED beam → back to their ground state by means of **stimulated emission**
- High depletion laser powers (often >250 MW/cm^2) → **instantaneously** to the ground state
- Reducing laser power → formation of a **non-fluorescent state** different mechanisms (metastable triplet state, formation of charge-transfer states, or photoswitching)

OPTICAL SETUP

- Synchronized ultrafast **lasers** pulses: the STED beam is red-shifted, 10-300 ps width (exc. shorter)
- **Phase plates** for the doughnut shape → quenching of the molecules at the periphery
- **Raster scanning** across the sample
- Single photon **detectors** (Single Photon Avalanche Diodes, SPADs)
- **Dichroic mirror**, to overlay excitation and depletion lasers and to separate fluorescence signal from excitation light
- Objective

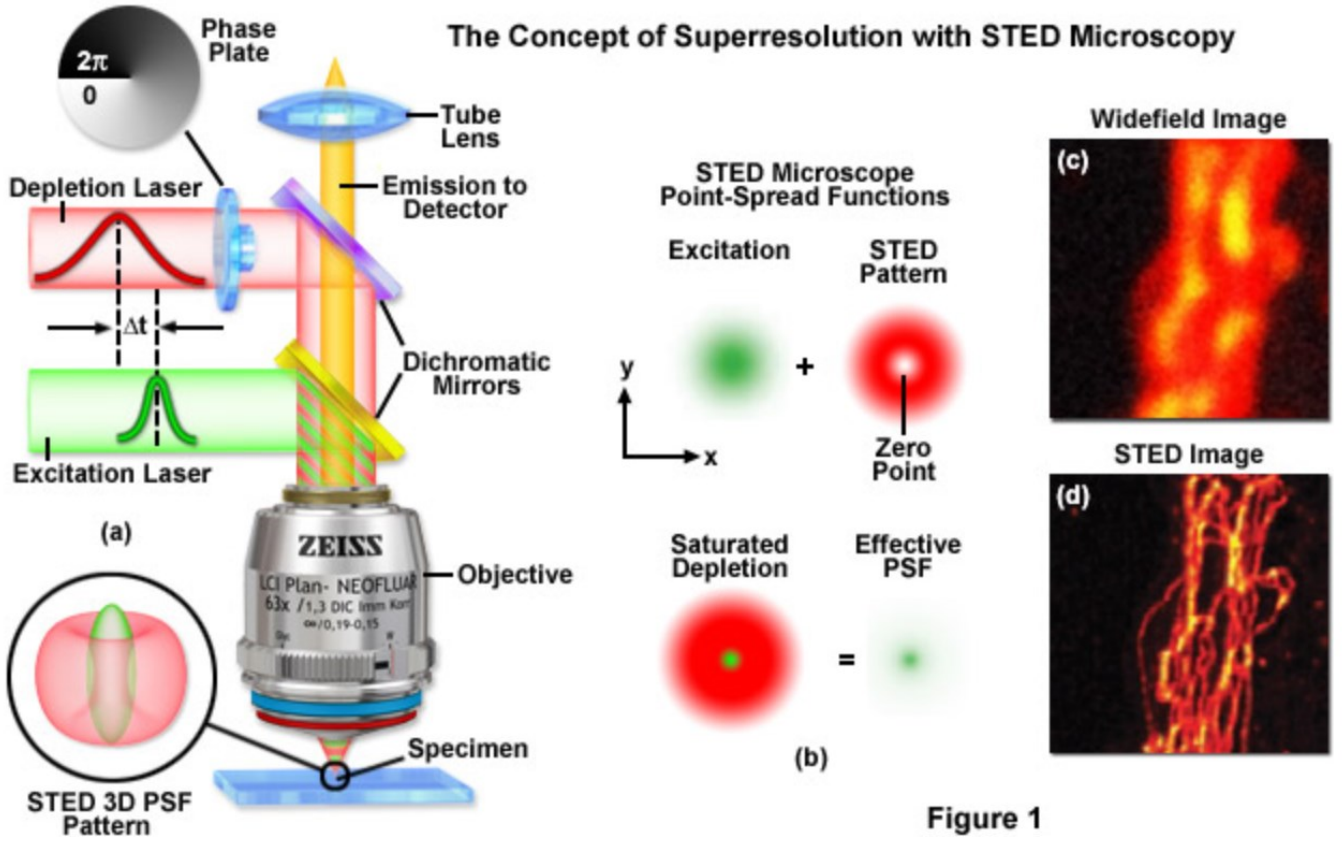


Figure 12 [8] – excitation and depletion lasers, focal spots produced by those lasers and comparison of widefield and STED images of microtubules (with Alexa 594)

REMARKS

- Further resolution enhancement → **time gates** to the collected data (gSTED)
- Another benefit: resolution increased only due to the **experimental configuration and laser power**
- Image **acquisition times** can approach the speed of any laser scanning confocal microscope
- The effective resolution increase is proportional to the **power** of the depletion laser → problematic at extremely high laser powers
- Wide range of fluorophores have been successfully used with STED, including fluorescent proteins, ATTO dyes, Alexa Fluors, DyLights, and several other synthetics.

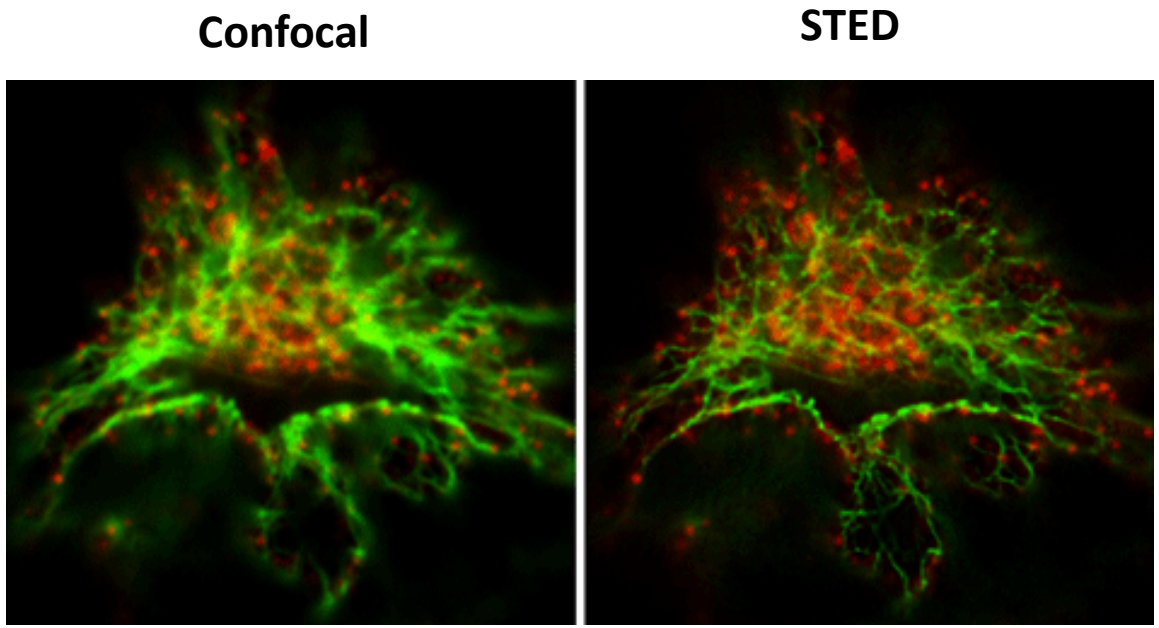


Figure 13 [16] – Comparison of confocal microscopy and STED microscopy. Vimentin and clathrin were visualized by immunohistological co-staining.

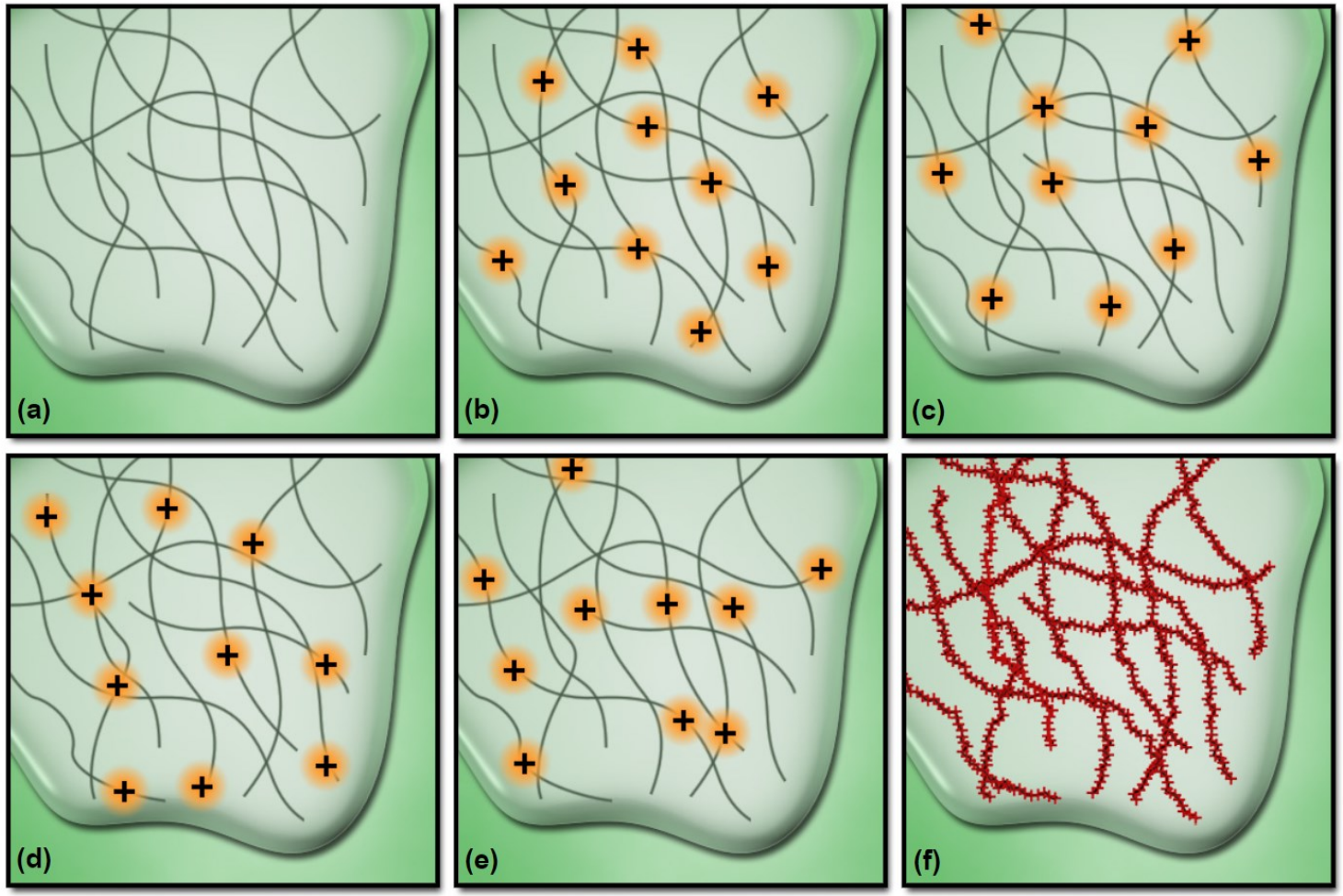


Figure 14 [1] – Basic principles of STORM Superresolution Imaging

Principle: the activated state of a ***photoswitchable*** molecule must lead to the consecutive emission of sufficient photons to enable ***precise localization*** before it enters a dark state or becomes deactivated by photobleaching

STORM

Stochastic Optical Reconstruction Microscopy

GENERAL FEATURES

Methodology: turn on and off single fluorophores (stochastically) then **fit** the PSF to finde their center, until they are alle exhausted

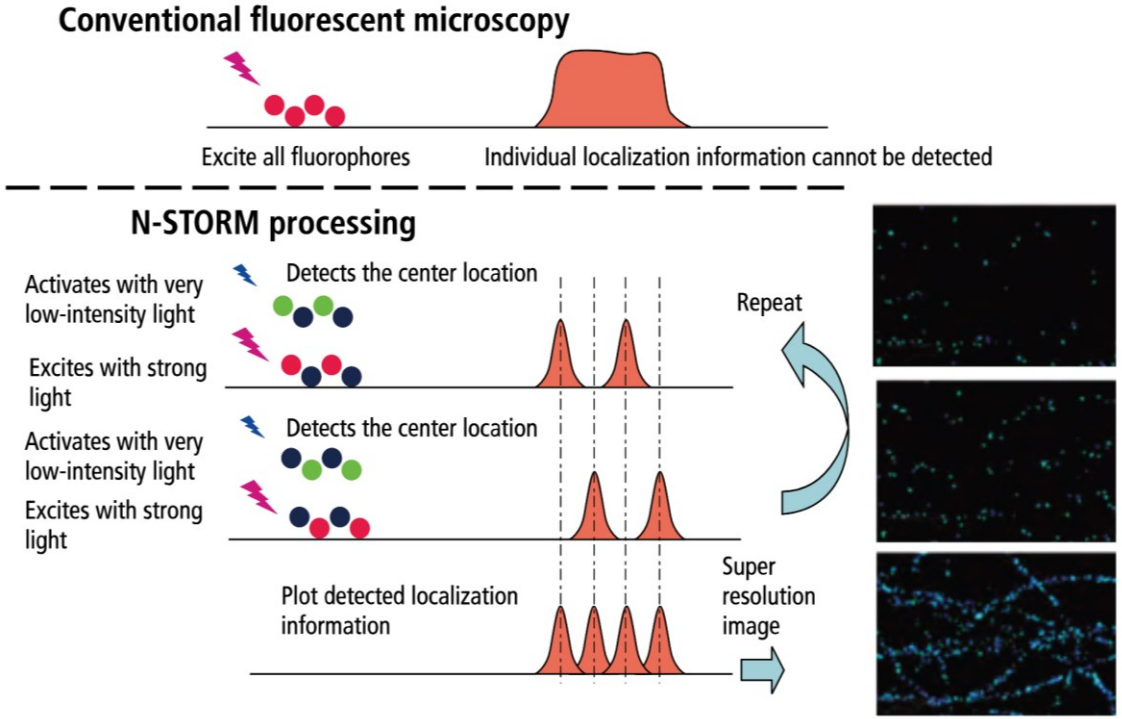


Figure 15 [2] – STORM process

» **Widefield microscopy** → faster than a confocal, good for the membrane

» Distance between molecules must exceed the Abbe diffraction limit ($\gtrsim 250nm$) → **parallel recording** of many individual emitters

» The dye needs to have a short **duty-cycle** (es. Cy3-Cy5) → lots of photons for high resolution

» **3D-storm**: exploits a cylindrical lens that modifies the shape of the focus → easier to recognize the location

OPTICAL SETUP

- Widefield microscope
- 2 **continuous lasers** at different wavelengths
- Dichroic mirror
- Digital camera

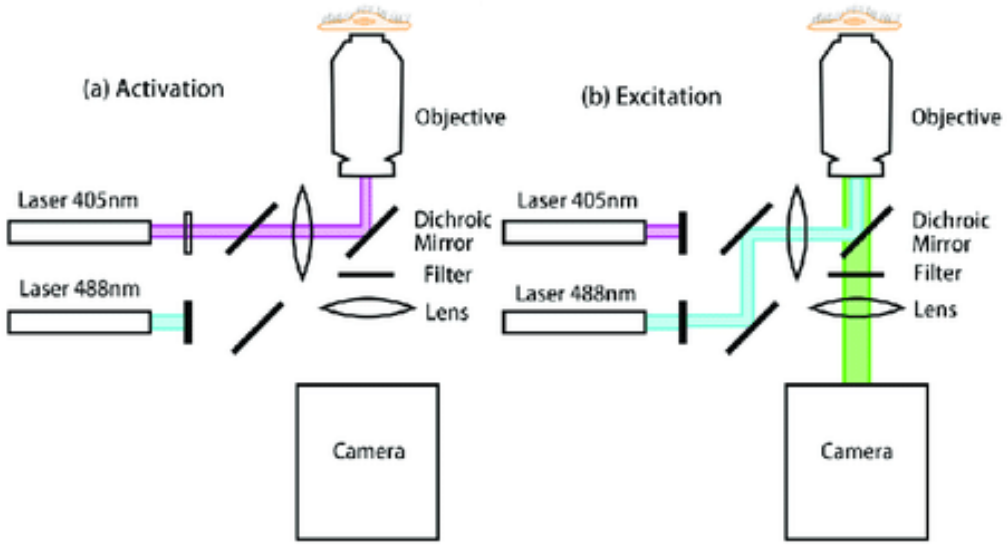


Figure 16 [3] – STORM optical setup

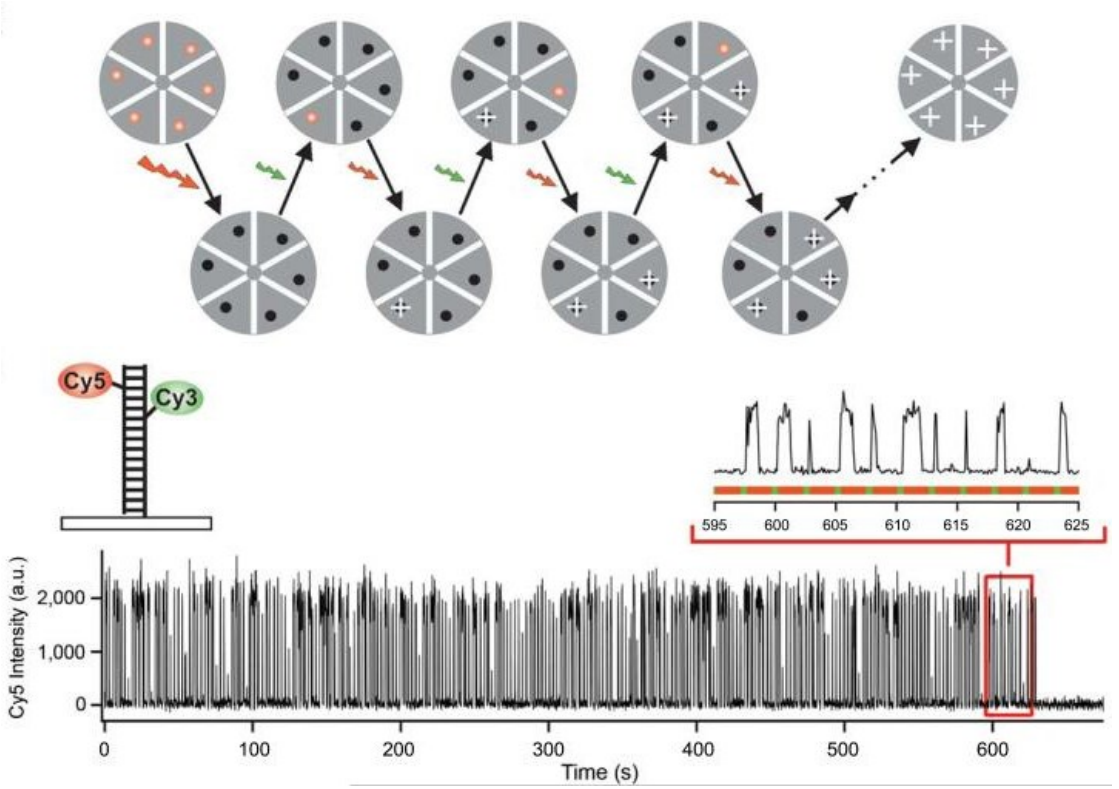


Figure 17 [4] – imaging sequence and example with Cy5 switch on DNA

REMARKS

- Most widely used super-resolution microscopy technique for **single-molecule imaging** or for structures like cytoskeletal biopolymers
- Limited uses invivo because of its **toxicity** (PALM is better, also for particle tracking)
- There can be problems with **localization** and structure if you consider the dimension/uncertaininty of the labels

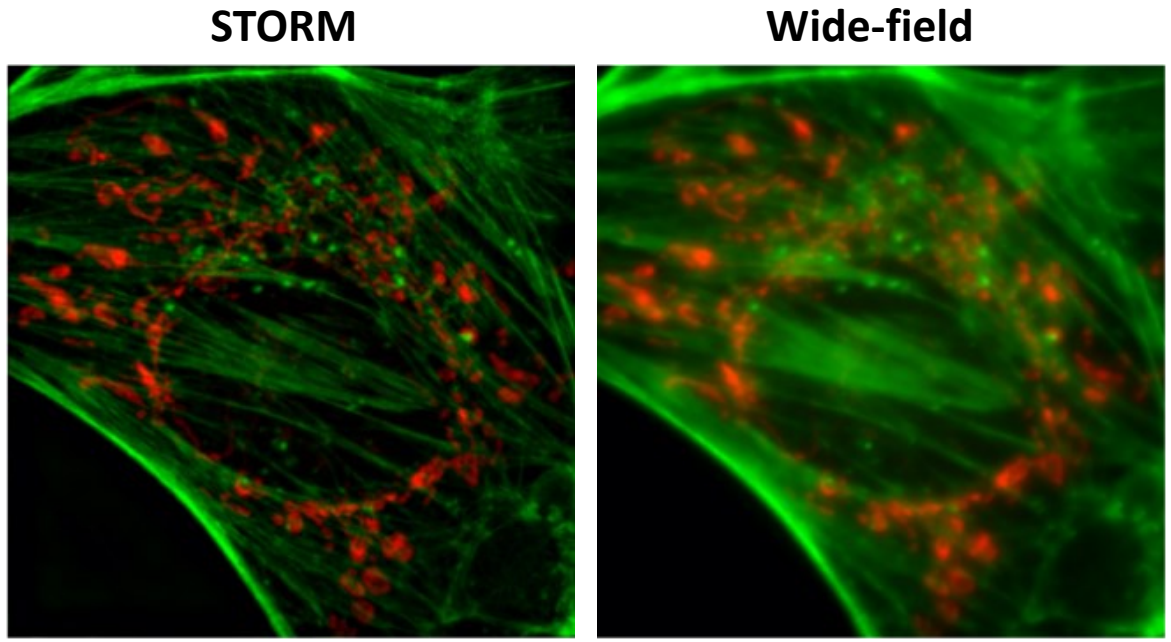


Figure 18 [17] – Comparison of wide-field microscopy and STORM microscopy.

- Obtained resolution around **20nm**
- Non pumped lasers → fluorophore's duty cycle limits the signal time, no need for the pumped laser
- Cyanine dye, Cy5 and Cy3 have been used for the first experiments, having this switching properties

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FLUORESECE & PSF

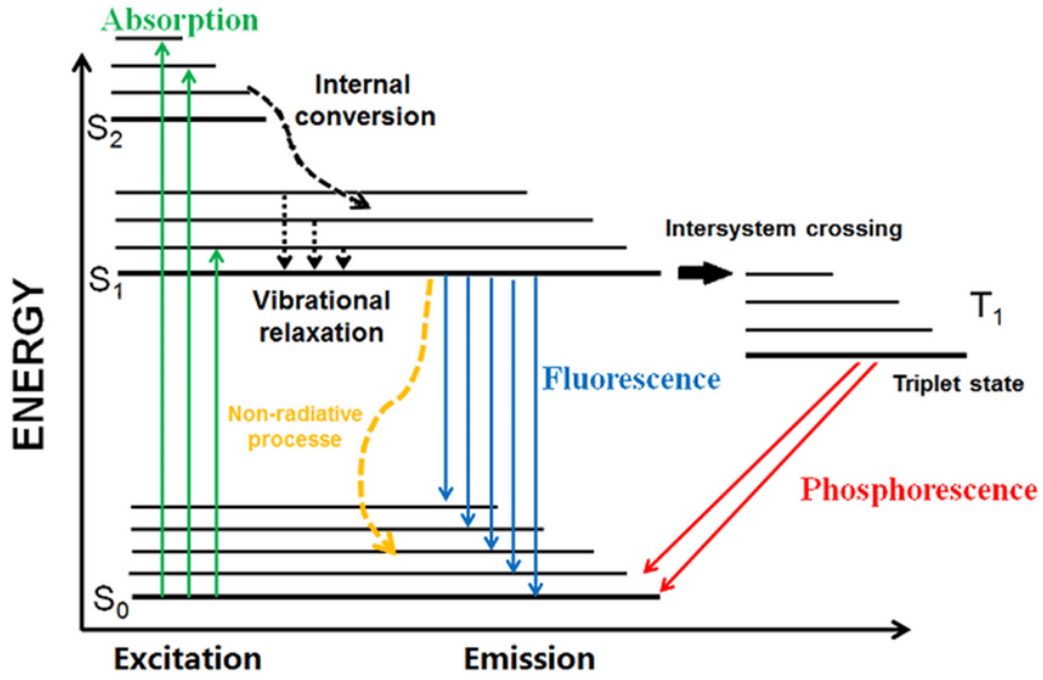
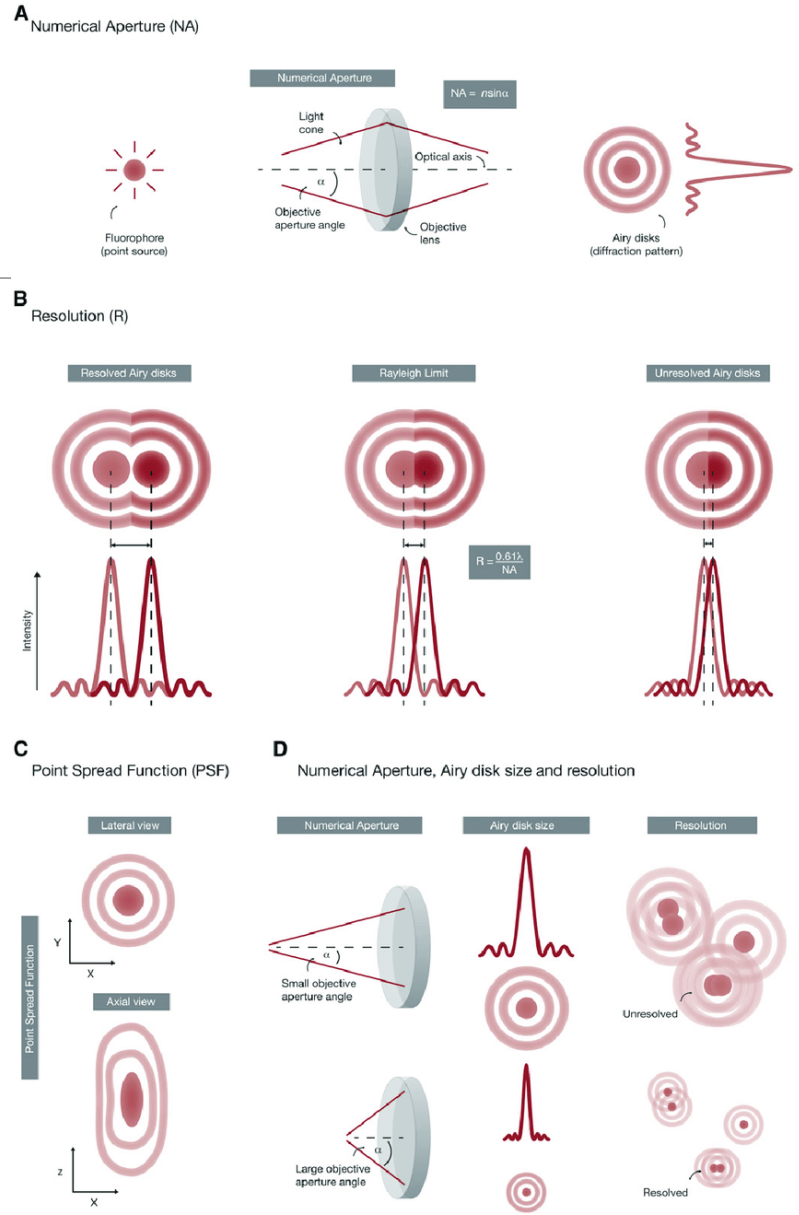


Figure 19 [18] – Jablonsky diagram



SIM mathematical expalantion

Illumination pattern

$$I_{\theta,\varphi}(r) = \frac{I_0}{2}[1 + \cos(2\pi k_\theta \cdot r + \varphi)]$$

Emission signal

$$D(r) = (s(r) \cdot I_{\theta,\varphi}(r)) \otimes h(r)$$
$$= D_0(r) + \cos(\varphi) \cdot D_C(r) - \sin(\varphi) \cdot D_S(r)$$

Fourier Transform of the emission signal

$$D_{\theta,\varphi}(k) = [S(k) \otimes I_{\theta,\varphi}(k)] \cdot H(k)$$
$$= \frac{I_0}{2}[S(k) \cdot H(k) + \frac{1}{2}e^{-i\varphi}S(k - k_\theta) \cdot H(k) + \frac{1}{2}e^{i\varphi}S(k + k_\theta) \cdot H(k)]$$

FT emission for each orientation

$$D_{\theta,\varphi_1}(k) = \frac{I_0}{2}[S(k) \cdot H(k) + \frac{1}{2}e^{-i\varphi_1}S(k - k_\theta) \cdot H(k) + \frac{1}{2}e^{i\varphi_1}S(k + k_\theta) \cdot H(k)]$$
$$D_{\theta,\varphi_2}(k) = \frac{I_0}{2}[S(k) \cdot H(k) + \frac{1}{2}e^{-i\varphi_2}S(k - k_\theta) \cdot H(k) + \frac{1}{2}e^{i\varphi_2}S(k + k_\theta) \cdot H(k)]$$
$$D_{\theta,\varphi_3}(k) = \frac{I_0}{2}[S(k) \cdot H(k) + \frac{1}{2}e^{-i\varphi_3}S(k - k_\theta) \cdot H(k) + \frac{1}{2}e^{i\varphi_3}S(k + k_\theta) \cdot H(k)]$$

Image in the Fourier space

$$\begin{bmatrix} S(k) \cdot H(k) \\ S(k - k_\theta) \cdot H(k) \\ S(k + k_\theta) \cdot H(k) \end{bmatrix} = \frac{2}{I_0} M^{-1} \begin{bmatrix} D_{\theta,\varphi_1}(k) \\ D_{\theta,\varphi_2}(k) \\ D_{\theta,\varphi_3}(k) \end{bmatrix}$$