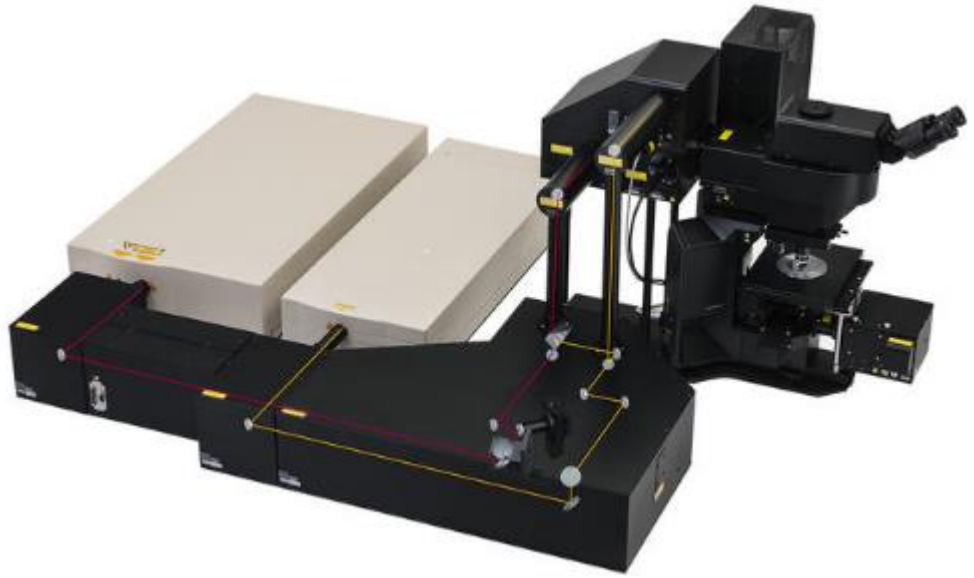
Lezione 02 – Microscopia a fluorescenza

Cosa e’ un microscopio

Leeuwenhoek microscope (inizio 1600)

https://youtu.be/4o3Q2ueh6uI



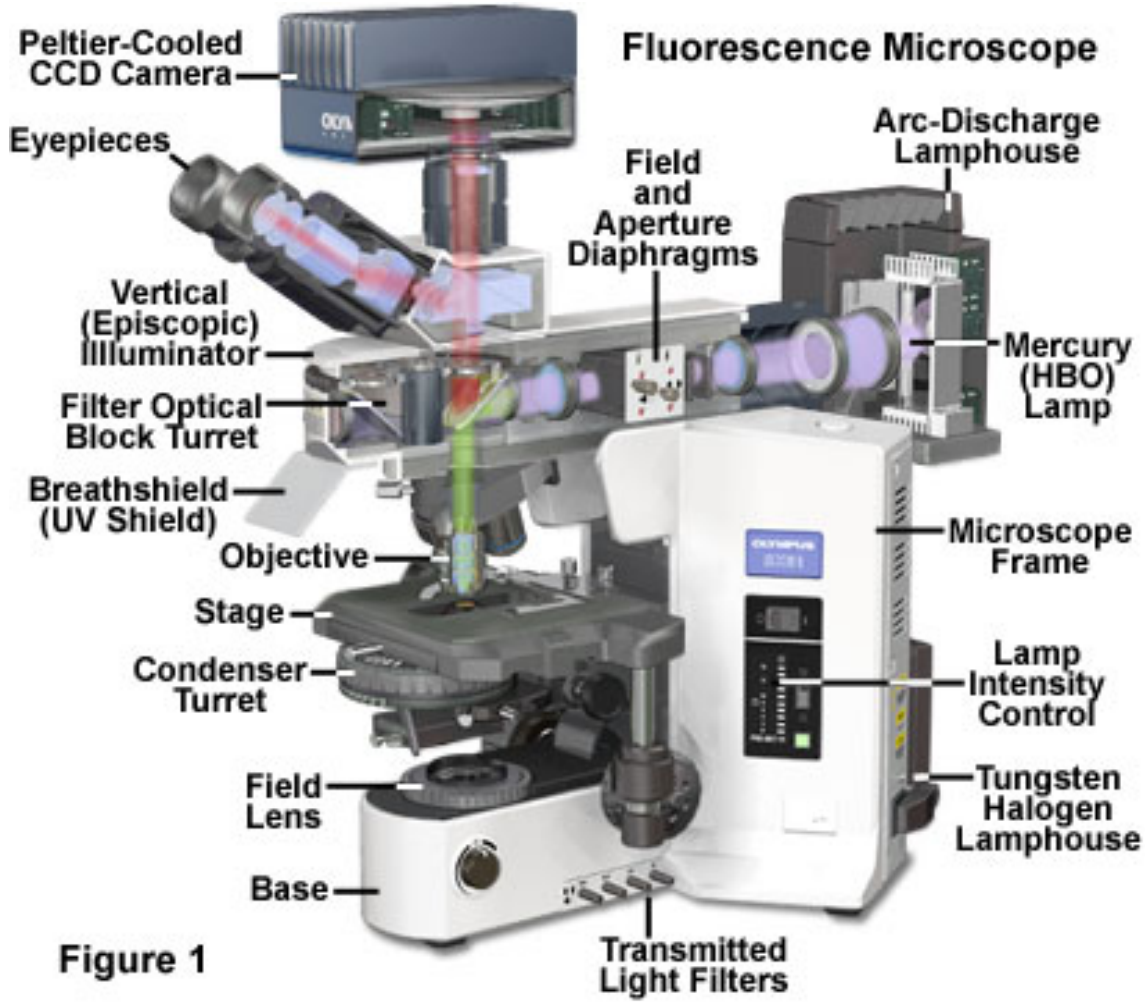
Olympus FV1200 (2015)

Un dispositivo in grado di investigare spazialmente un campinoe in un intervallo di um to nm

- Ottico (fluorescenza)
- Acustico
- Elettronico
- Forza

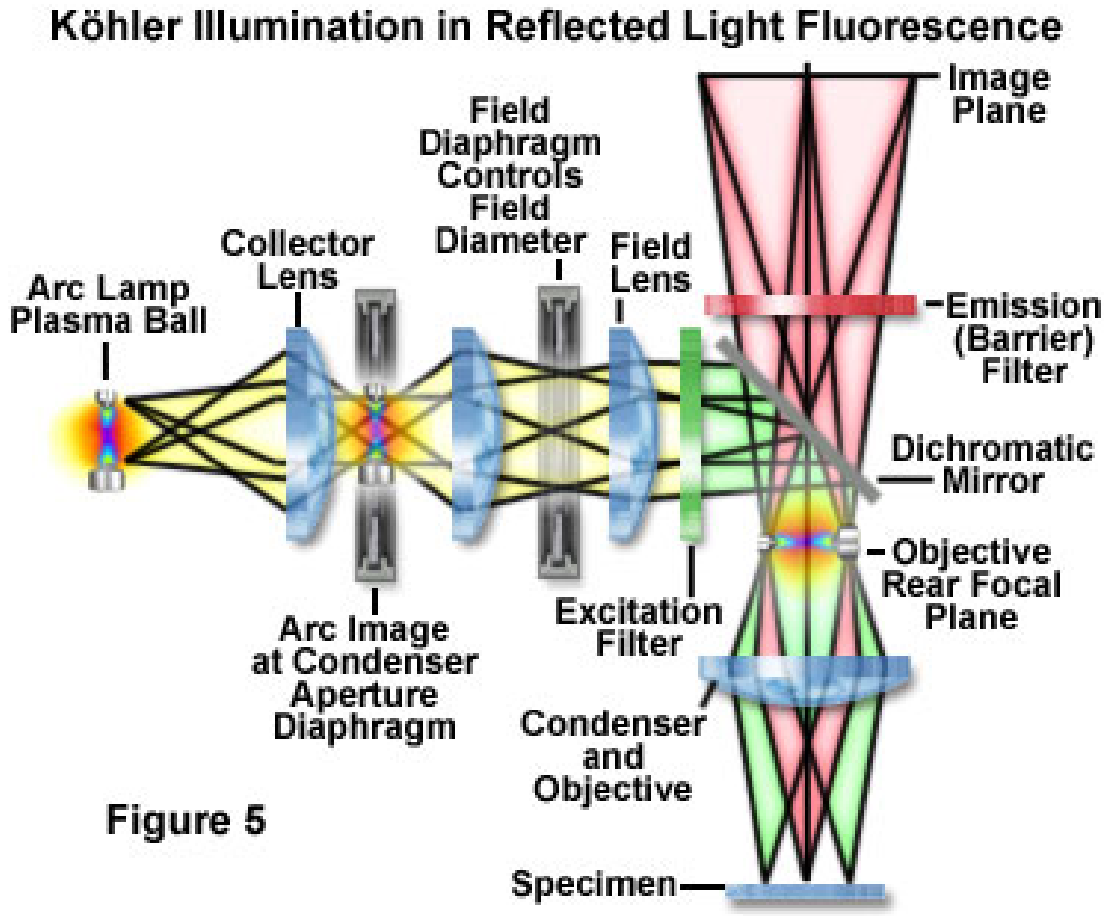
Microscope for widefield epifluorescence

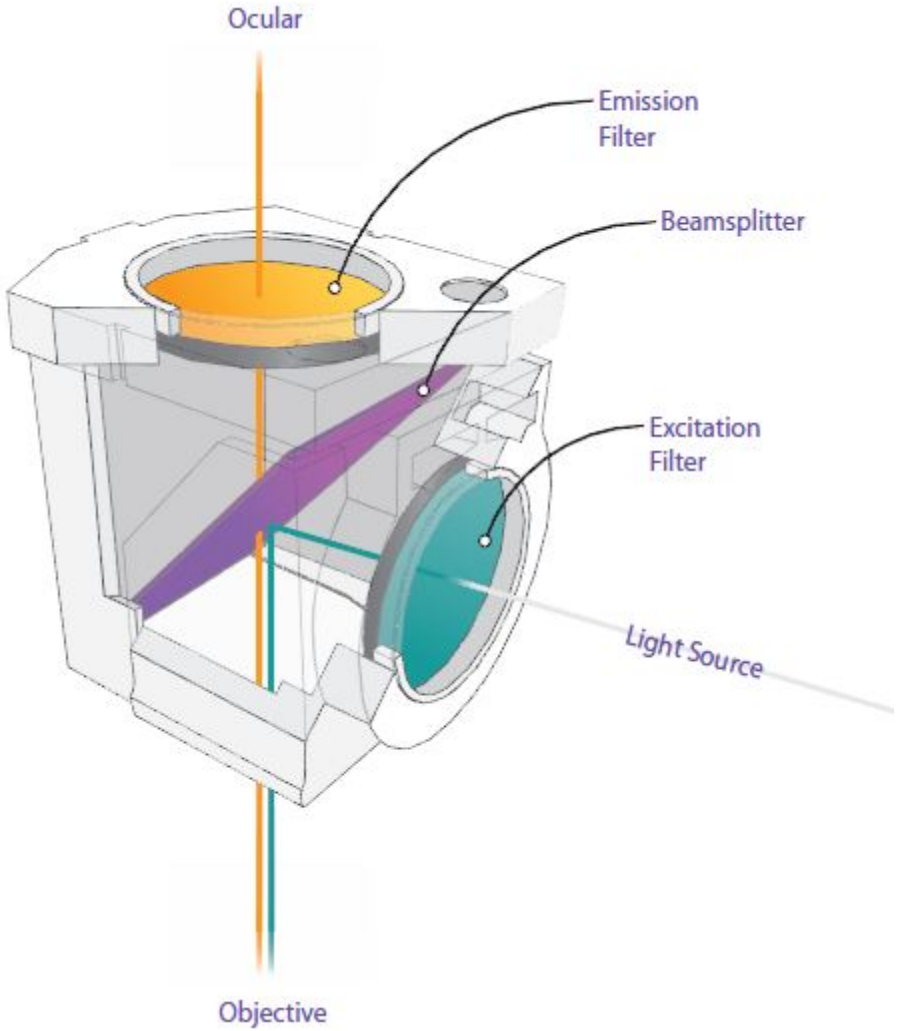
- Lamp
- Dichroic Reflector
- Objective
- Sample Stage
- DIC analyser
- Dichroic Reflector
- CCD camera



https://www.olympus-lifescience.com/en/microscope-resource/primer/java/lightpaths/ix70fluorescence/

Light path of a Epi-fluorescent Microscope



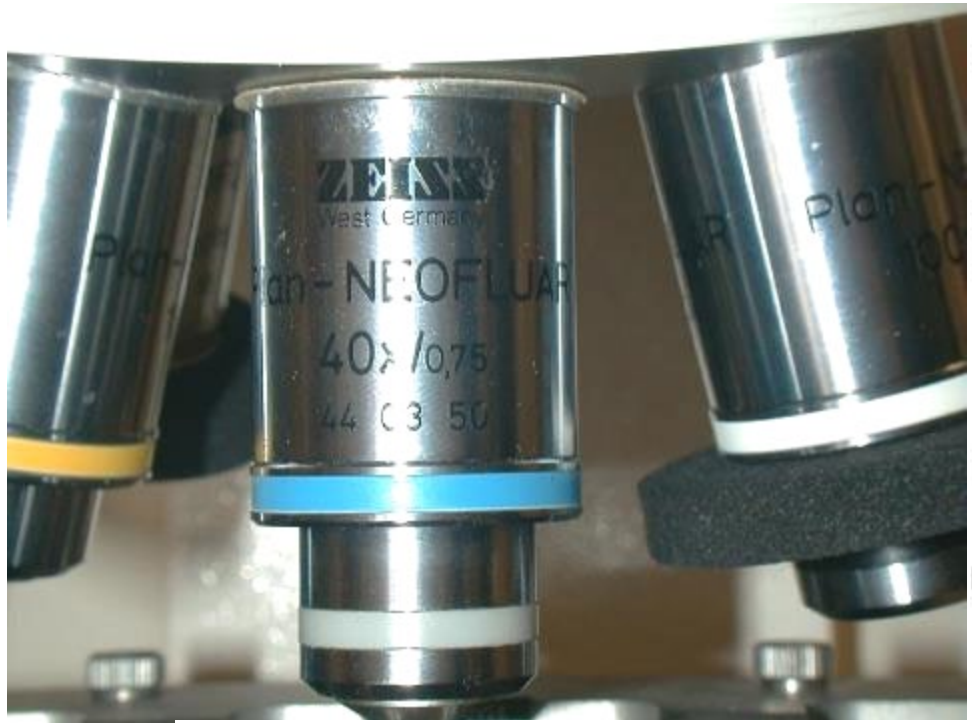


http://microscopy.fsu.edu/primer/techniques/fluorescence/anatomy/fluoromicroanatomy.html

The objective determines the content of your image!

- The objective is critical to the efficiency of light collection (your signal)
- And determines the accuracy of the image (inaccuracy is called aberration).
- The objective determines resolution.

Microscope Objectives



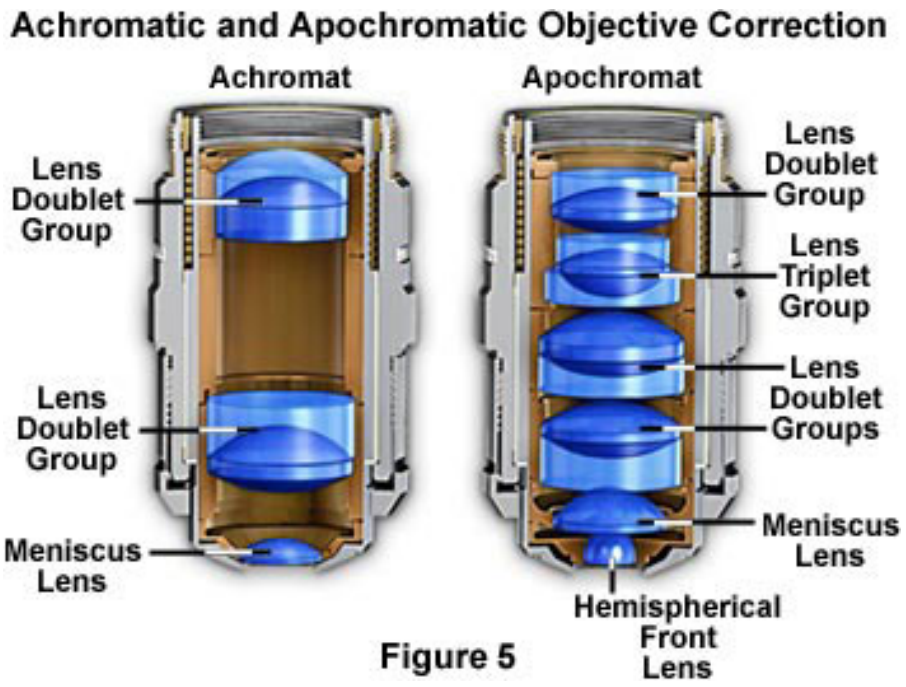


Figure 5

TABLE 1.2 Common objective descriptions

Achromats	corrects chromatic aberration for blue and red wavelengths; also corrects spherical aberration for green
Fluorites	corrects chromatic aberration for blue and red wavelengths; also corrects spherical aberration for two colors
Apochromats	corrects chromatic aberration for blue, green, and red wavelengths; also corrects spherical aberration for two colors
Plan	corrected to provide flat field

x.html

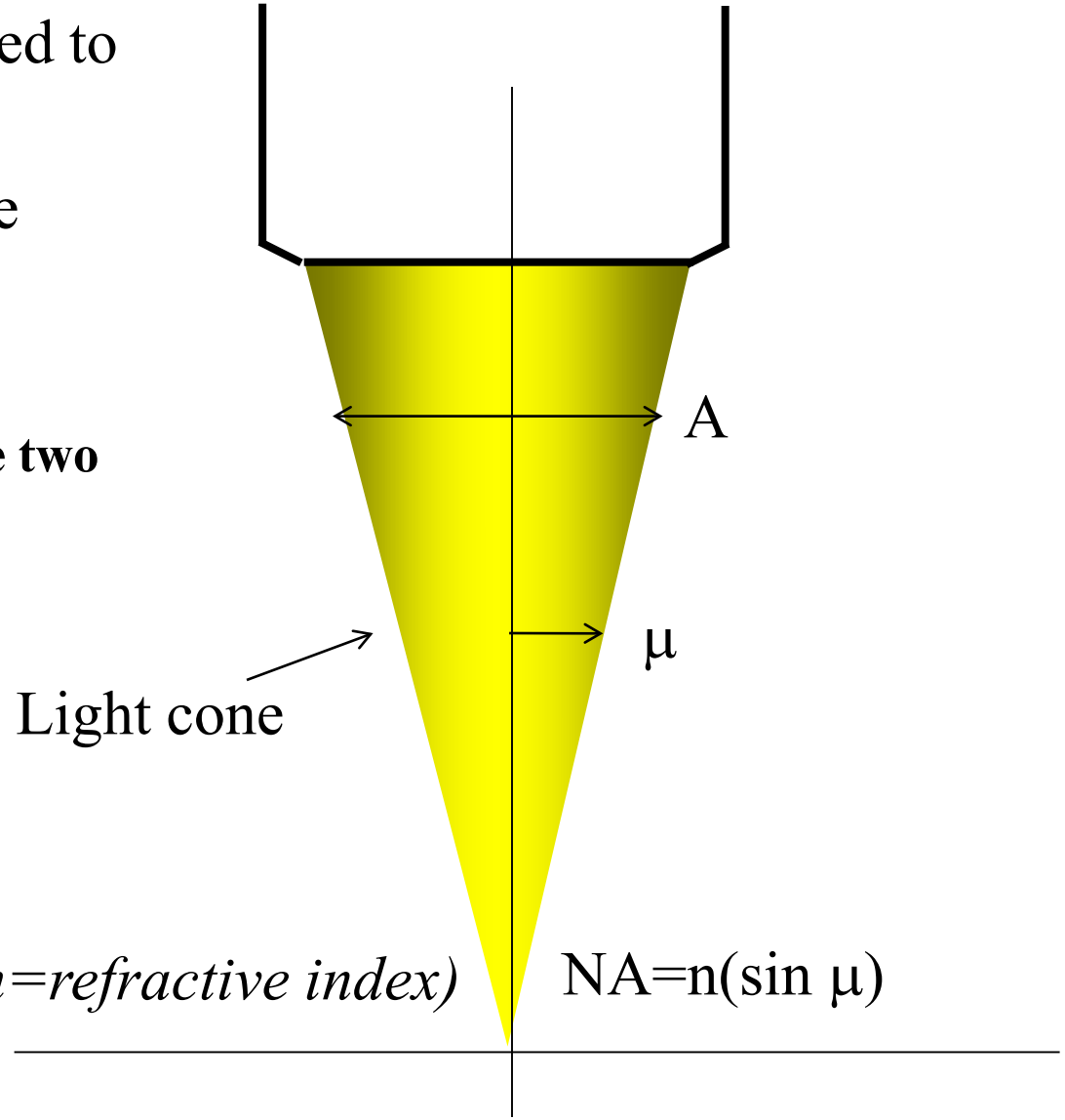
Numerical Aperture

- Resolving power is directly related to numerical aperture.
- The higher the NA the greater the resolution
- Resolving power:

The ability of an objective to resolve two distinct lines very close together

$$NA = n \sin \mu$$

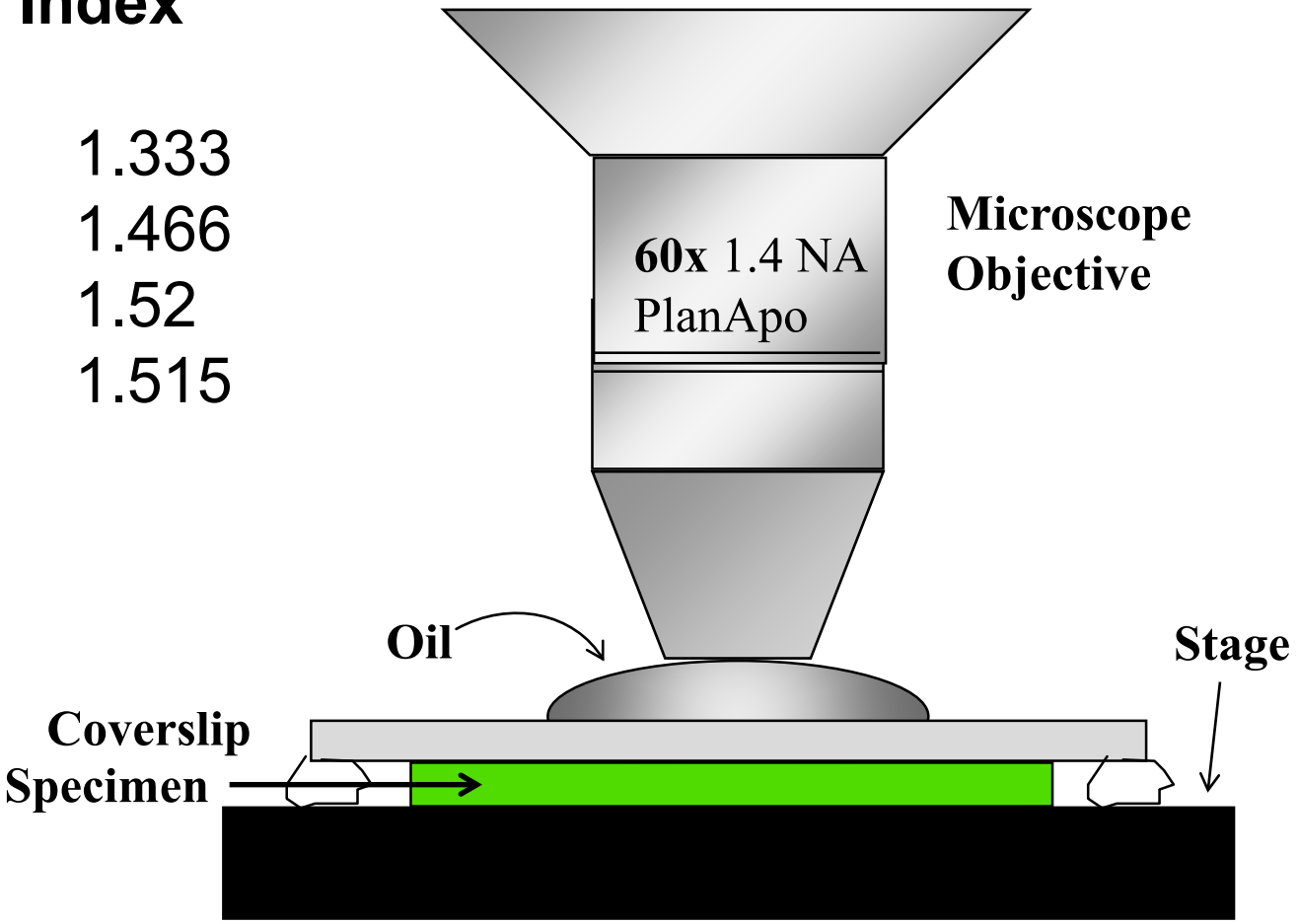
- μ is 1/2 the angular aperture of the objective



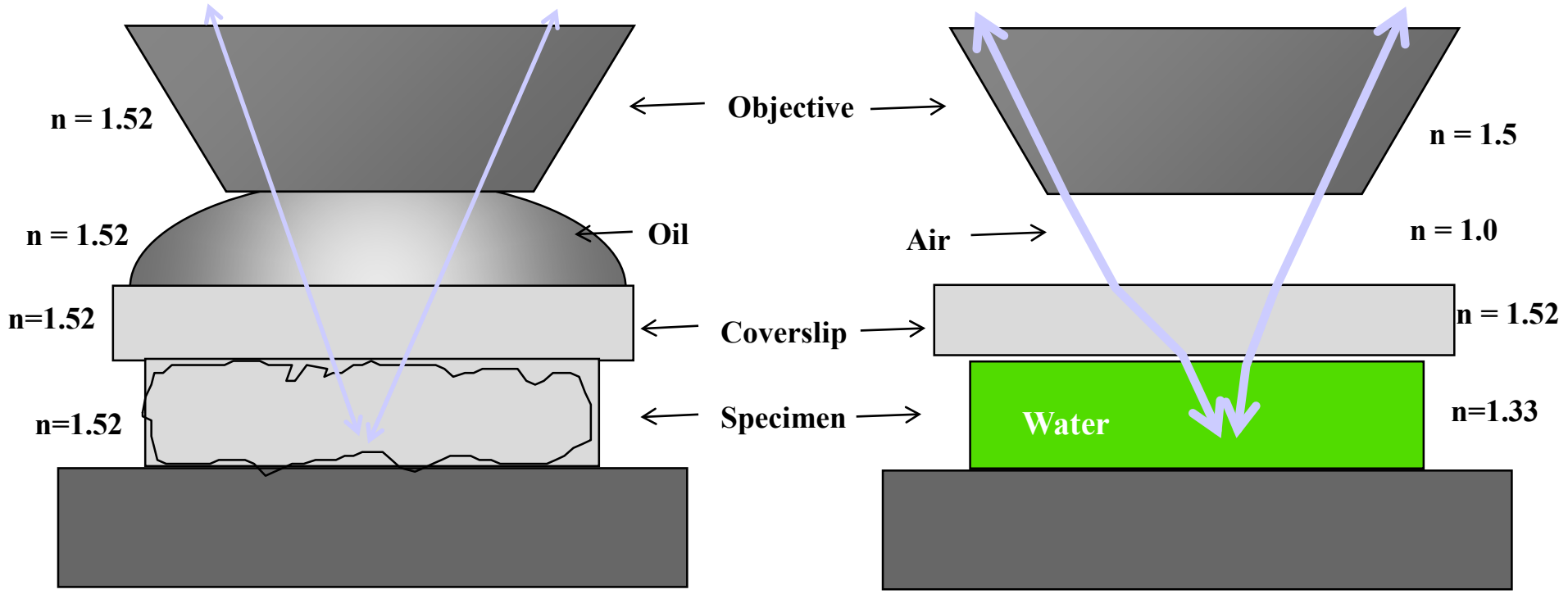
Microscope Objectives

Refractive Index

Cells	
Water	1.333
glycerol	1.466
Glass	1.52
Oil	1.515



Refractive Index



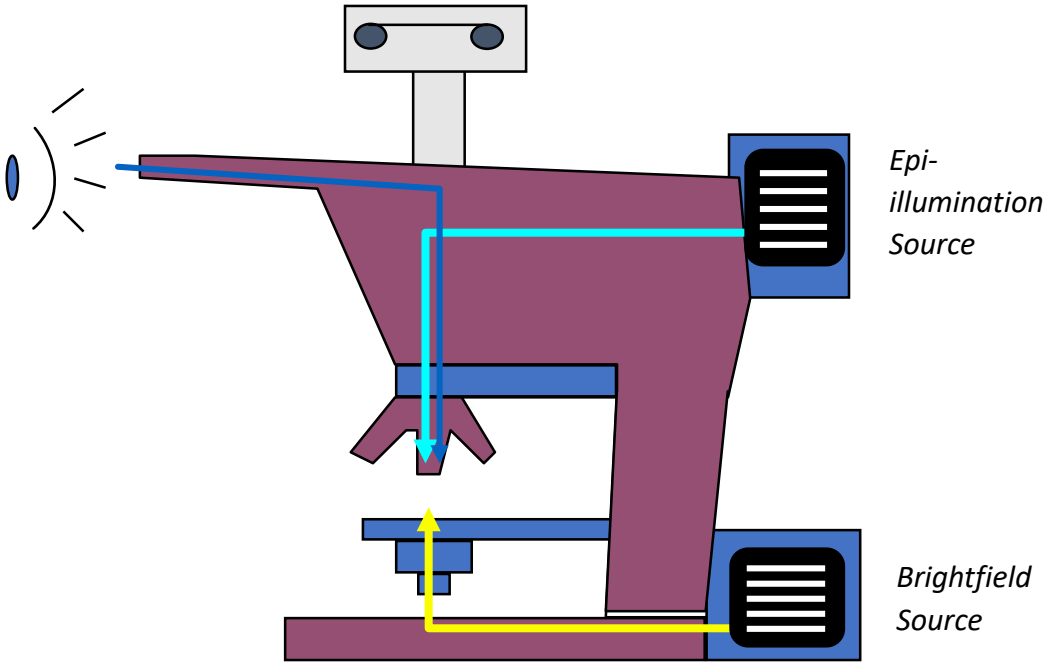
Simulazione indice di rifrazione/percorso ottico

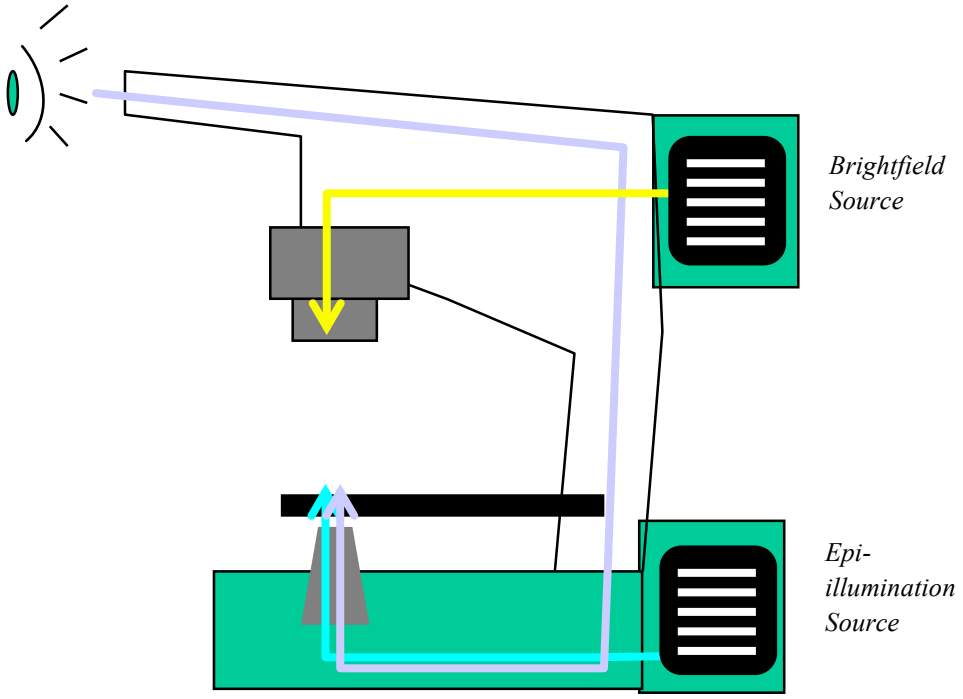
https://www.olympus-lifescience.com/en/microscope-resource/primer/java/microscopy/immersion/

Simulazione indice di rifrazione/risoluzione

https://www.microscopyu.com/tutorials/imageformation-airyna

Microscopio dritto VS rovesciato





Imaging a Fluorescenza

Principles and Advantages

- Range dinamico elevato
- Permettere la localizzazione di un fenomeno ad alta risoluzione
- Piu'fenomeni contemporaneamente.

All the dimension of Fluorescence imaging

Live imaging

Principles

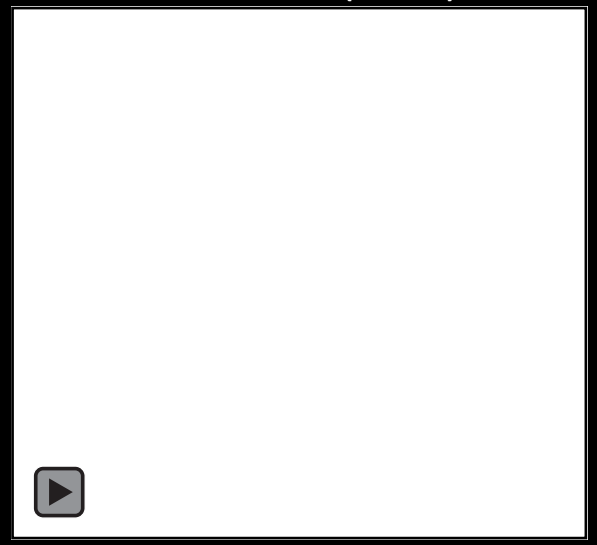
“Ottimizzazione per non stressare il campione”

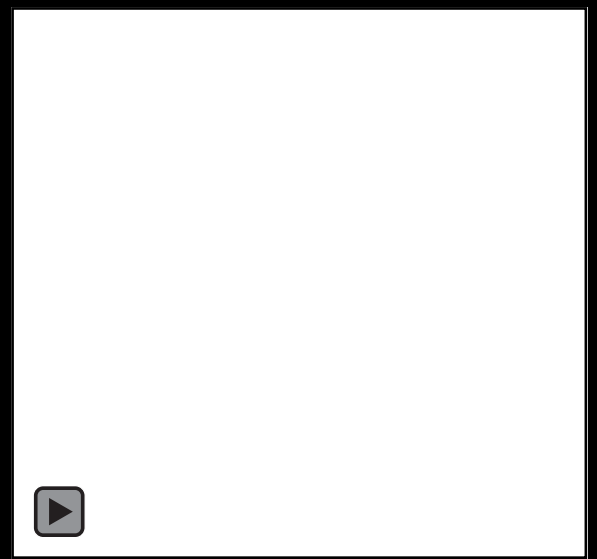
- High sensitivity camera,
- Alta velocita’di lettura
- Controllo ambientale

Il range temporale del live imaging

Fast Live (ms-s)

- Signalling intracellulare
- Traslocazione di protein
- Produzione di metaboliti
- Riarrangamenti di organelle/citoscheletro
- Cinetiche enzimatiche
- vescicolazione

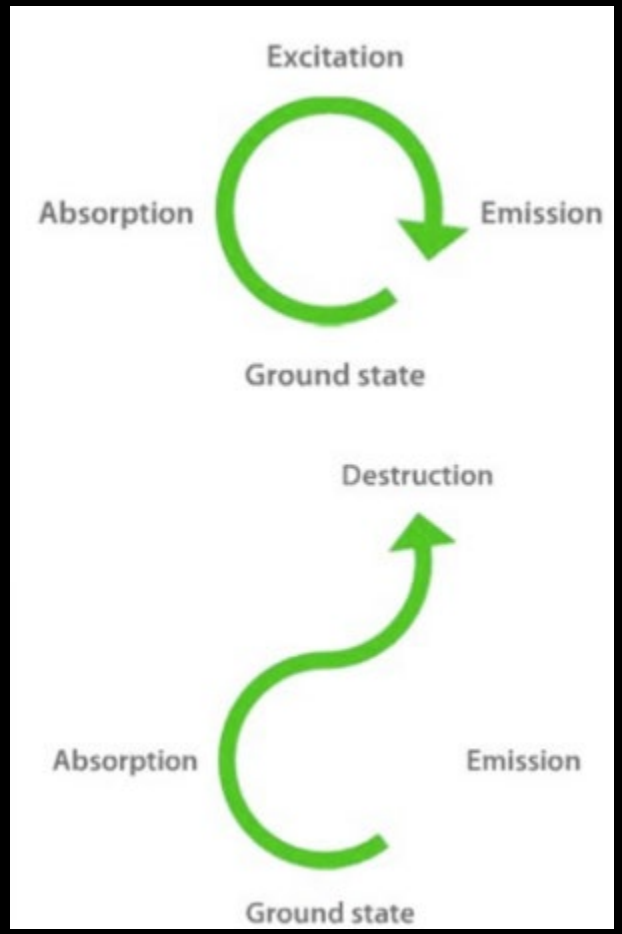


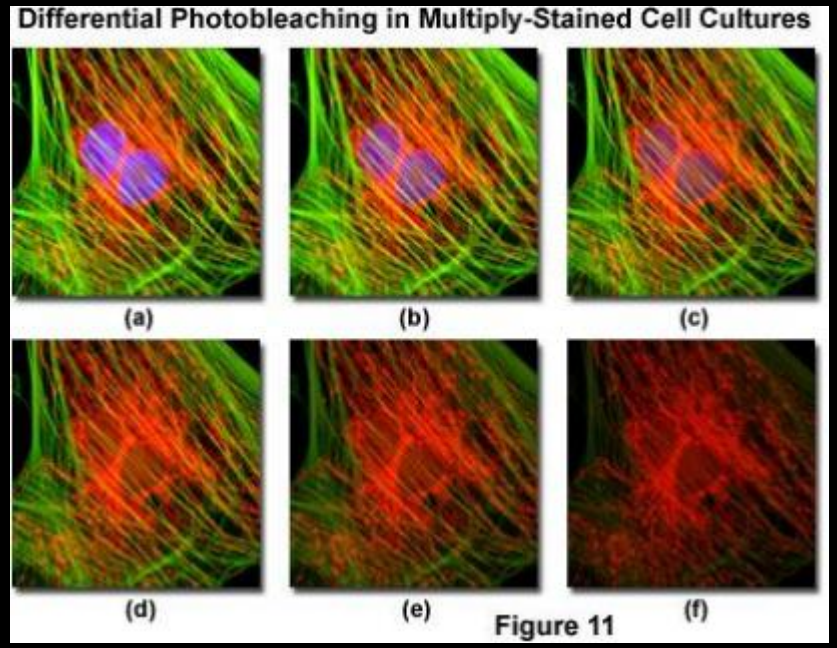


Long Live (ore-giorni)

- Ciclo cellulare
- Migrazione cellulare
- Interazione tra cellule

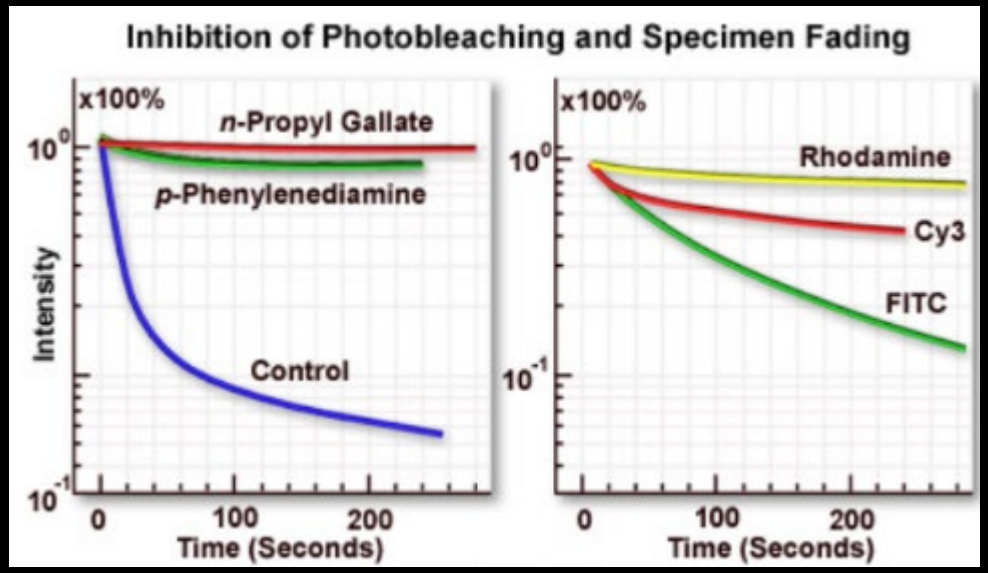
Degradazione temporale del segnale: photobleaching



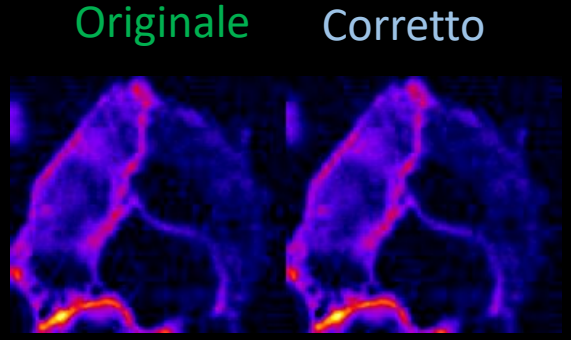


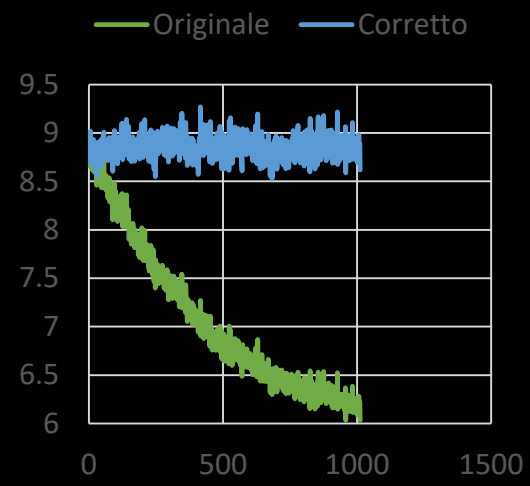
L'instabilita' dello stato eccitato puo' degradare il fluoroforo

Prevenire/Correggere il photobleaching



- Utilizzare composti che promuovono la stabilita' del fluoroforo
- Scegliere un fluoroforo molto stabile (e.g. EGFP)
- Limitare l'illuminazione (e.g. Potenza eccitazione, temo di esposizione)
- Correggere matematicamente il decadimento
- Imaging raziometrico

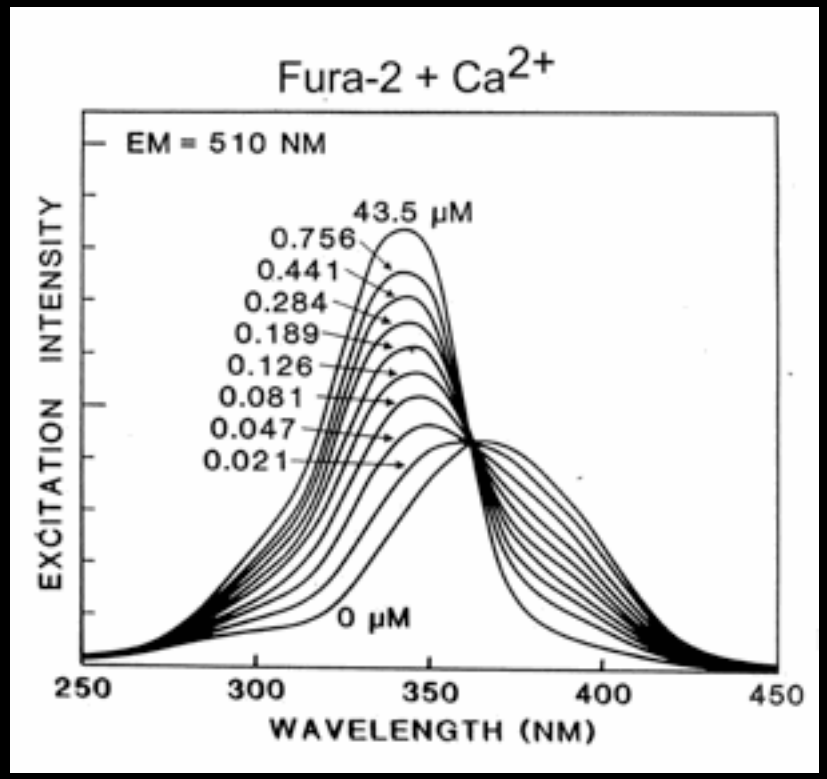




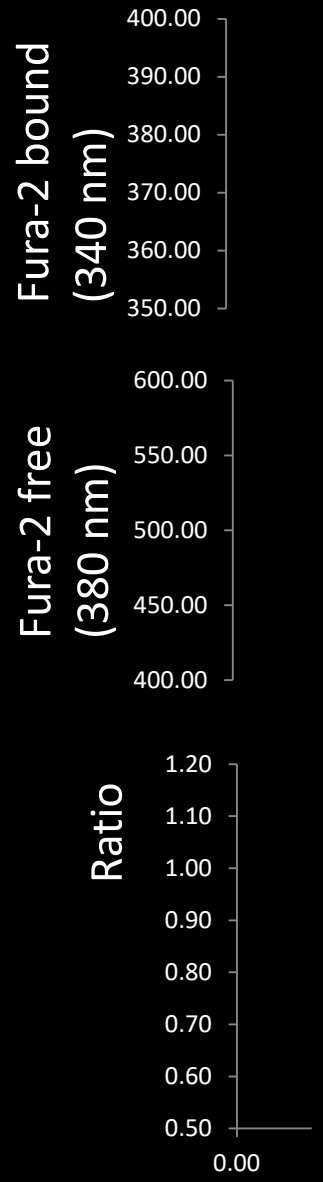
Imaging Raziometrico

Principio

Alcuni composti cambiano picco di eccitazione/assorbimento in risposta all'interazione con fattori sperimentali



Imaging Raziometrico



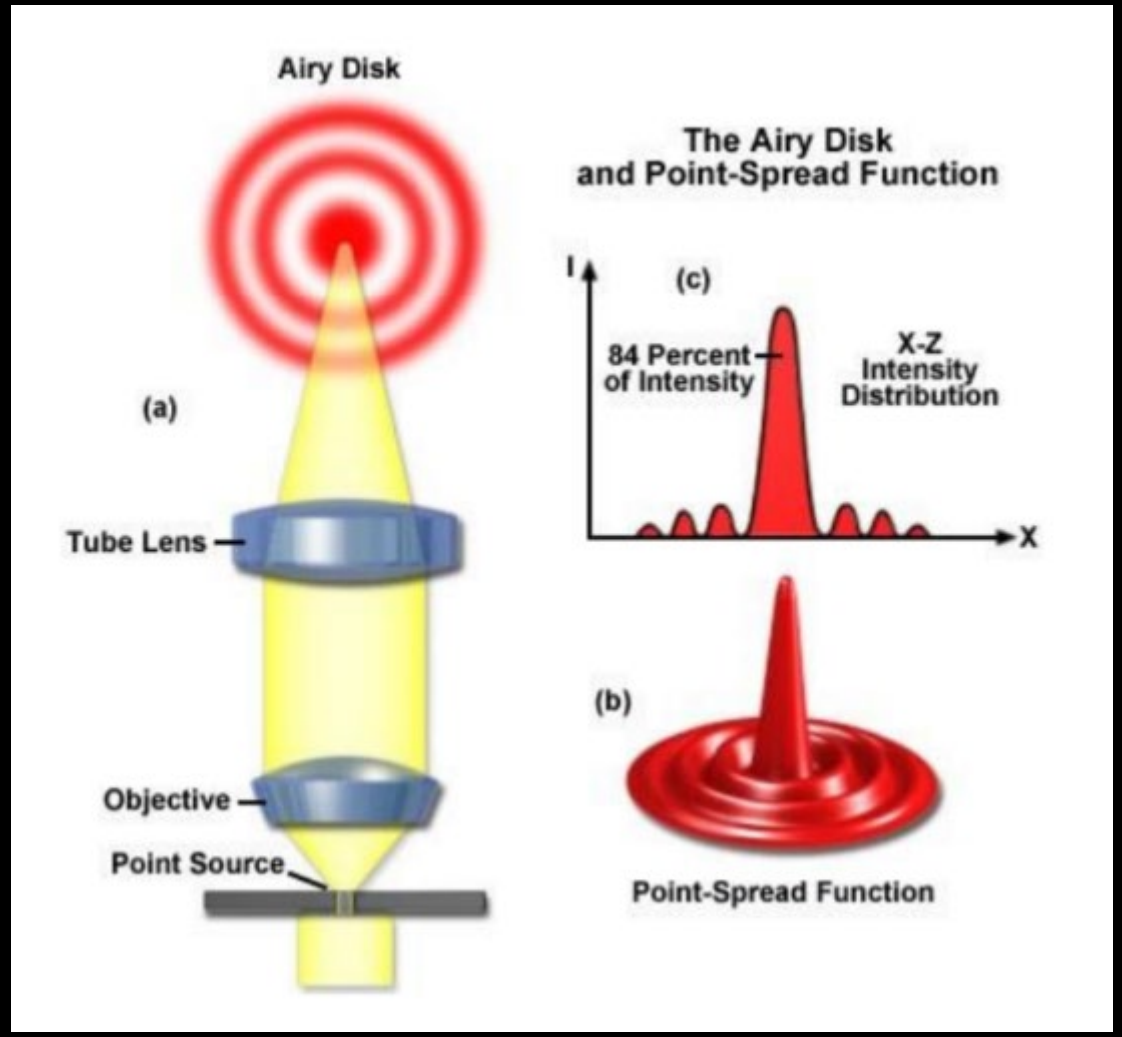
Imaging Raziometrico

Pros VS Cons

- Alta sensibilita’
- Limitata aberrazione dell’immagine

- Velocita’ della cinetica dimezzata
- Funziona solo per alcuni fluorofori

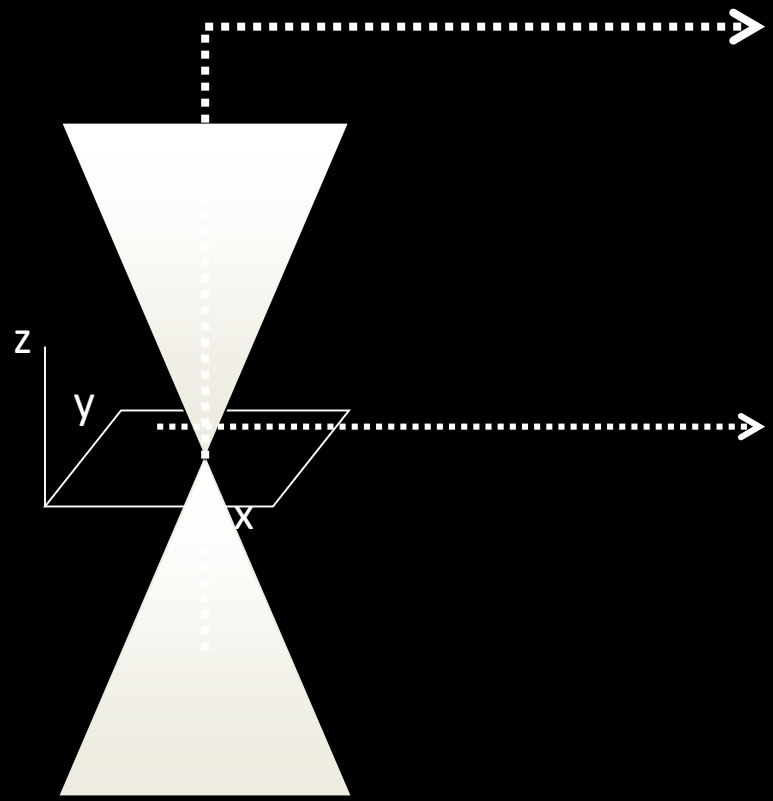
Degradazione spaziale del segnale: PSF

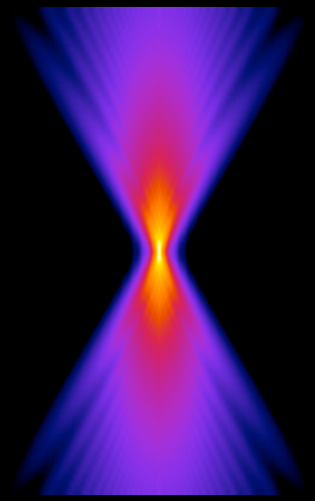


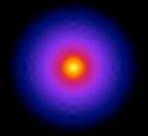
- La diffrazione spontanea della luce genera una deformazione del segnale
- PSF (point spread function), descrive come il percorso ottico trasforma il segnale reale in segnale misurato

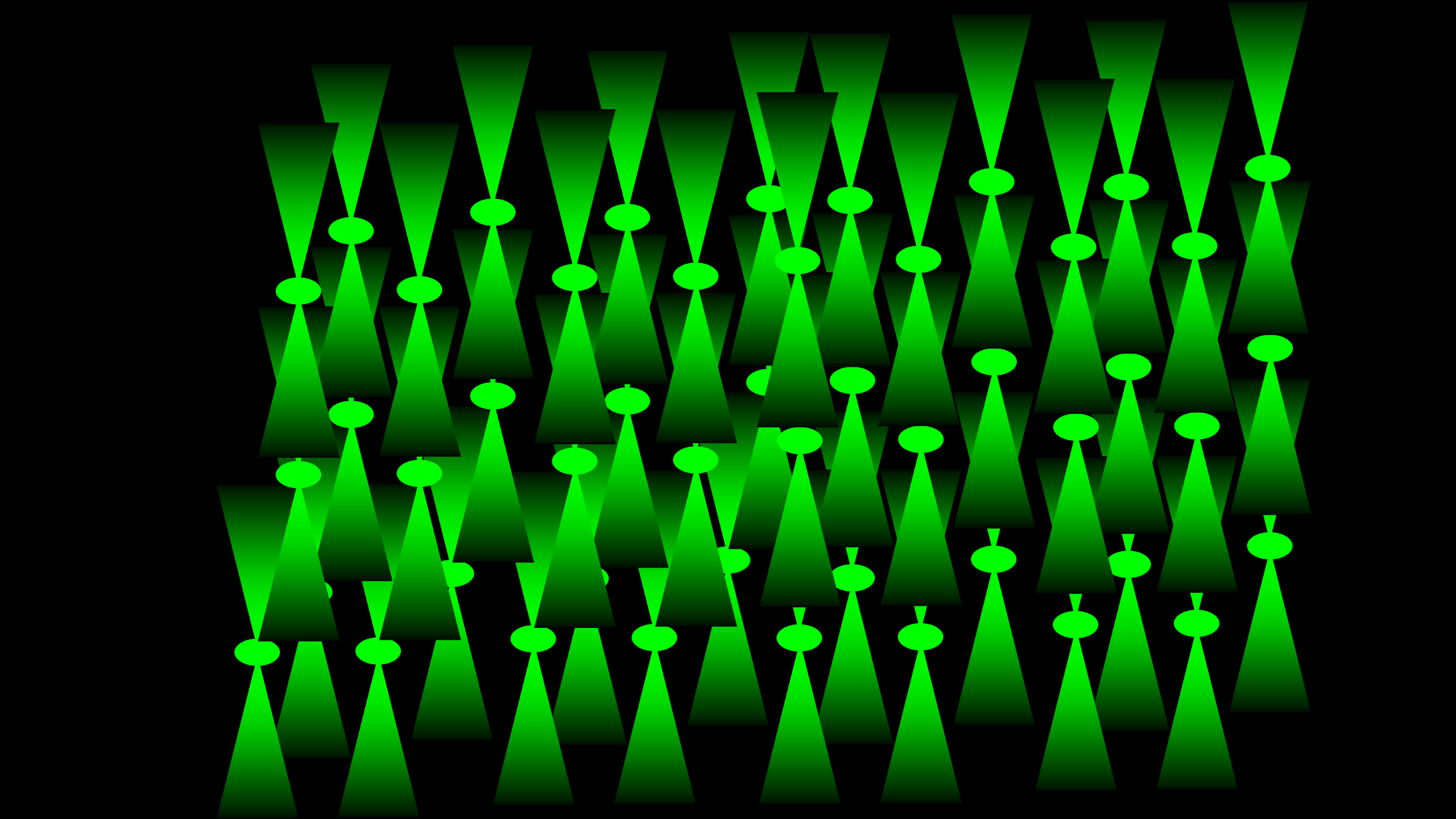
Forma della PSF in 3D

Light distribution in a Volume

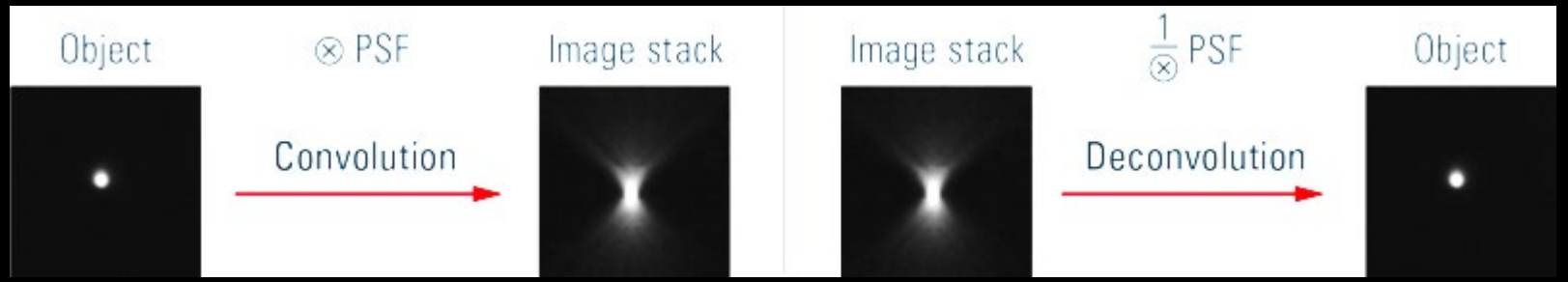




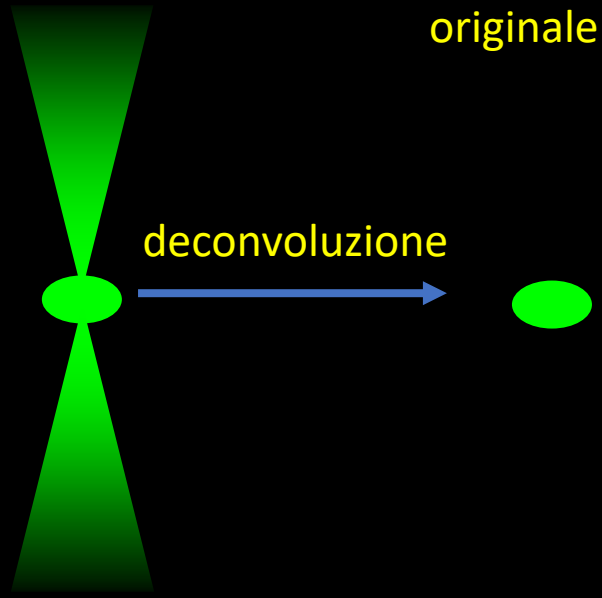


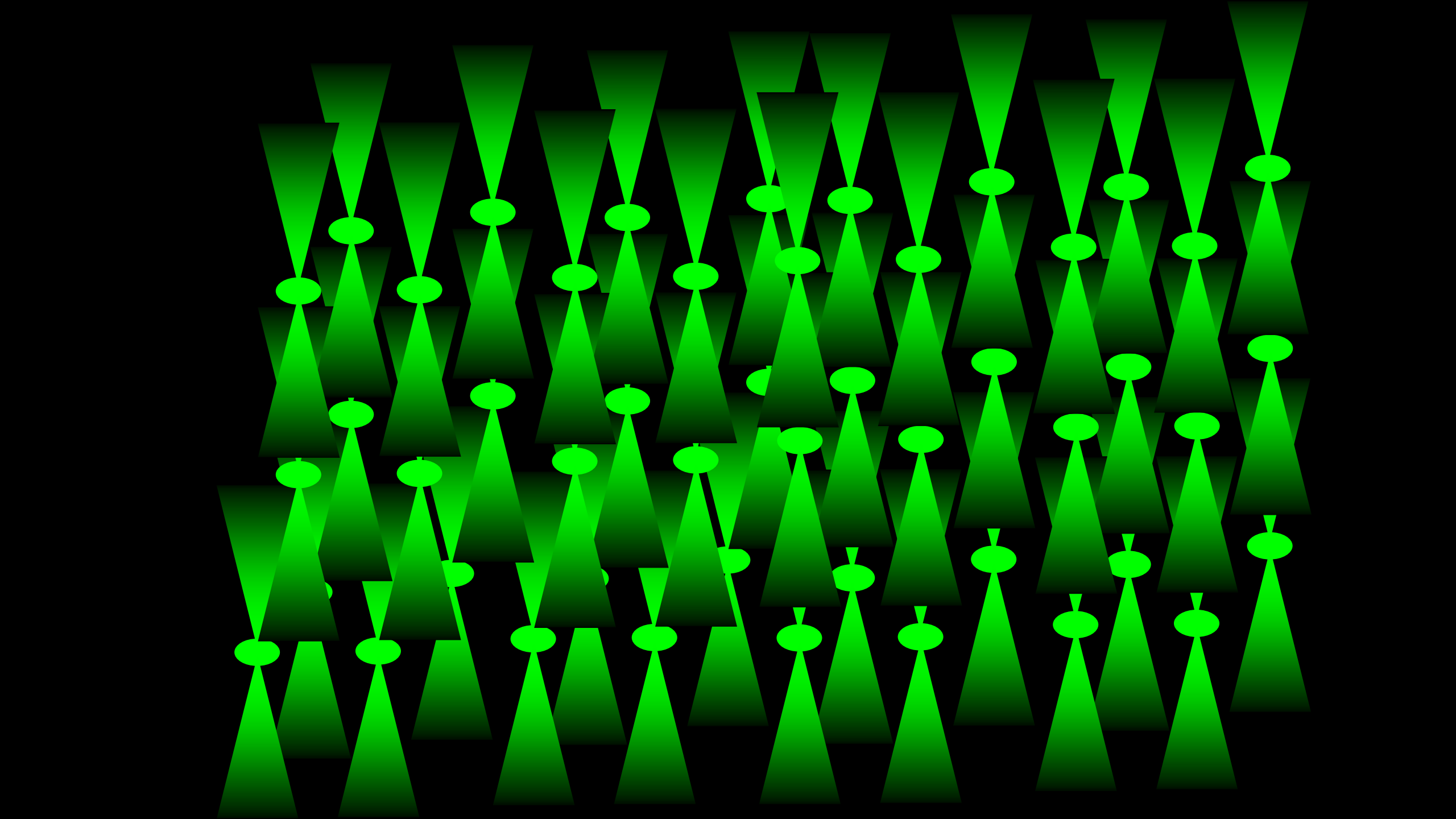


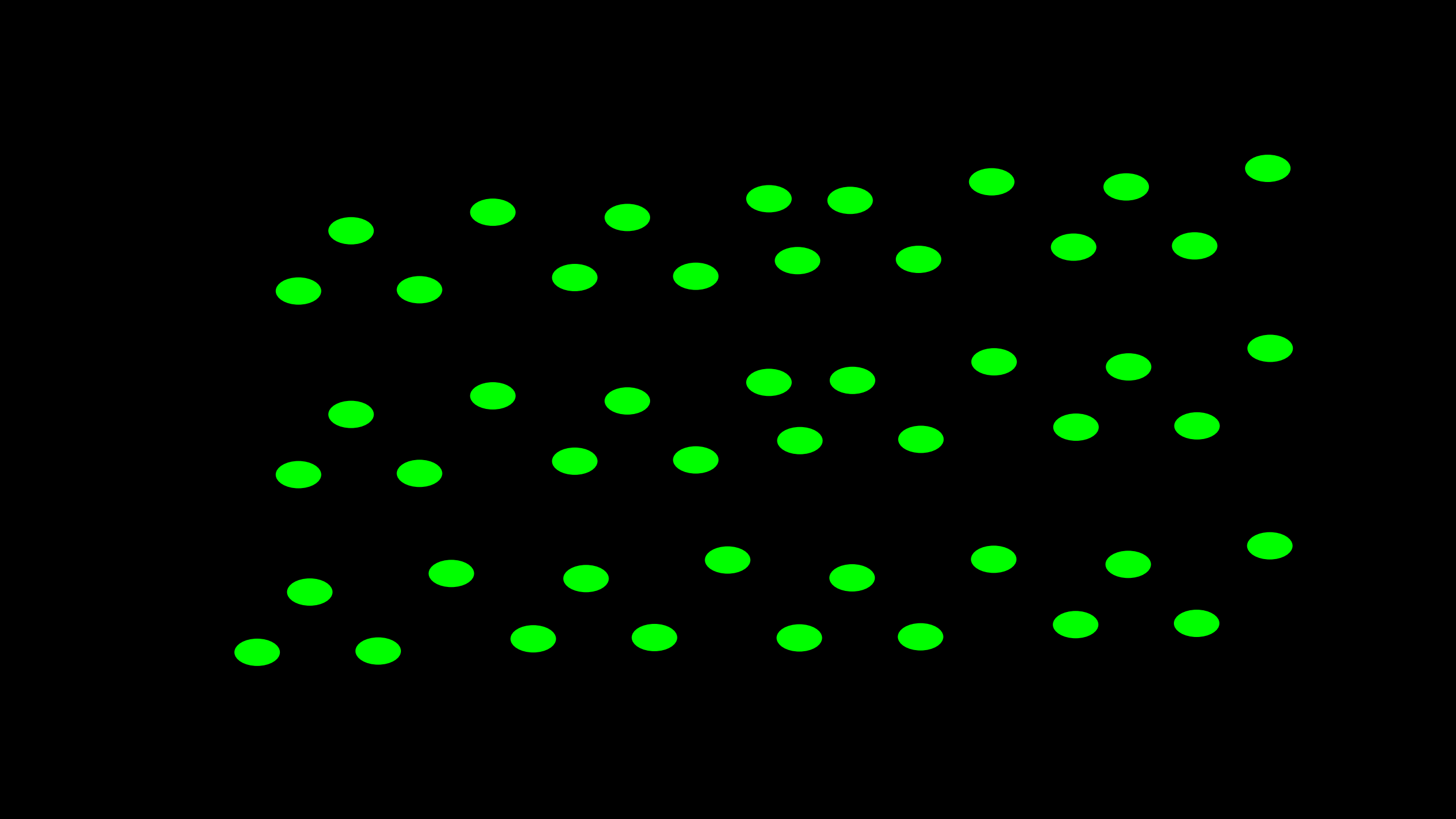
Deconvoluzione digitale



- Se conosci il segnale misurato e quello reale puoi calcolare PSF
- Se conosci il segnale misurato e PSF puoi calcolare il segnale originale







Digital Deconvolution

Pros VS Cons

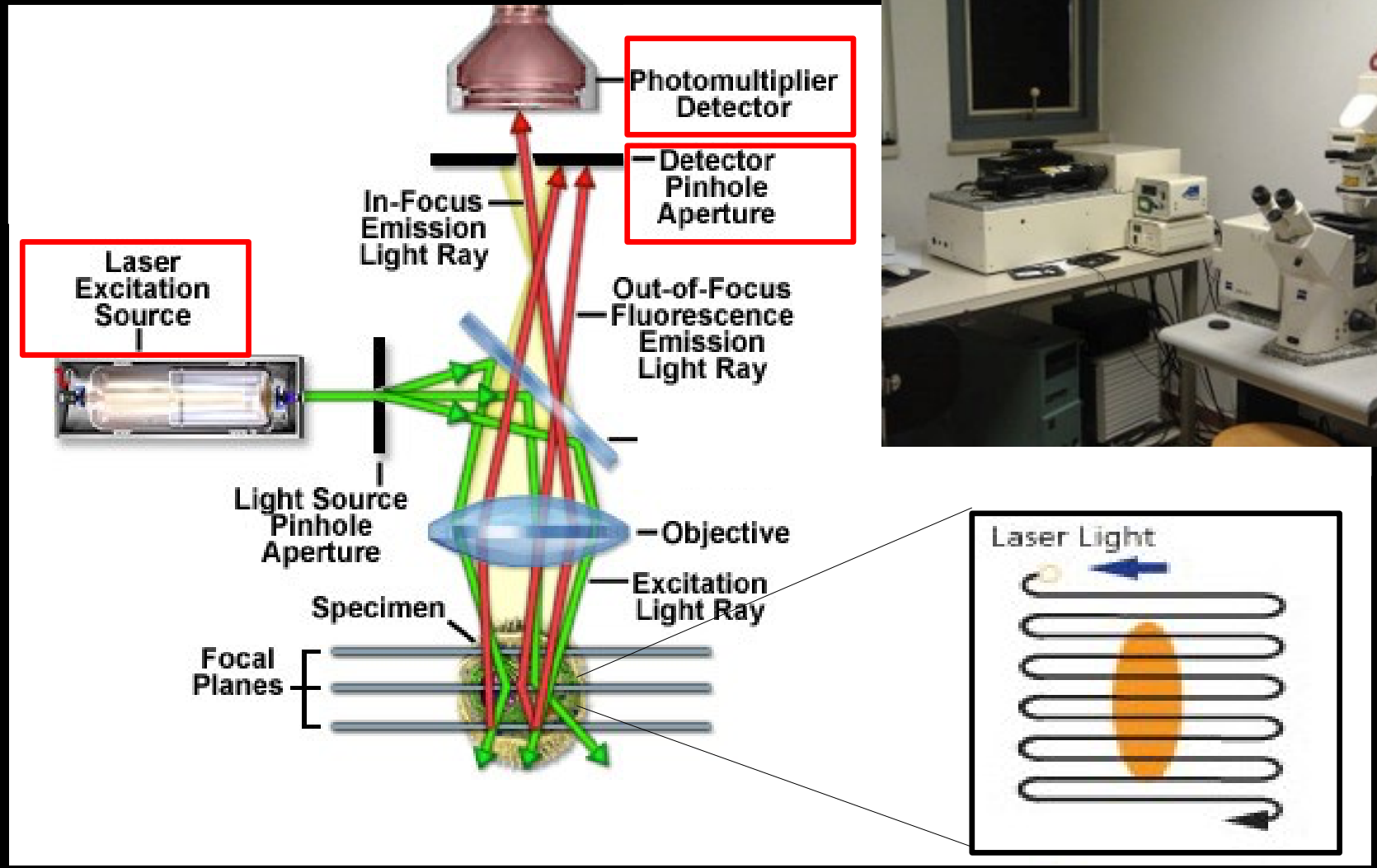
- Parziale correzione spaziale di qualunque lunghezza d’onda
- Poco invasiva

- Richiede un ampia capacita’ di calcolo (non e’ in diretta)
- Funziona al massimo in una profondita’ di 50 um
- Mappatura precisa di PSF

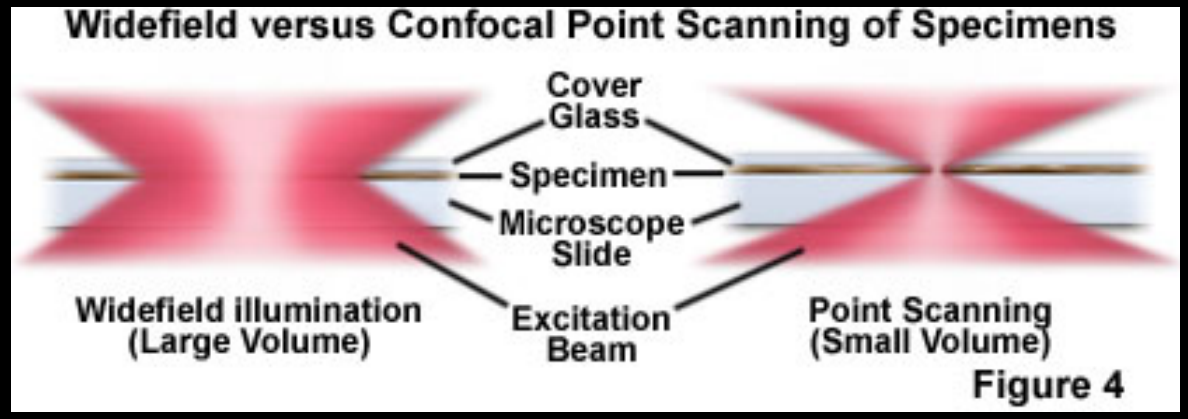
Laser Scanning Confocal imaging

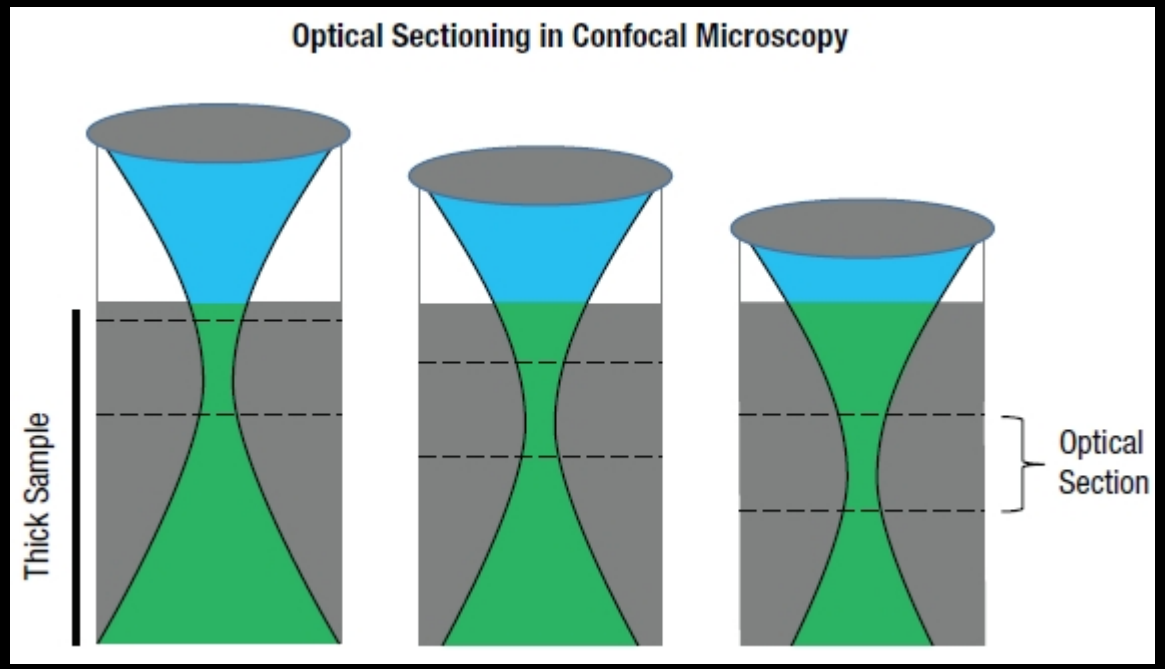
Principles

Rimozione meccanica della luce fuori fuoco

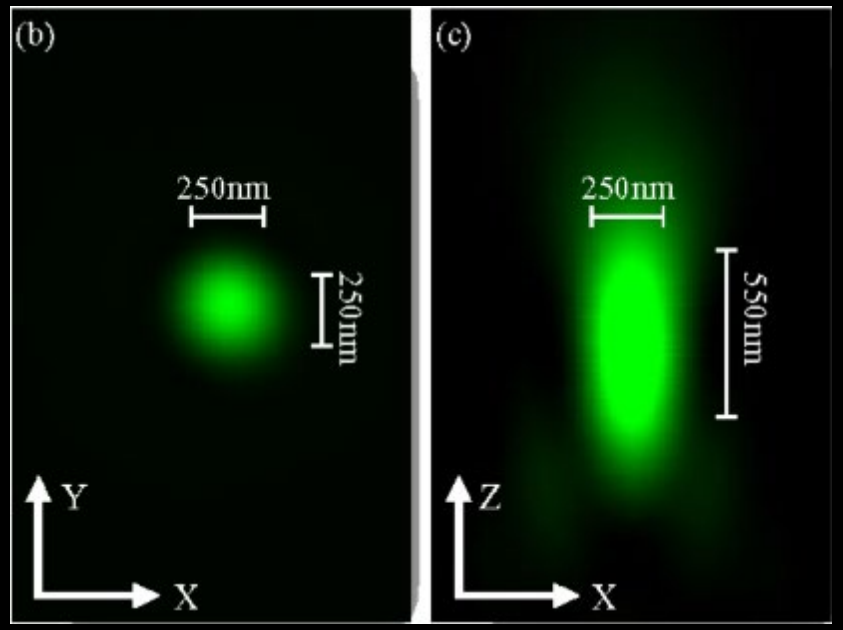


Laser Scanning Confocal imaging





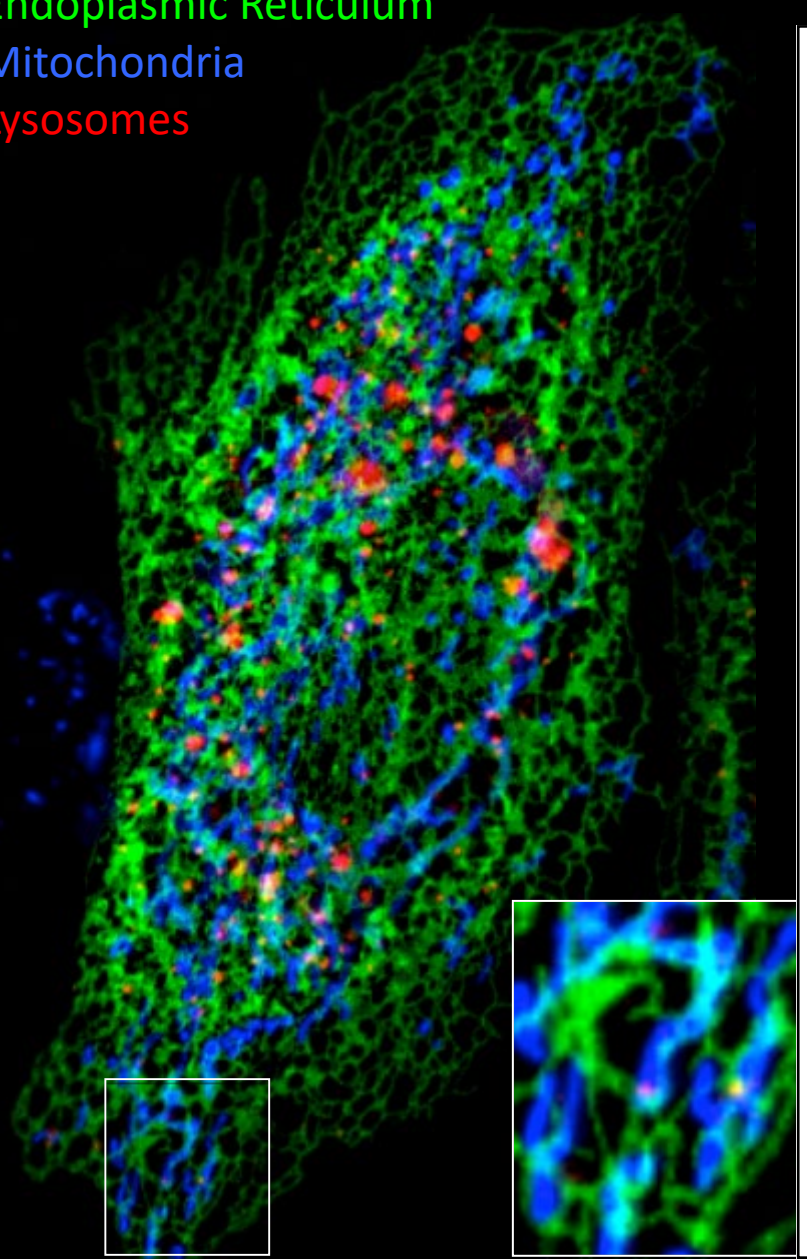
Punti chiave:
- Punto di fuoco piu' piccolo,
- Sezionamento ottico



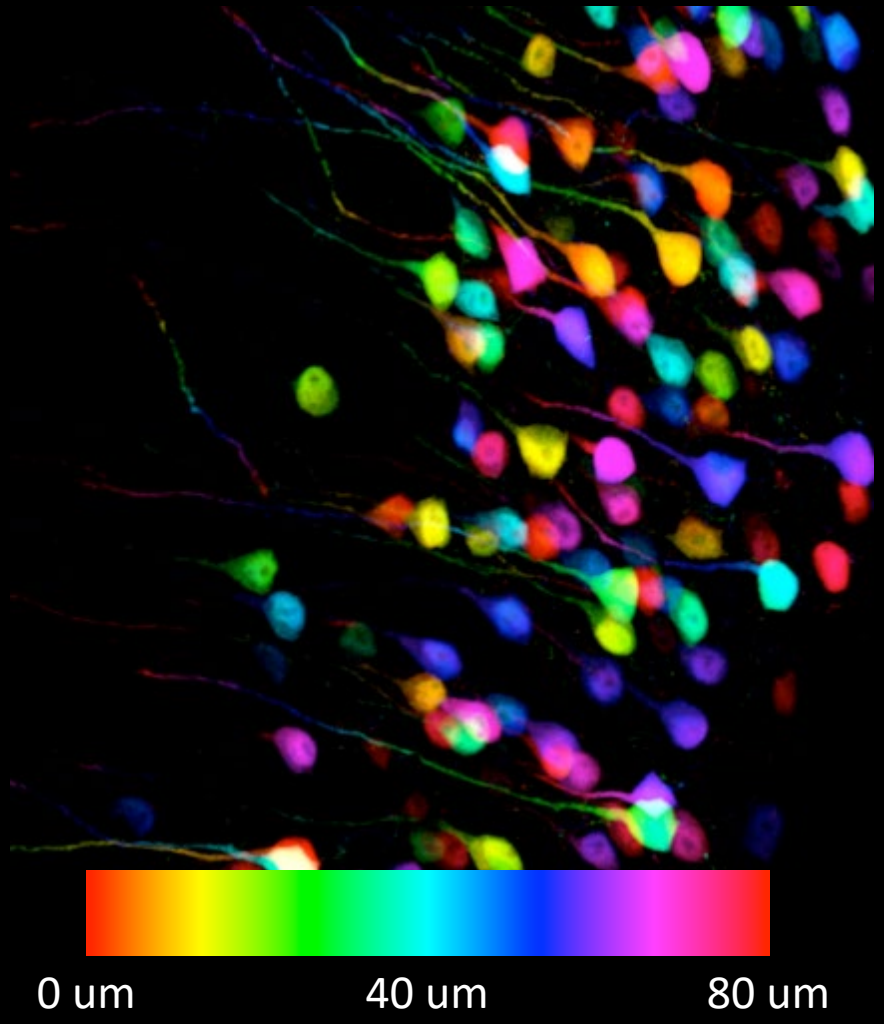
https://www.microscopyu.com/tutorials/laser-scanning-confocal-microscopy

High resolution imaging of small cell districts

Endoplasmic Reticulum
Mitochondria
Lysosomes



High Resolution imaging of thick samples



Laser Scanning Confocal imaging

Pros VS Cons

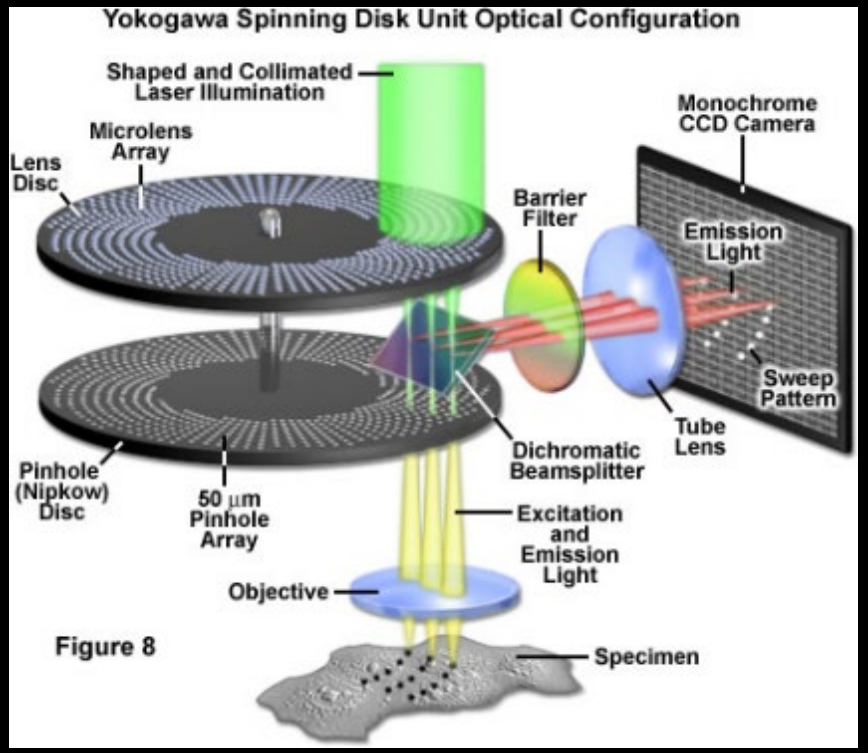
- Altissima risoluzione spaziale (200 nm in xy and 350nm in Z)
- Alta penetranza (campioni spessi fino a 250 – 300 um)
- Non richiede processamento post acquisizione
- Molto Lento
- Richiede luce potente (stressa il campione)

Live Fast Confocal imaging

Principles

microlenti

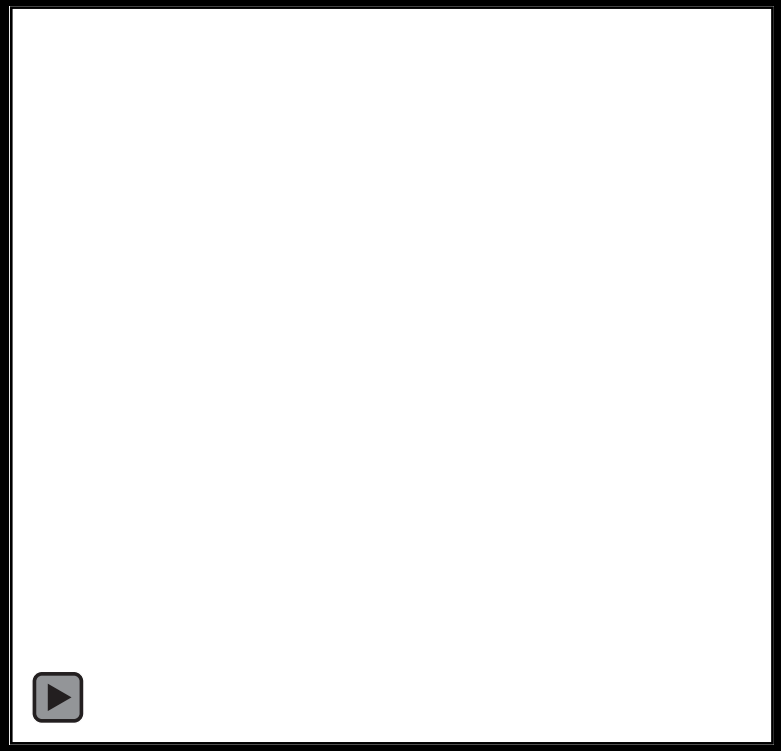
Disco di pinholes



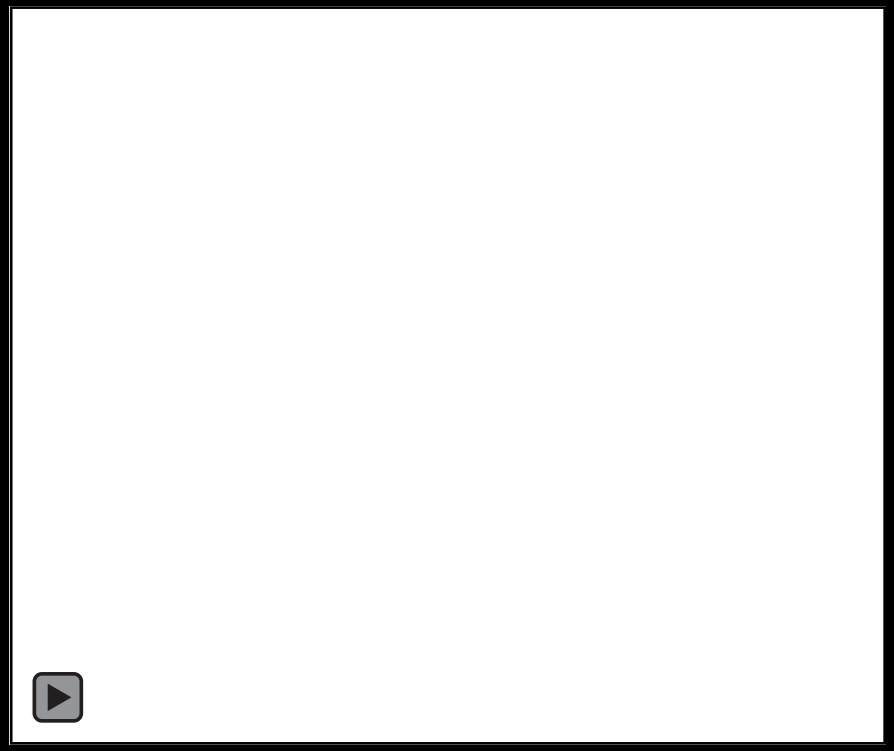
"mechanical removal of out of focus ligh by an array of pinholes"

Live Fast Confocal imaging

Real Time Z stack acquisition of Lysosomal GFP



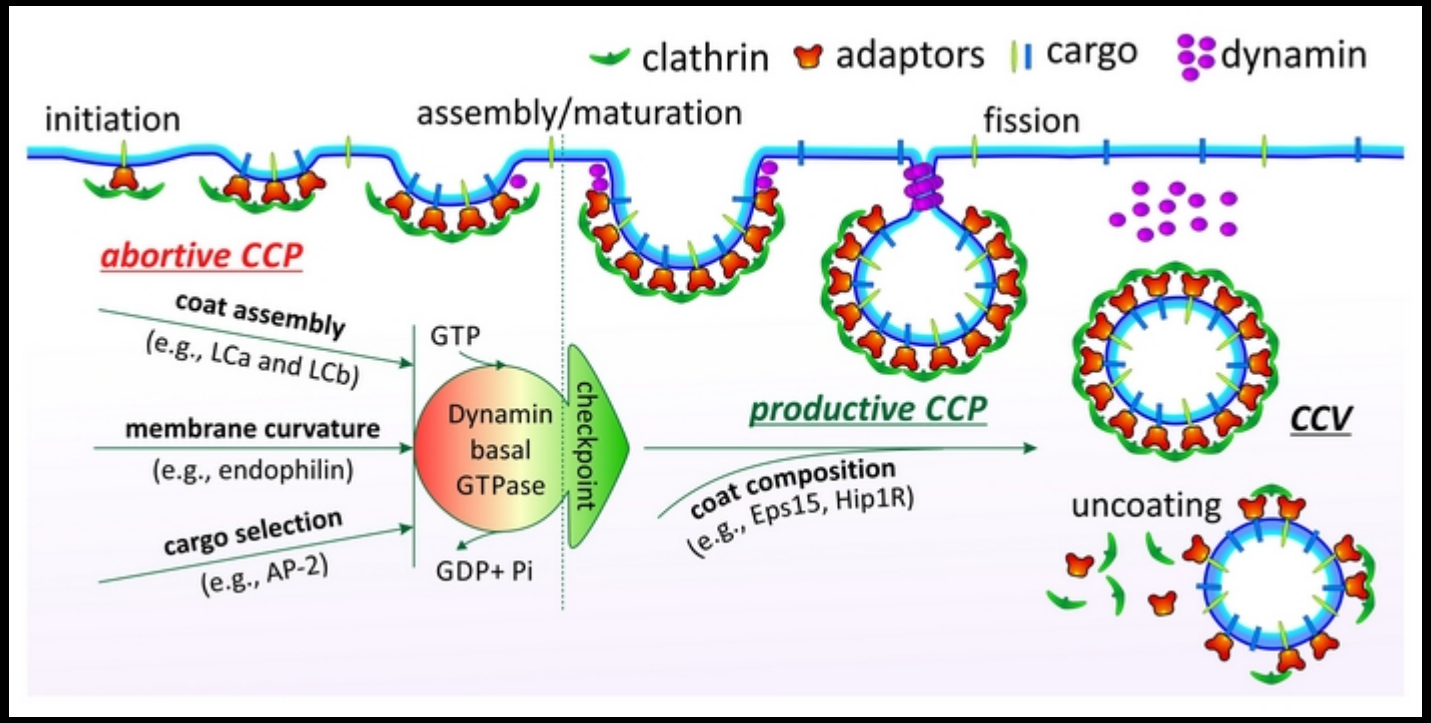
3D lysosome tracking

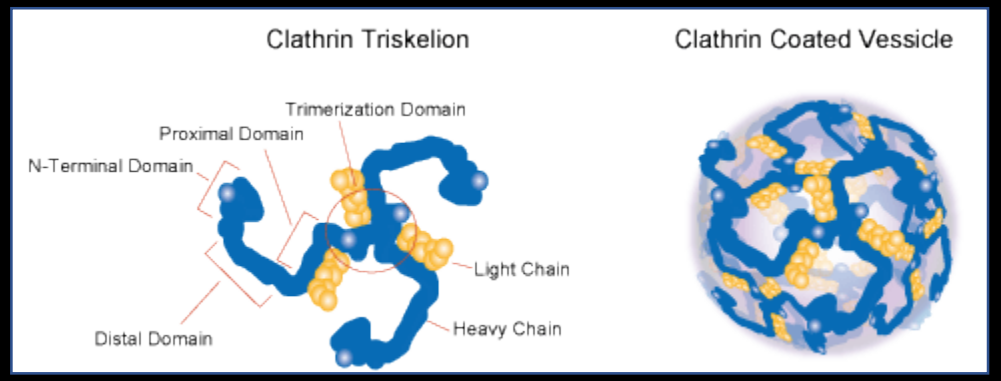


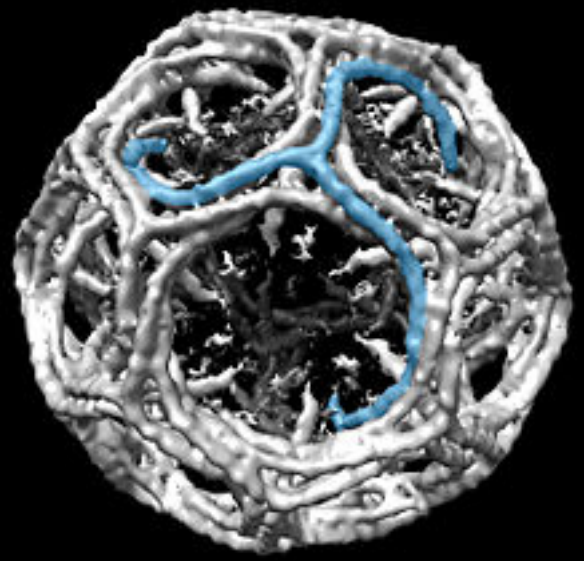
Live Fast Vs Laser Scanning Confocal

- High Speed of Acquisition (ms instead of s)
- Investigation of fast kinetics with precise 3D sampling,
- Lower sample stress,
- Lower sensitivity

Esempio Dinamiche Molecolari: la nascita di vescicole di clatrina







La formazione del triskelion e' catalizzata dall'interazione con l'adattatore?

Esempio Dinamiche Molecolari: la nascita di vescicole di clatrina

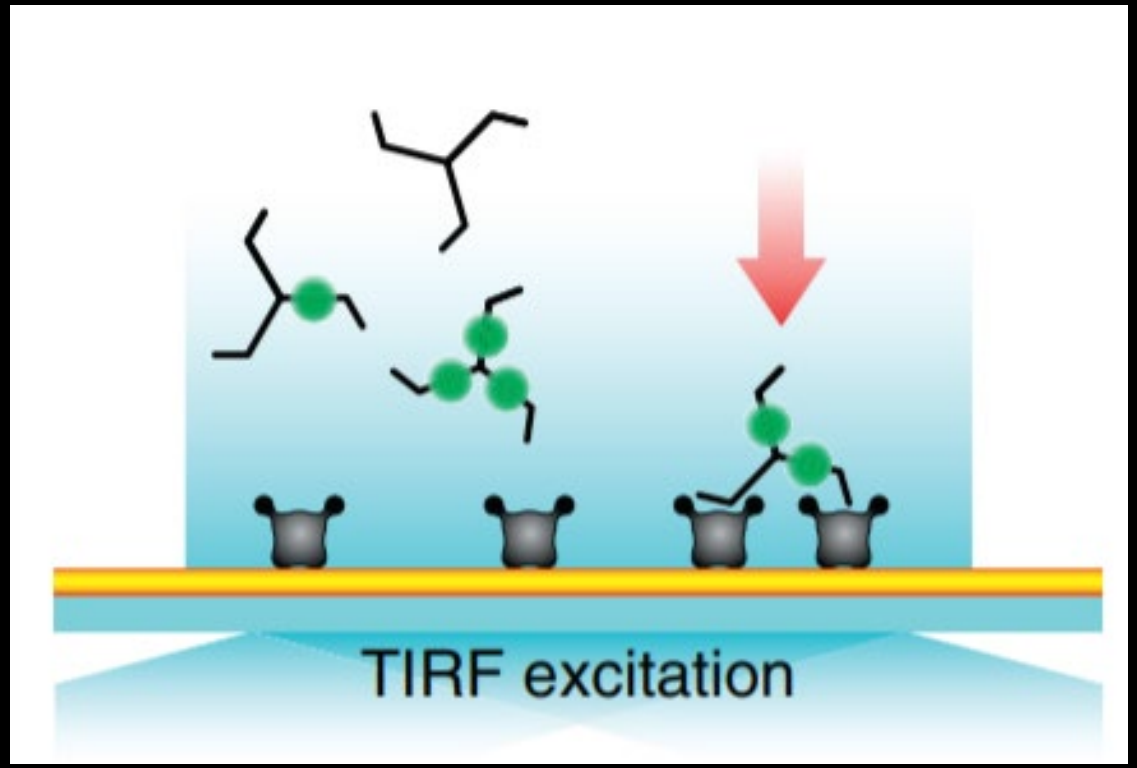
Cell

The First Five Seconds in the Life of a Clathrin-Coated Pit

Emanuele Cocucci,[1,2] François Aguet,[1] Steeve Boulant,[1,2,3] and Tom Kirchhausen[1,2,*]
[1]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
[2]Immune Disease Institute and Program in Cellular and Molecular Medicine at Boston Children's Hospital, Boston, MA 02115, USA
[3]Present address: Laboratory of Viral Infection and Innate Immune Sensing Dynamics, Ruprecht-Karls-Universität, Heidelberg 69117, Germany
*Correspondence: kirchhausen@crystal.harvard.edu
http://dx.doi.org/10.1016/j.cell.2012.05.047

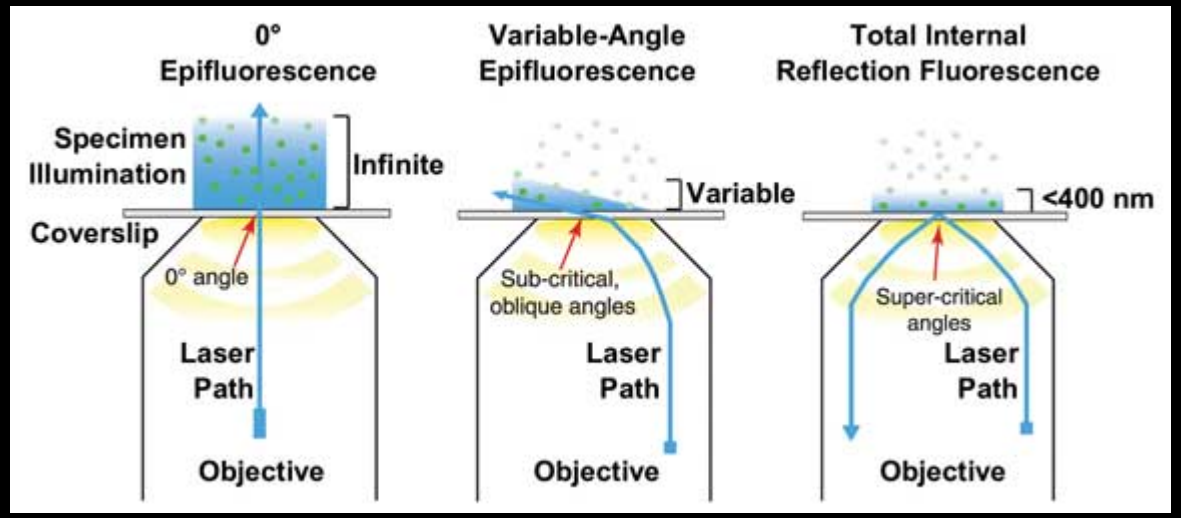
Set-up sperimentale

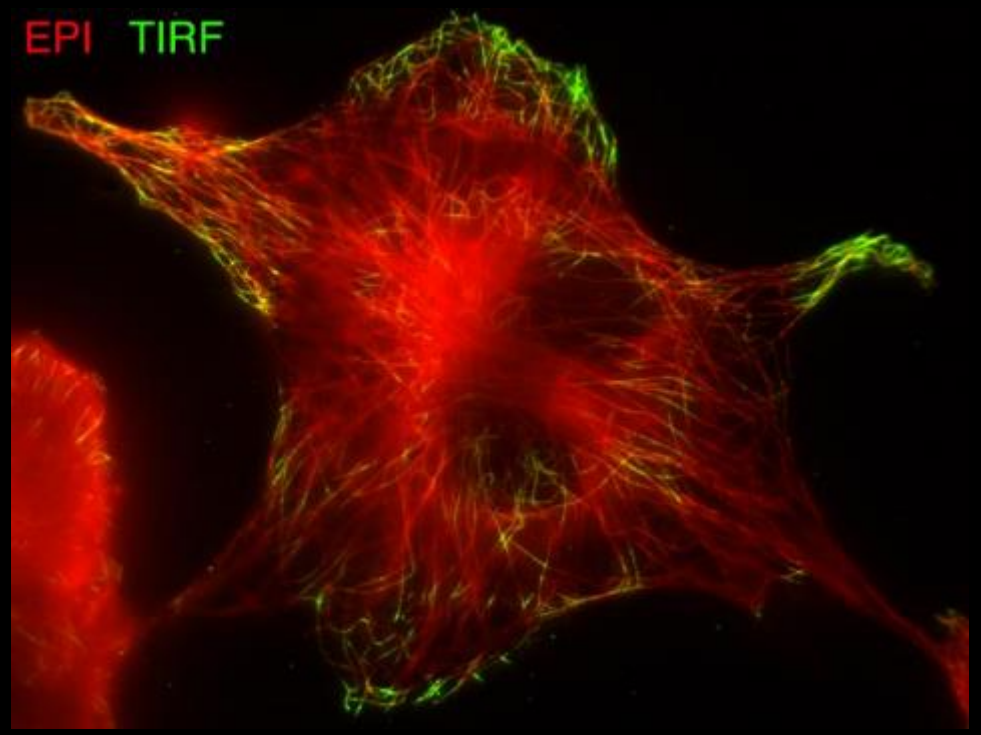
Contiamo il numero di molecule di light chain che si assemblano nella vescicola nascente



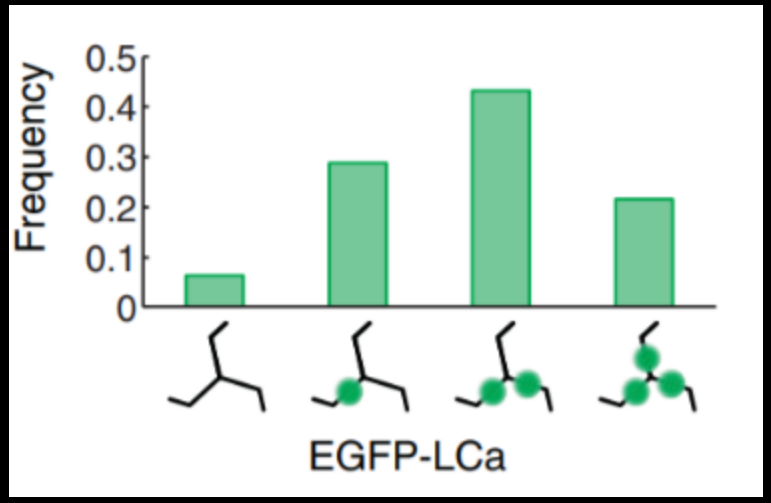
TIRFM

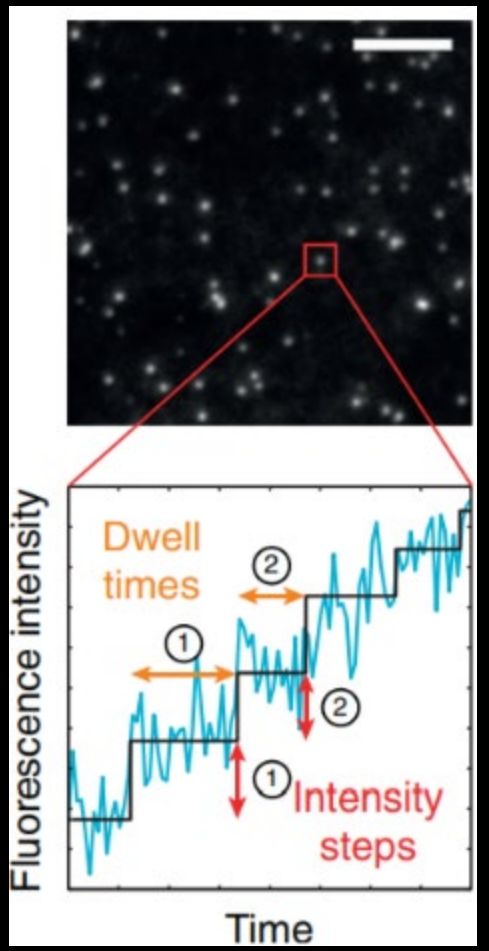
In condizioni particolari la luce di eccitazione puo' essere mandata fuori dall'obiettivo con una profondita' massima di 400nm



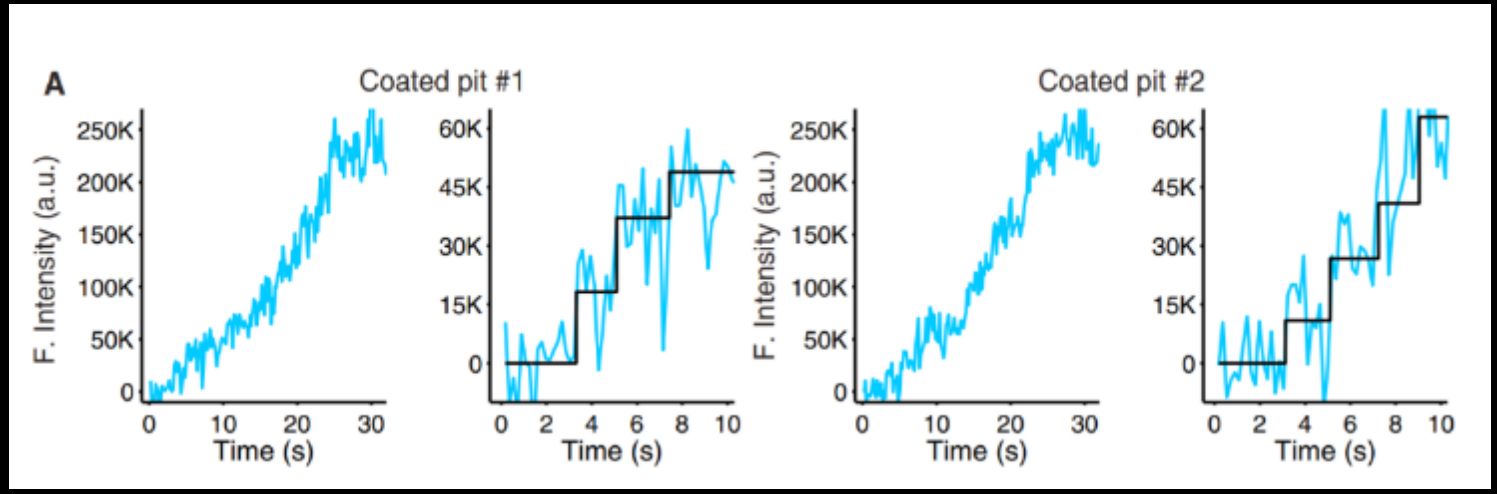


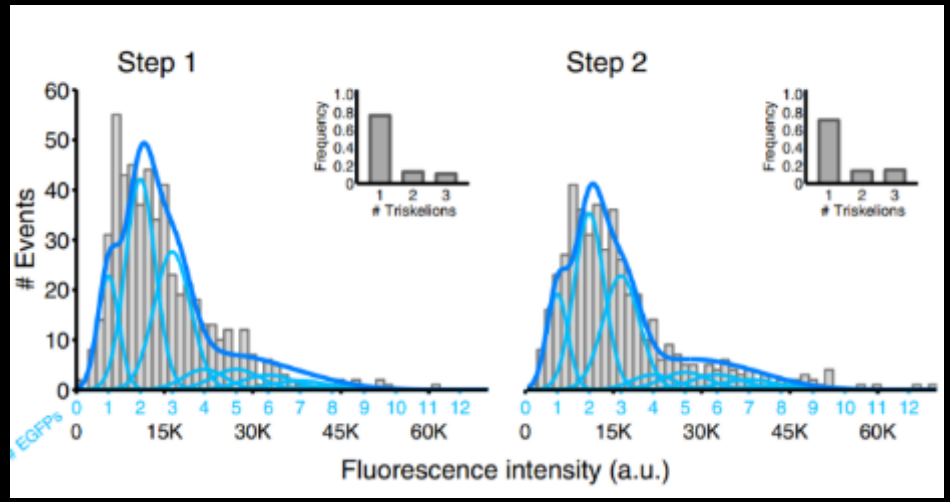
Cinetica a singola molecola



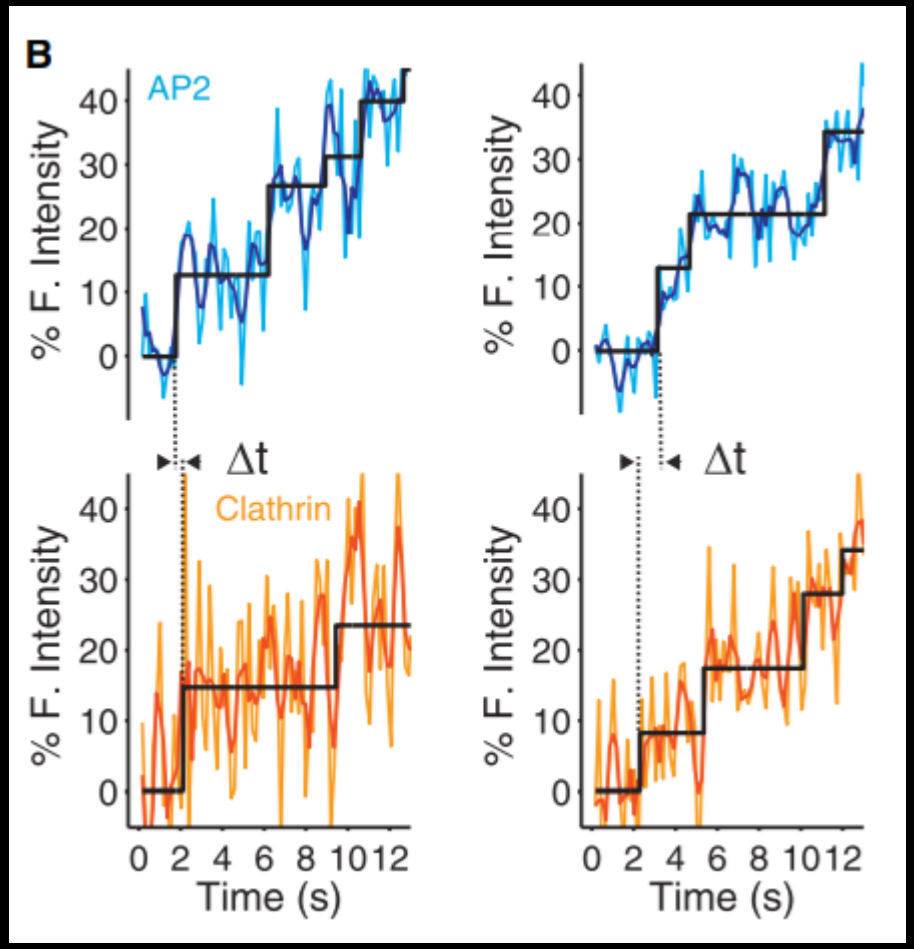


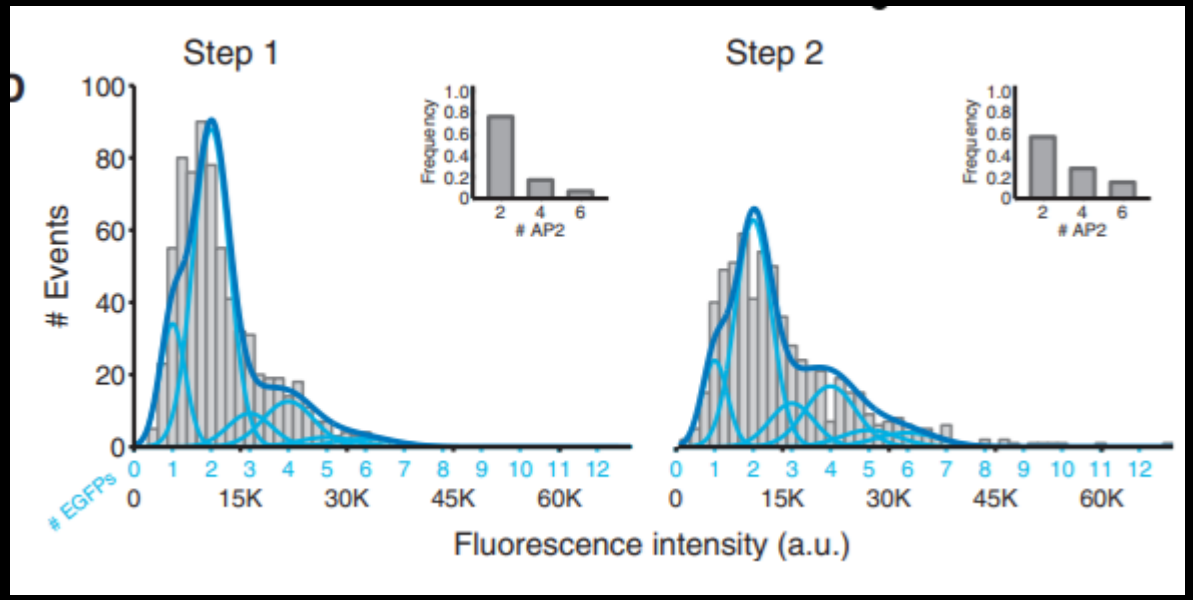
Step di multipla intensita’ nei primi secondi di vita della vescicola



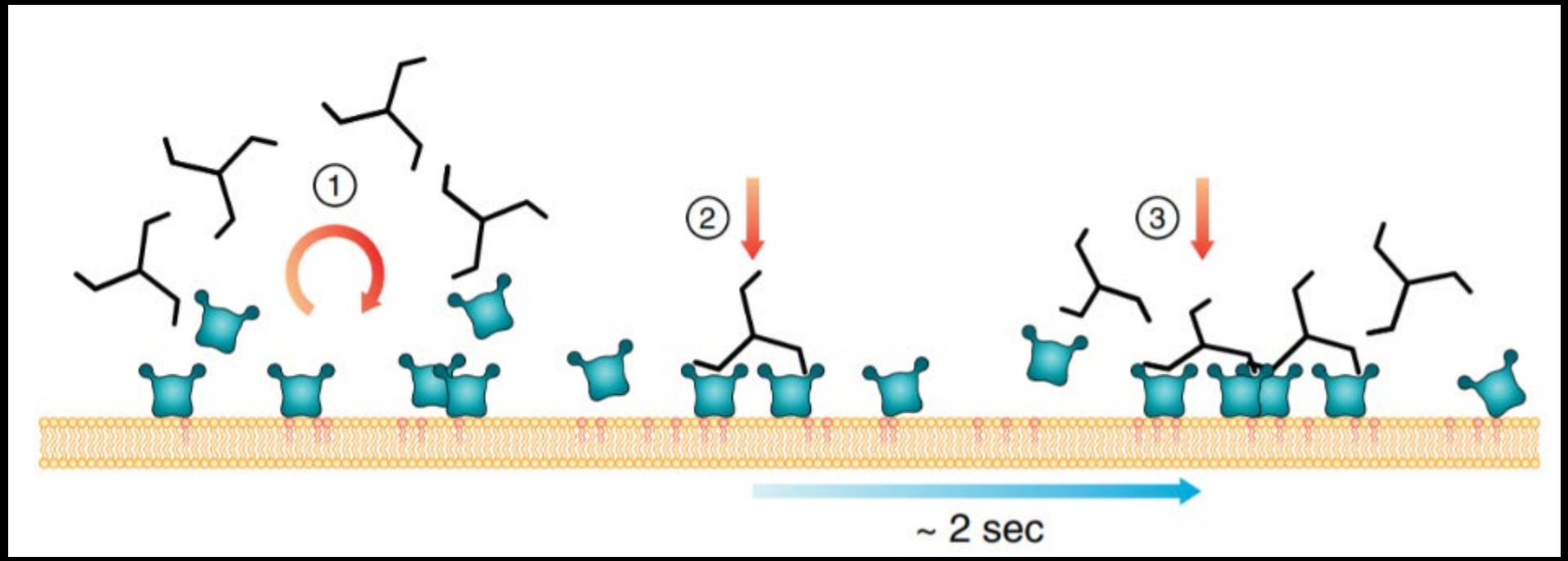


Il recettore AP2 aggrega dopo o assieme a clatrina



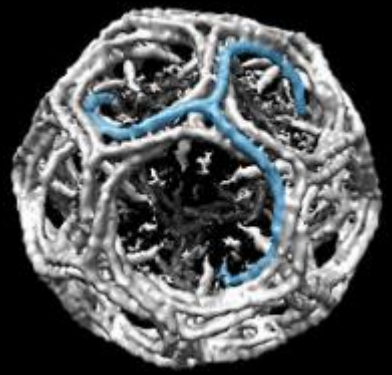


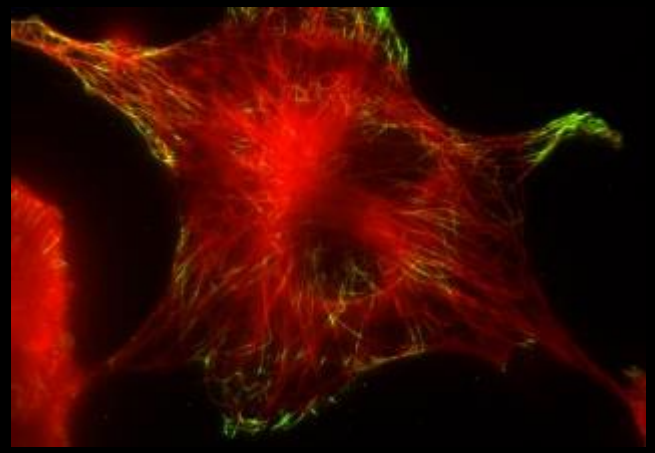
Modello di formazione del complesso di clatrina



Sotto I 200 nm: SUPER RESOLUTION

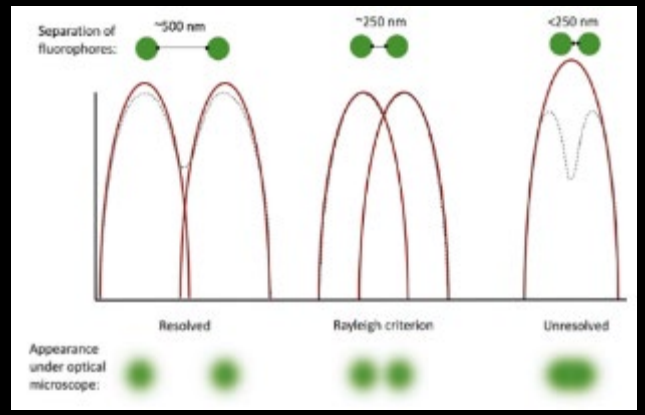
Biologia cellulare → Biochimica



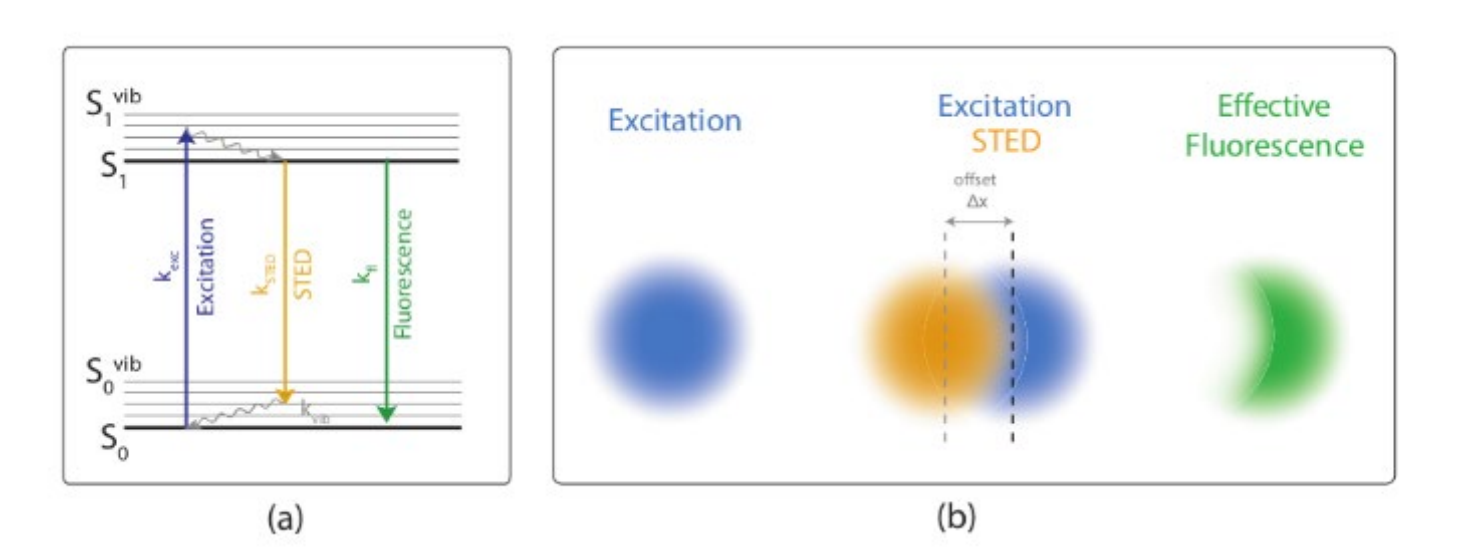


<200-250 nm
Limite di Abbe

Un oggetto illuminato non puo' essere risolto con un dettaglio minore di: $d=\lambda/2NA$

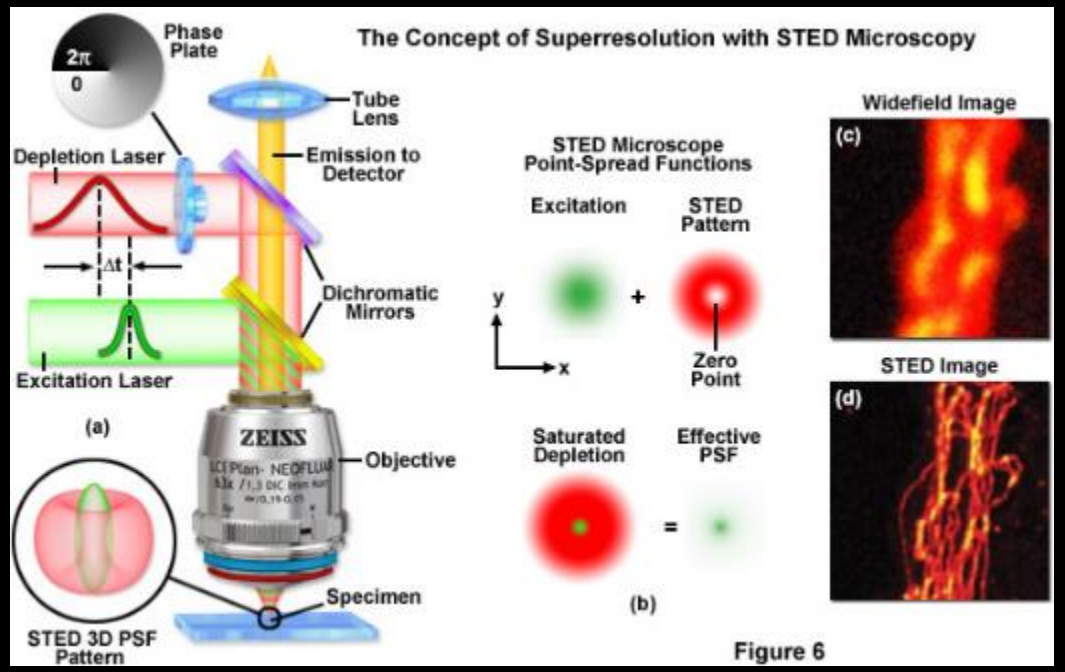


STED: Stimulated emission depletion

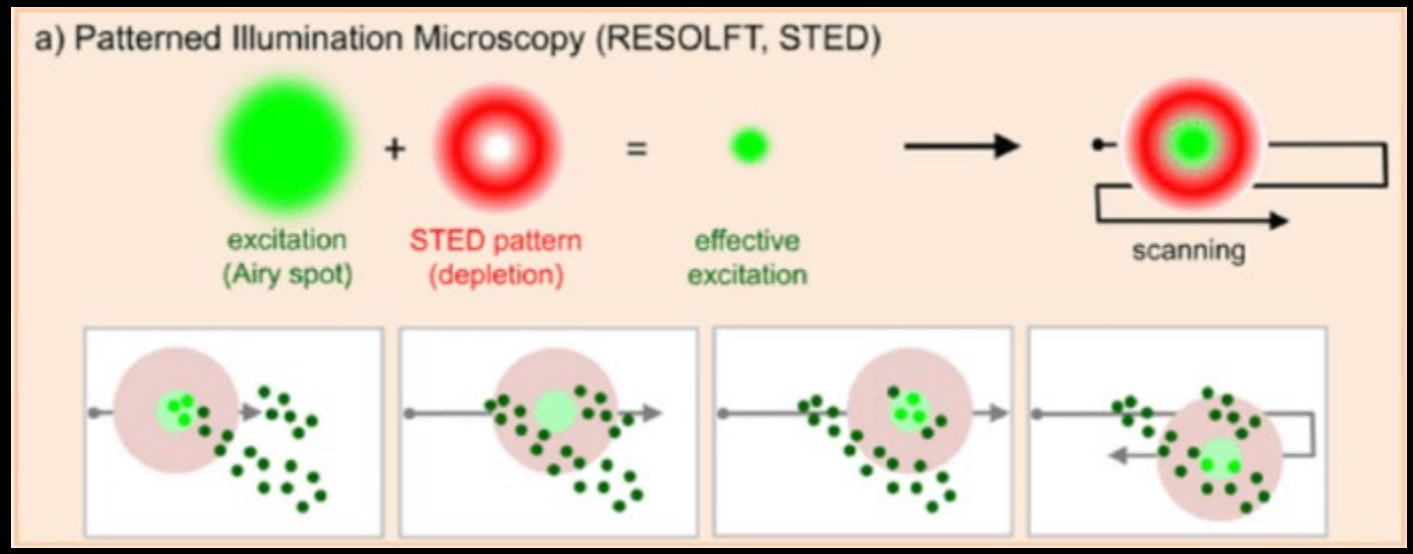


- Costringere l'ettrone eccitato a rientrare nello stato basale fornendo ulteriore energia
- L'emissione forzata é a lunghezza d'onda diversa da quella emessa spontamneamente (tipicamente, ma non necessariamente maggiore)

STED: Stimulated emission depletion

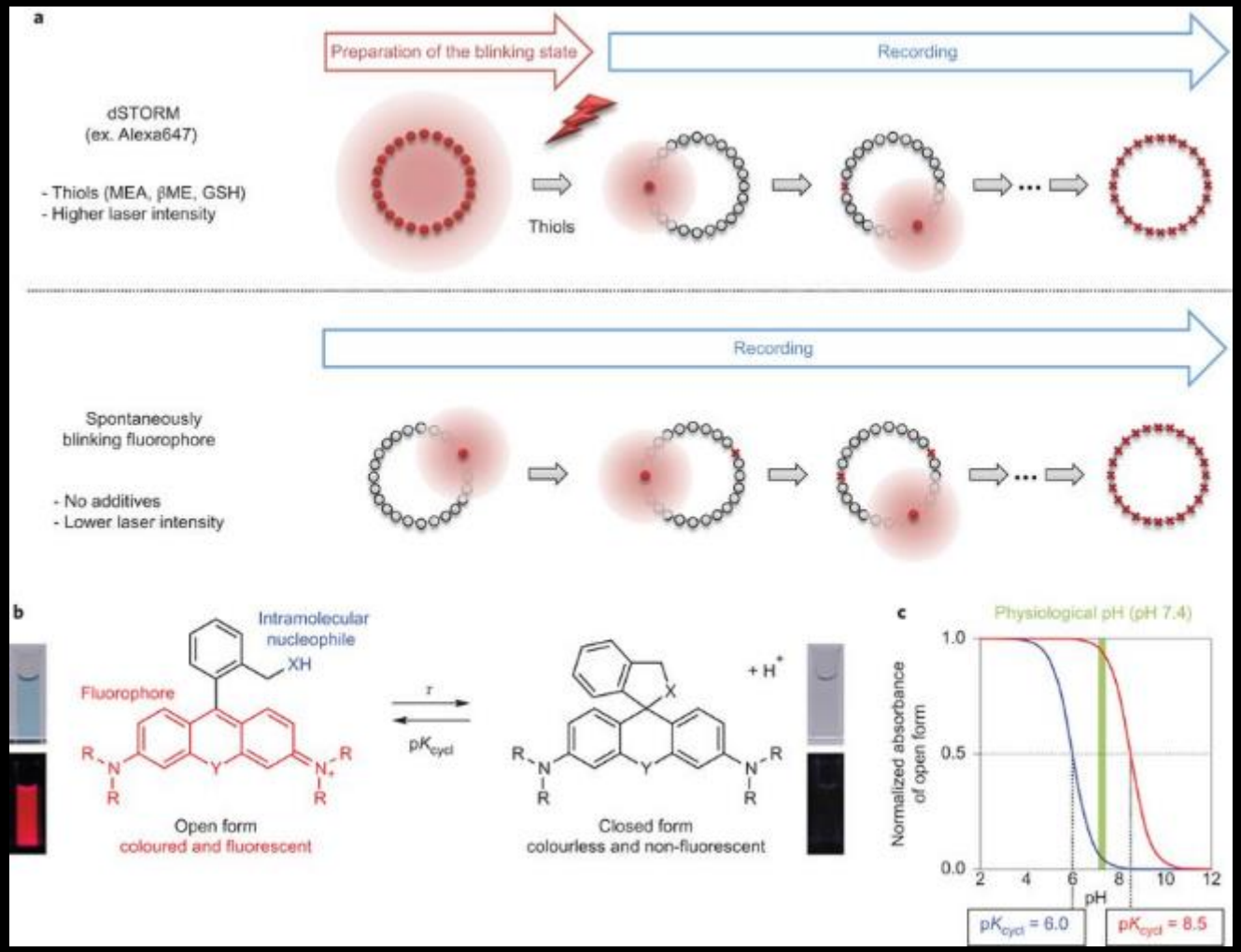


- Accoppiare un percorso ottico di emissione ad uno di deplezione per ridurre la zona di eccitamento
- Raggiunge tipicamente I 20 nm di risoluzione



http://zeiss-campus.magnet.fsu.edu/tutorials/superresolution/stedfundamentals/indexflash.html

Alcuni fluorofori “lampeggiano”



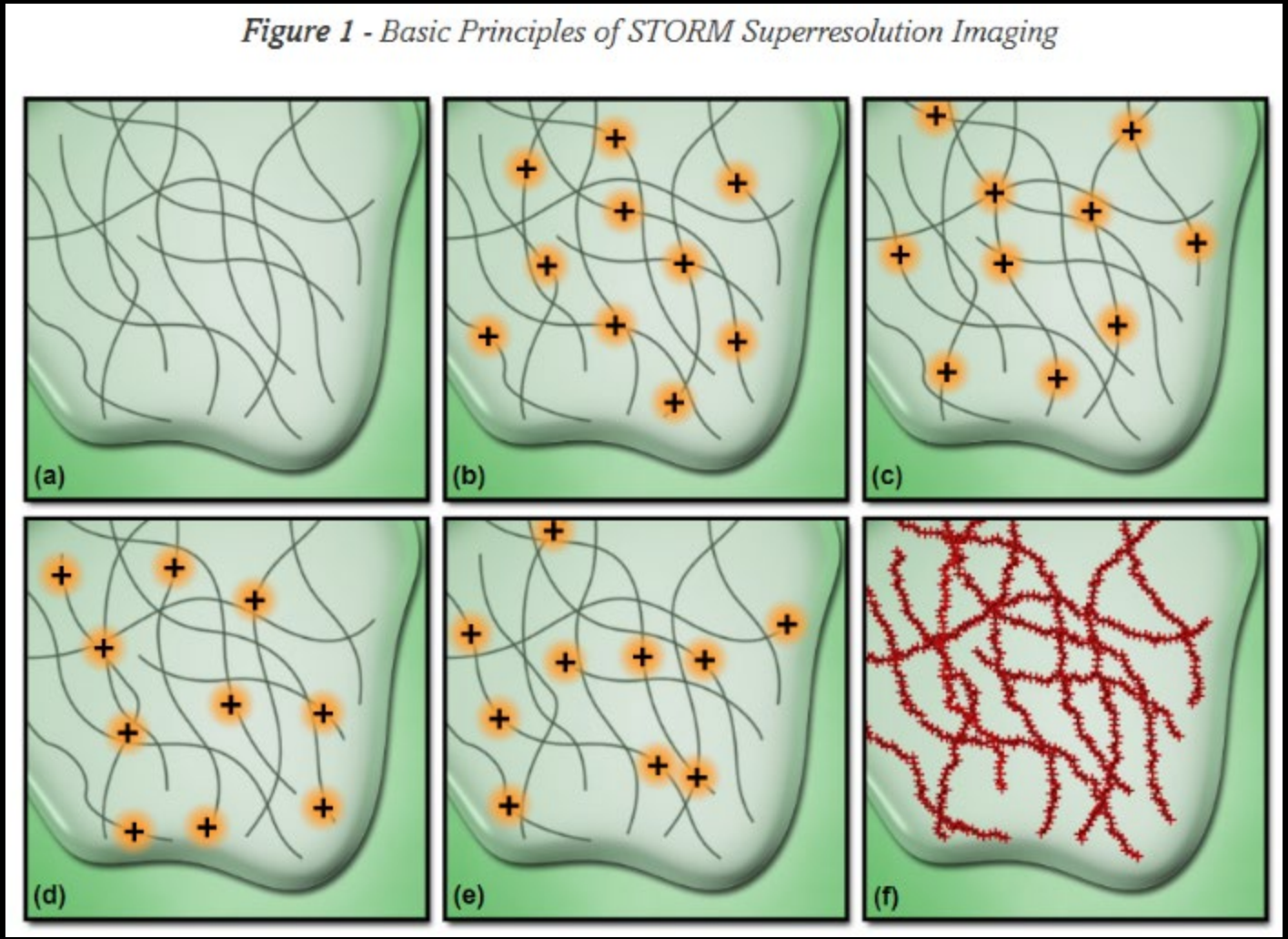
La fluorescenza puo’ essere uno stato transitorio e reversibile (spontaneamente o stimolato).

$$\text{fluo} \underset{K'}{\overset{K}{\rightleftharpoons}} \text{Non fluo}$$

Determinanti K e K’:

- *Tioli,*
- *Luce UV,*
- *proprieta’ intrinseca del fluoroforo,*
- *TBD*

Stochastic Optical Reconstruction Microscopy (STORM)



https://www.microscopyu.com/tutorials/stochastic-optical-reconstruction-microscopy-storm-imaging


That's all Folks!