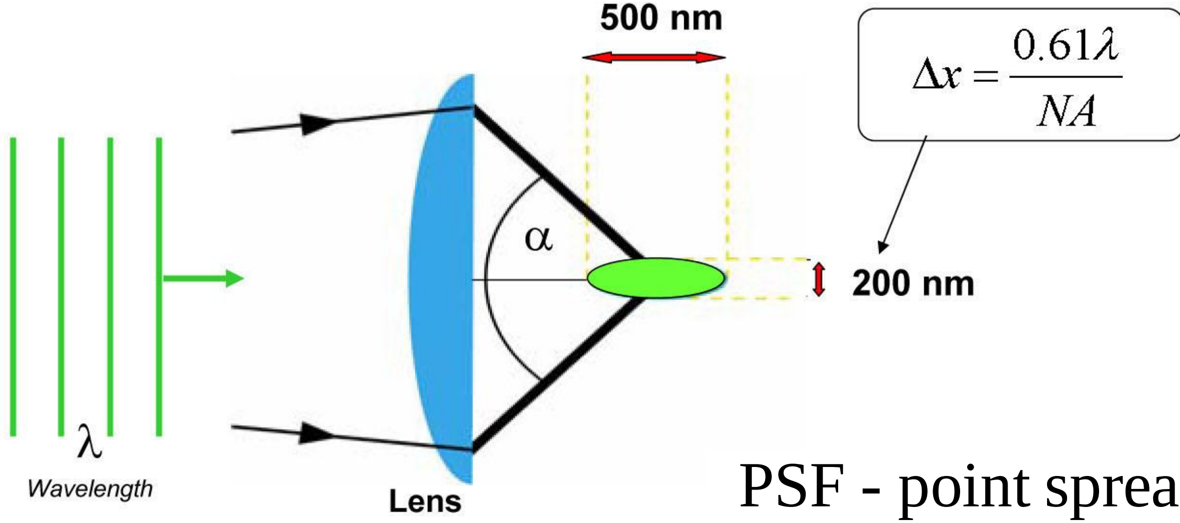
Mikroskoobiga nanoskoopiasse?

Difraktsioonipiir

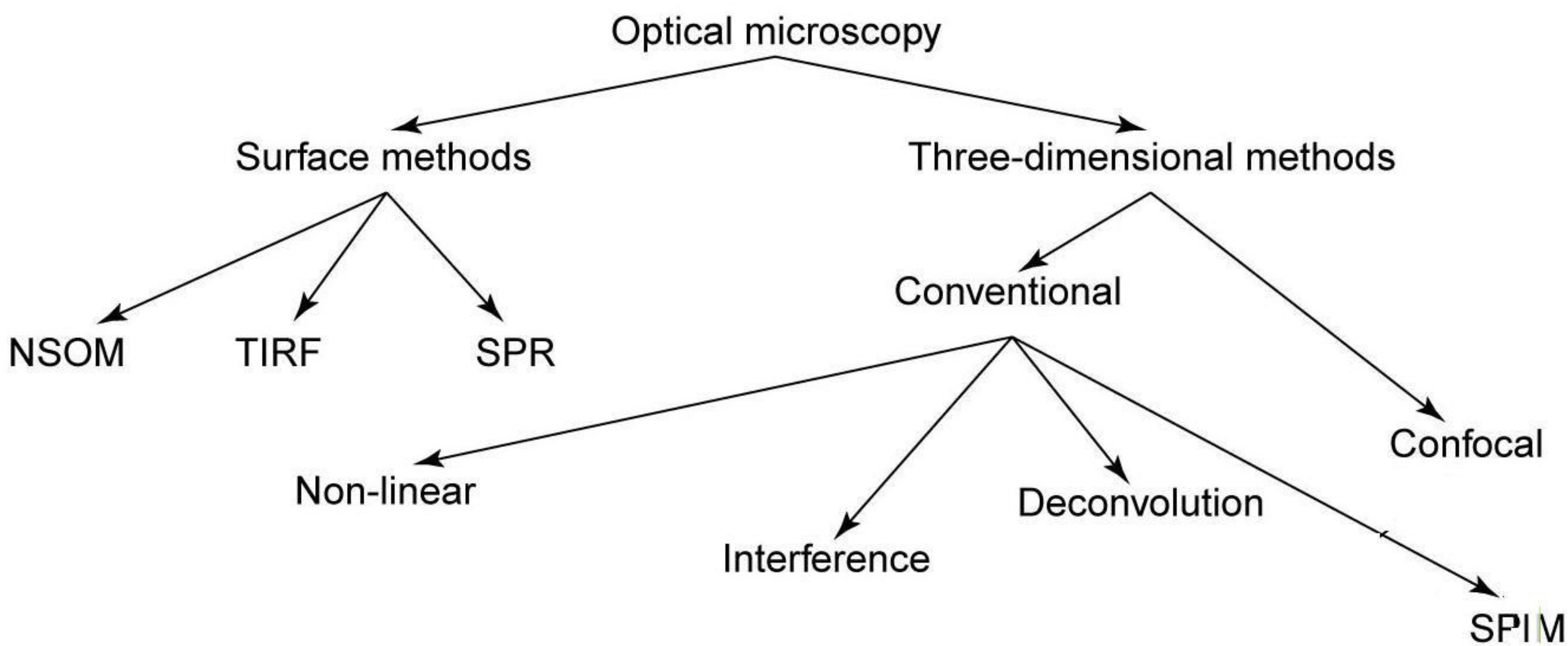
$$\Delta z = \frac{2\lambda}{NA^2}, \quad NA = n \sin \alpha$$

$$\Delta x = \frac{0.61\lambda}{NA}$$

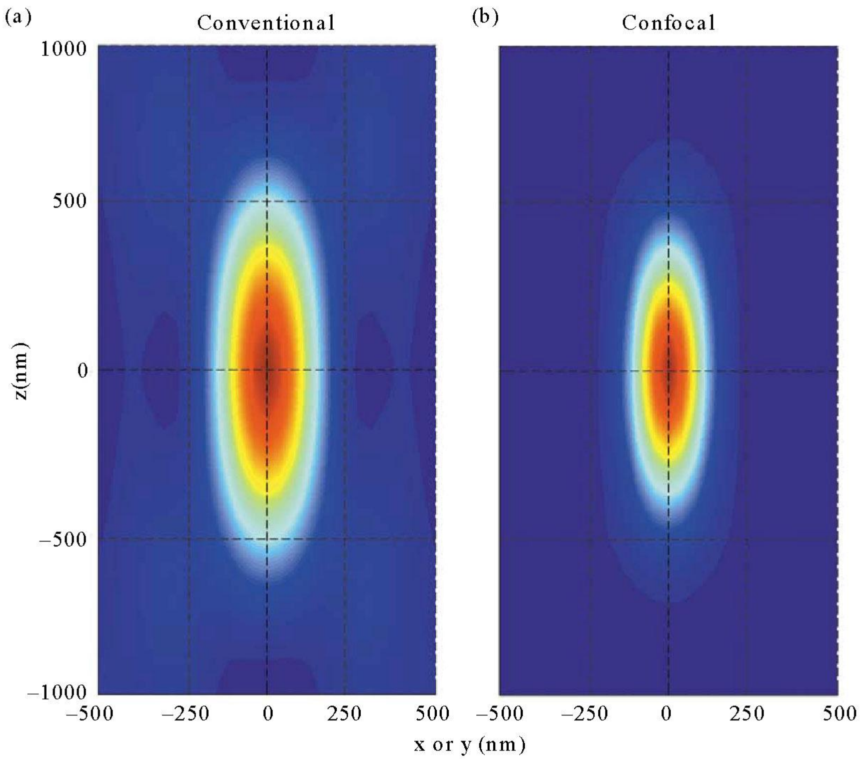


PSF - point spread function
(punkti ülekandejaotus)

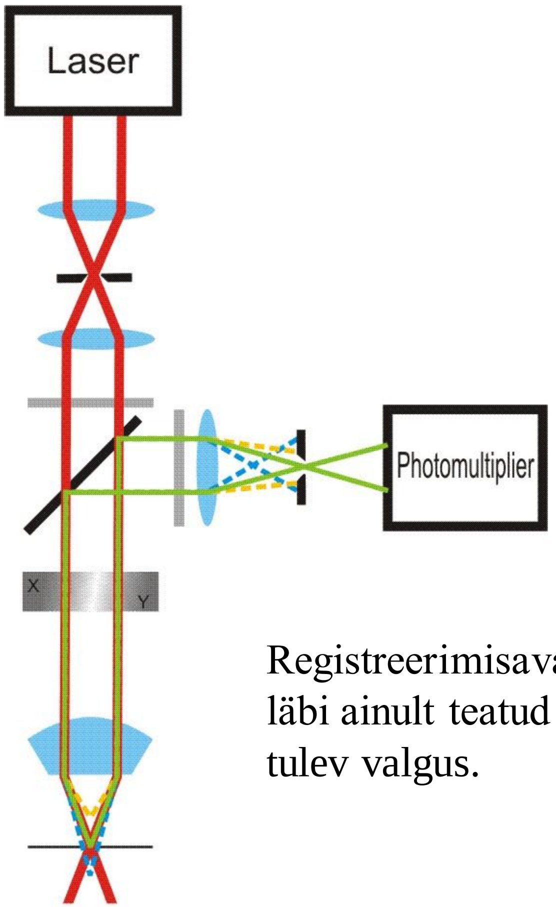
Kuidas difraktsioonipiiri ületada?



Konfokaalmikroskoopia

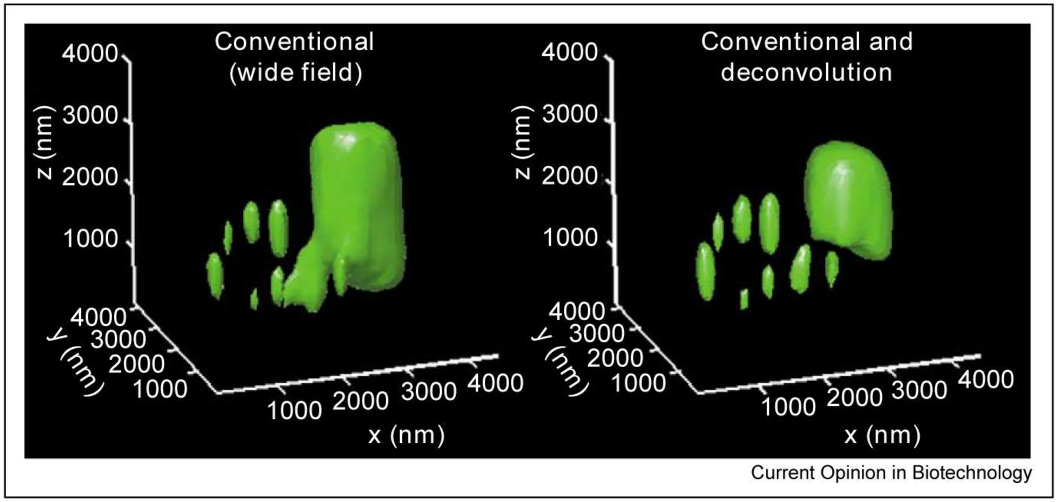


PSF: NA=1, λ=500 nm



Registreerimisavast pääseb läbi ainult teatud tasandist tulev valgus.

Dekonvolutsioon



The deconvolution effect. **(a)** Raw data and **(b)** deconvolved data of a small part of the nucleus where telomeres have been labeled with Cy3. Fluorescence images were measured with Axioplan 2 (Zeiss) with a Planapo 63×/1.4 oil immersion lens. In the raw image, some of the telomeres cannot be resolved (touching), but in the deconvolved image the telomeres are completely resolved. Deconvolution method is based on [5].

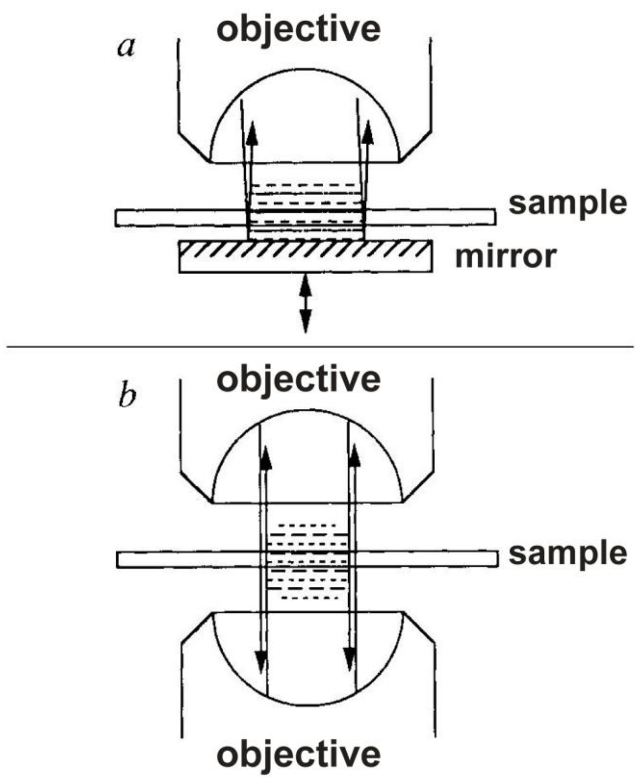
- Vaja täpsemalt teada PSF (aparaatfunktsiooni)
- Müravaba signaal

Interferentsmeetodid

Lihtsaim idee: seisulained

(SWM – standing wave microscopy)

- 4Pi
- I^5M
- ApoTome
- HELM

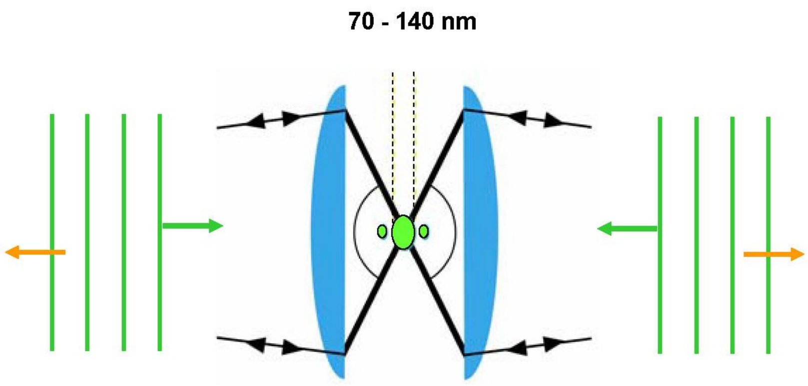


Kõrvalmaksimumide segavat mõju raske elimineerida.

Seetõttu kasutatakse sageli kombinatsioonis teiste meetoditega.

4Pi mikroskoopia

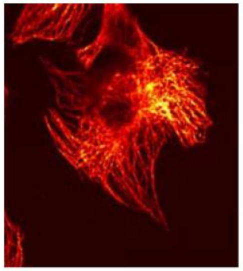
4Pi- Microscopy:



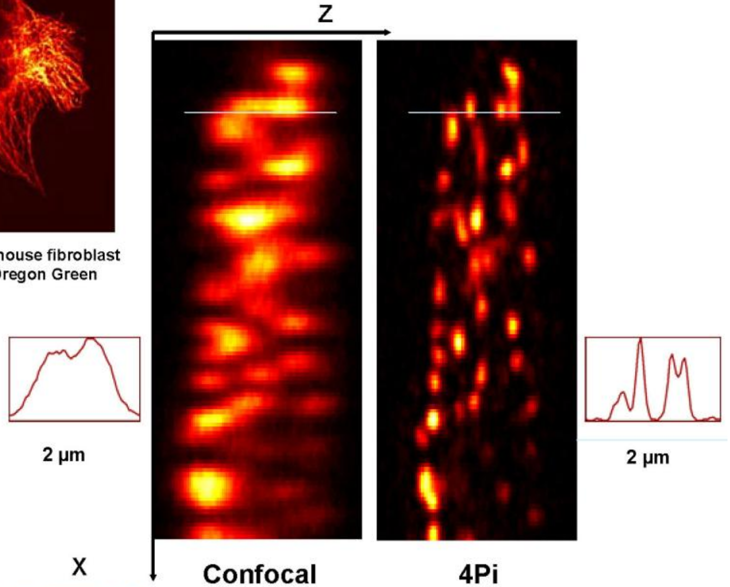
$$\vec{E}^{4Pi}(r,z,\varphi)=\vec{E}_1(r,z,\varphi)+\vec{E}_2(r,-z,\varphi)$$

Coherent illumination and/or fluorescence detection

S.W. Hell (1990), *Europ. Patent* OS 0491289.
S.W. Hell, et al. (1992), *Opt. Commun.* **93**, 277.
M. Schrader, et al. (1998), *Biophys. J.* **75**, 1659.
H. Gugel, et al. (2004), *Biophys. J.* **87**, 4146.



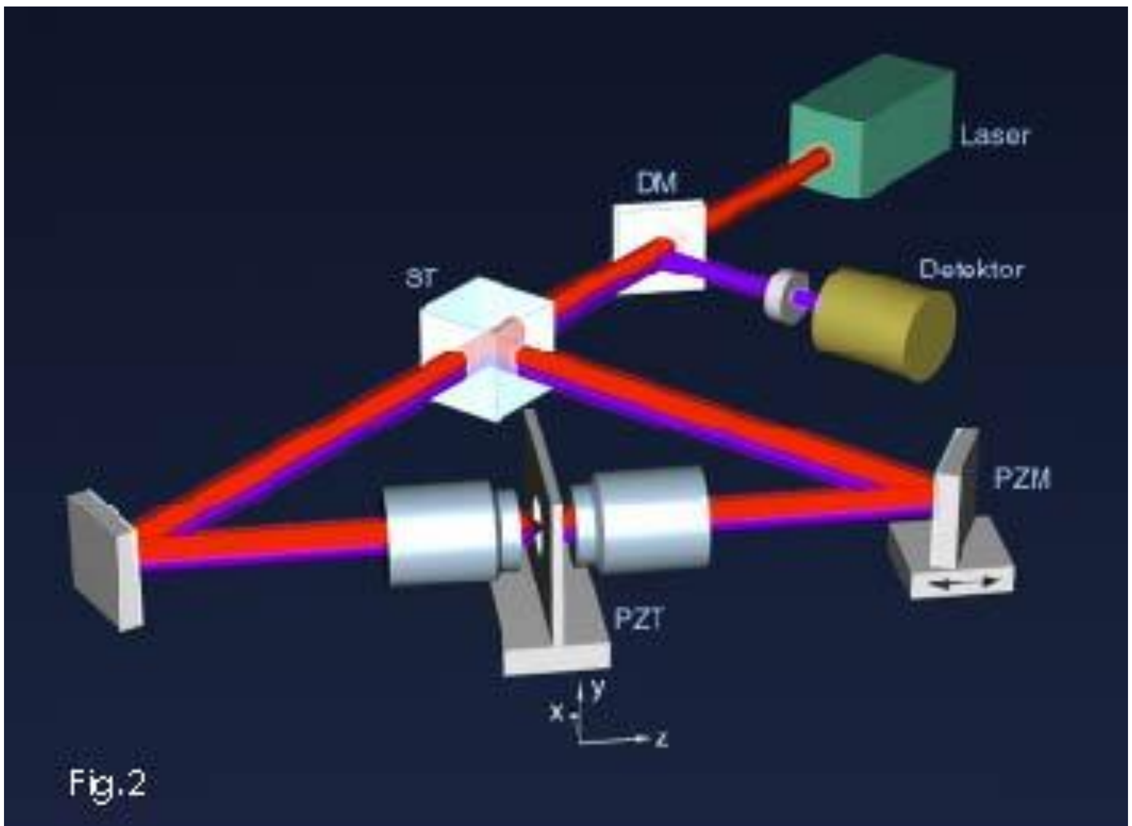
Microtubules, mouse fibroblast
Immunofluor, Oregon Green



S.W. Hell, et al. (1992), *Opt. Commun.* **93**, 277.
M. Schrader, et al. (1998), *Biophys. J.* **75**, 1659.
H. Gugel, et al. (2004), *Biophys. J.* **87**, 4146.

4Pi mikroskoopia

•**4Pi-A**: interfereeruvad ainult ergastava kiirguse lainefrondid; lahutus pikitelje suunas paraneb 3-4 korda (~140 nm),
•**4Pi-C**: ka signaal registreeritakse mõlemas kanalis, mis interfereeruvad detektoril; lahutus pikitelje suunas paraneb 5-7 korda (kuni **70 nm**).



I^2M, I^3M, I^5M

Image Interference Microscopy = I^2M

\+ Incoherent Interference Illumination Microscopy = I^3M

= I^5M

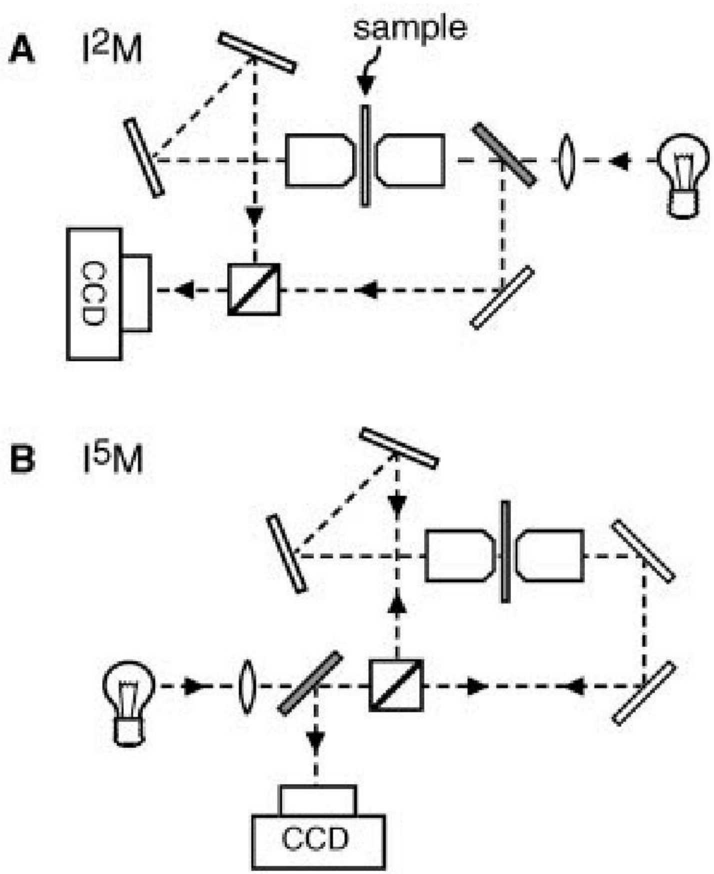


Fig. 1. Concept of I^2M, where the sample is observed from both sides (A), and I^5M, where it is also illuminated from both sides (B).

Kasutatakse mittekoherentset valgusallikat (lampi) Köhleri režiimis, s.o. objektiivi(de)st väljub kollimeeritud kiirtekimp.

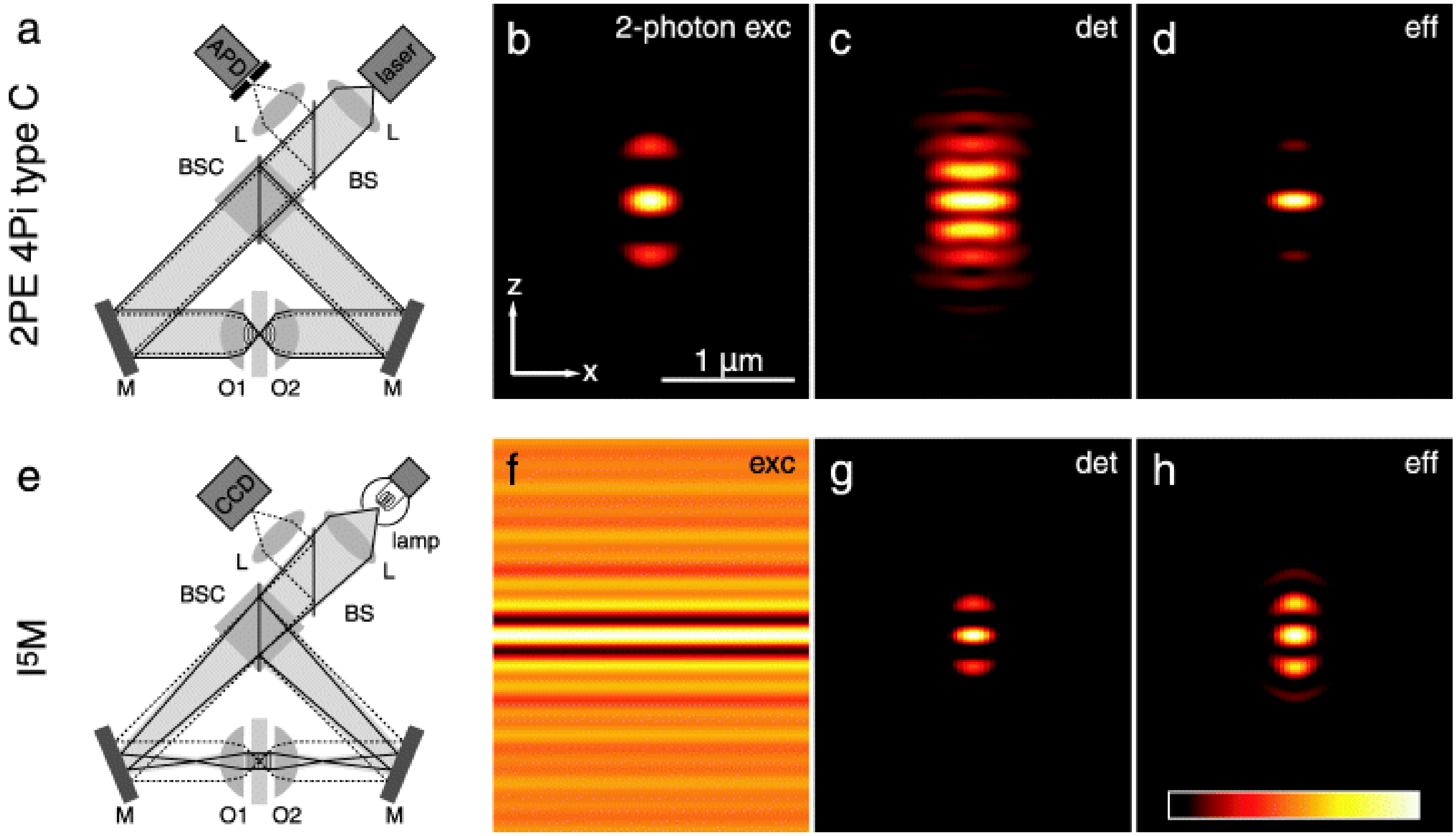
\+ korraga võimalik vaadata tasandilist pilti (ei vaja skaneerimist);

– kõrvalmaksimumid suuremad, raskem vabaneda.

• **Saavutatav pikilahutus ~100 nm**

4Pi ja I^{5}M võrdlus

Point spread function

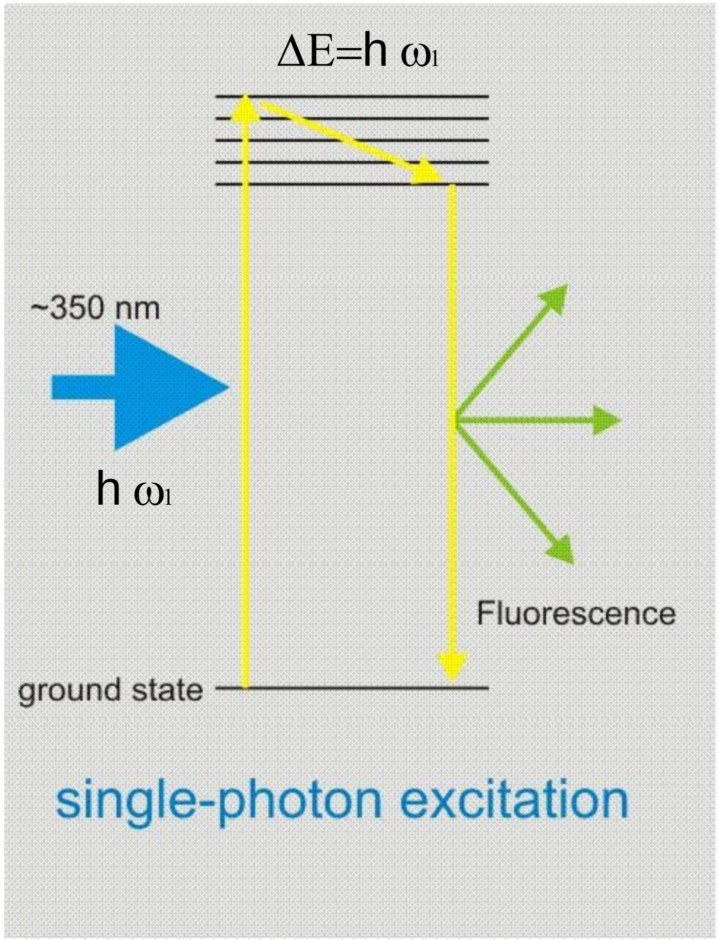


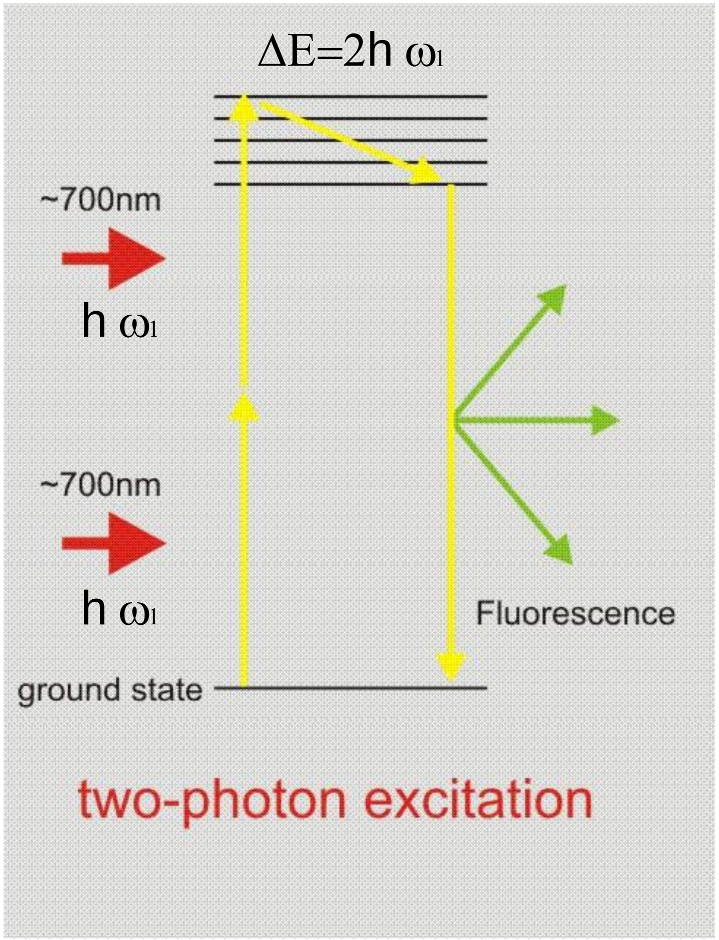
Mittelineaarsed meetodid

- multifotoonsed protsessid
    - 2- (mitme-) fotoonne ergastus
    - teise harmooniku genereerimine
    - CARS
- pööratav küllastumine (reversible saturation)
    - STED
    - SSIM

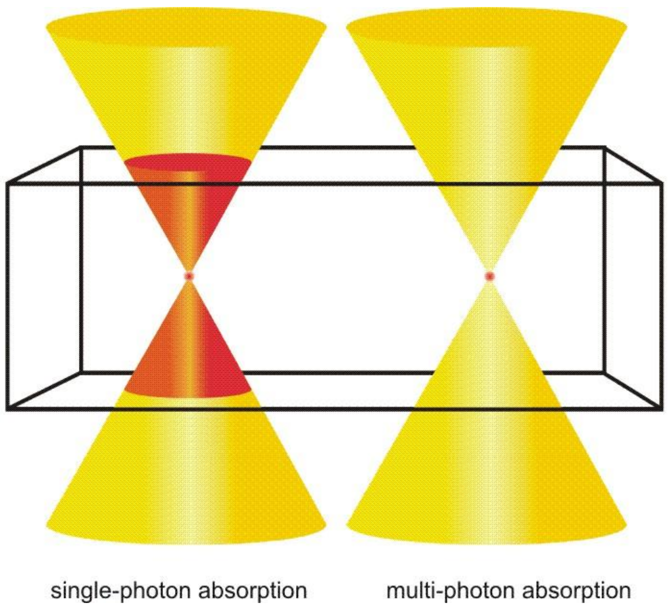
Idee: Signaali mittelineaarne sõltuvus ergastusest võib efektiivselt vähendada piirkonda, kus signaali arvestataval määral tekib.

Kahefotoonne ergastus (energeetiliselt)

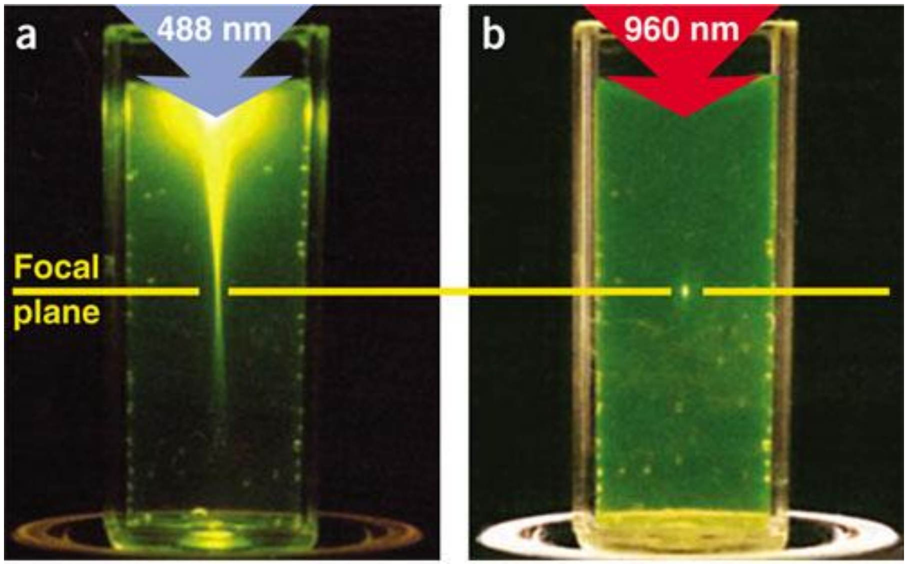




Kahefotoonne ergastus (ruumiliselt)



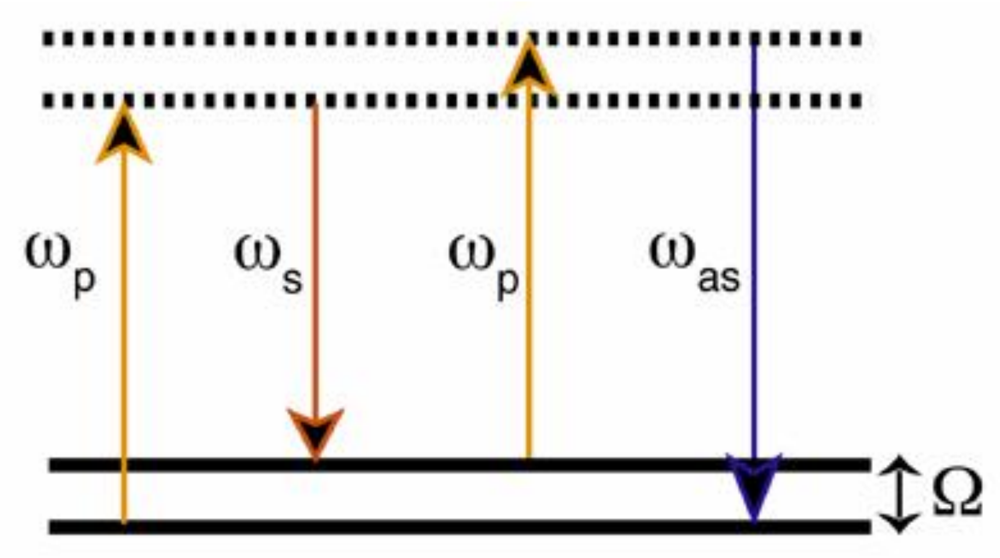
$I_{flu} \sim I_{ex}$ $\qquad$ $I_{flu} \sim I^2_{ex}$



Lahutus vastab umbkaudu konfokaalsele juhule, kuna lainepikkus on suur. Fookusevälise kiirguse tõhusama elimineerimise tõttu on efektiivne lahutus kahefotoonsel juhul siiski sageli parem.

CARS mikroskoopia

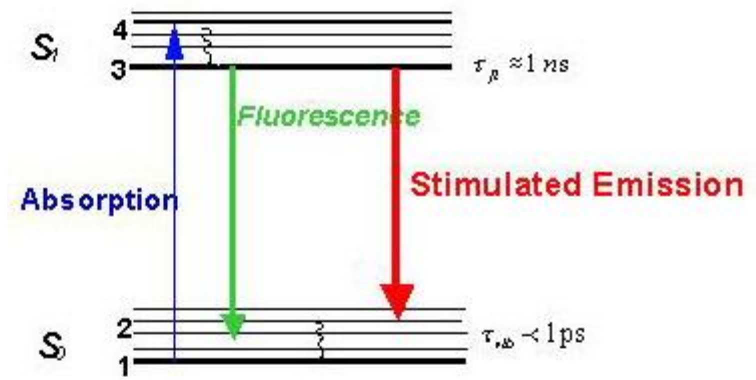
(Coherent Anti-Stokes Raman Scattering)



- 3. järku mittelin. protsess → lahutus
- Samas resonantne → väiksemad ergastusvõimsused
- Detekteeritakse otseselt objektis leiduvate struktuuride võnkesagedusi → pole vaja markereid

- Ergastatakse kahe sagedusega ω_p ja ω_s
- Kui objektis mingi võnkesagedus $\Omega = \omega_p - \omega_s$, tekib resonantne signaal anti-Stokesi sagedusel $\omega_{as} = 2\omega_p - \omega_s$

STED
(Stimulated emission depletion)

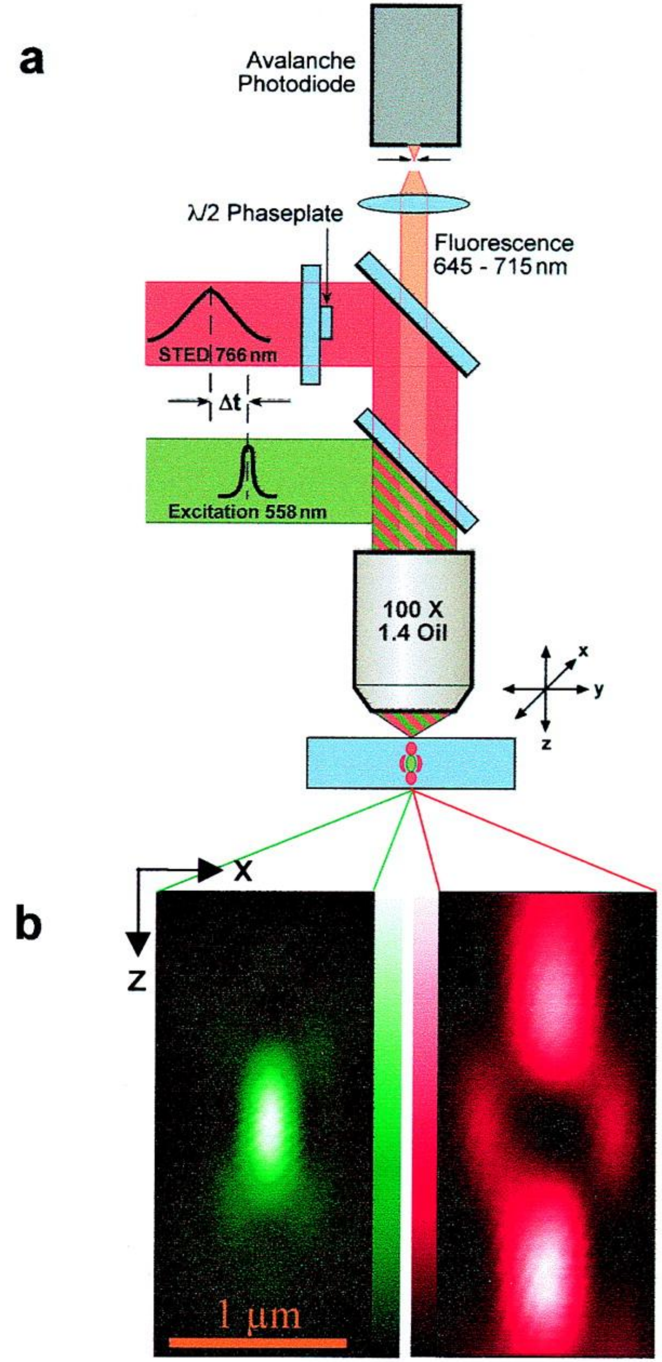


• Ergastuspulss tekitab ergastuse difraktsioonpiiratud alas;

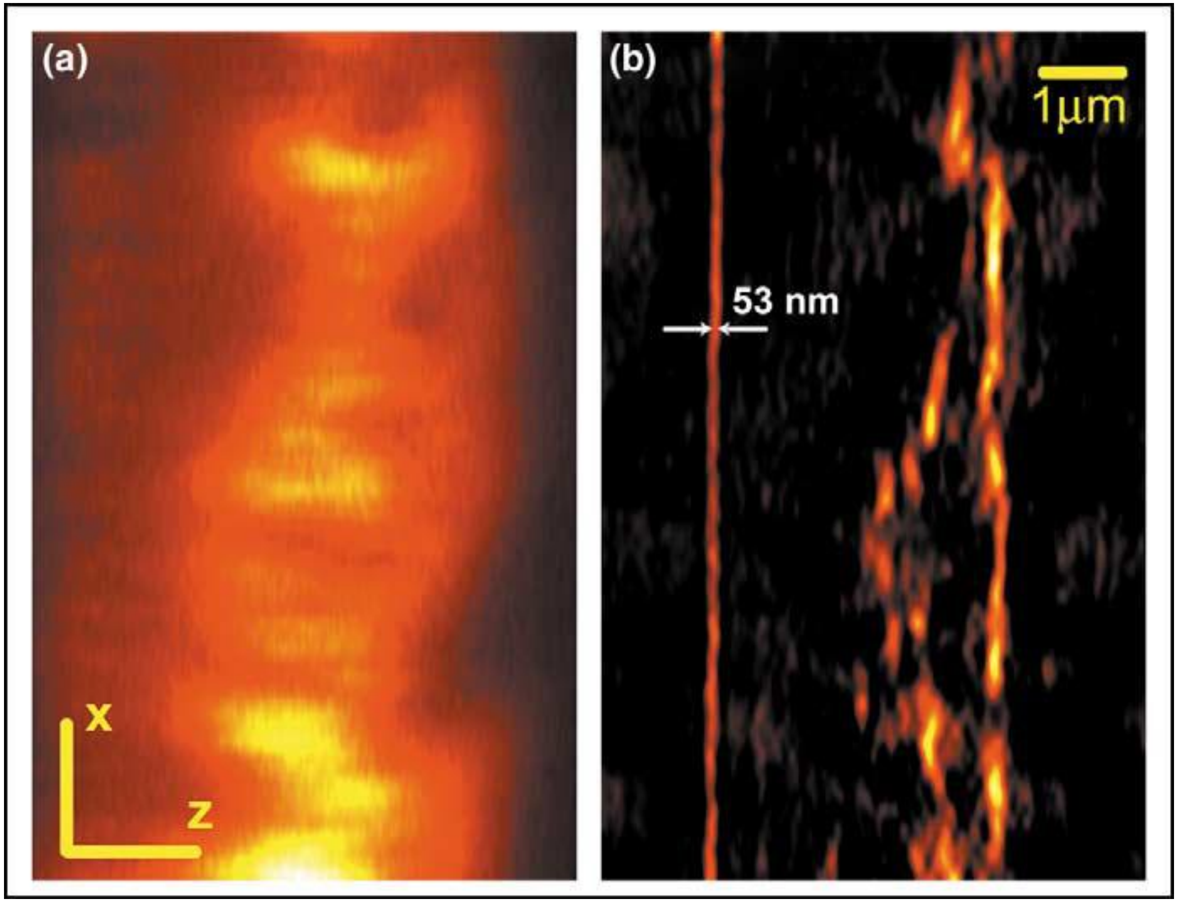
• talle järgnev tühjenduspulss (STED-pulss) tühjendab ergastatud tasemed rõngakujulises alas ümber ergastuse keskme.

• PSF kuju saavutatakse STED-kiire keskosa laskmisega läbi ringikujulise λ/2 plaadi.

Põhimõtteliselt võib keskmine osa olla kuitahes väike.



STED



confocal STED-4Pi

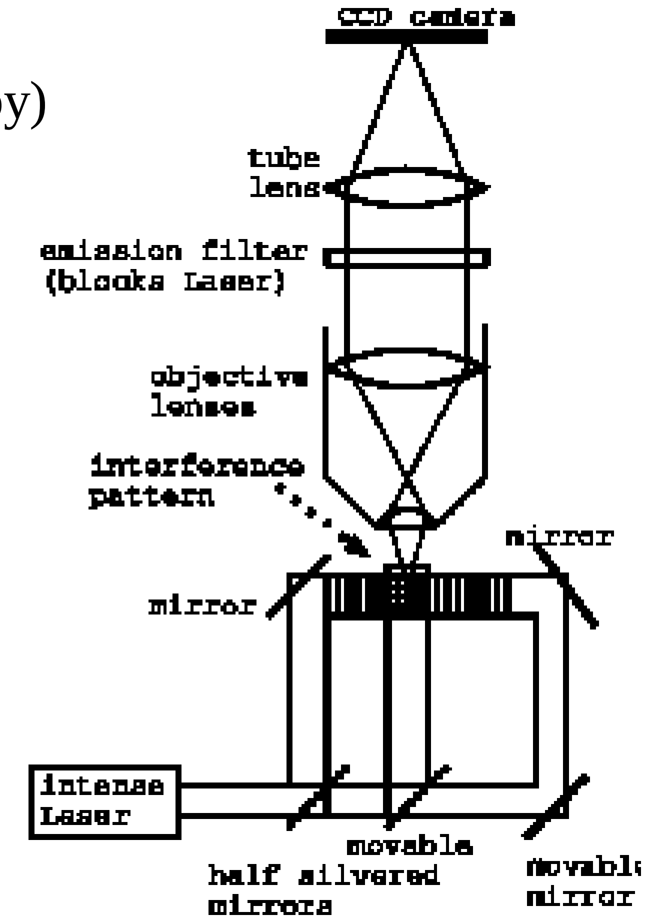
Fluorescently labeled microtubule of a human embryonic kidney labeled with a red-emitting dye (MR 121SE)

Koos 4Pi-ga saavutatud lahutus 30 nm suurusjärgus.

SSIM

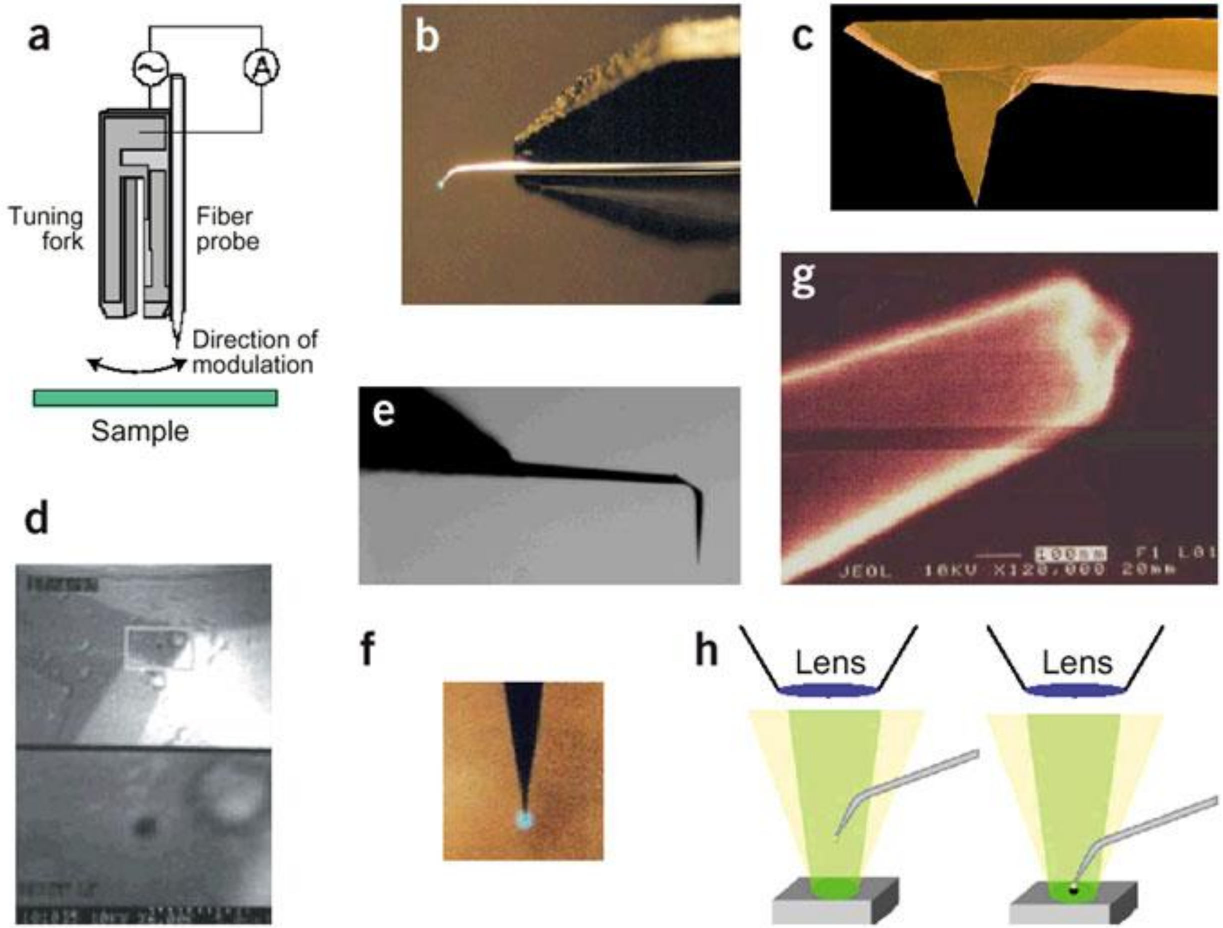
(Saturated structured illumination microscopy)

Objekti peal tekitatakse tugeva valguse abil interferentsipilt, mis küllastab kogu kiirguse, v.a. väikestes ruumiosades maksimumide vahel. Intensiivsuse kasvades muutuvad need väga väikesteks, võrreldavateks STED abil saadud PSF-dega (~50 nm).



üks võimalikest skeemidest

Pinnameetodid: NSOM (SNOM)

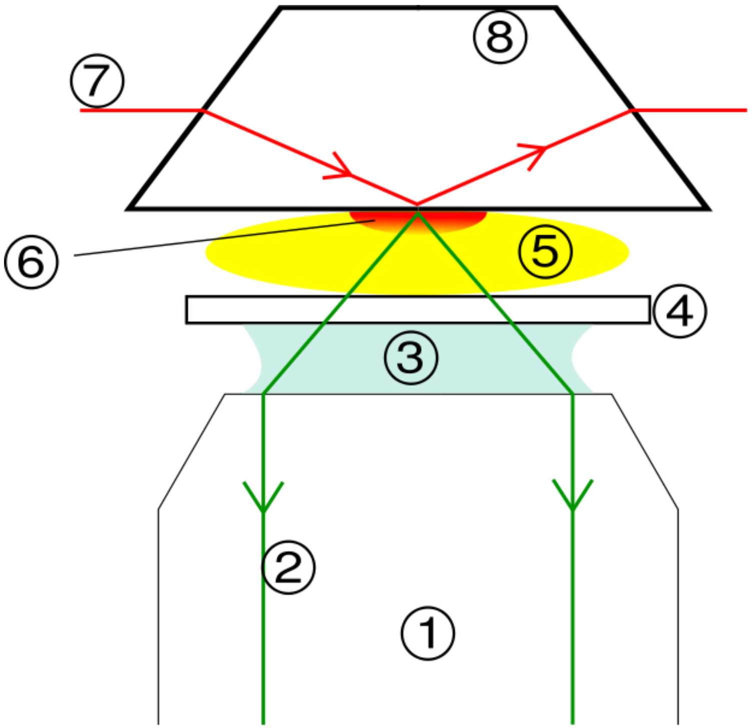


Fiibriotsa läbimõõtu saab vähendada ~20 nm-ni

Pinnameetodid: TIRF

(Total Internal Reflection Fluorescence)

1.Objective
2.Emission beam (signal)
3.Immersion oil
4.Cover slip
5.Specimen
6.Evanescent wave range
7.Excitation beam
8.Quartz prism



Evanescent wave penetration depth ~100 nm

Kokkuvõtteks