

Methoden der Strukturenuntersuchung

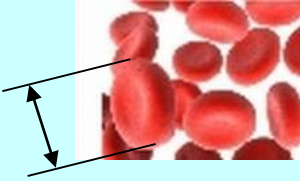
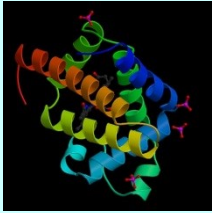
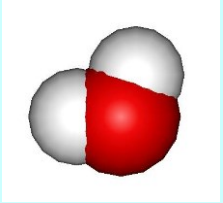

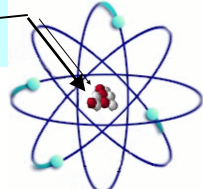
Lichtmikroskopische Techniken
Rastermikroskope

Gusztáv Schay

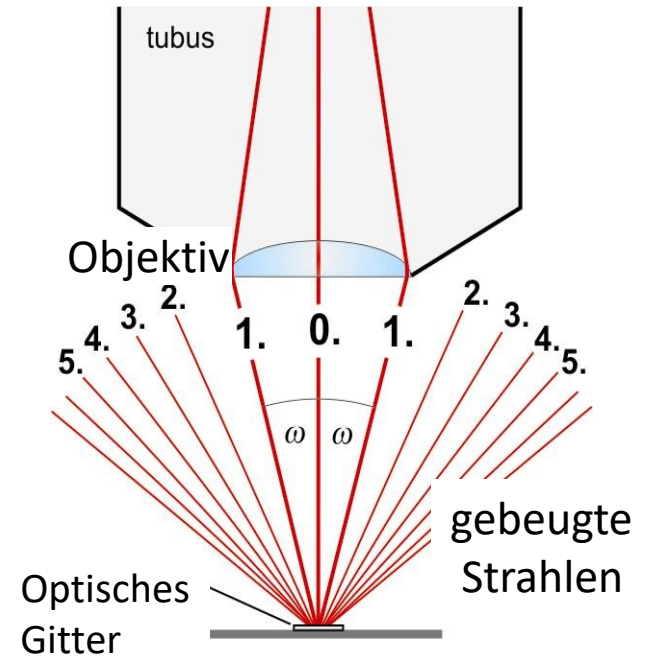
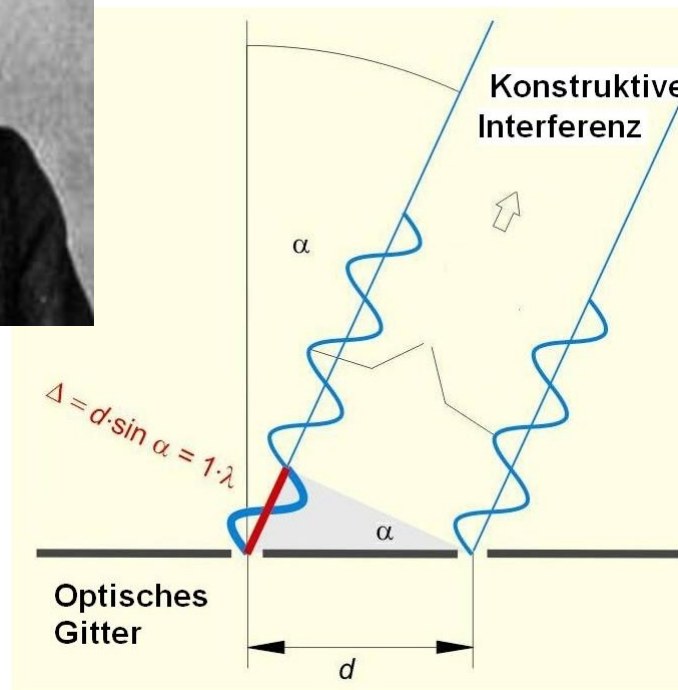


SEMMELWEIS
UNIVERSITY 1769

Typische Grössen

m			
10^0	meter	Mann	
10^{-3}	millimeter	Abstand der man mit Auge sehen kann	
10^{-6}	mikrometer	Zelle (z.B. Blutkörpern)	
10^{-9}	nanometer	Protein	
10^{-10}	– Angström	Durchmesser des Atoms, H Atom $\varnothing \approx 1$ Angström (Å)	
10^{-12}	pikometer	Wellenlänge der Röntgenstrahlung	
10^{-15}	femtométer	Atomkern	

Auflösungsgrenze des Lichtmikroskops



Auflösungsgrenze: $\delta = 0,61 \cdot \lambda / (n \cdot \sin \omega)$

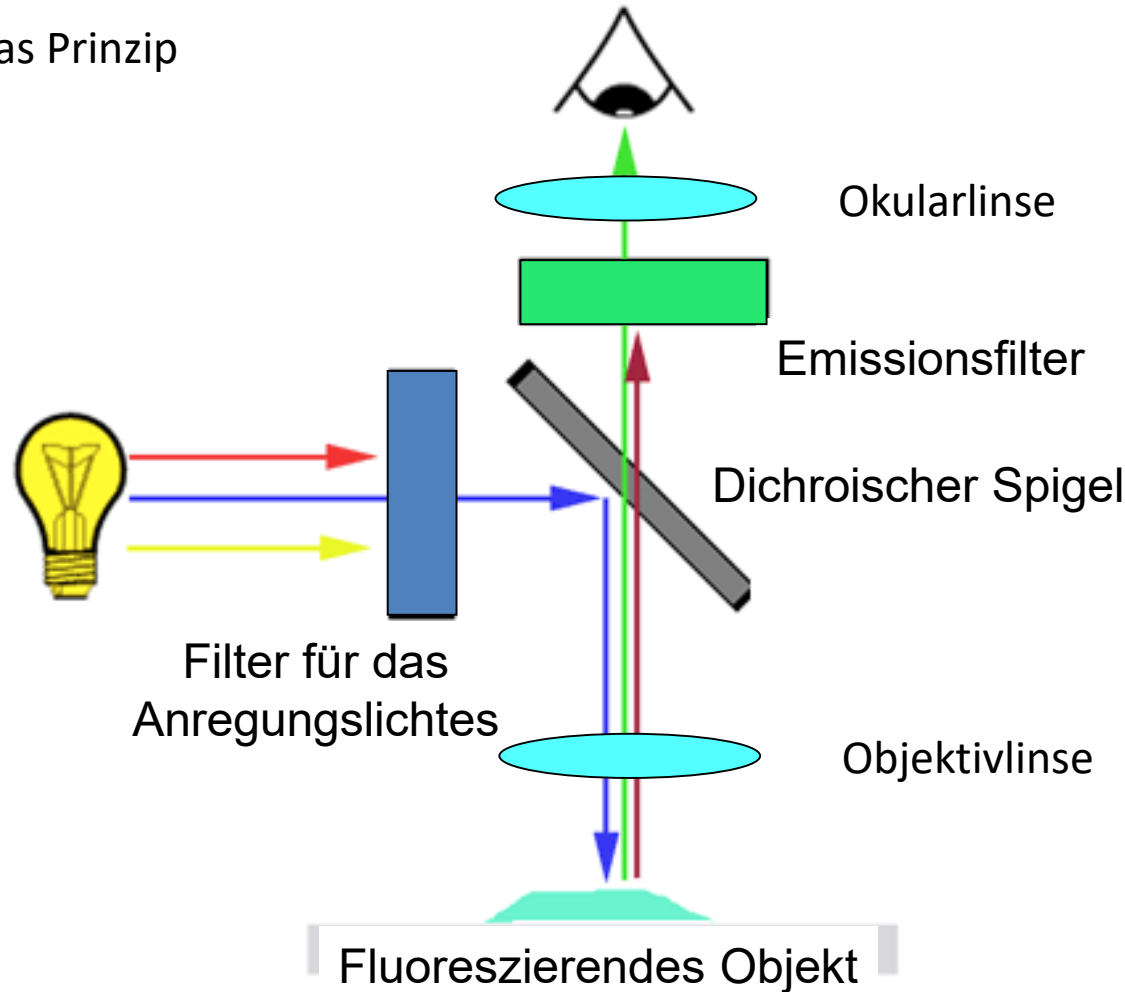
Mit $\lambda = 400 \text{ nm}$, $n = 1,6$ und $\omega \approx 90^\circ$ ist $d \approx 150 \text{ nm}$

Spezielle Lichtmikroskopische Techniquen

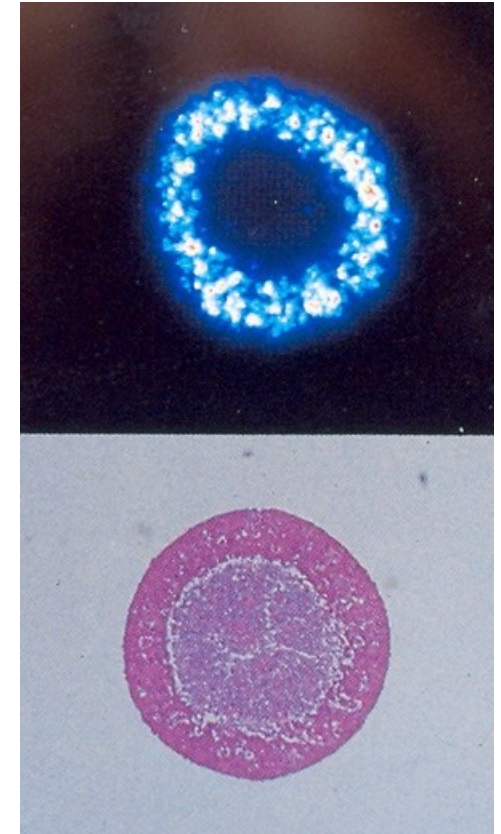
- Schon gelernt beim Praktikum:
 - Stereomikroskop
 - Phasenkontrast Mikroskop
 - Immersionsmikroskop
 - Dunkelfeldmikroskop
- Konfokales Mikroskop
- Zweiphotonenmikroskop
- Fluoreszenzkorrelationsmethode

Fluoreszenzmikroskop

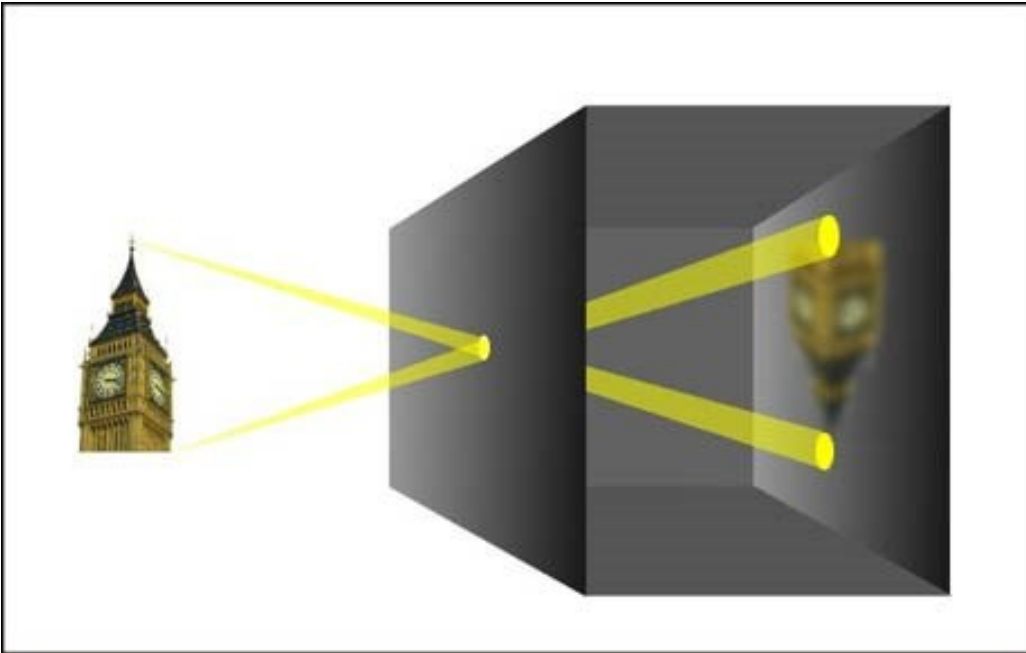
Das Prinzip



ATP-Verteilung
visualisiert mit Luciferin

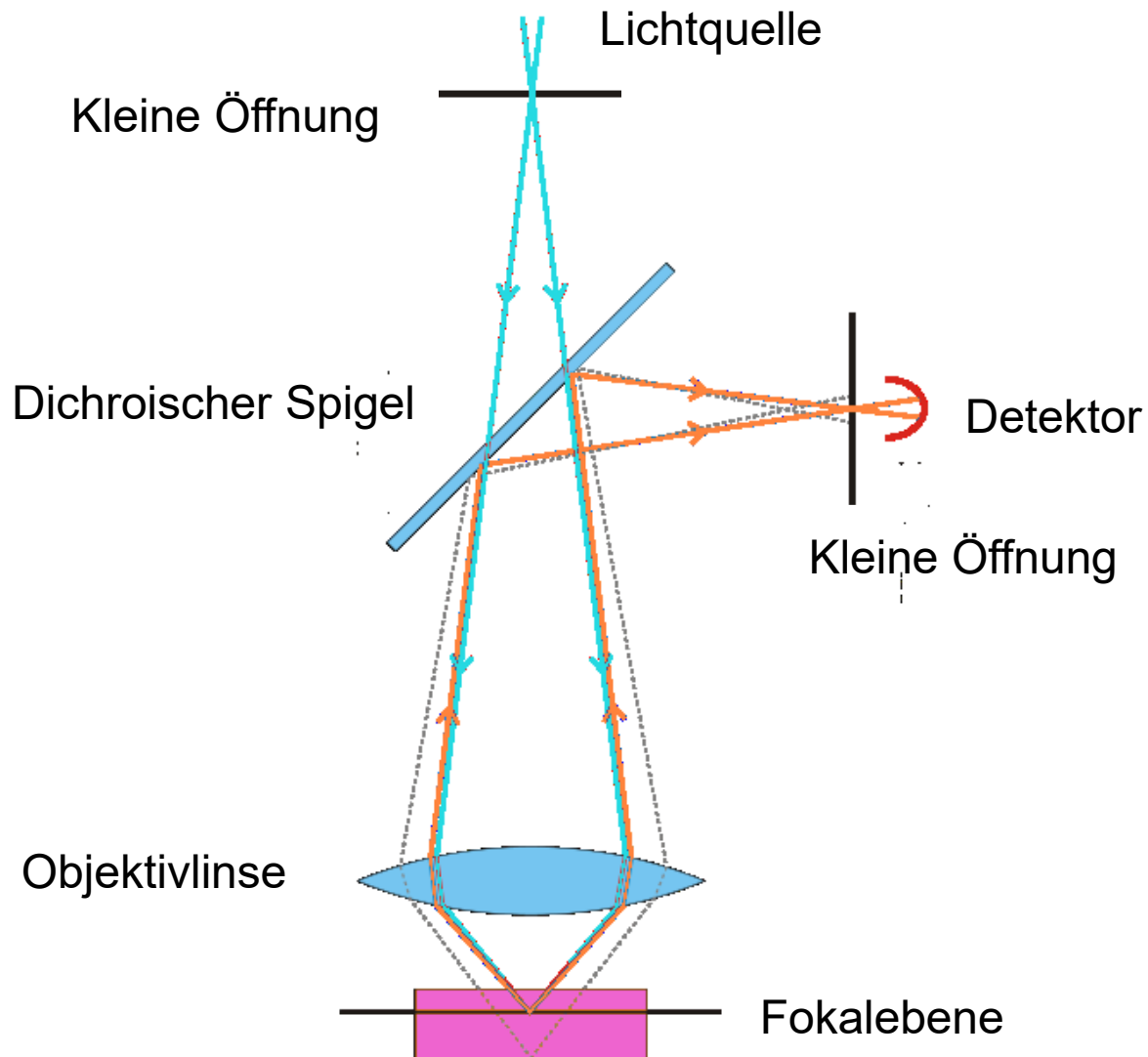


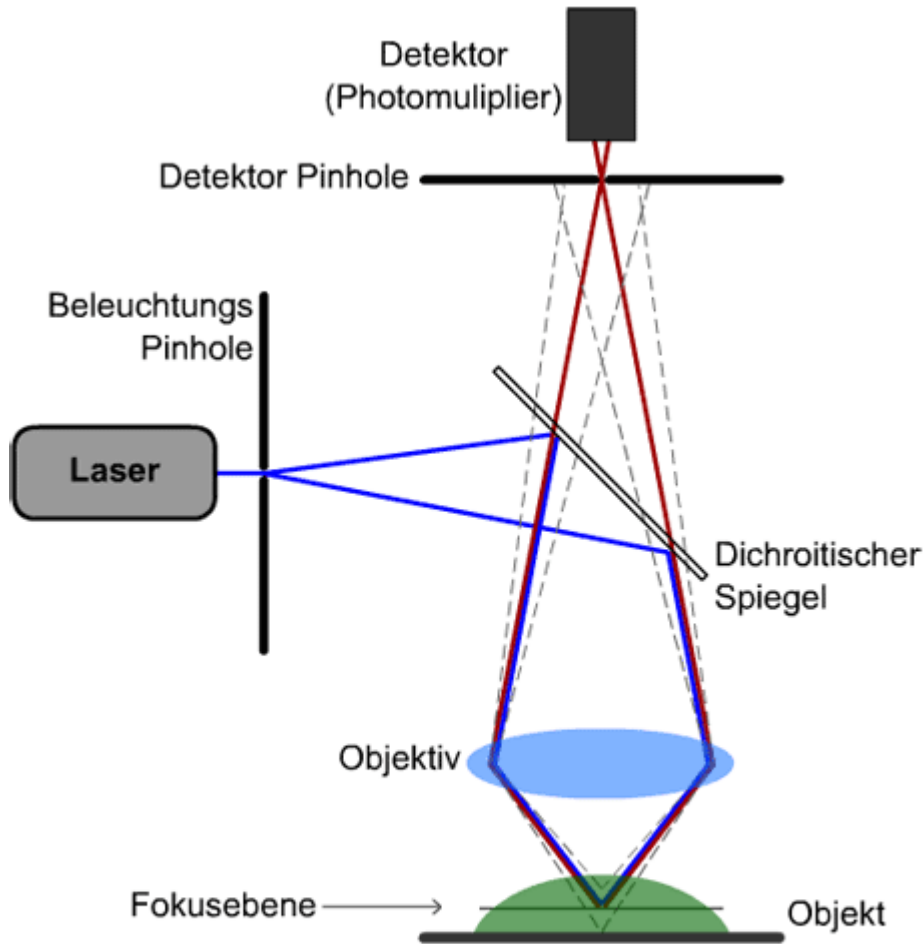
Konventionelle mikroskopische Aufnahme



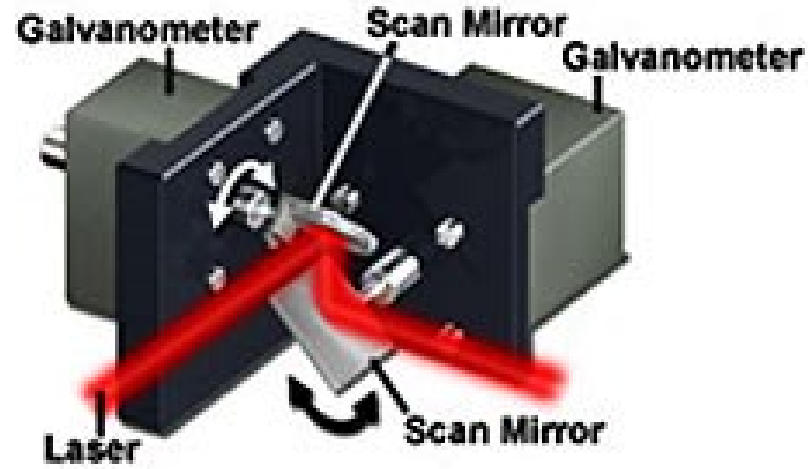
Lochkamera

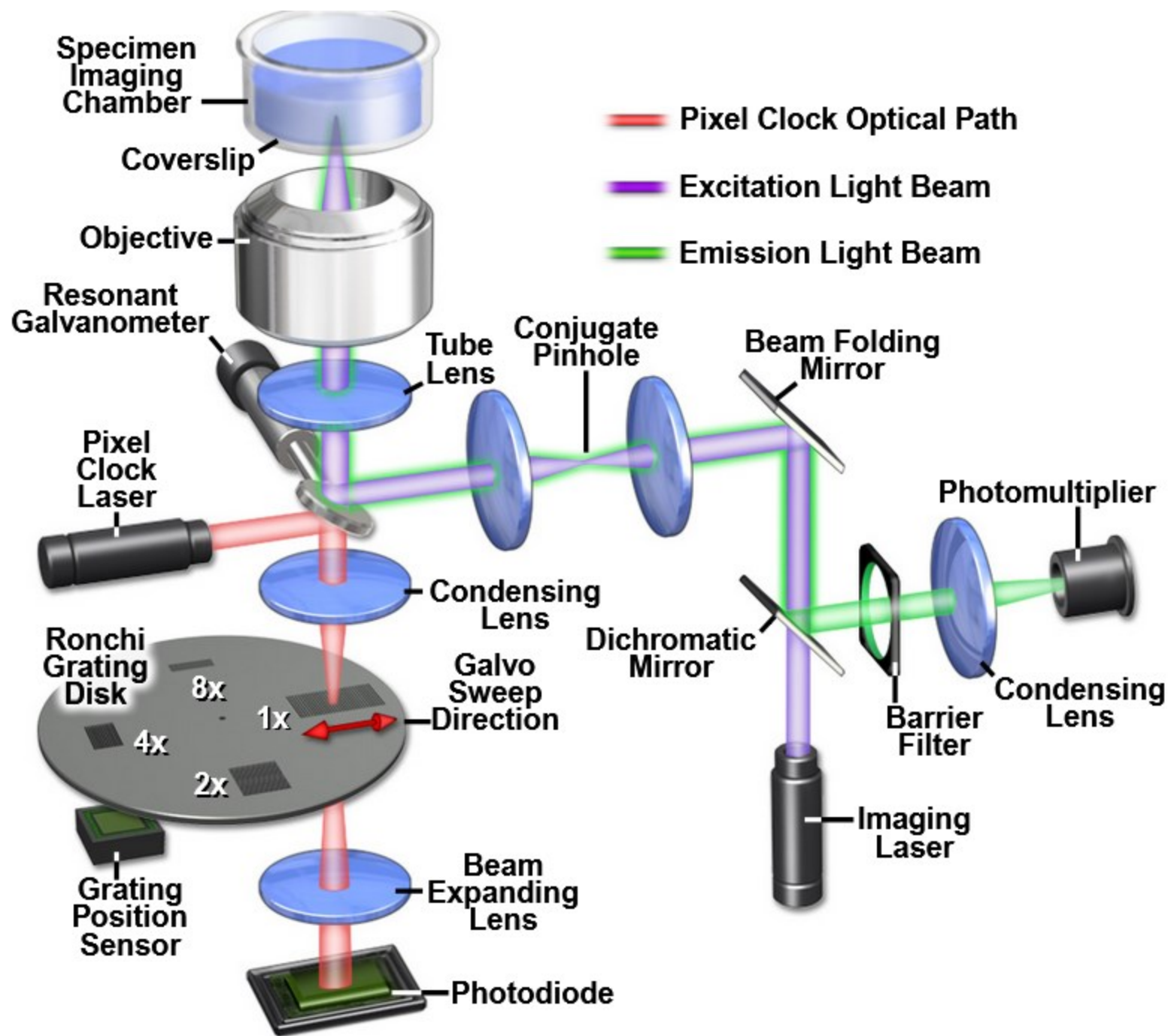
Konfokales Mikroskop





— Fluoreszenzlicht aus der Fokusebene
 - - - Out of Focus - Licht





Airy Disks and Diffraction Pattern Intensity Profiles

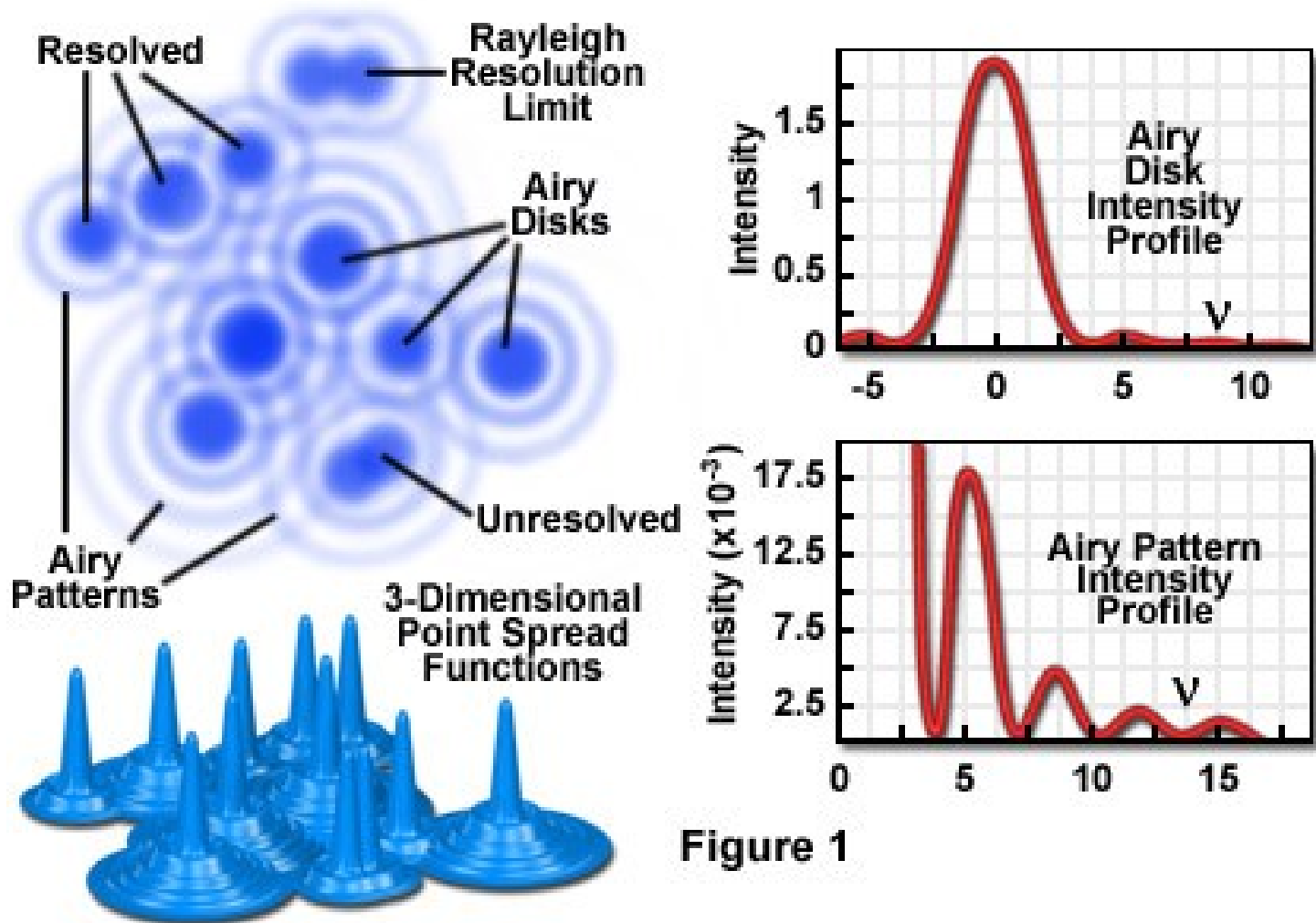
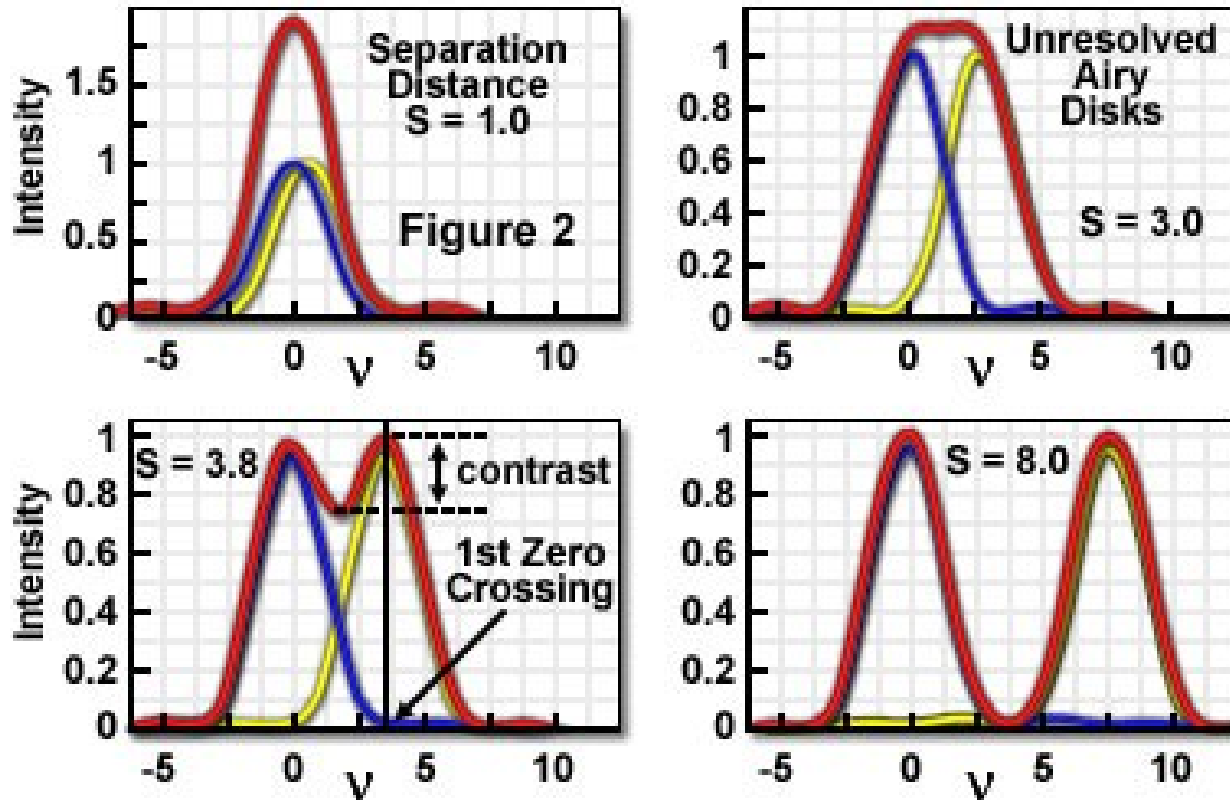


Figure 1

Contrast and Resolution in Fluorescence Microscopy



Axial PSF Intensity Profiles

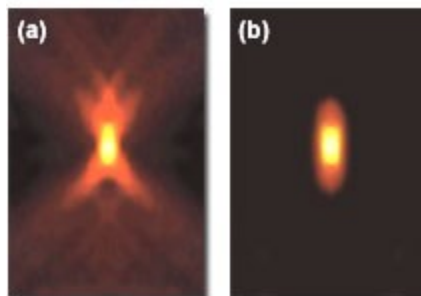


Figure 3

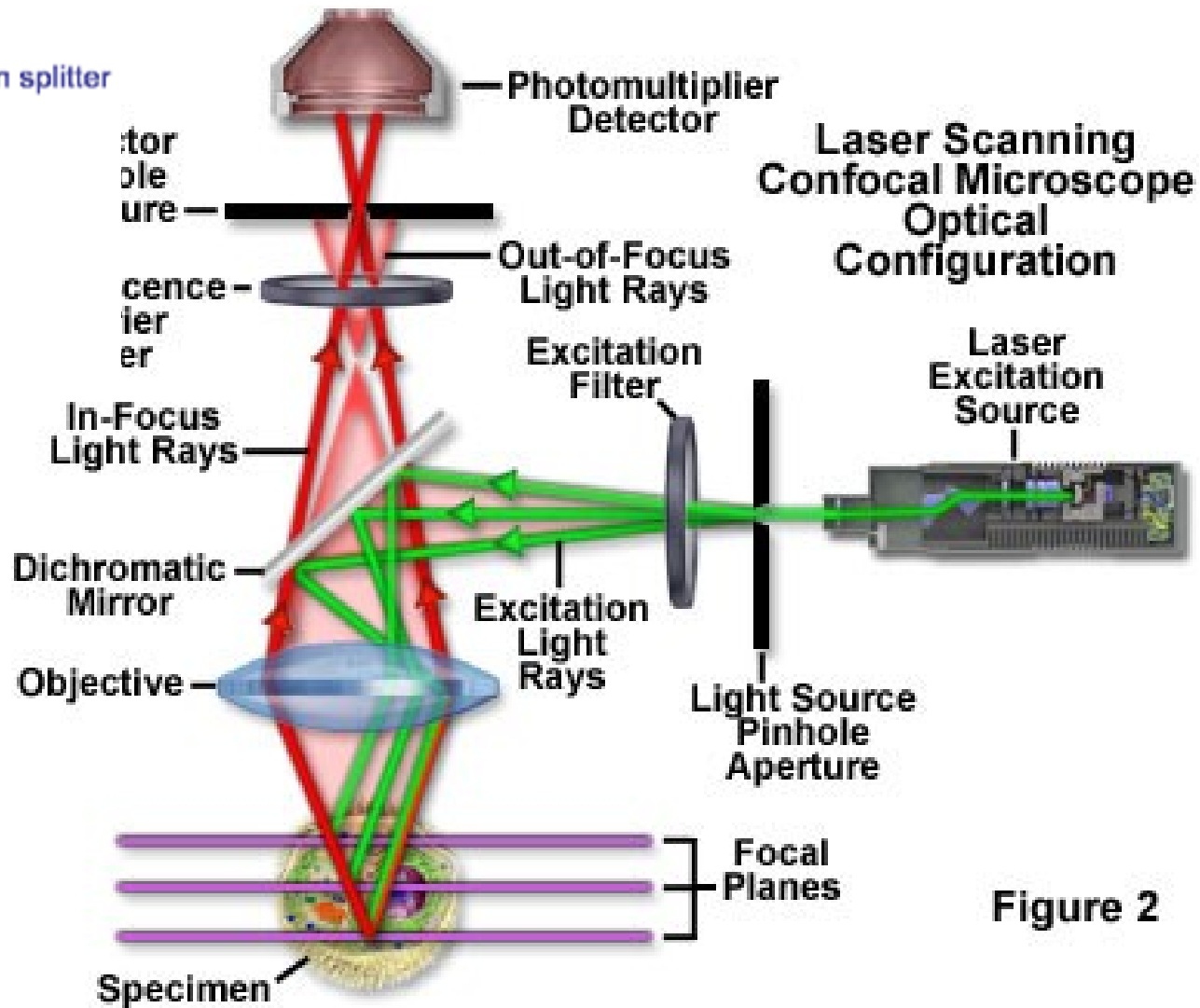
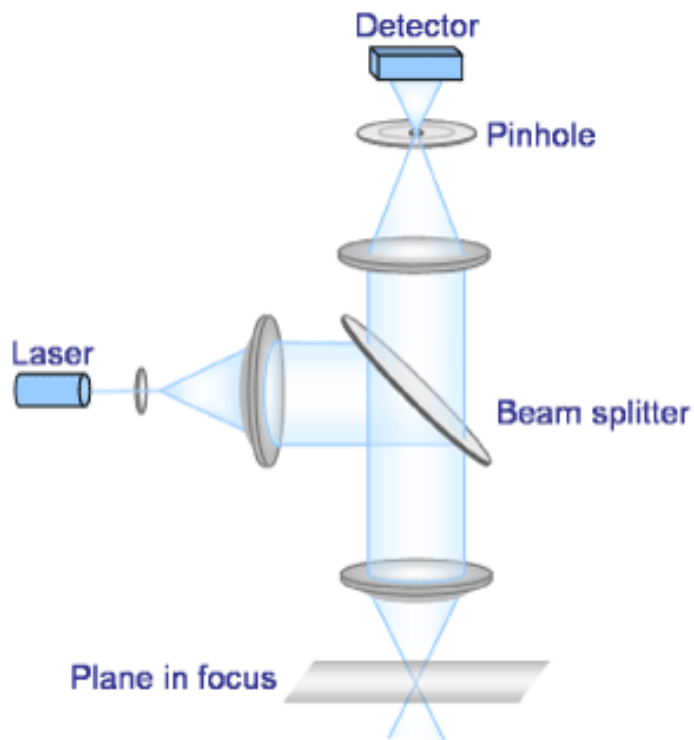


Figure 2

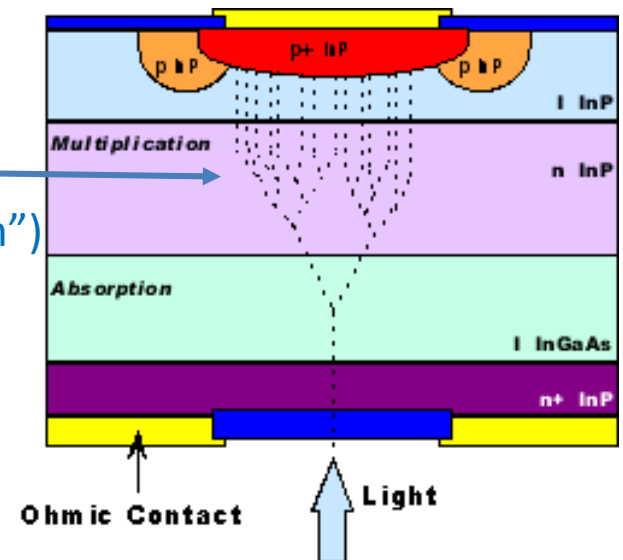
PMT



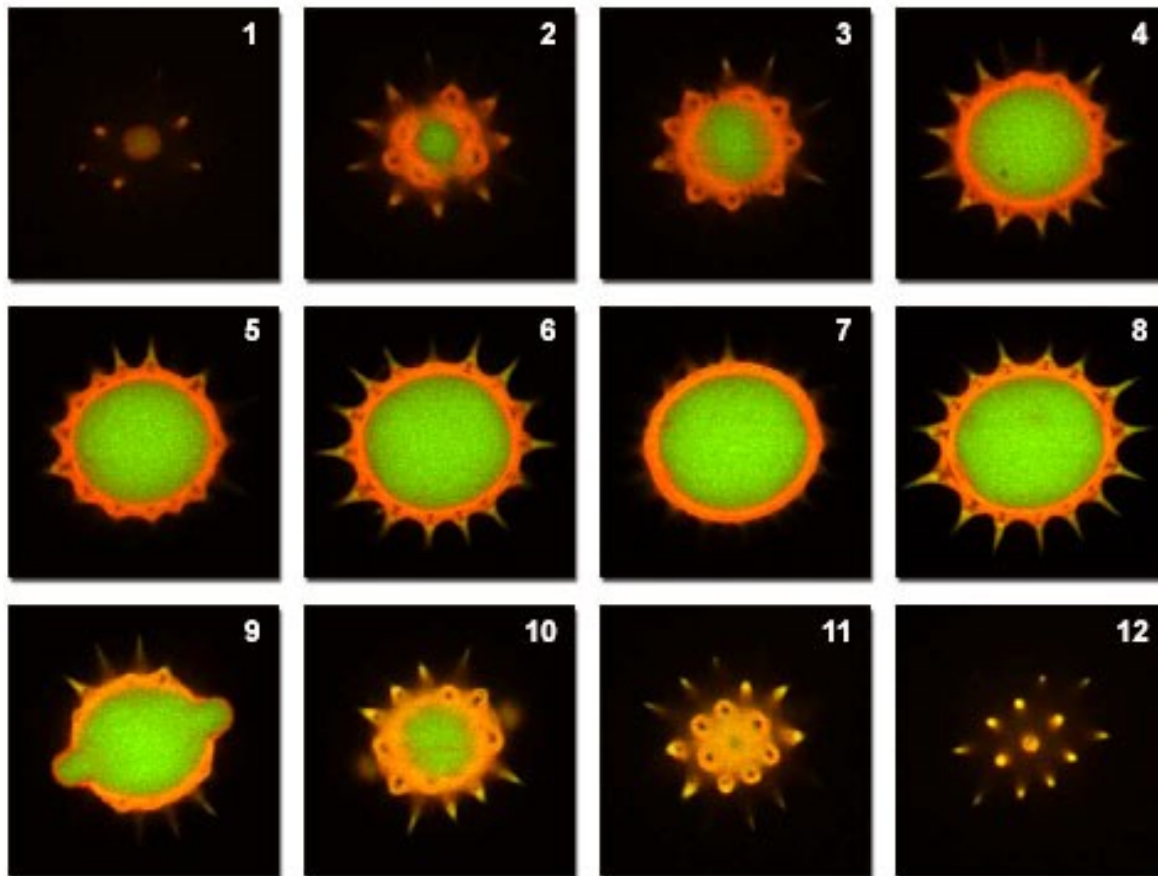
APD: Avalanche Photodiode
(Lavinenphotodiode)



Vervielfachung
(„sekundäre Ionisation“)



Pollen Grain Serial Optical Sections by Confocal Microscopy



Three-Dimensional Volume Renders from Confocal Optical Sections

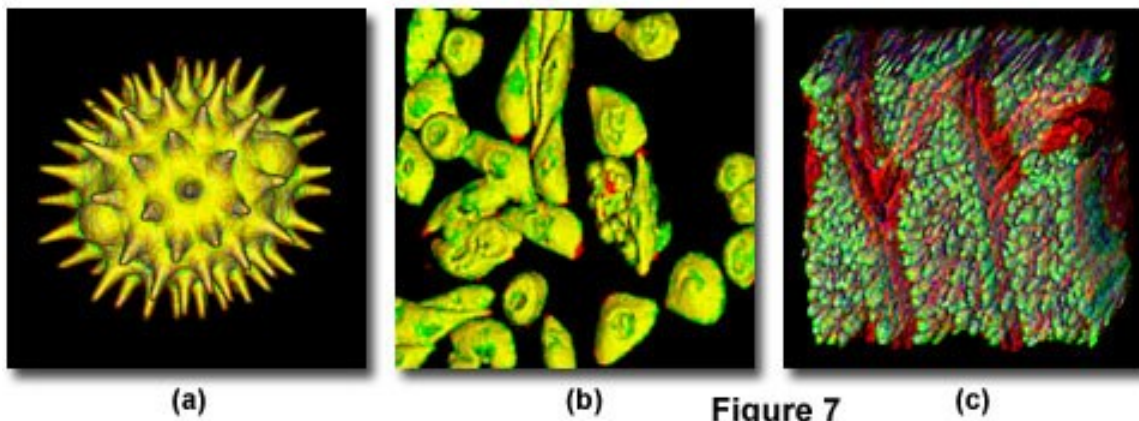
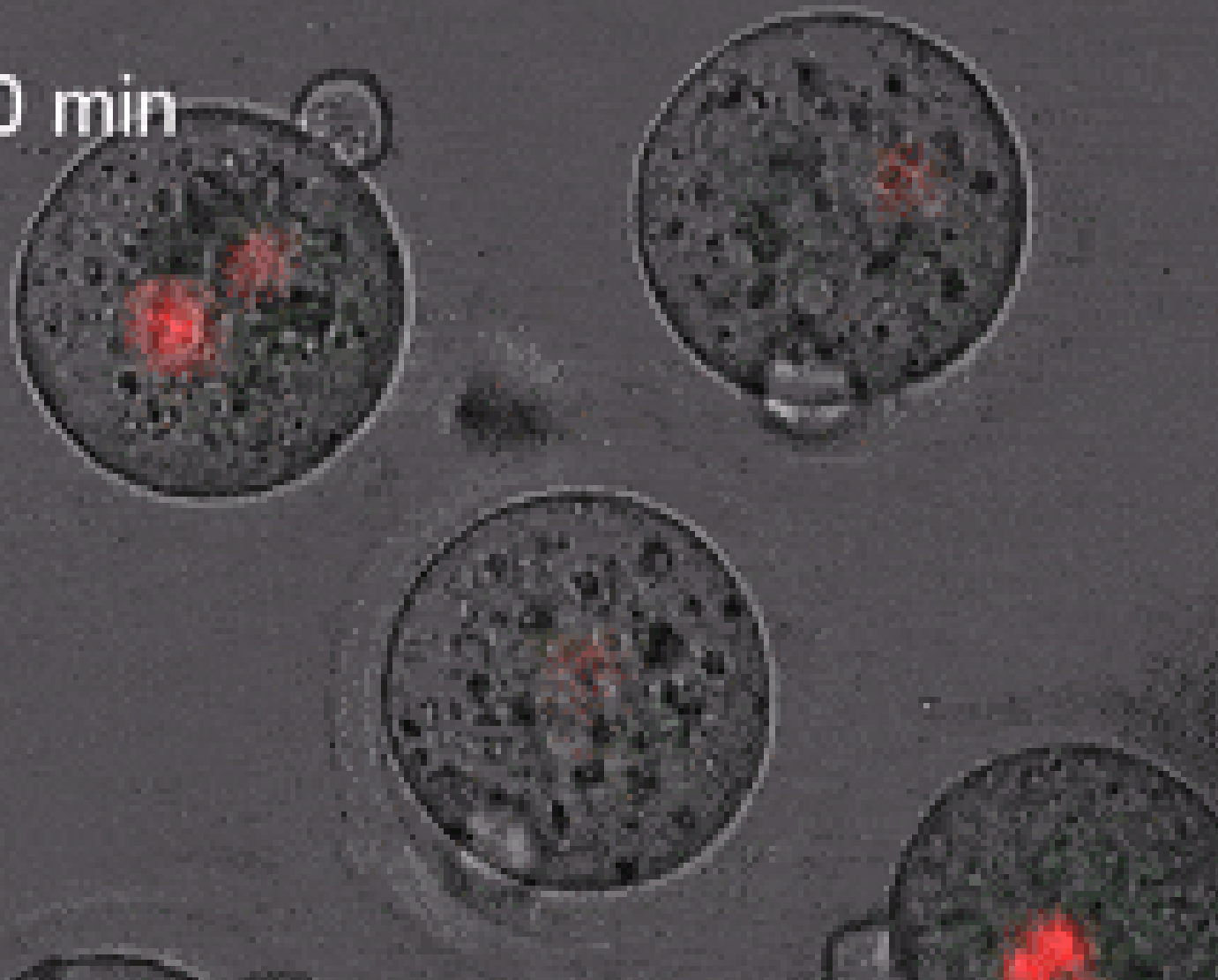
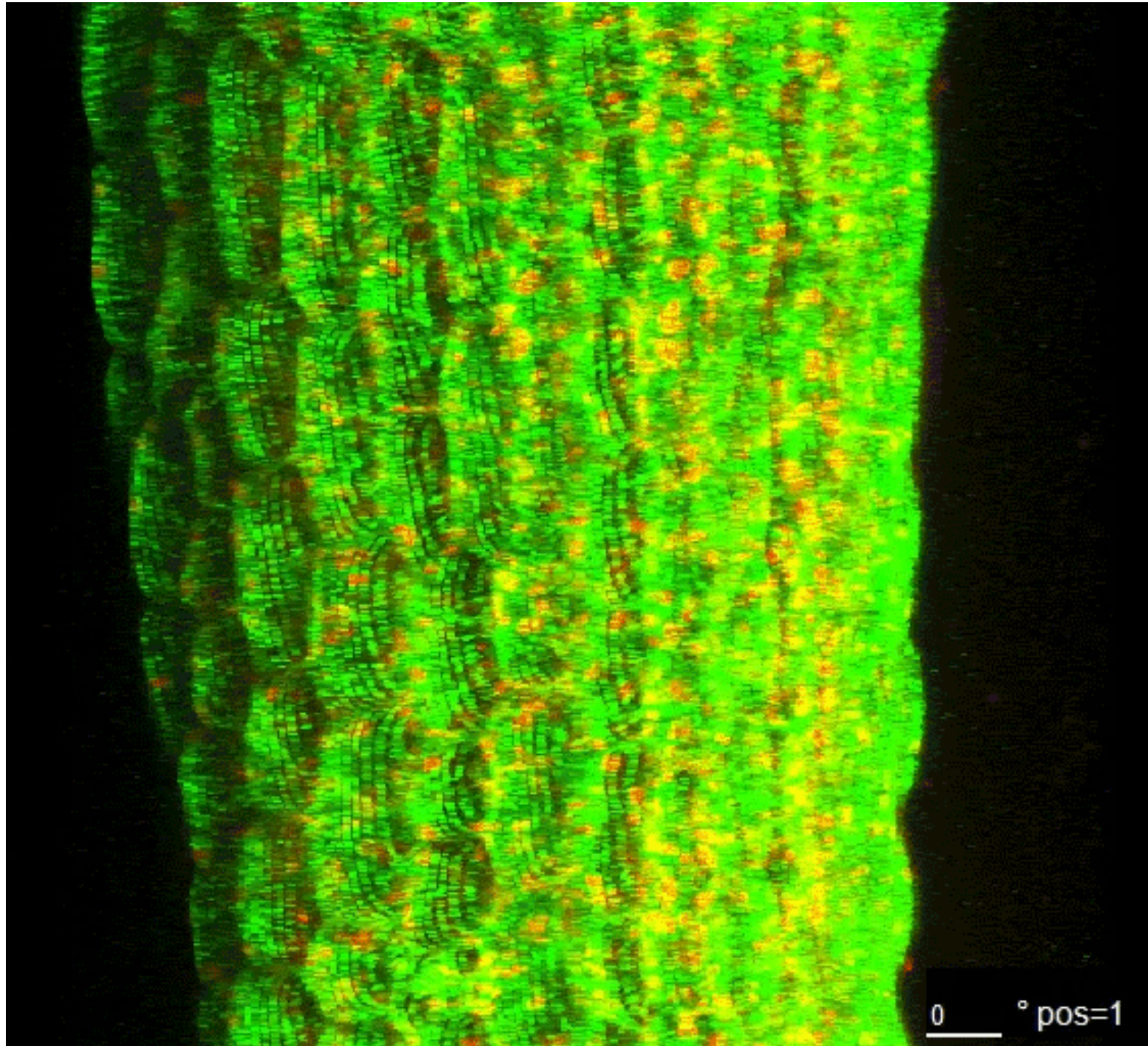


Figure 7

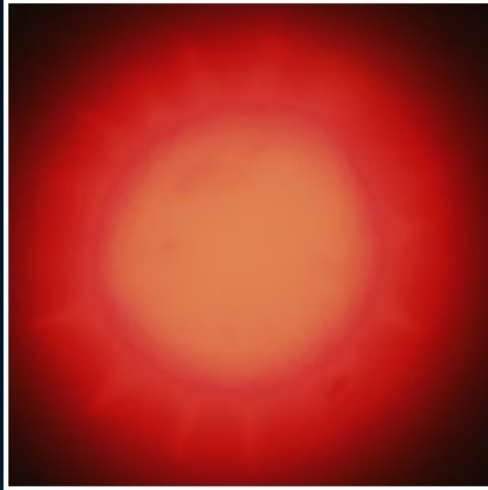
50 min



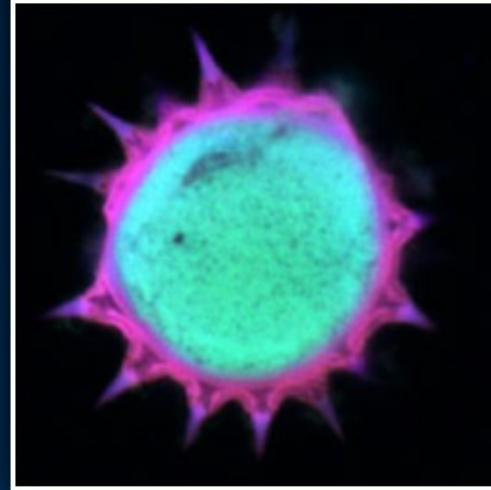


Comparing Confocal and Widefield Fluorescence Microscopy

Widefield Image



Confocal Image



Red: 25%



Green: 25%



Blue: 25%



Magnification:

100X

Specimen Set:

Specimen Set 1

Specimen Set 2

Specimen Set 3

Lock Button



Focus



Brightness



Z-Axis Position



Choose A Specimen

Pollen Grain

Pinhole Aperture Size:

Small Medium Large

Scan Line Speed: Medium



Spectral Overlap in Paired Alexa Fluor Probes

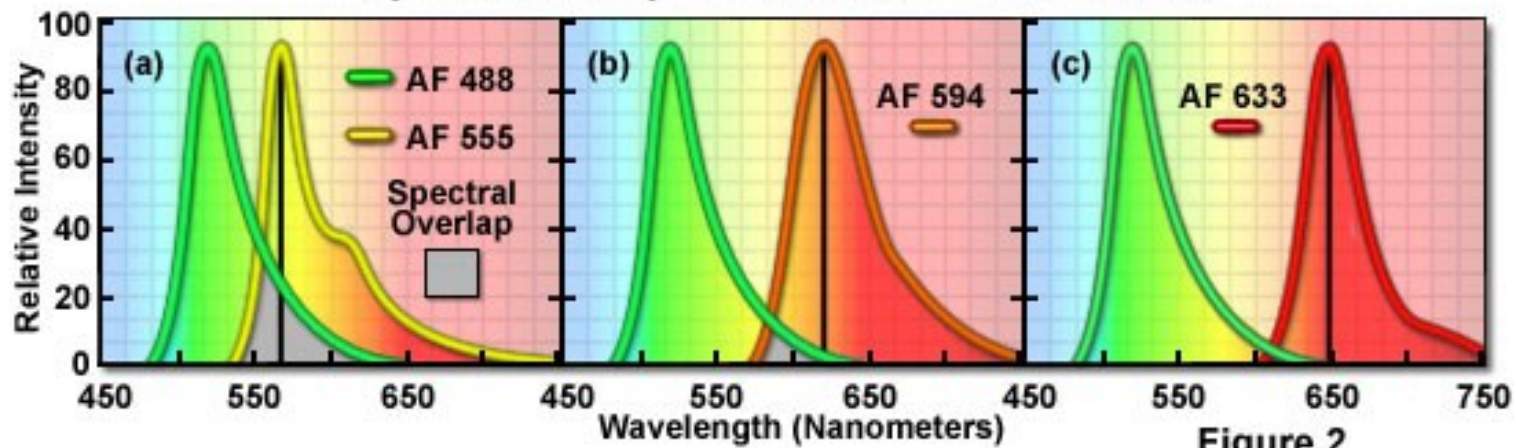


Figure 2

Fluorophore Emission Bleed-Through in Confocal Microscopy

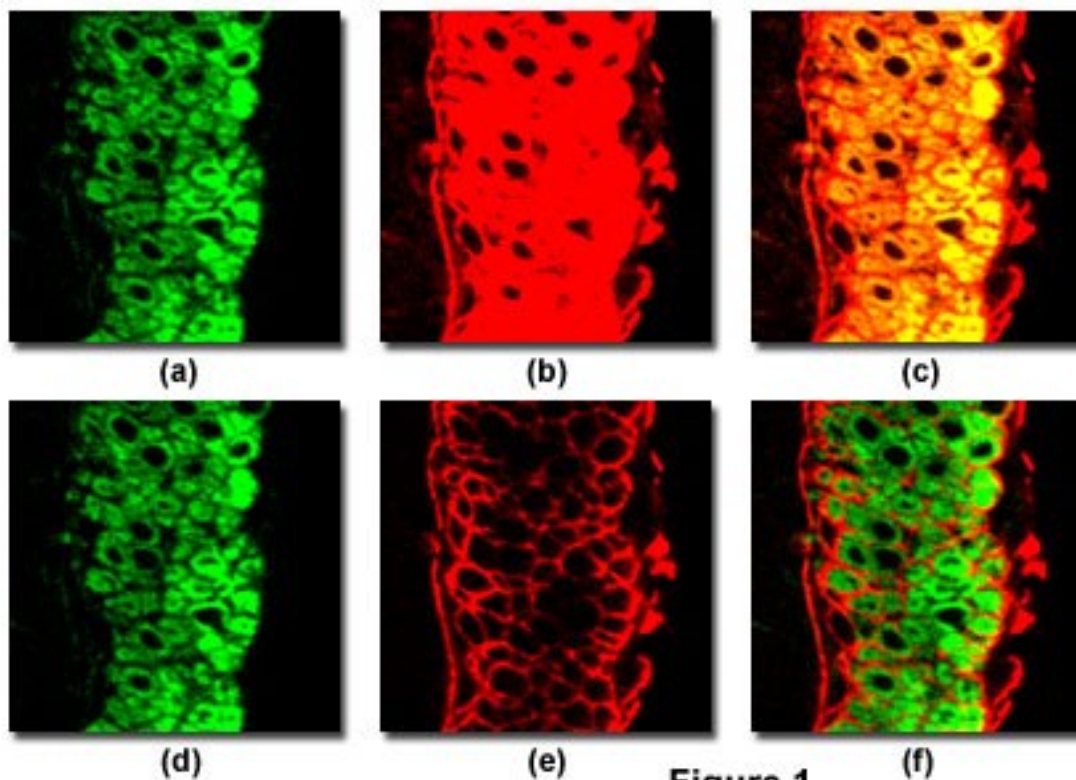
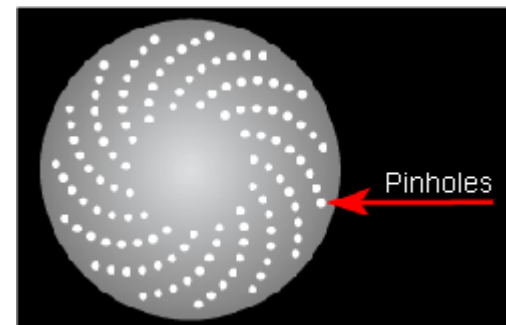
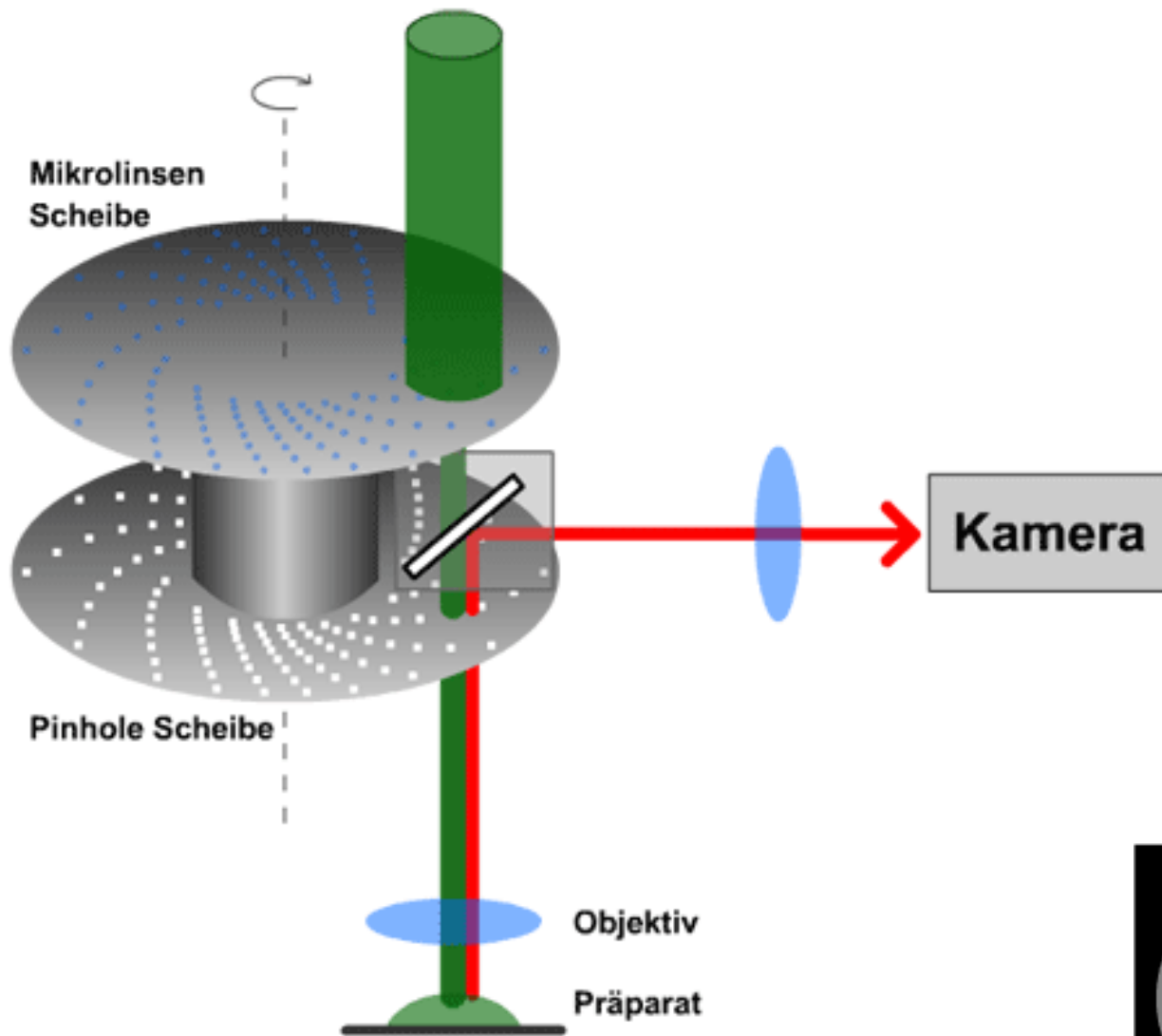
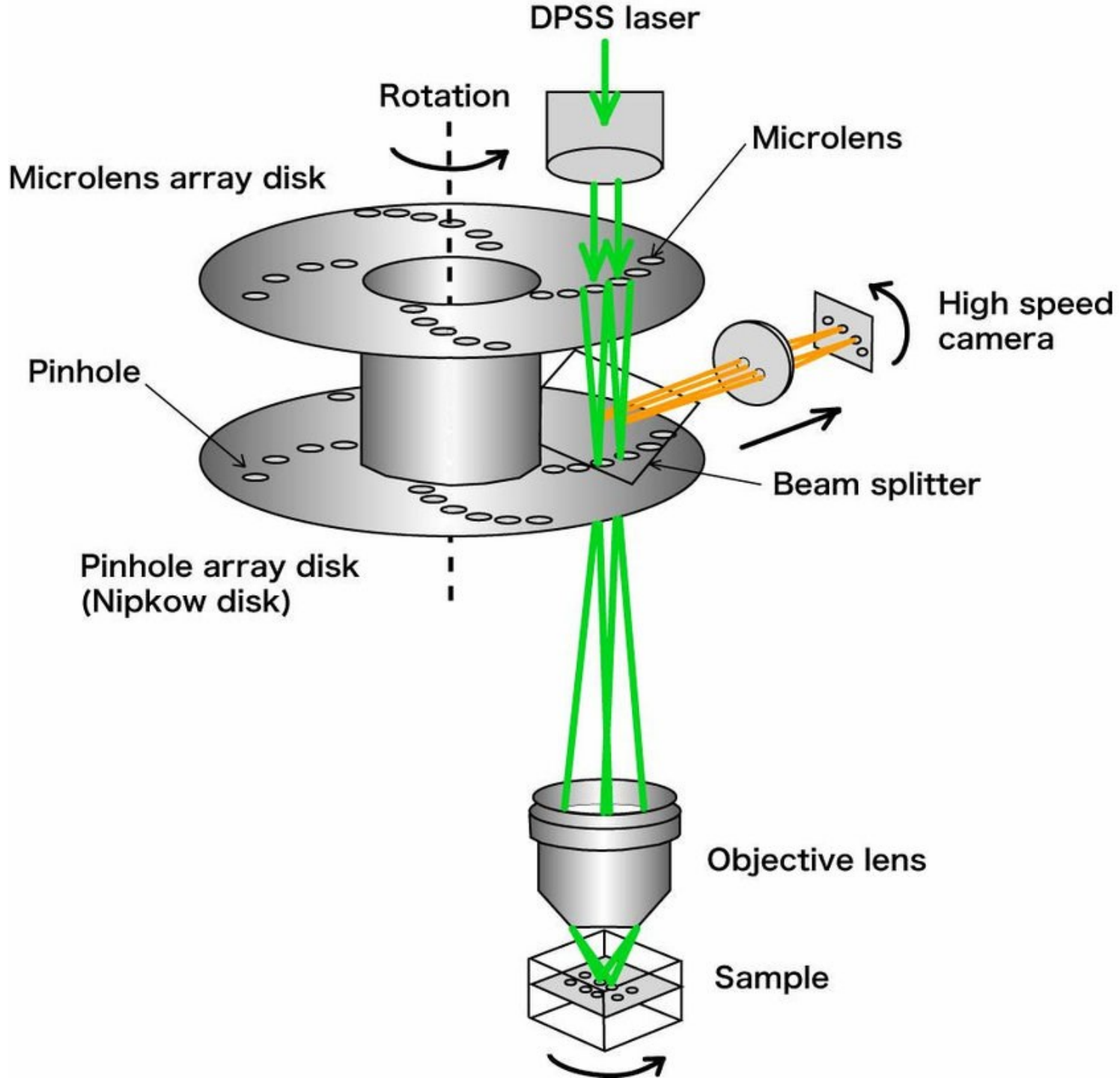


Figure 1

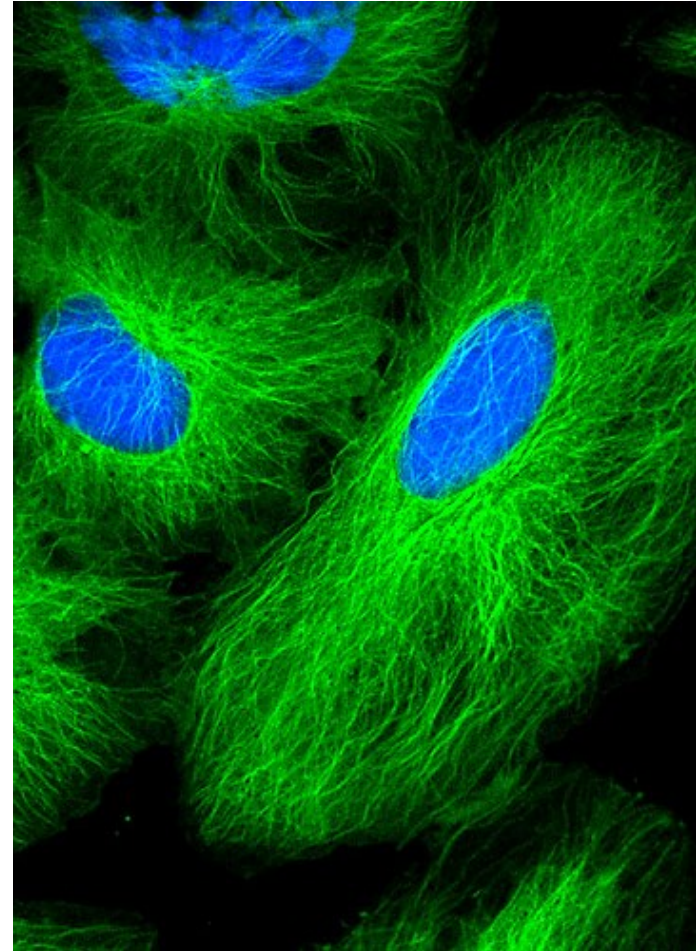


Nipkow-Disk



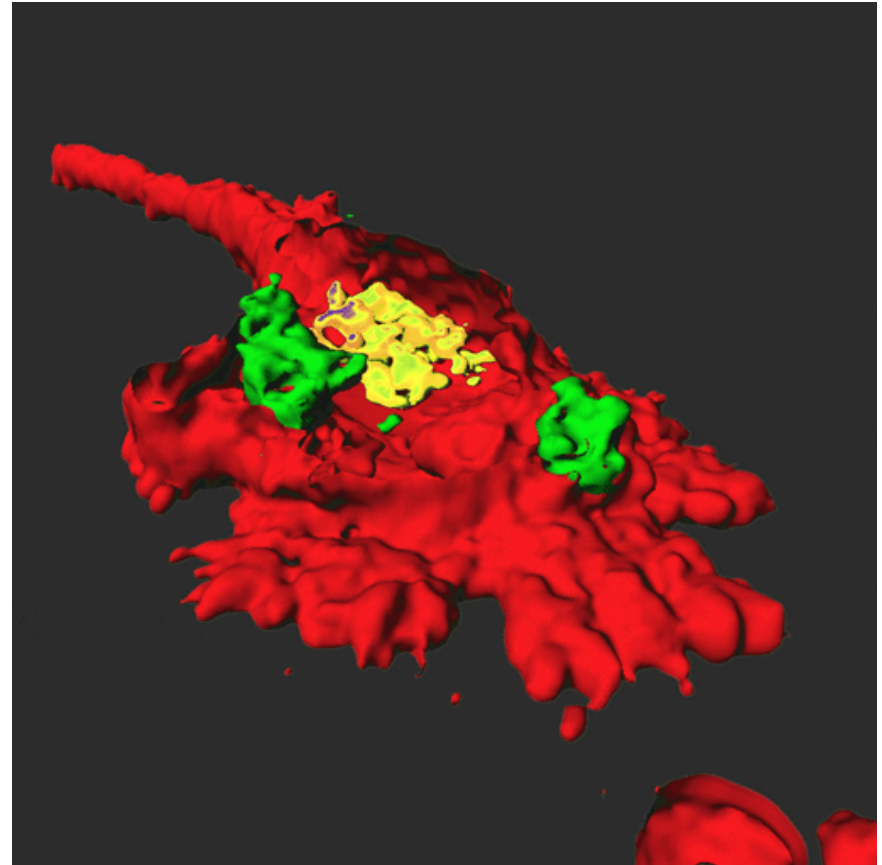
Konfokales Mikroskop

Aus Tubulin bestehende
Mikrotubuli in Zellen

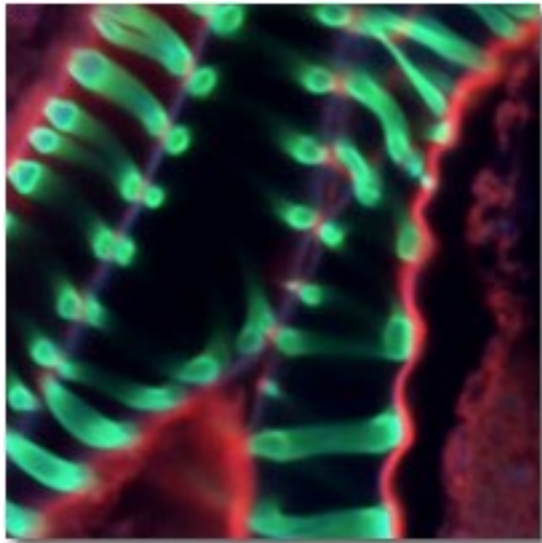
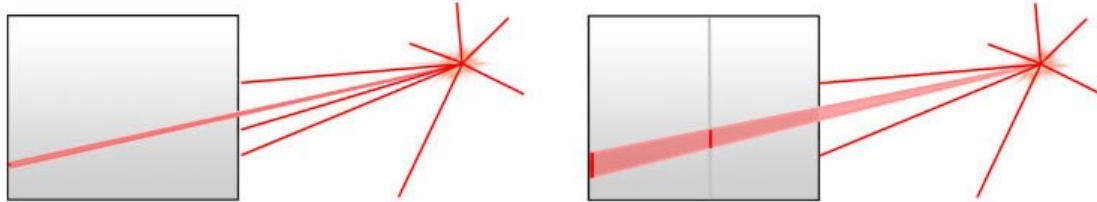


Konfokales Mikroskop

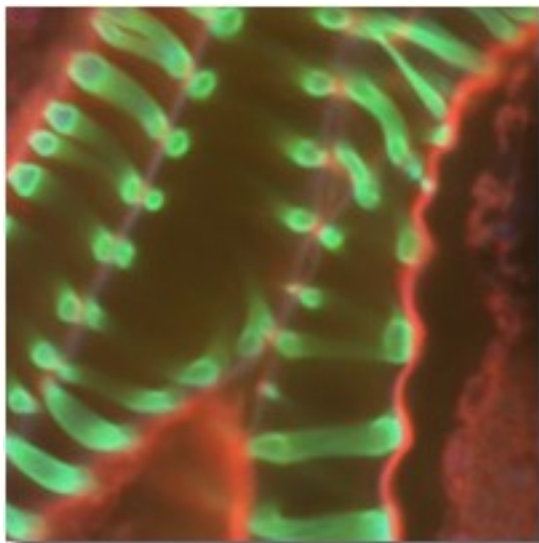
Dendritische Zelle mit
Pollenteilchen.
3D Aufnahme mit konfokalem
Mikroskop.



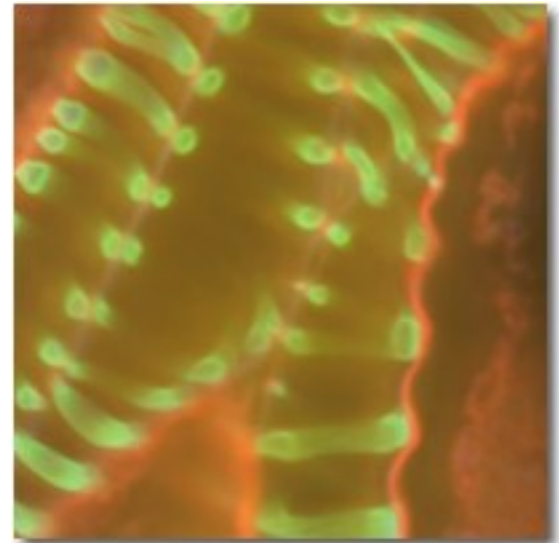
Lochblendengrösse (pinhole size)



1 Airy-Einheit



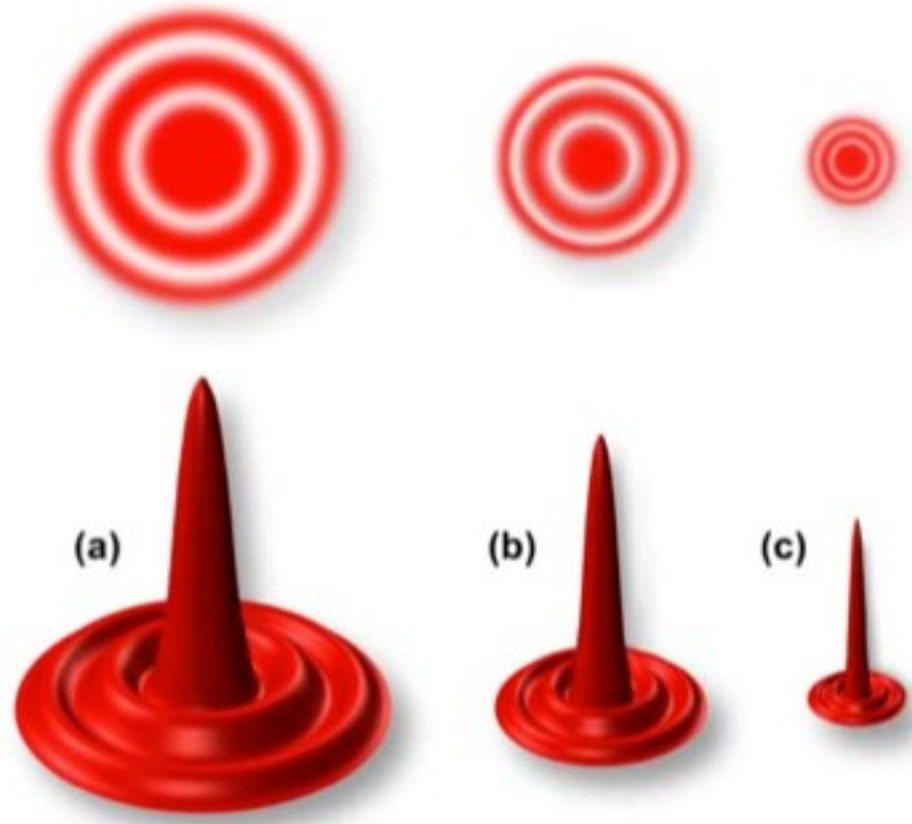
4 Airy-Einheiten



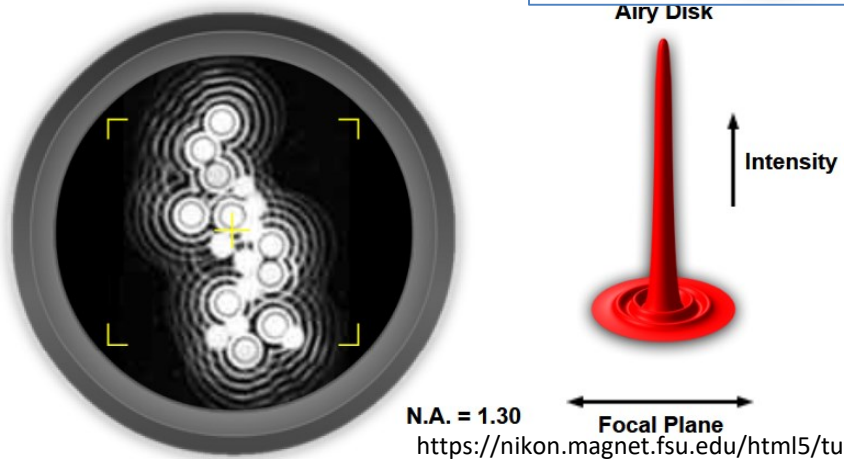
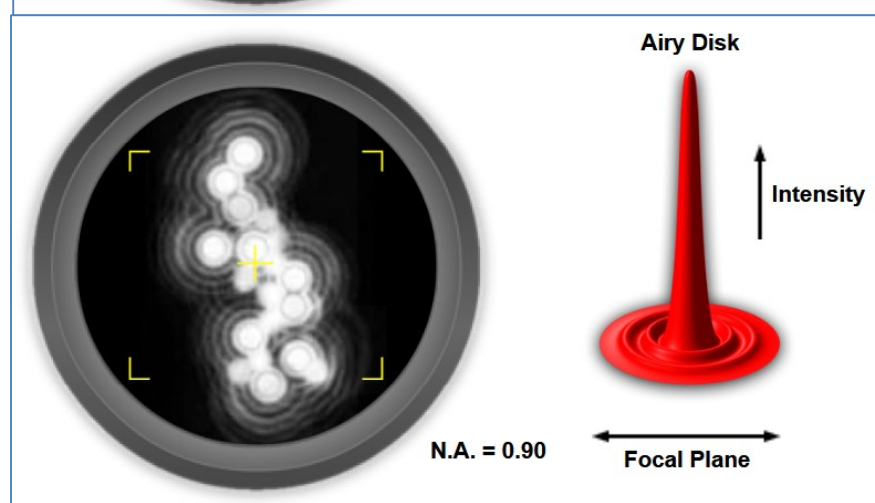
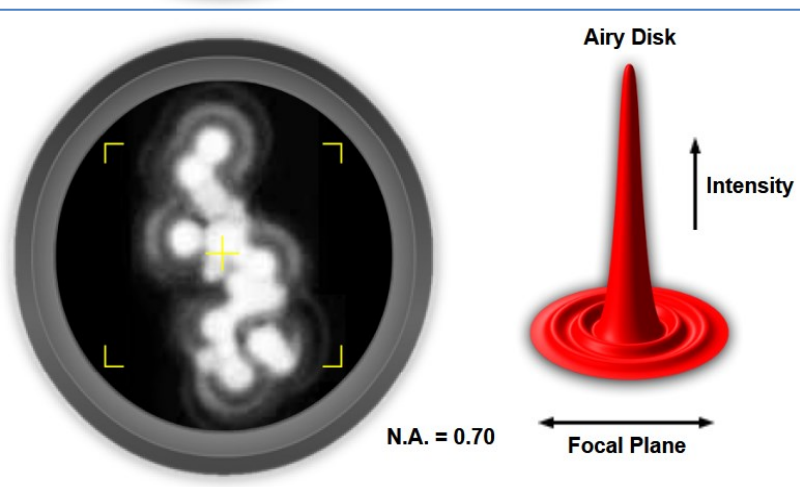
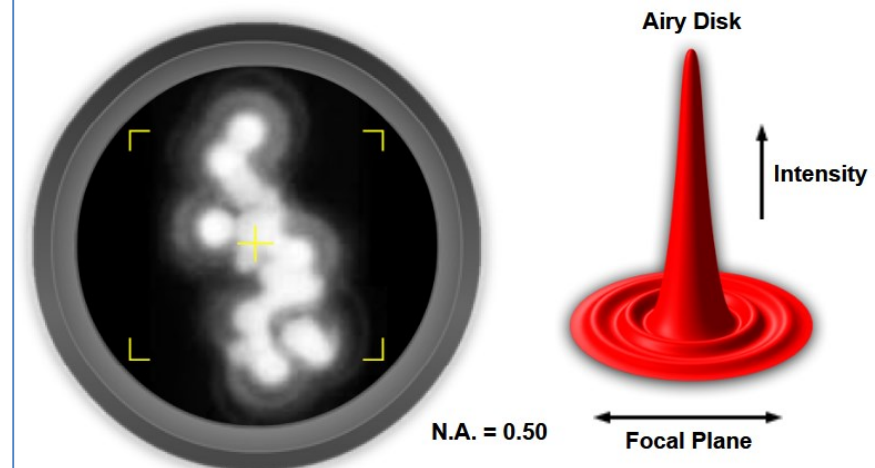
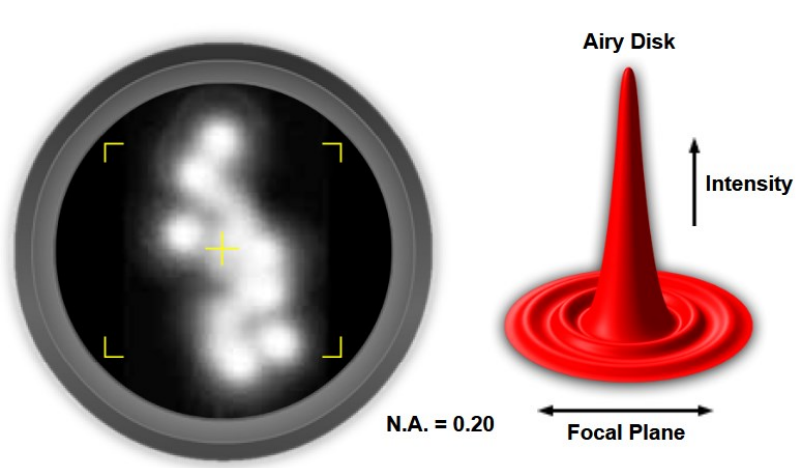
20 Airy-Einheiten

Airy-Einheit $1AE = \frac{1.22 \cdot \lambda}{NA}$

Die Numerische Appertur beeinflusst die Scheibengrösse:



$$1AE = \frac{1.22 \cdot \lambda}{NA}$$

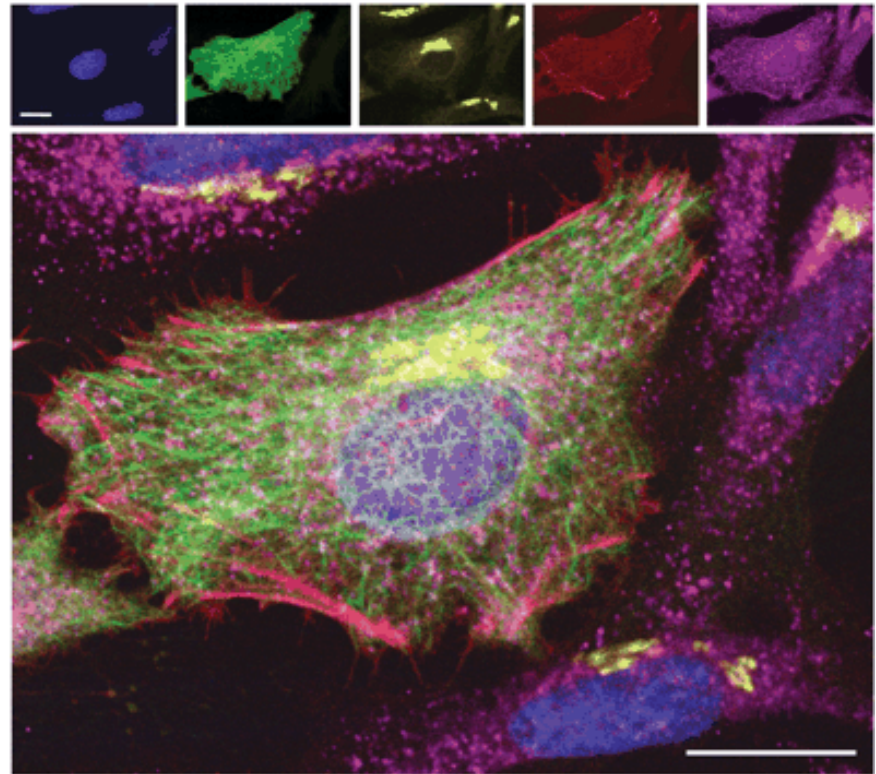


Gleichzeitige Anwendung von mehreren fluoreszierenden Markierungen

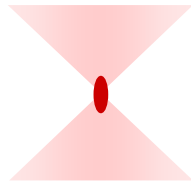
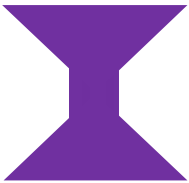
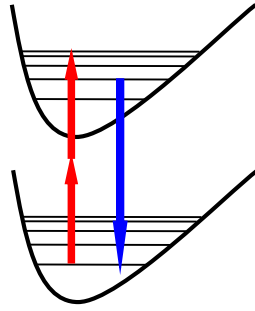
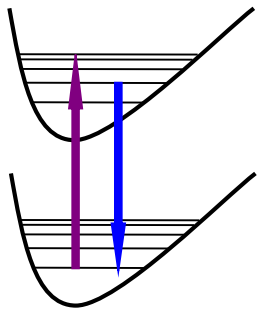
He-La Zellen markiert mit fünf unterschiedlichen Fluoreszenzmethoden.

Der Masstab ist 20 μm .

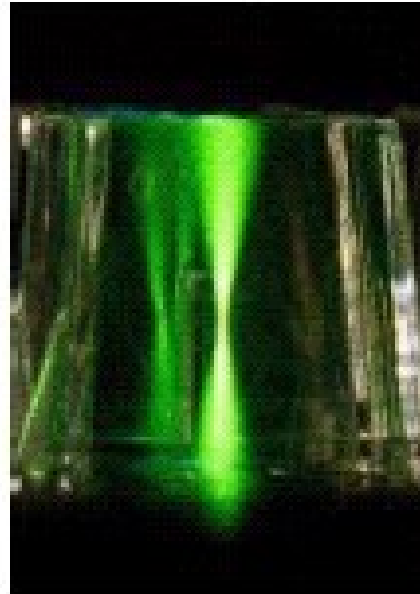
Excitation (nm):	800 (2 photon)	488	432	568	637
Emission (nm):	410-490	500-530	555-565	580-620	>660
Fluorophore:	Hoechst	GFP	QD565	ReAsH	Cy5
Targeting:	direct affinity	genetic	immuno	genetic	immuno
Target:	DNA	α -tubulin	giantin	β -actin	Cytochrome c
Structure:	nucliel	microtubules	golgi	stress fibers	mitochondria



Fluoreszenzanzregung mit zwei Photonen Zweiphotonenmikroskop



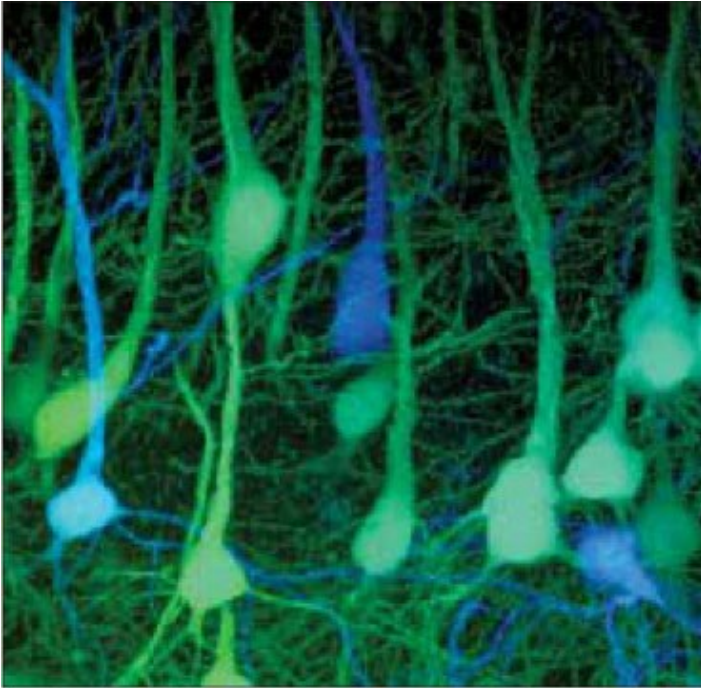
IR Laser



Fluoreszenzmission bei
Einphoton- und
Zweiphotonenanregung.

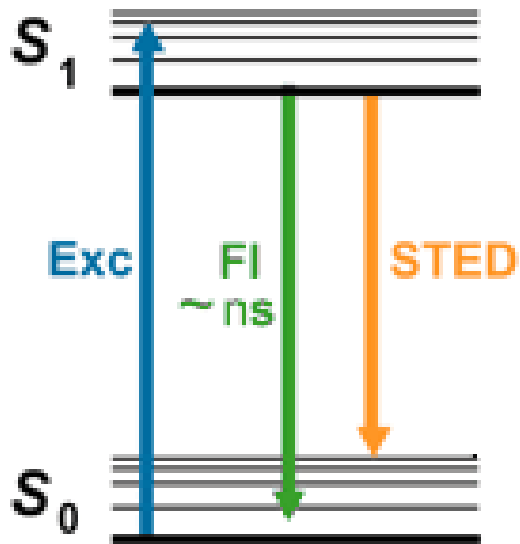
Auflösung!

Zweiphotonenmikroskopie

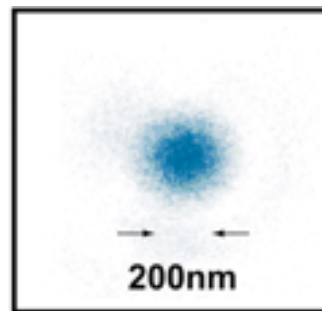


Visual Cortex von genetisch manipulierten Mause die GFP produzieren.

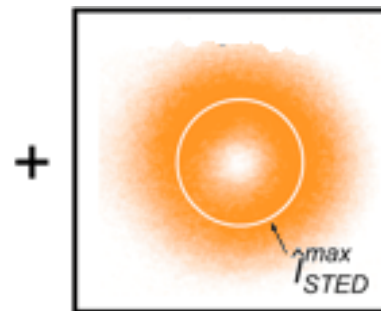
STimulated Emission Depletion (STED) Mikroskop



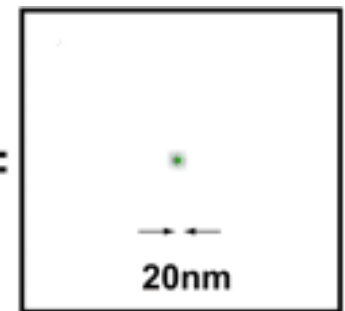
Stefan Hell (Nobelpreis 2014)



Anregung

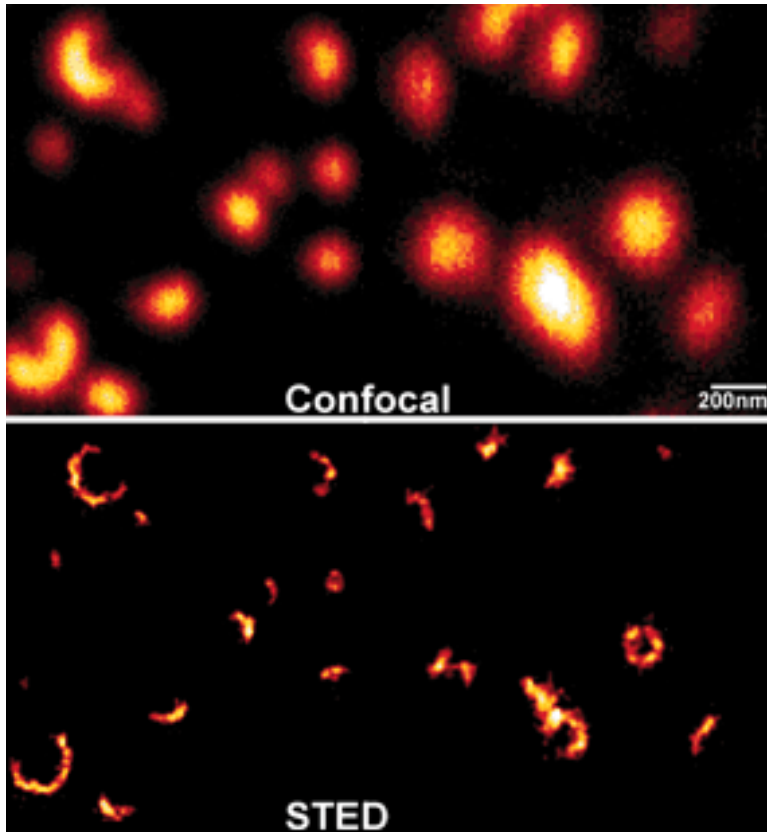


Auslöschung



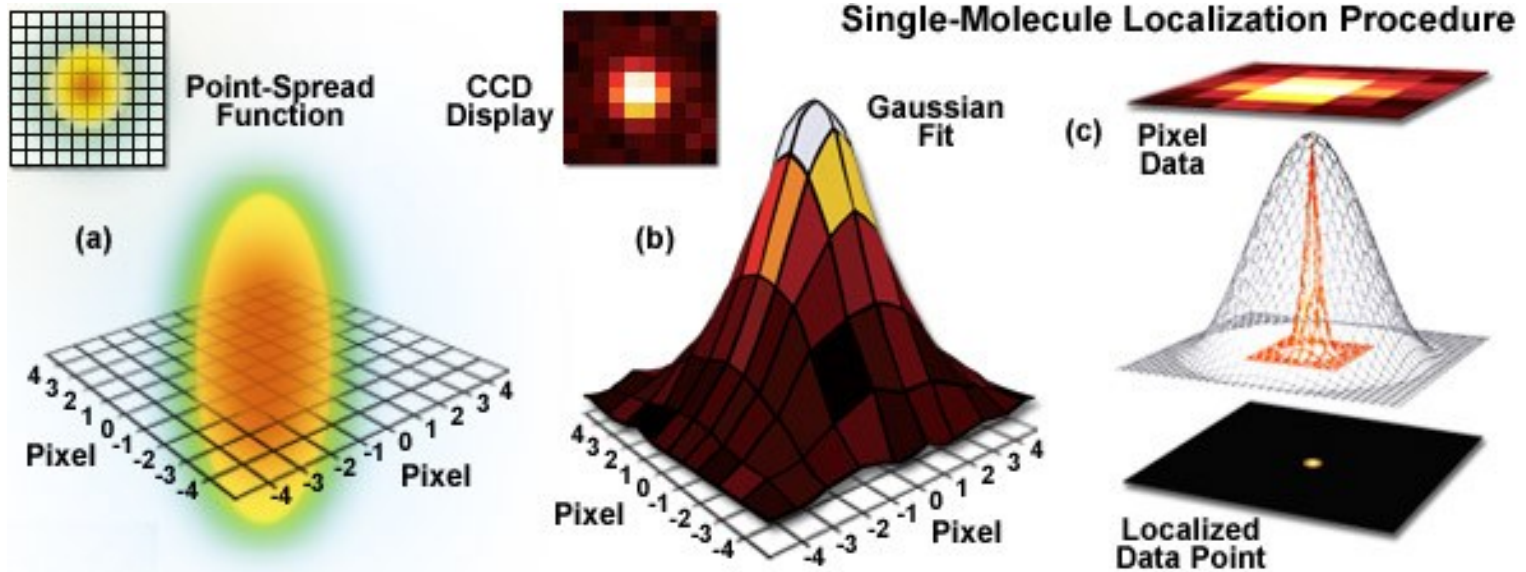
Lumineszenz nach dem Auslöschung

STimulated Emission Depletion (STED) Mikroskop

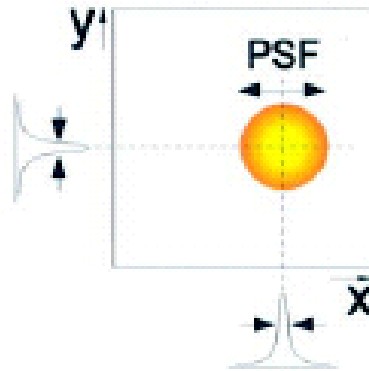
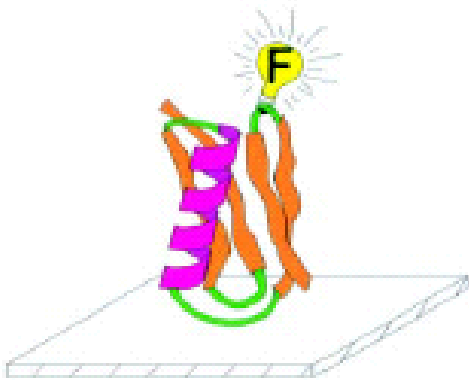


**Reorganization des
Synaptolysins in
synaptischen Vesikeln**

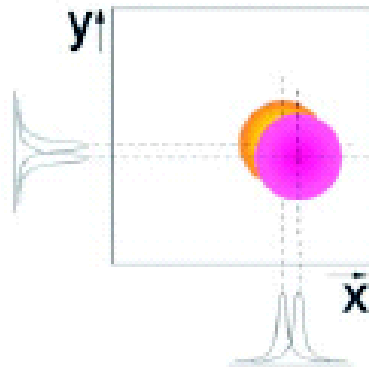
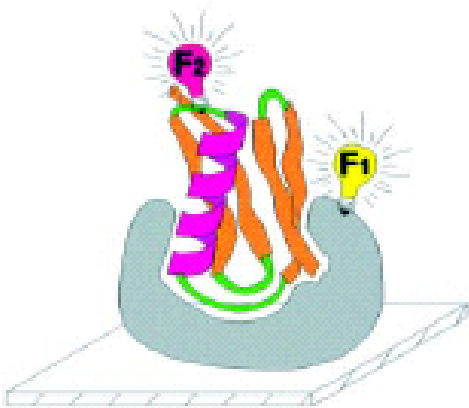
STED: Lokalization



STED: Lokalisation und Kolokalisation



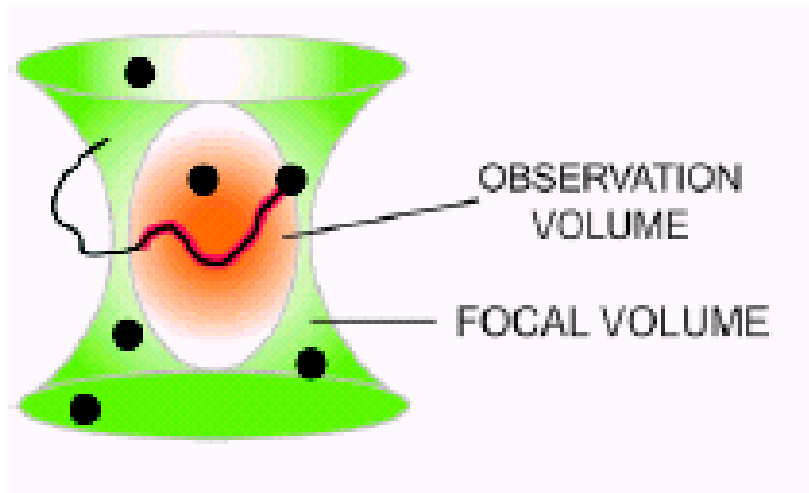
Die Position des Eiweisses kann mit nm Genauigkeit angenommen werden.



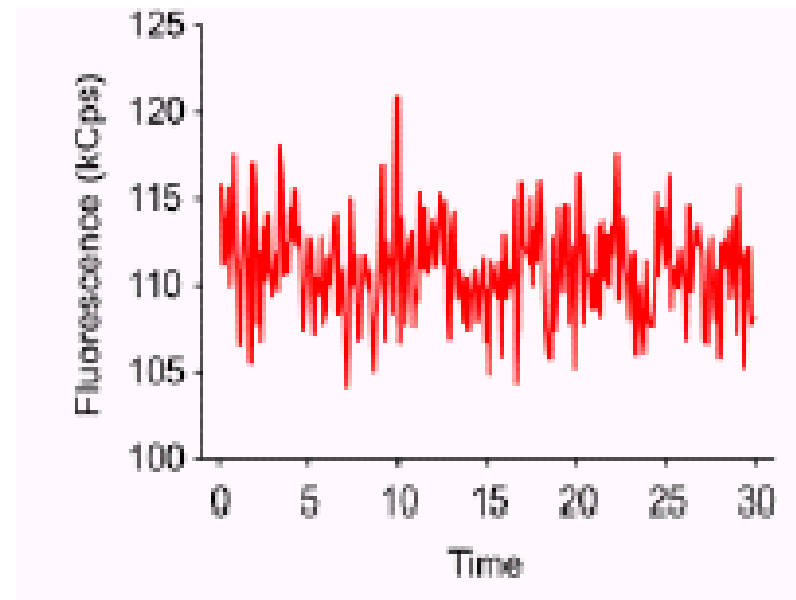
Kolokalisation bedeutet nicht unbedingt eine Wechselwirkung!

Fluoreszenzkorrelationspektroskopie (FCS)

Fluktuation der Molekülen in einem
sehr kleinen Volumen: fl
Konzentration: 10 nM
Anzahl der Moleküle in
Beobachtungsvolumen beträgt
durchschnittlich: 6



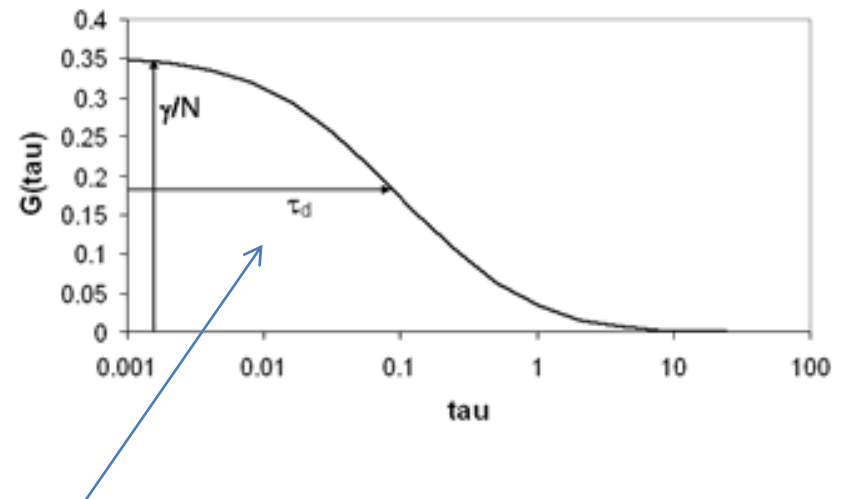
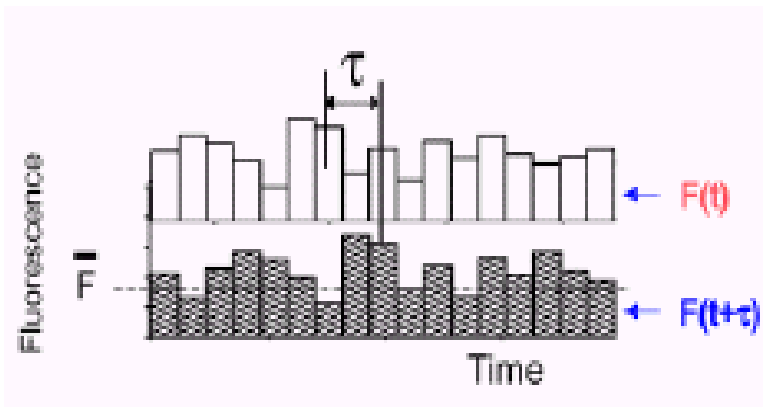
Fluktuationen des
Fluoreszenzlichtes:



Ähnlich zur dynamischen Lichtstreuung, aber mit Fluoreszenz

FCS: Autokorrelationsfunktion

$$G(\tau) = \frac{\langle \delta I(t) \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2} = \frac{\langle I(t) I(t + \tau) \rangle}{\langle I(t) \rangle^2} - 1$$



τ_d – charakteristische Zeit der Diffusion eines Moleküls

Diffusionskonstante ist abhängig von der Molekülengröße!

FCS: Welche Information kann man erhalten?

Ligandenbindung

Kleines Ligandmolekül mit Fluoreszenzmarkierung + großes Eiweißmolekül: ***Diffusionskonstante*** ändert sich

Aggregation

Markierte Proteine: ***Lichtintensität*** von Dimere, Tetramere... ist höher

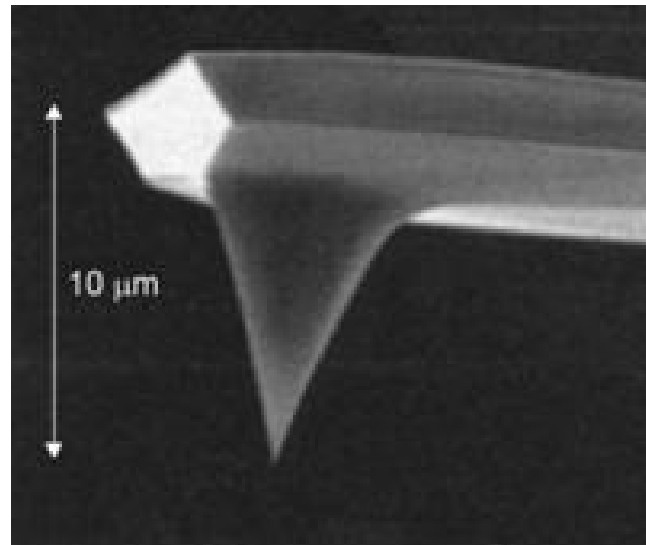
Konzentration

Reaktionsgeschwindigkeit

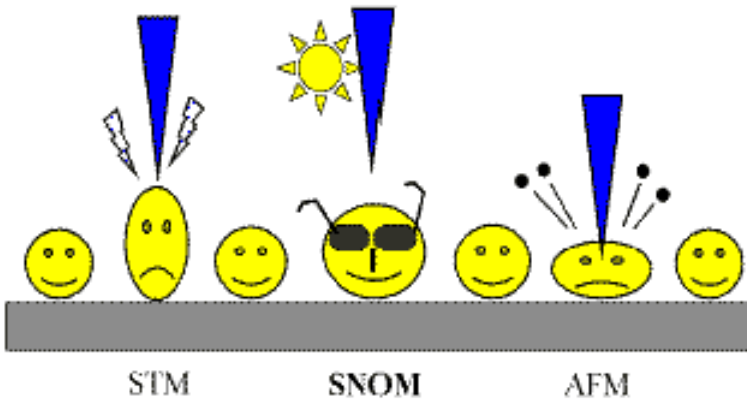
Diffusion in der Inneren der Zellen

Die Autokorrelationsfunktion muss zu einer Modellfunktion angepasst werden um diese Informationen aus der Parametern der angepasste Funktion zu erhalten.

RASTERSONDENMIKROSKOPE



Rastermikroskope (Scanning Probe Microscopes)



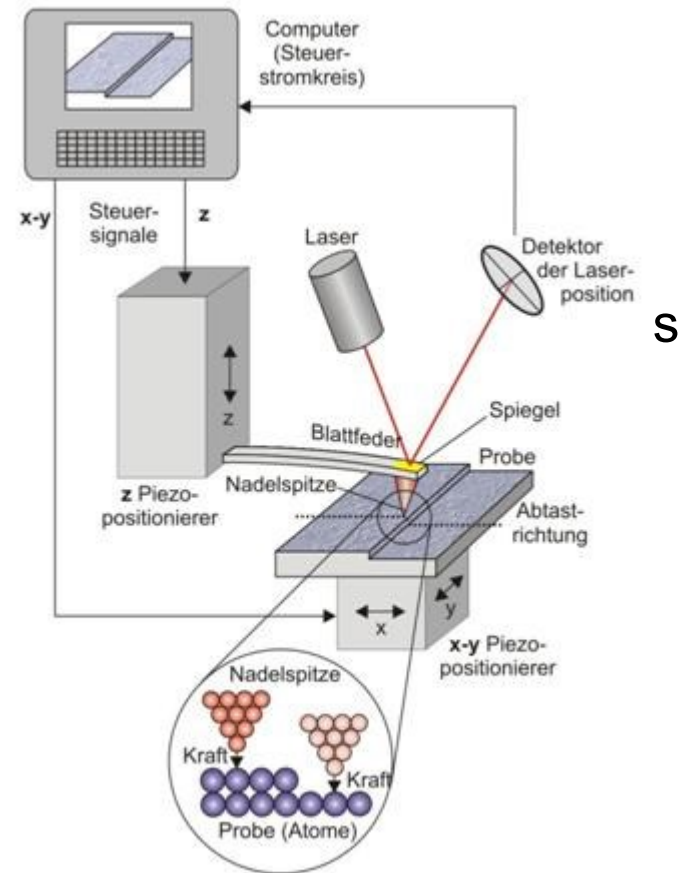
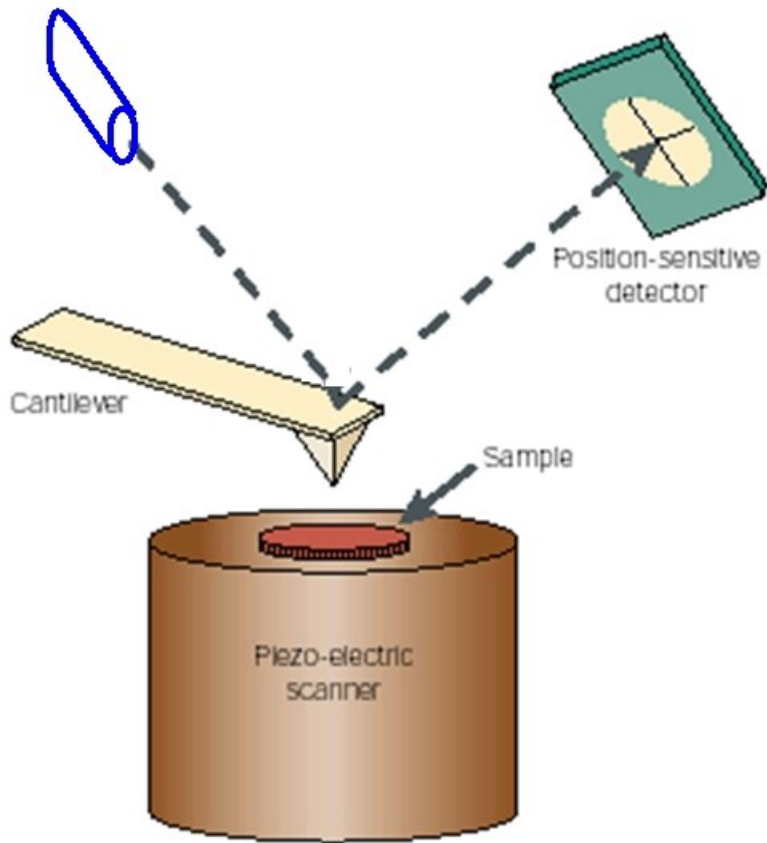
STM:
Scanning Tunneling Microscope
Rastertunnelmikroskop

SNOM:
Scanning Nearfield Optical Microscope

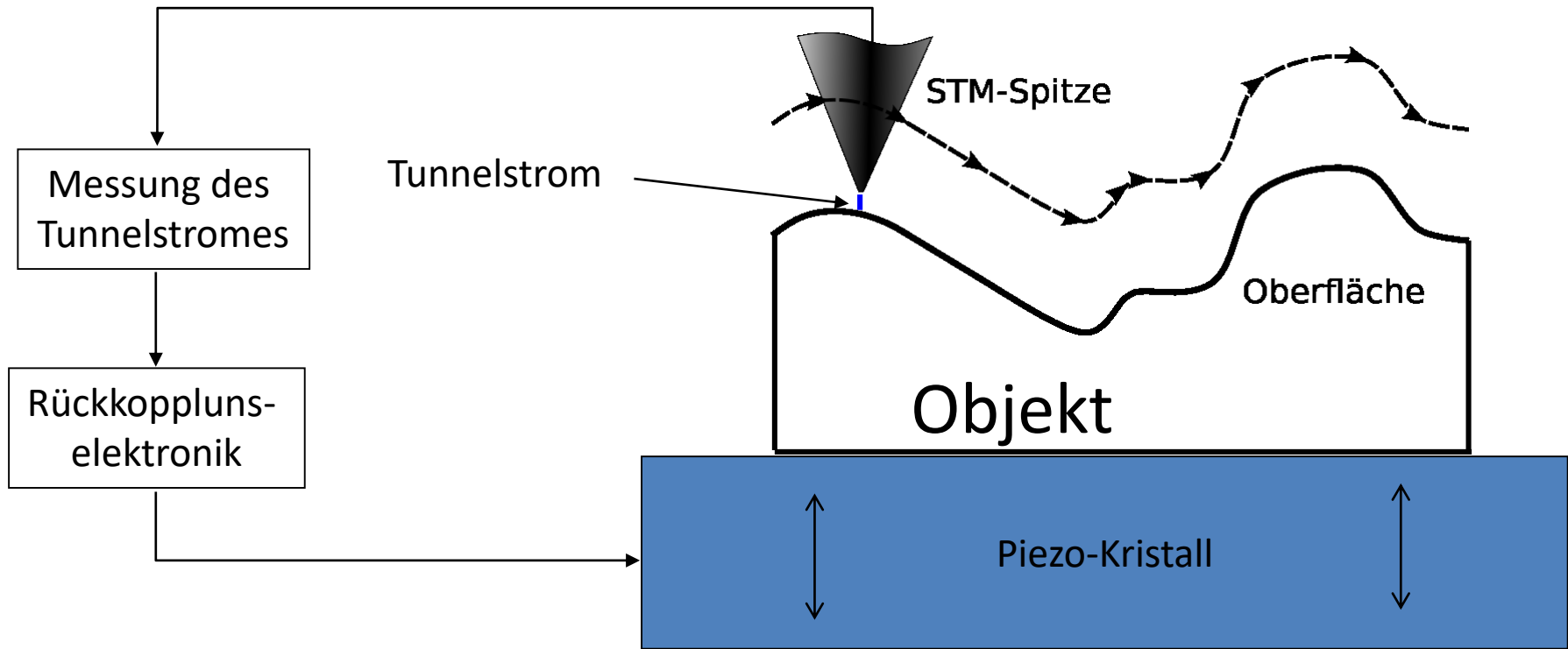
AFM:
Atomic Force Microscope
Rasterkraftmikroskop
(Atomkraftmikroskop)

Das Rastertunnelmikroskop wurde in 1981 von Heinrich Rohrer und Gerd K. Binnig entwickelt. Fünf Jahre später erhielten sie den Nobel-Preis.

Rasterkraftmikroskop (Atomkraftmikroskop) (Atomic Force Microscope-AFM)



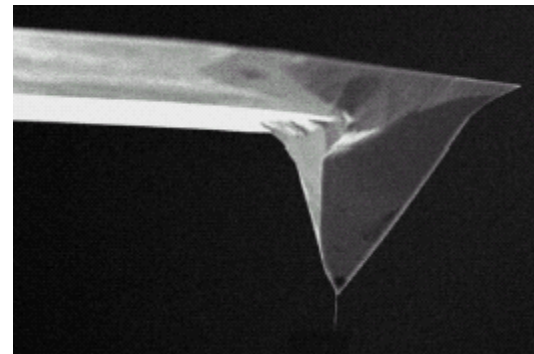
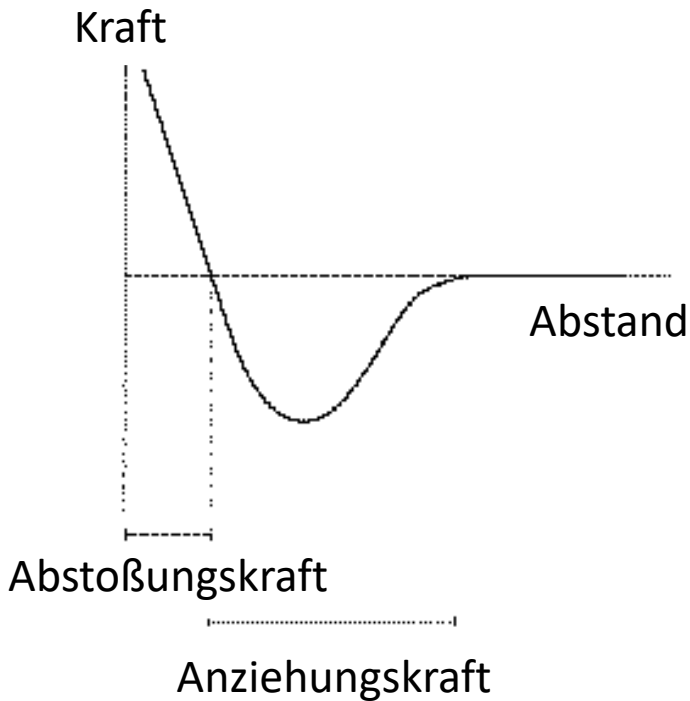
Rastertunnelmikroskop



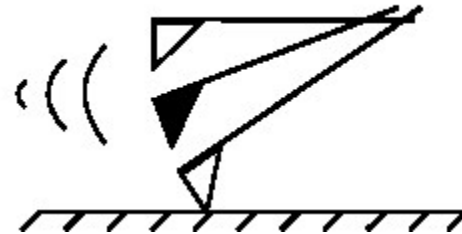
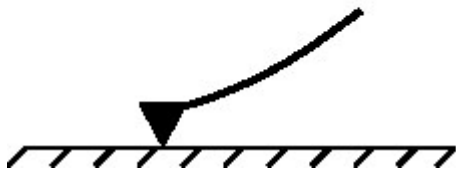
Der Tunnelstrom ist konstant gehalten mit der vertikalen Bewegung des Objektes.

Die Kraft zwischen der Nadel und dem Objekt

- eine sehr spitze, nadelartige Sonde
- Krümmungsradius bei der Spitze $\approx 10\text{-}20\text{ nm}$ \Rightarrow x-y Auflösung!



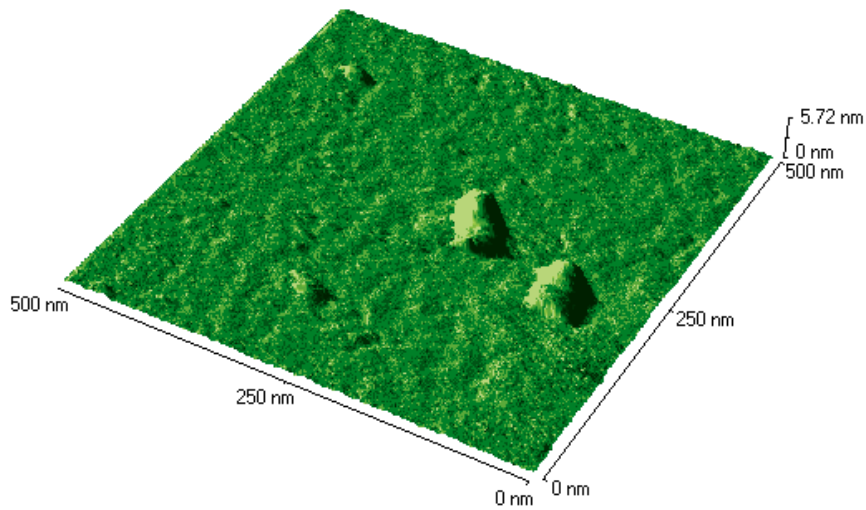
AFM Messmethoden



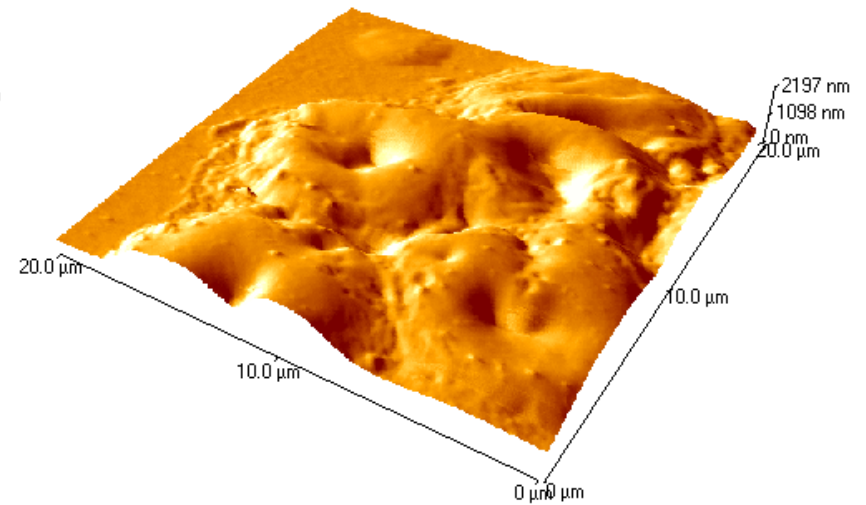
- Kontakt-Modus
- Der intermittierende Modus
(engl.: *intermittent contact mode*, oder *tapping mode* genannt)

AFM Aufnahmen

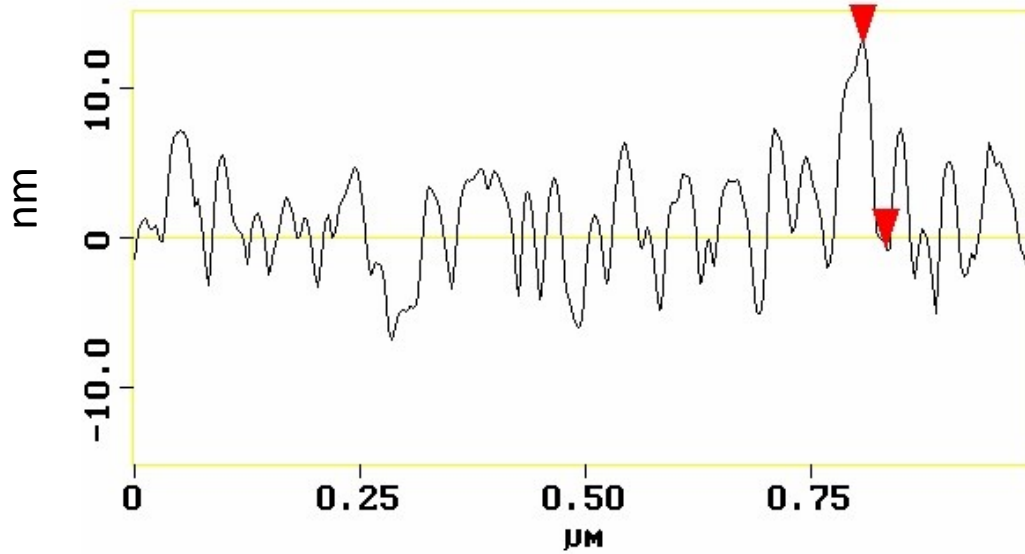
Hitzeschockproteine



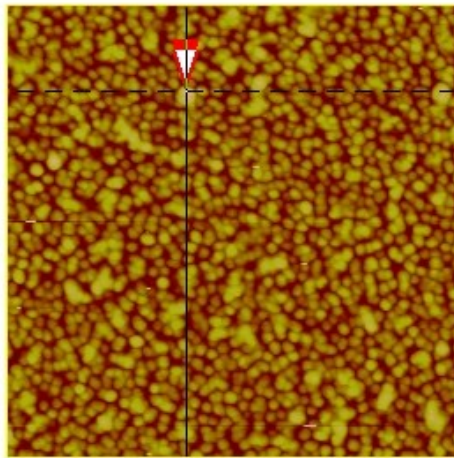
Rote blutzellen



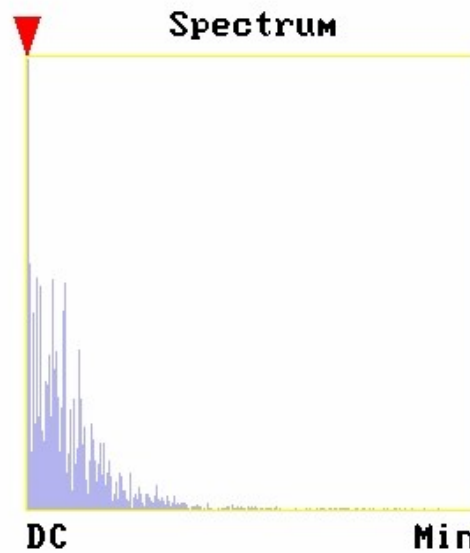
AFM Aufnahmen



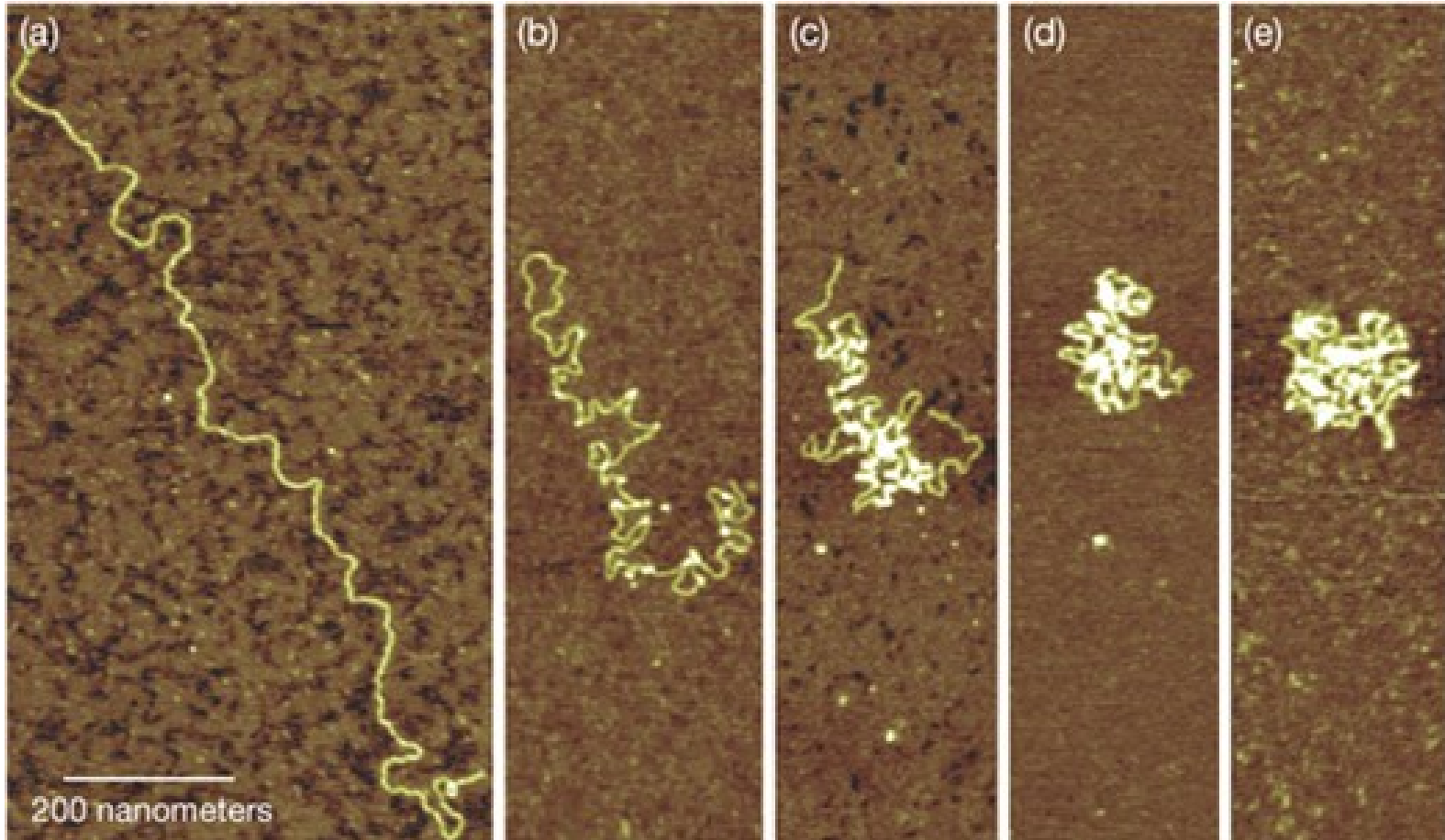
Alpha-Crystallin
Aggregate



acryins1.004



DNS



Progressive images from atomic force microscopy show the compaction of DNA caused by a protein called AbF2. (<https://www.llnl.gov/str/May04/DeYoreo.html>)