Principles & Practice of Light Microscopy 5

Special Techniques

(TIRF, FRET, FRAP , FLIP , FLIM , FCS, molecular sensors...)

Edited by: Zvi Kam, Weizmann
For Advance Light Microscopy course

Total Internal Reflection Fluorescence (TIRF)

TIRF

Total Internal Reflection Microscopy

Problem: background light from other planes

One approach: confocal

Even more powerful
solution: TIRF
IF the object of interest
is at the surface

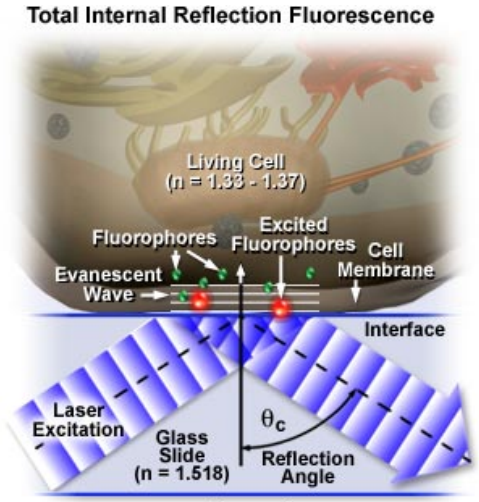
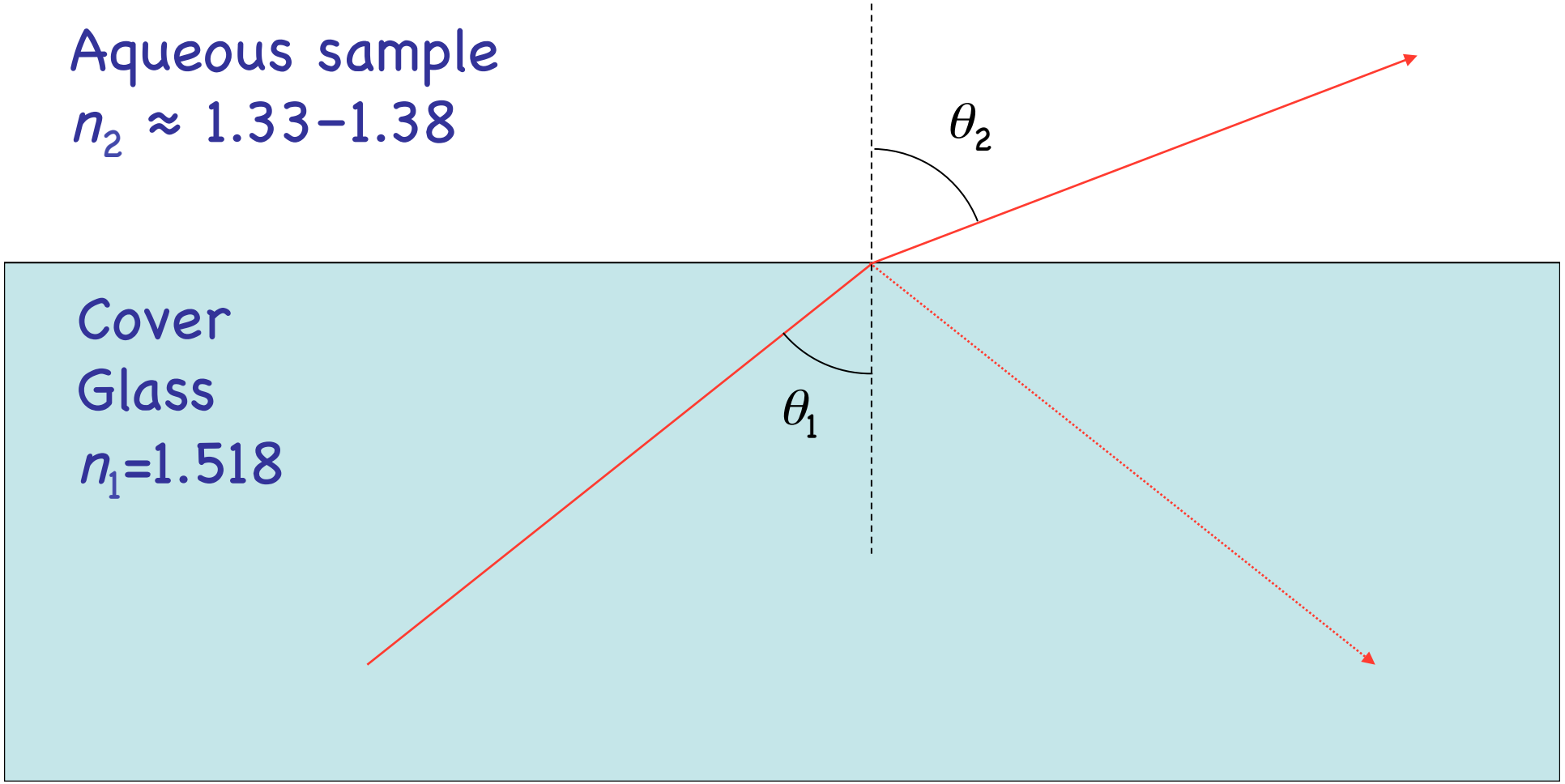


Figure 1

Total Internal Reflection

Snell's Law: $n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$



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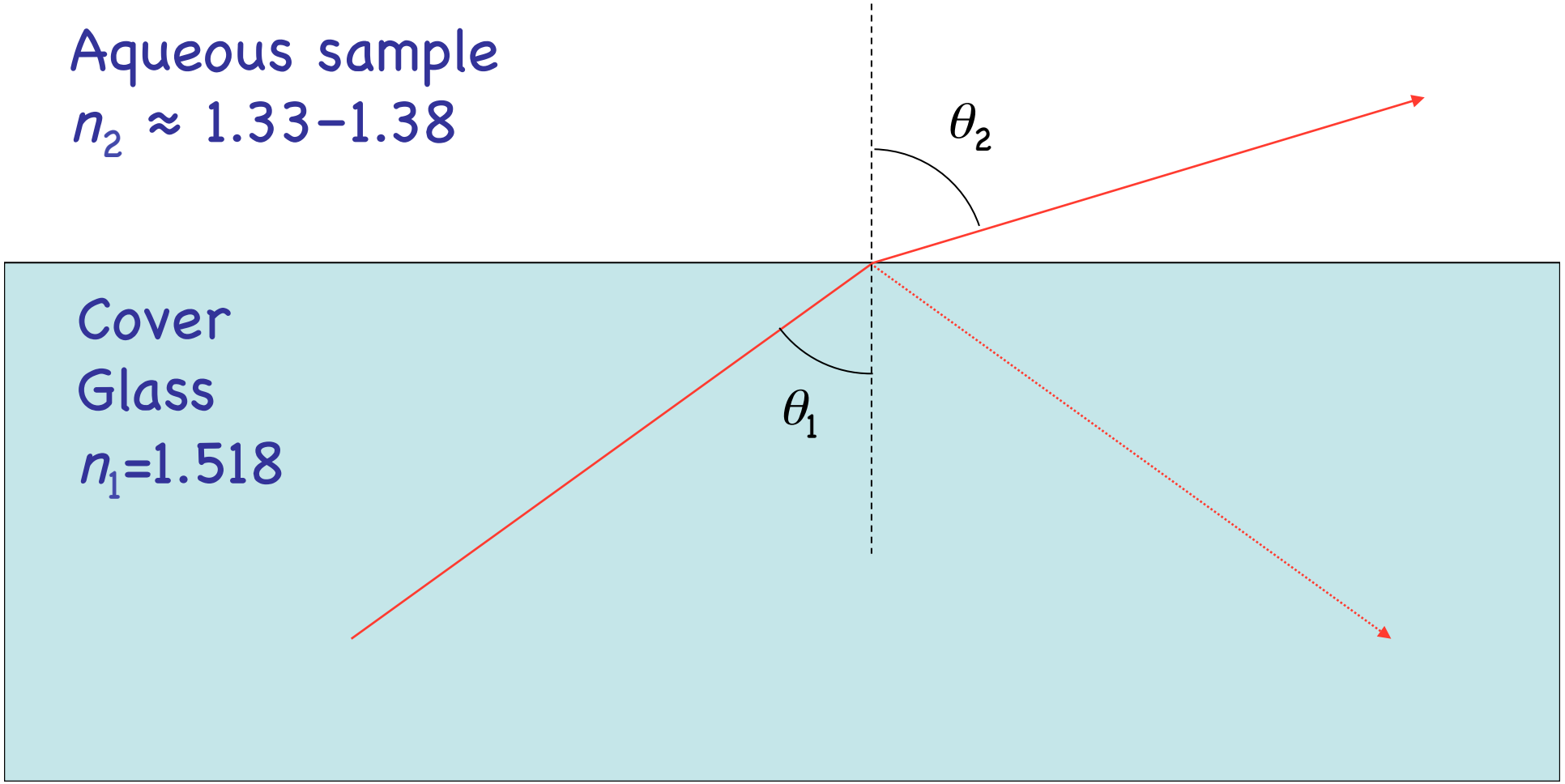

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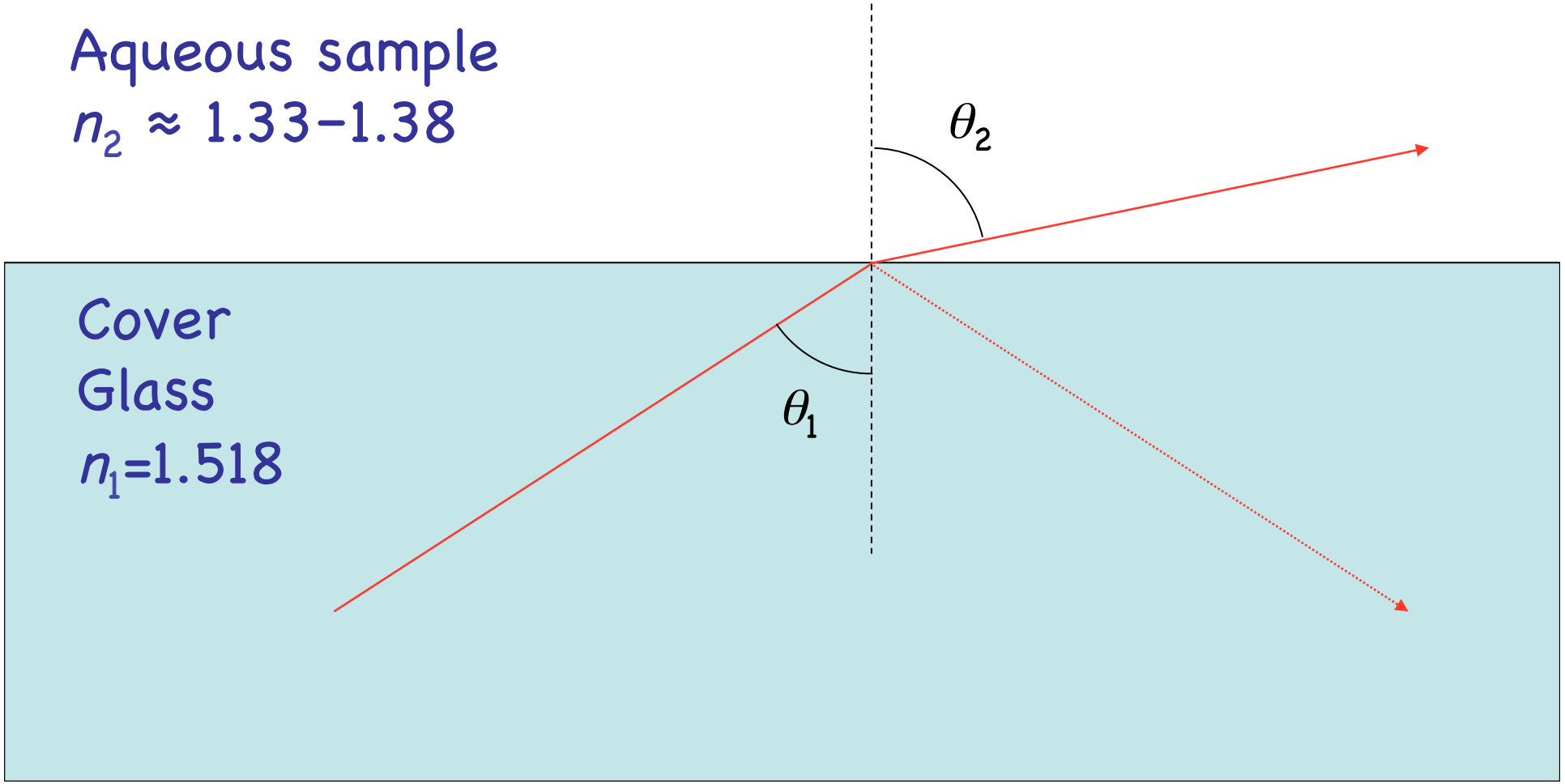

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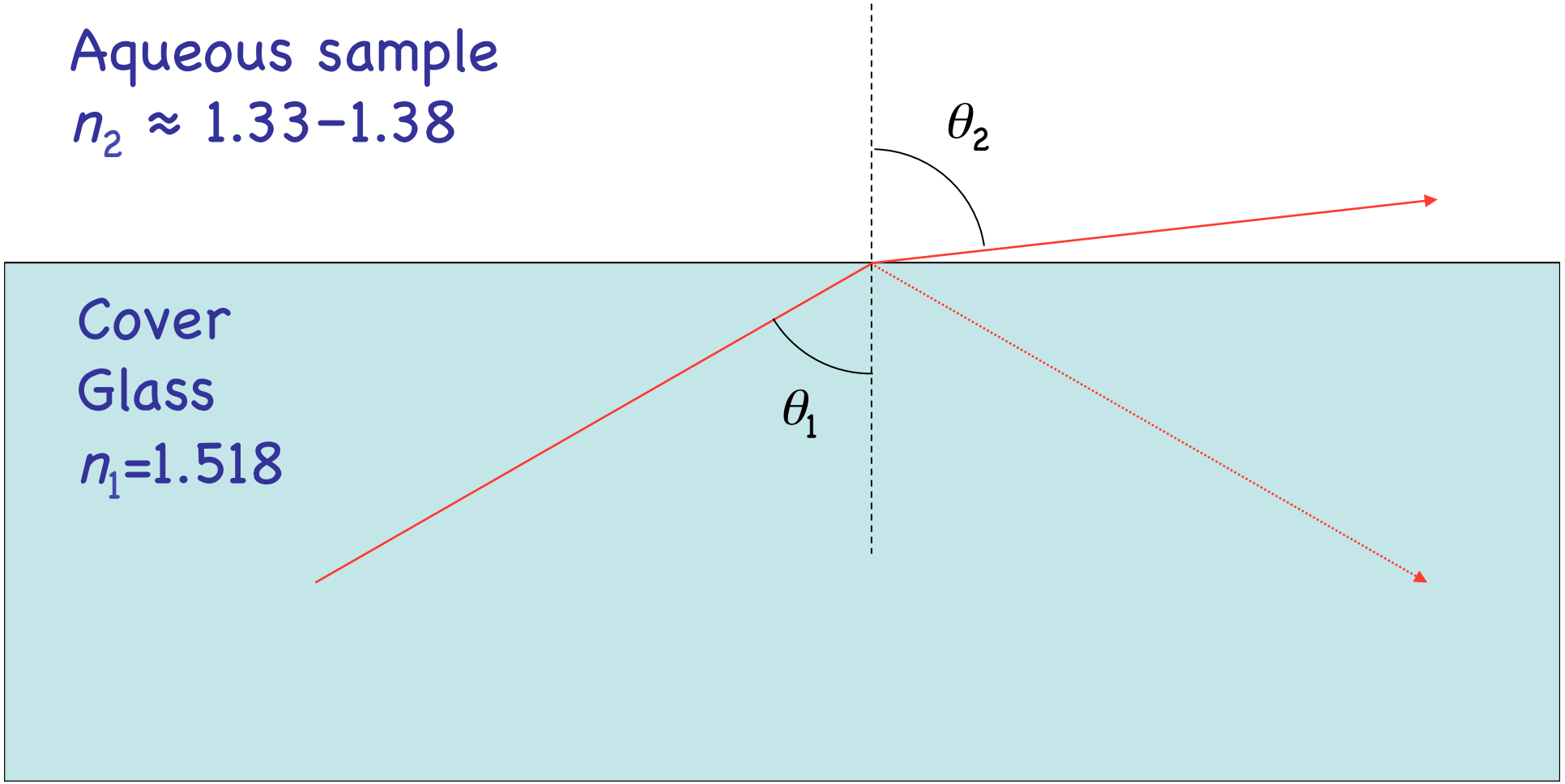

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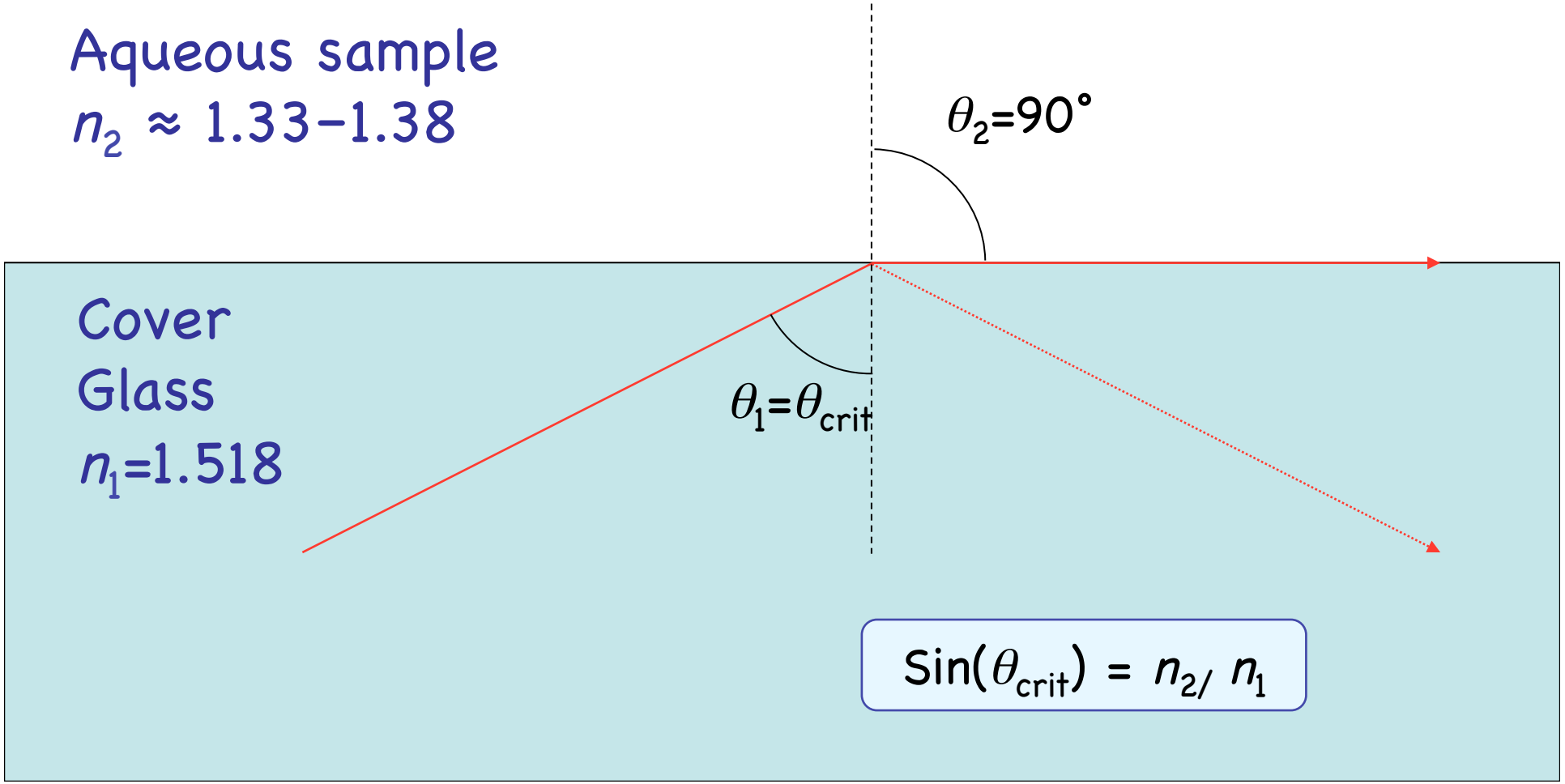

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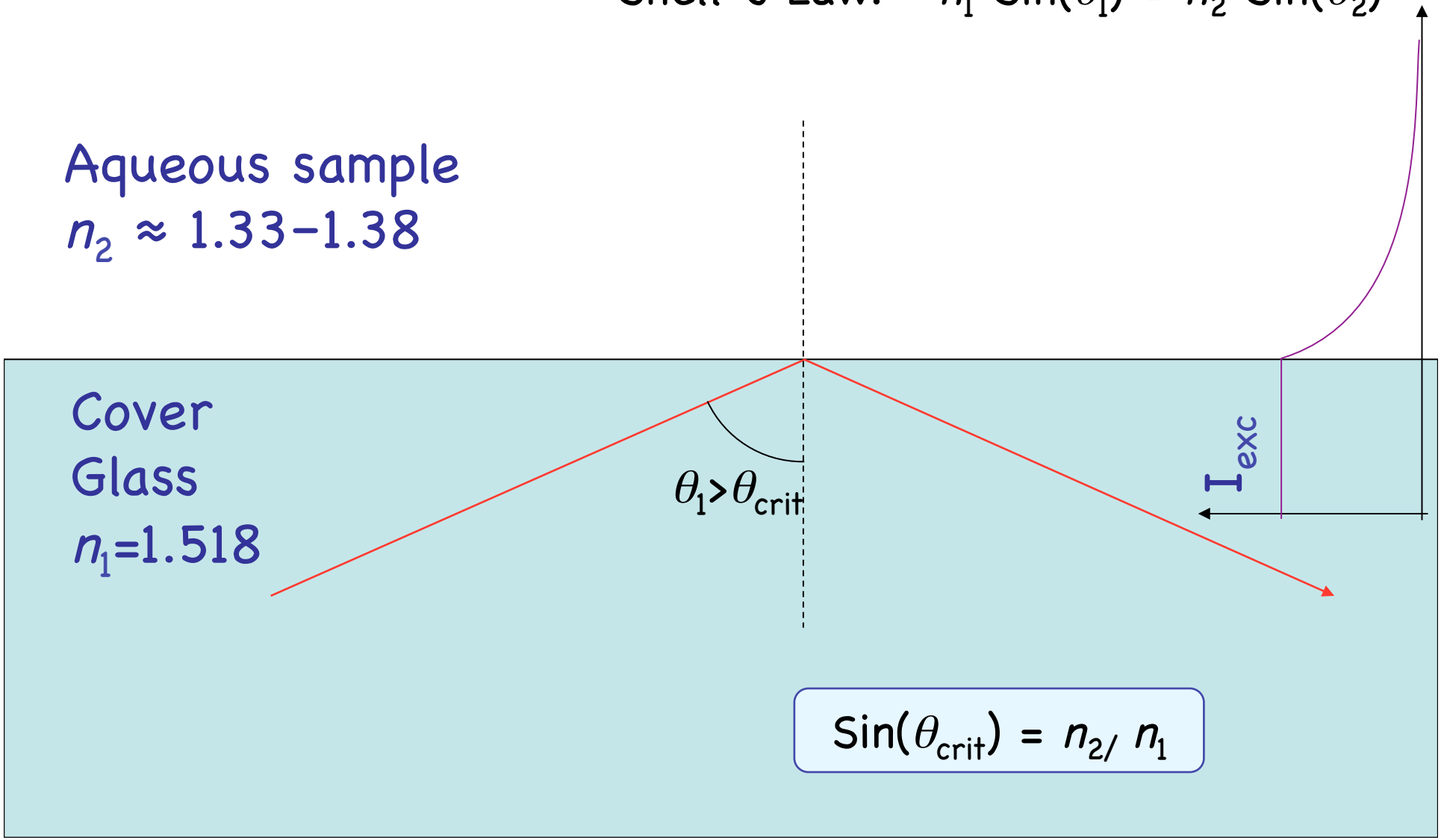


Total Internal Reflection

Decay length vs. angle

$$k_z = \sqrt{k_2^{\,2} - k_x^{\,2}} =$$

$$= \sqrt{\left(\frac{n_2}{\lambda}\right)^2 - \left(\frac{n_1 \mathrm{Sin}(\theta_1)}{\lambda}\right)^2}$$

$$= \frac{1}{\lambda}\sqrt{n_2^{\,2} - n_1^{\,2}\mathrm{Sin}^2(\theta_1)}$$

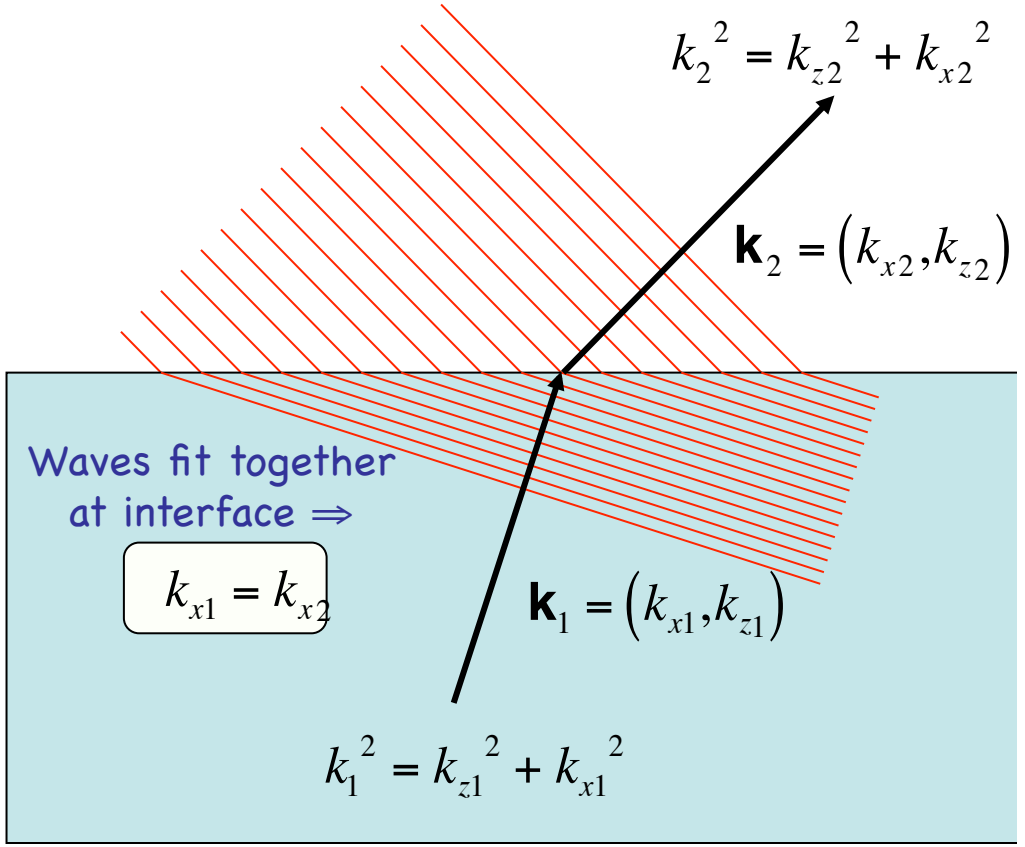
If $n_1 Sin(\theta_1) > n_2 \qquad \theta_1 > \theta_{crit}$

then k_z is imaginary: $k_z = ib$

$$E \propto e^{2\pi i k_z z} = e^{-2\pi b z}$$

$$I \propto |E|^2 \propto e^{-4\pi b z} = e^{-z/z_{TIRF}}$$

$$z_{\mathrm{TIRF}} = 1/4\pi b = \lambda \Big/ 4\pi\sqrt{n_2^{\,2} - n_1^{\,2}\mathrm{Sin}^2(\theta_1)}$$

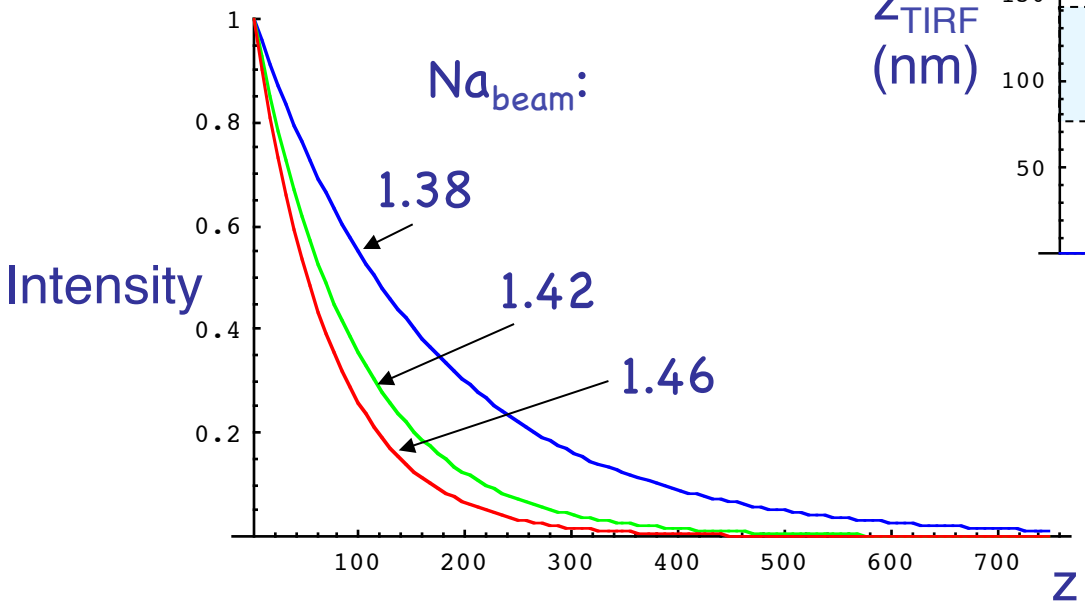


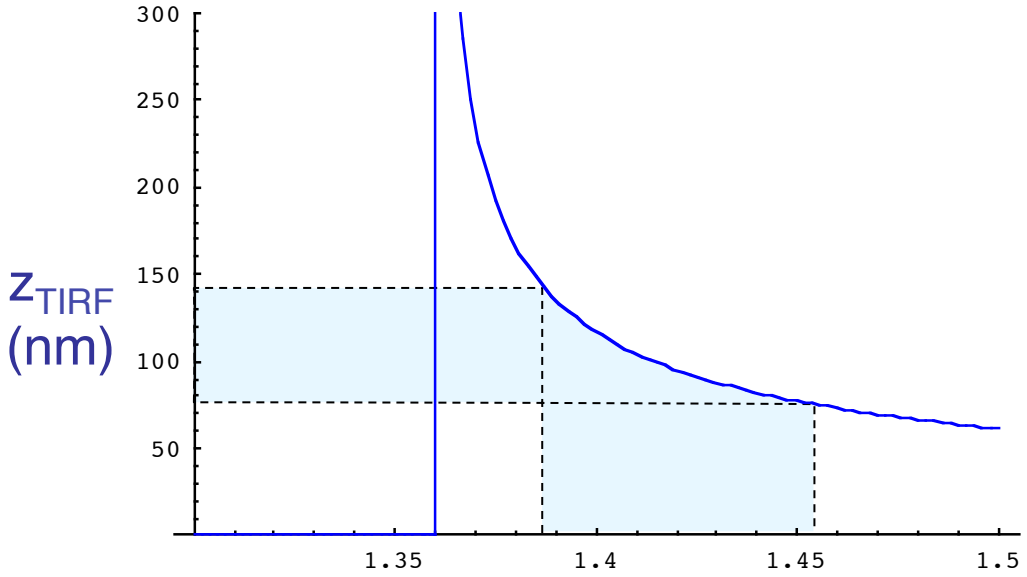
Total Internal Reflection

Decay length vs. angle

$$I \propto e^{-z/z_{TIRF}} = e^{-z\frac{4\pi}{\lambda}\sqrt{n_2{}^2 - n_1{}^2\,\mathrm{Sin}^2(\theta_1)}}$$

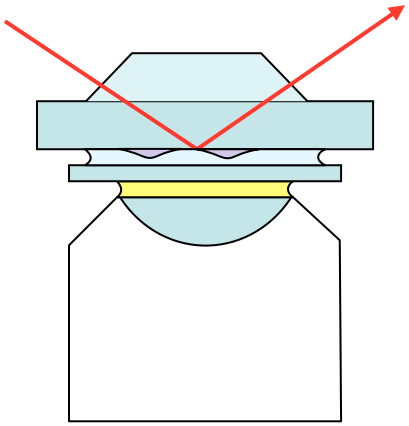
Typical TIRF depth
≈ 75–150 nm





Two forms of TIRF microscopy

Prism coupled

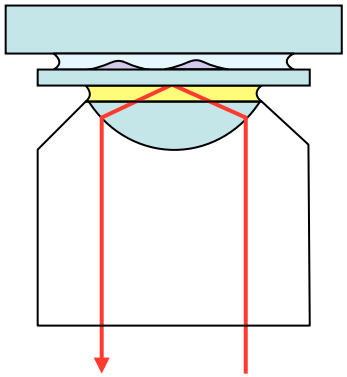


Sample on slide

- No excitation light in emission path
  ⇒ Very low background (if quartz slide)
- Needs separate, external beam path
  ⇒ Harder to align

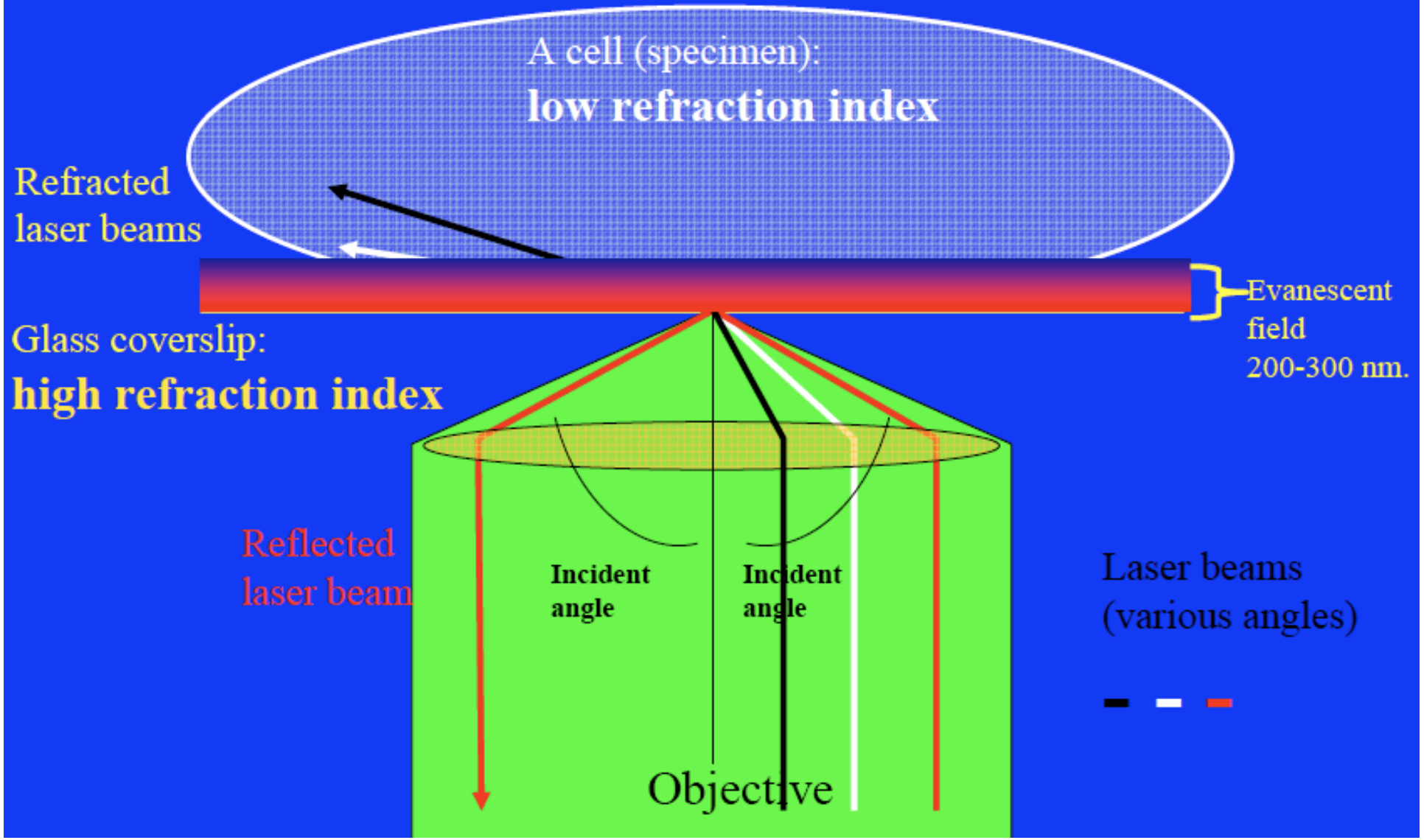
Through the objective

Sample on cover glass

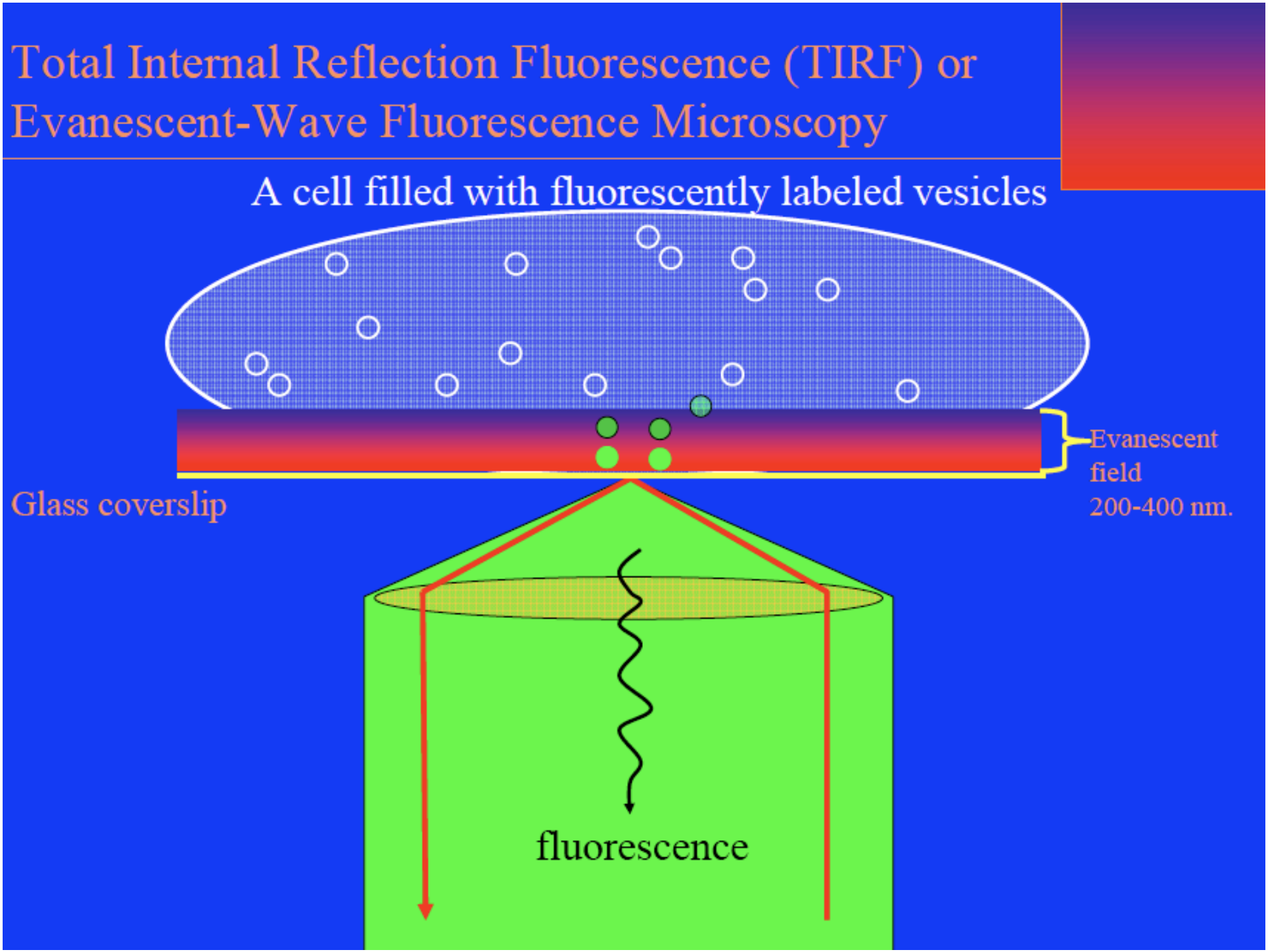


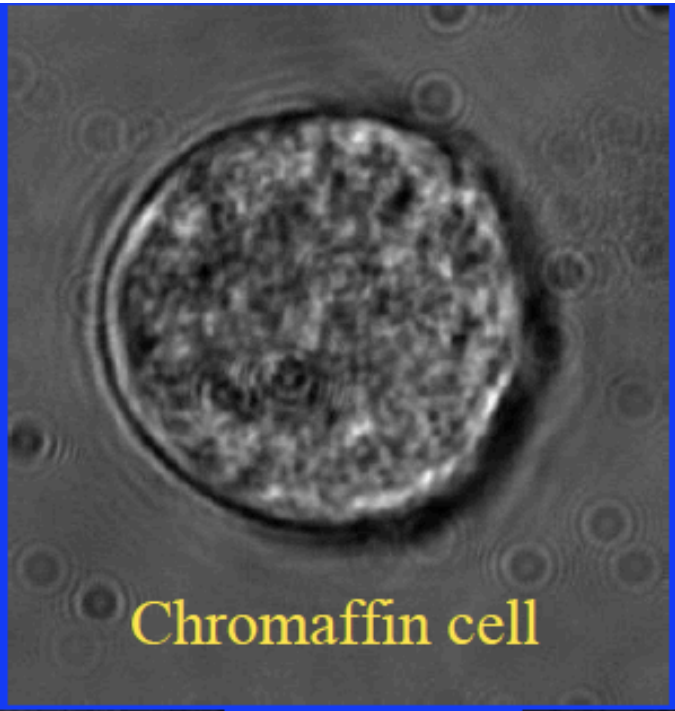
- Easy to align
- Excitation light in emission path
  ⇒ Vulnerable to autofluorescence in the optics
- Requires very high NA

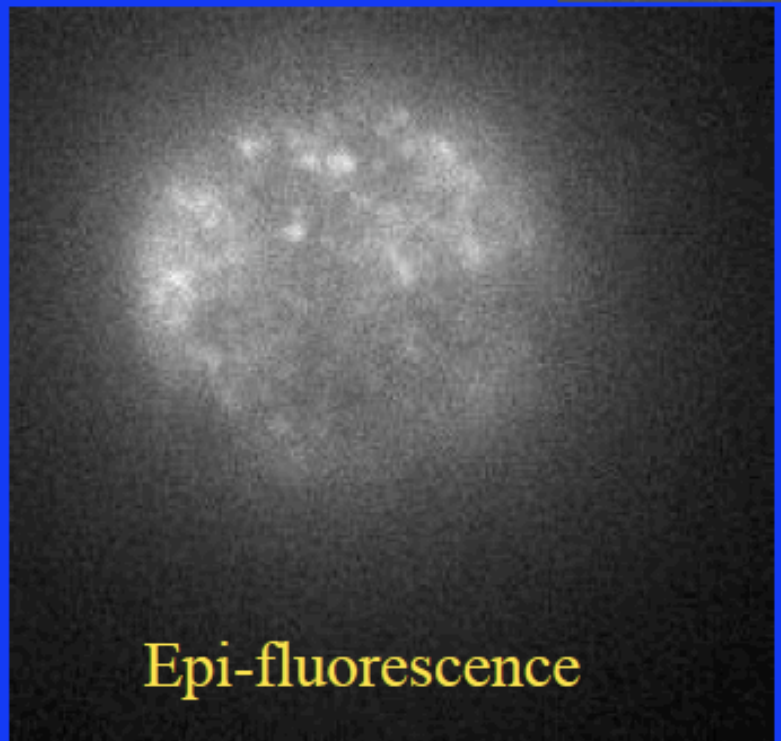
Total Internal Reflection Fluorescence (TIRF) or Evanescent-Wave Fluorescence Microscopy

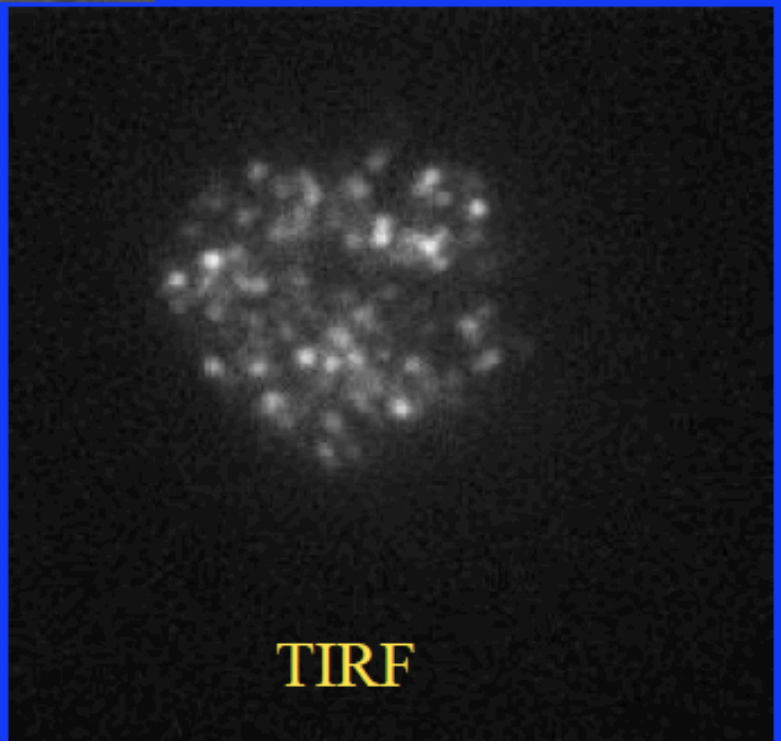


Total Internal Reflection Fluorescence (TIRF) or Evanescent-Wave Fluorescence Microscopy

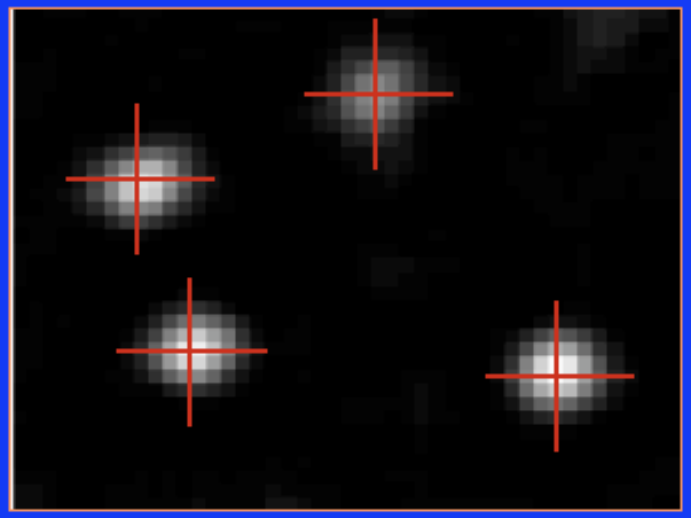



Chromaffin cell


Epi-fluorescence

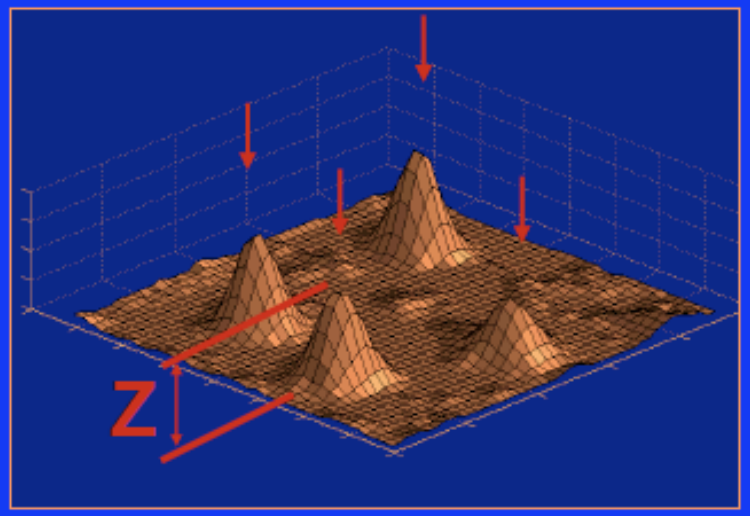

TIRF

Identification of individual vesicles



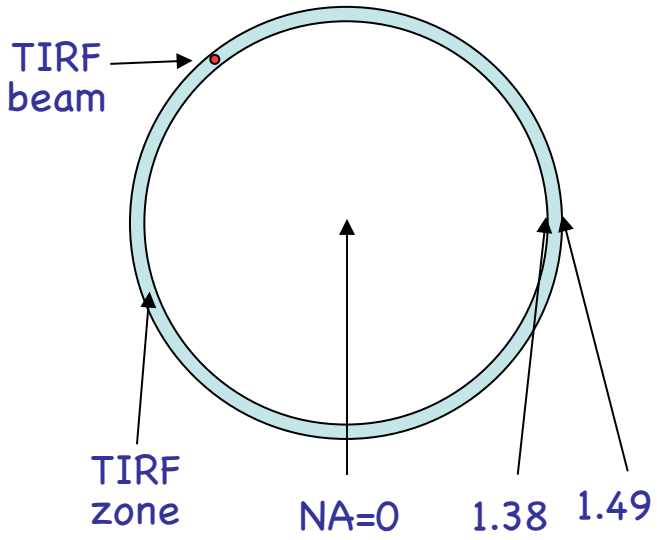
Y

X



TIRF Objectives

Back focal plane
$r \propto n \sin(\theta) \sim$ "NA"



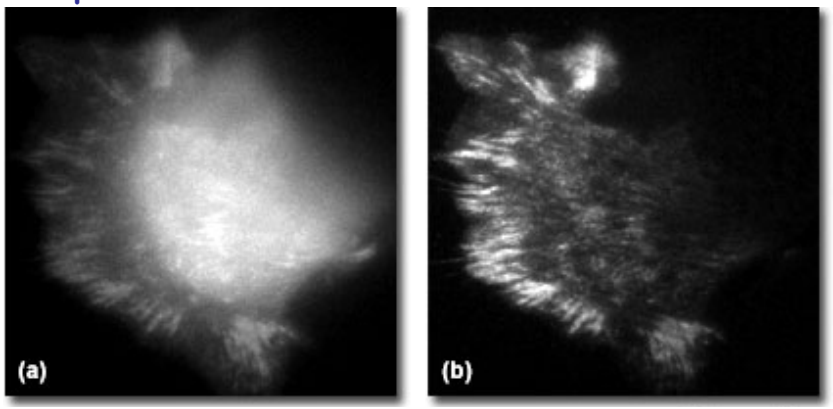
Typical NA 1.45–1.49

Extreme example: Olympus NA 1.65
Requires special high-index cover glass
and (volatile, toxic) immersion fluid

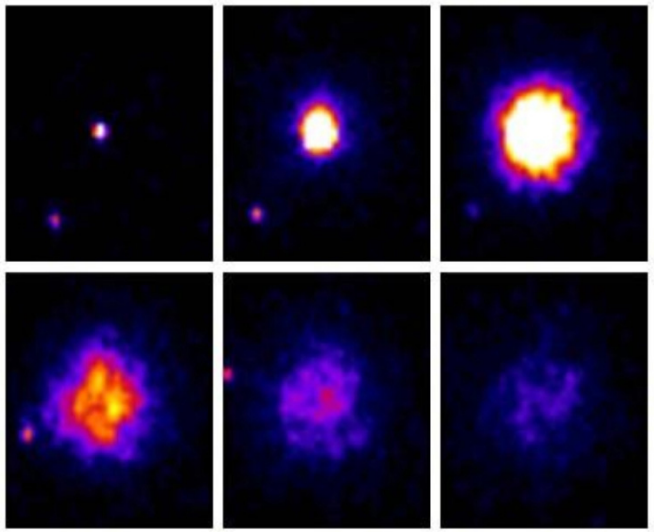
TIRF examples

Focal adhesions

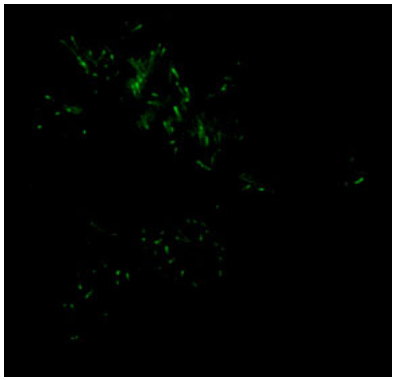
Epifluorescence TIRF



Single vesicle release
1 s intervals



Microtubule growth
movie

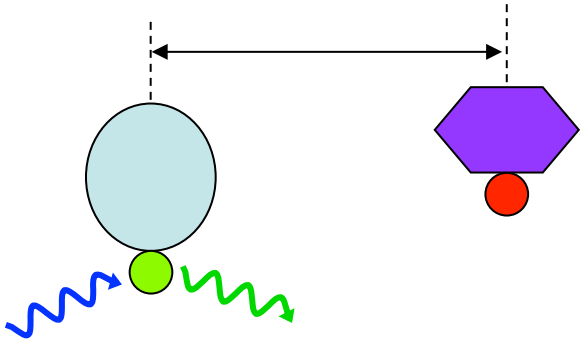


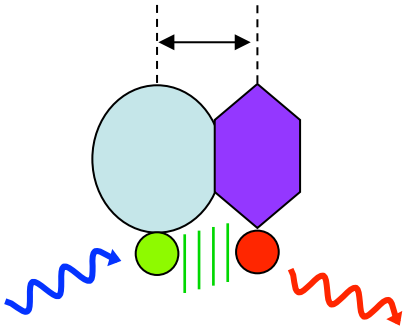
Fluorescence Resonance Energy Transfer (FRET)

FRET

Fluorescence Resonance Energy Transfer
or Förster Resonance Energy Transfer

Need: sense *interactions*
Idea: sense *distance*





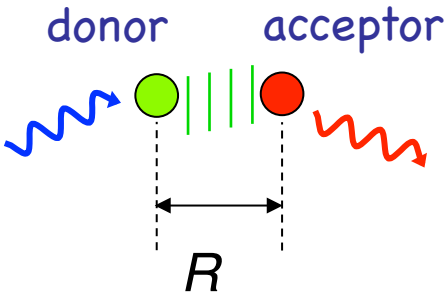
Excitation energy is transferred
from donor to acceptor

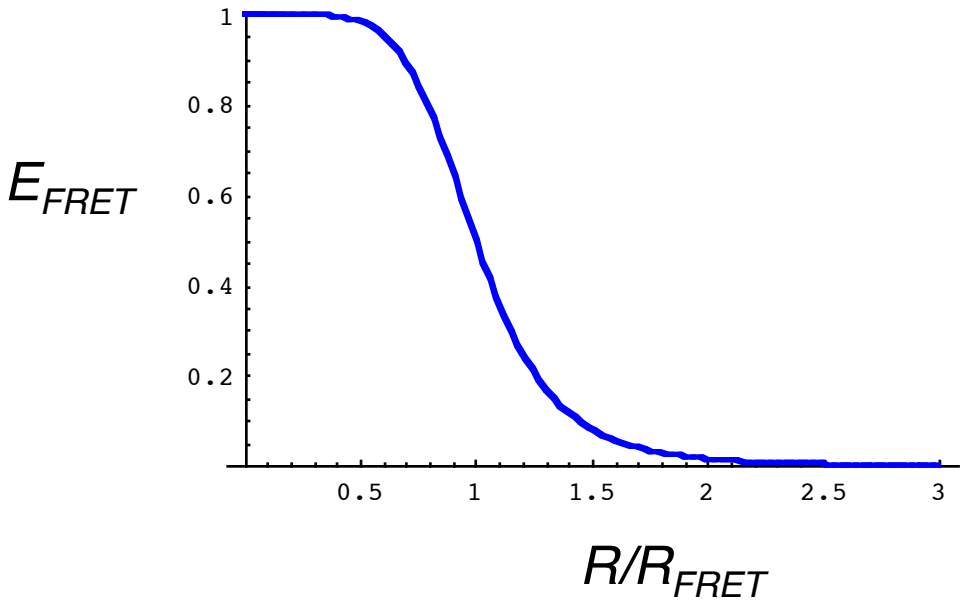
Efficiency depends on distance
(and on angles, motion...)

FRET efficiency vs. distance

Characteristic distance R_{FRET}

$$\text{FRET efficiency} \propto 1\Big/\left(1+\left(R/R_{FRET}\right)^6\right)$$

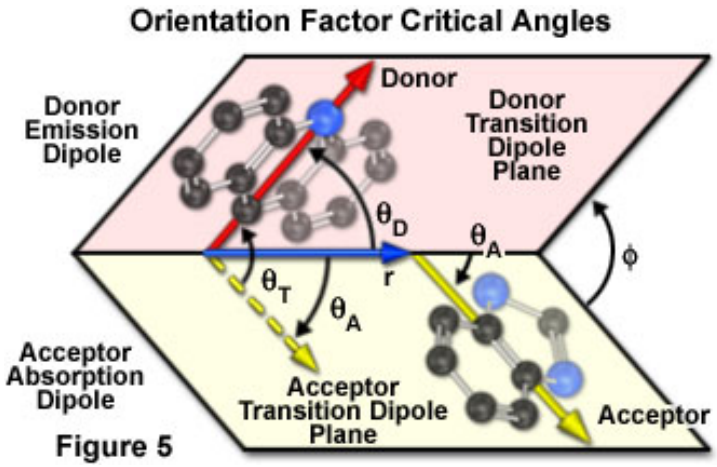




Example:
FITC→TRITC
R_{FRET}≈5.5 nm

Distance scale
~ 50 times smaller than
microscope resolution

FRET efficiency vs. angles

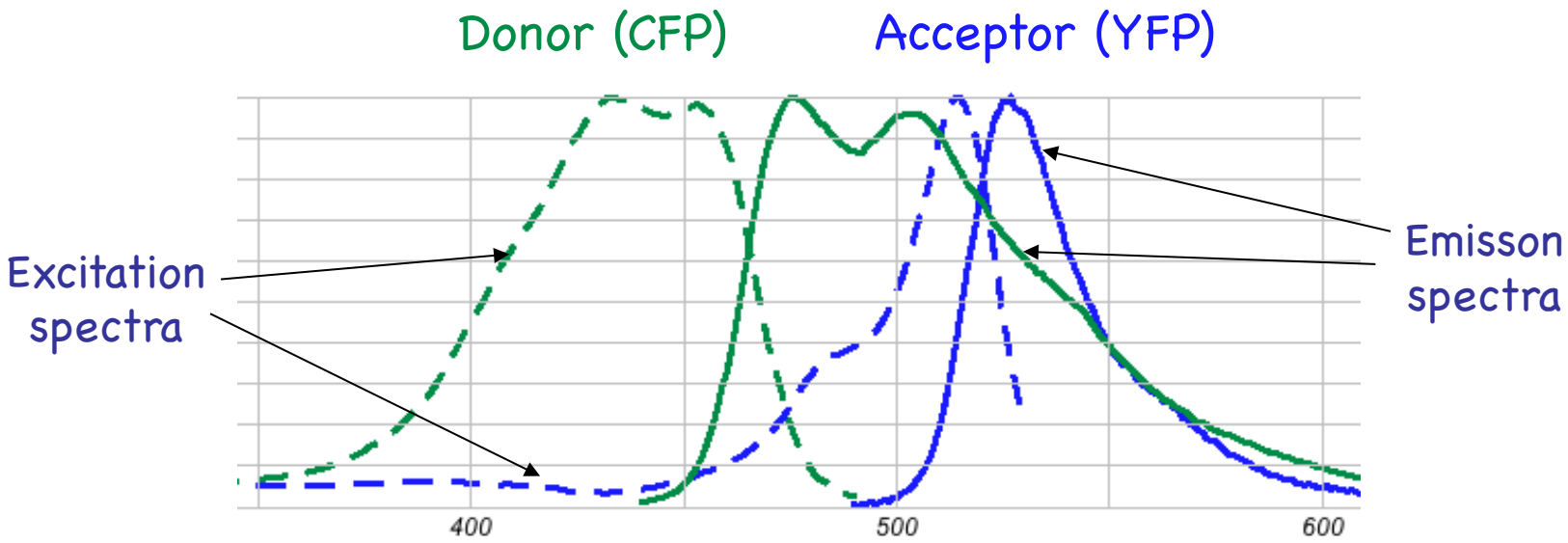


$$R_{FRET} \propto (\cos \theta_T - 3\cos \theta_D \cos \theta_A)^{1/3}$$

Angle effects less important if the fluorophores tumble freely faster than the fluorescent decay time

FRET Pairs

Förester Spectral overlap integral

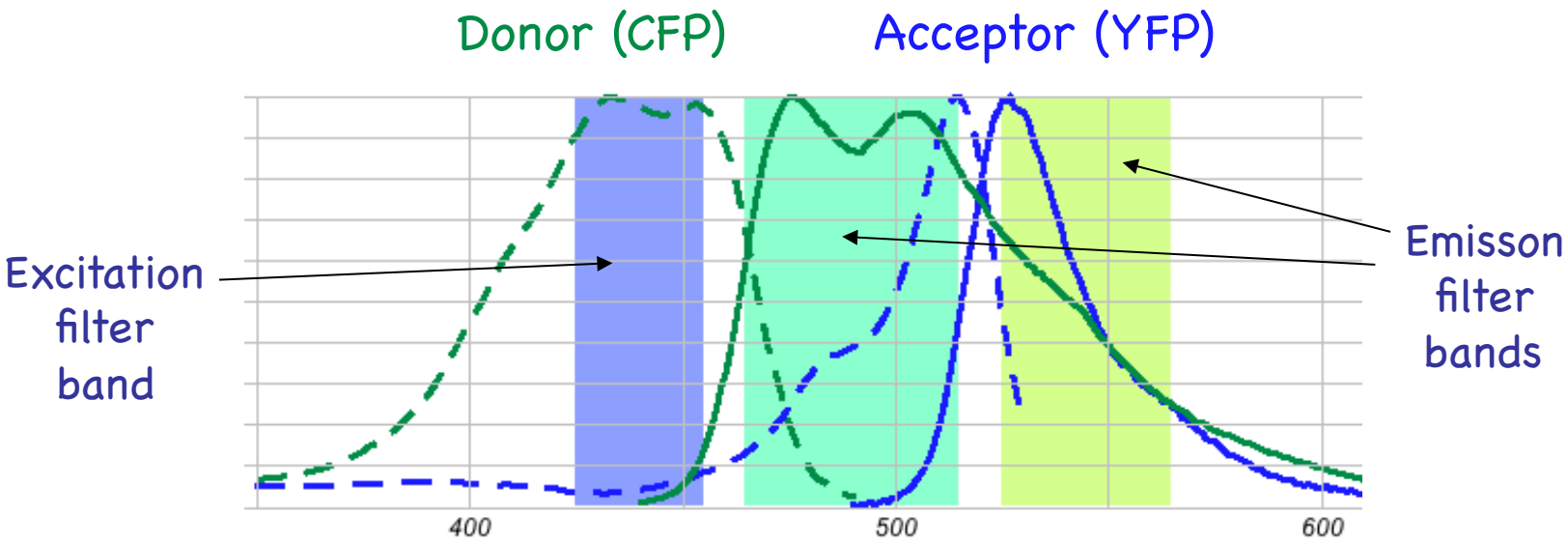


$$E_{FRET} \propto \int Em_{donor}(\lambda) Exc_{acceptor}(\lambda) d\lambda$$

Protein FRET pairs:

CFP / YFP
Cerulean / Citrine ?
CyPet / Ypet ?
EGFP / MKO / cherry ?

FRET detection



Unwanted crosstalk between bands

FRET

How can one detect it?

Want to know FRET efficiency separately from concentration

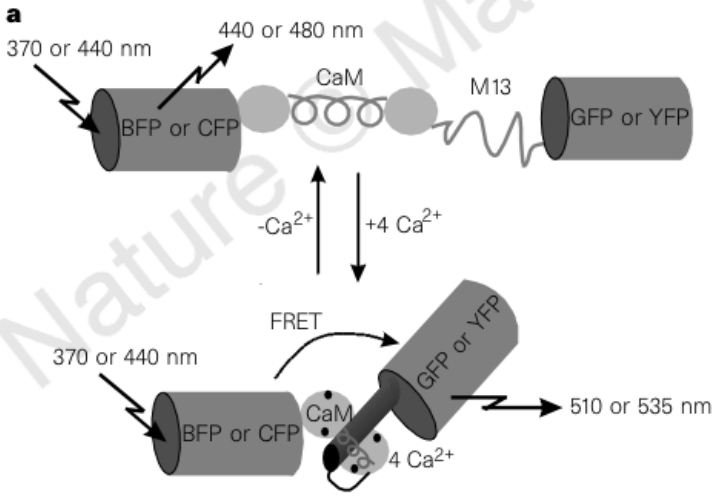
- Spectrally
  - Measure intensity of donor-donor, acceptor-acceptor & donor-acceptor (FRET)
- Donor lifetime
  - Lifetime is shortened by FRET
- Acceptor photobleaching
  - Donor intensity rebounds when acceptor is removed
- Donor photobeaching
  - Quenching byFRET slows photobleaching

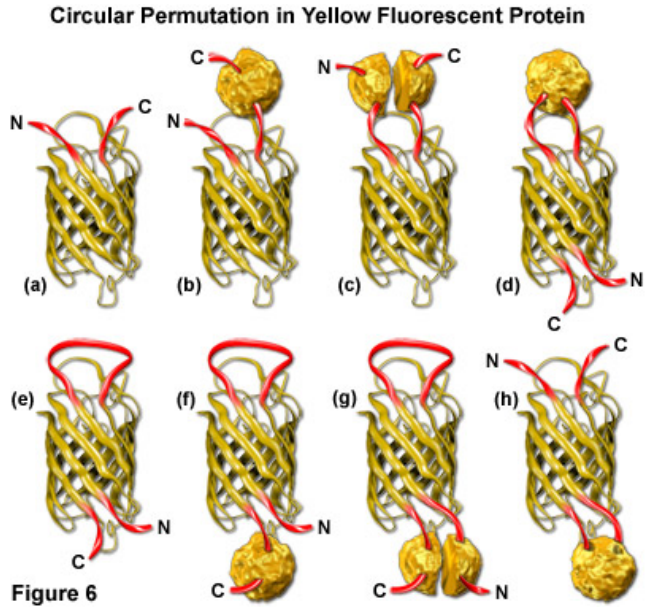
- Need to calibrate for background, crosstalk etc
- Environmental effects can change spectra & lifetimes
  Complicates calibration

FRET-based sensors

- Chameleons
  Conformation change alters FRET

- Camgaroos
  Perturbation of a (circularly permuted) FP by conformation change of a fusion partner





Fluorescence Recovery After Photobleaching (FRAP)

FRAP

Fluorescence Recovery after Photo-bleaching

Need: to probe transport
Idea: bleach in one area,
watch recovery by transport from other areas

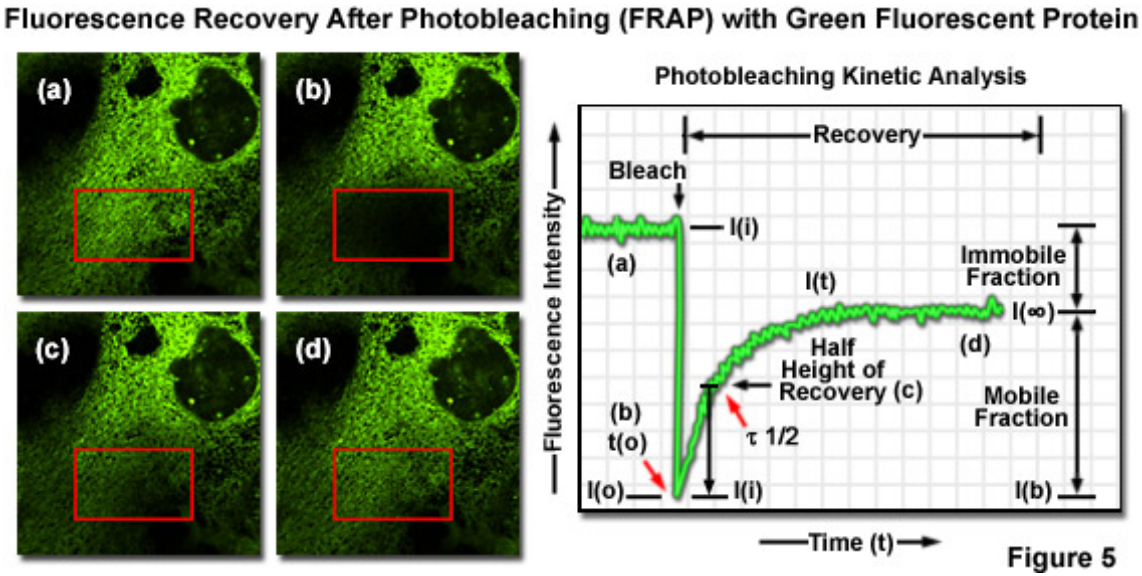
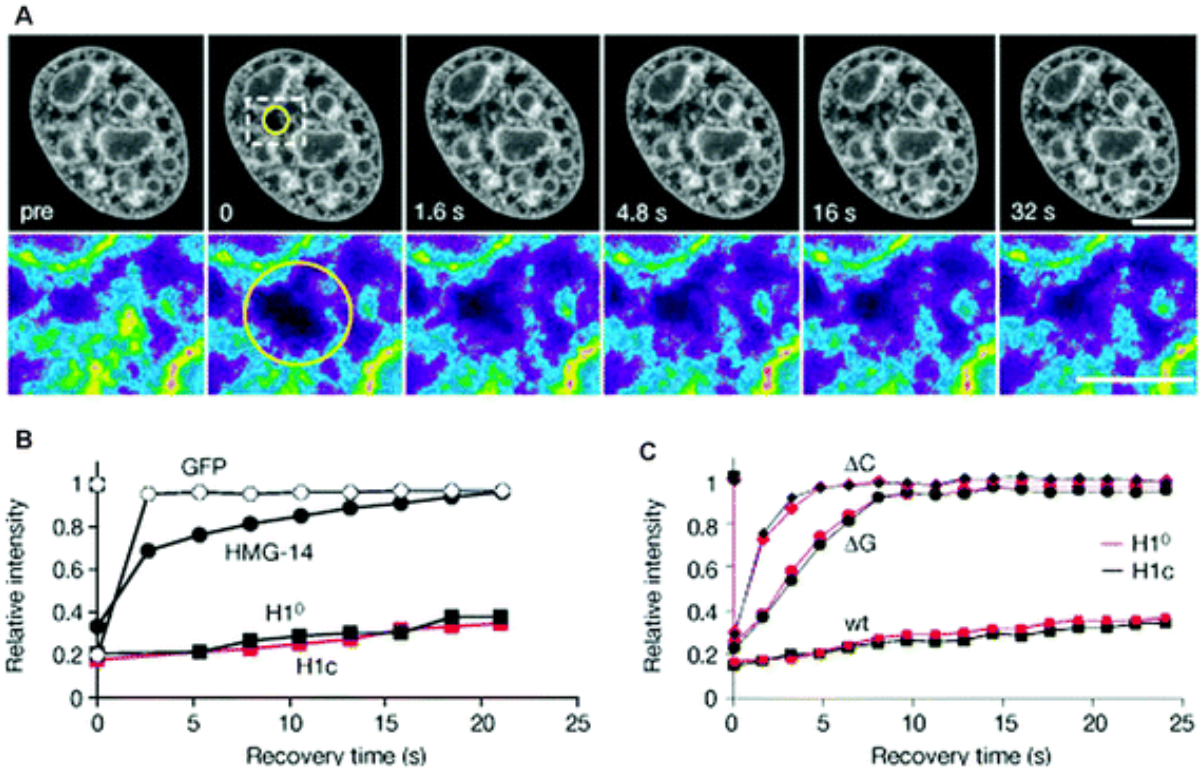


Figure 5

FRAP example



GFP-Histone H1

Wildtype H1 is immobile

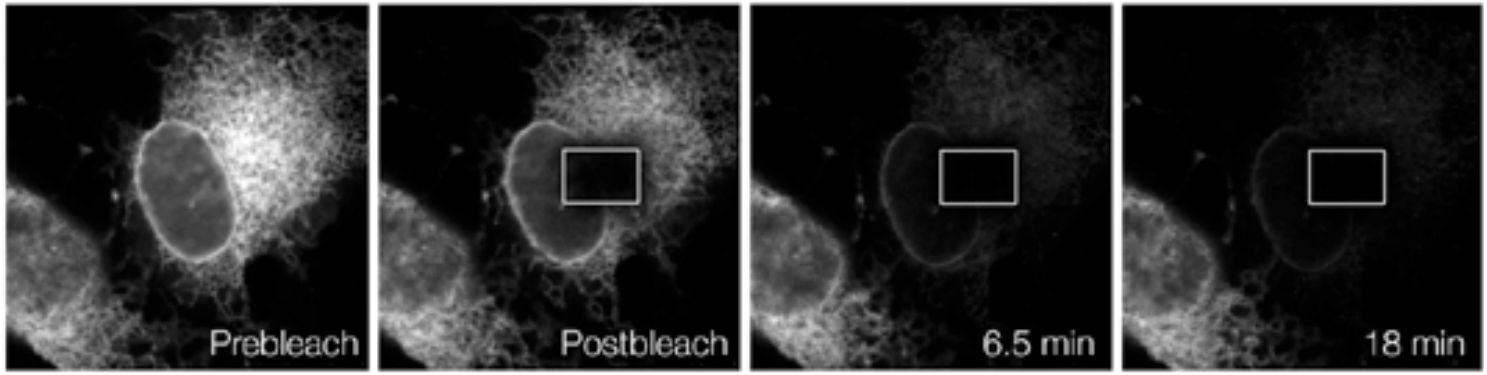
Partially deleted mutants exchange much faster

Fluorescence Loss In Photobleaching (FLIP)

FLIP

Fluorescence Loss in Photo-bleaching

Need: probe connectivity
Idea: bleach in one compartment,
watch loss in connected compartments by exchange



Bleach one area repeatedly. Entire ER dims.
$\Rightarrow$ ER is contiguous

Photo Activation (PA)

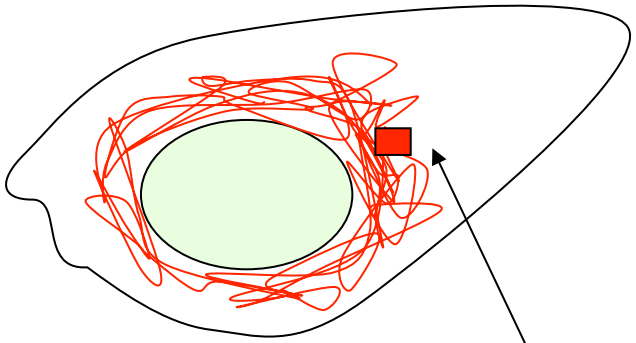
Photo-Activation

(Better?) FRAP/FLIP alternative

Some fluorophores can be activated by light

- Photo-uncagable dyes
- GFP-family proteins

Look for weak light
against dark background
Instead of slight dimming
of bright background



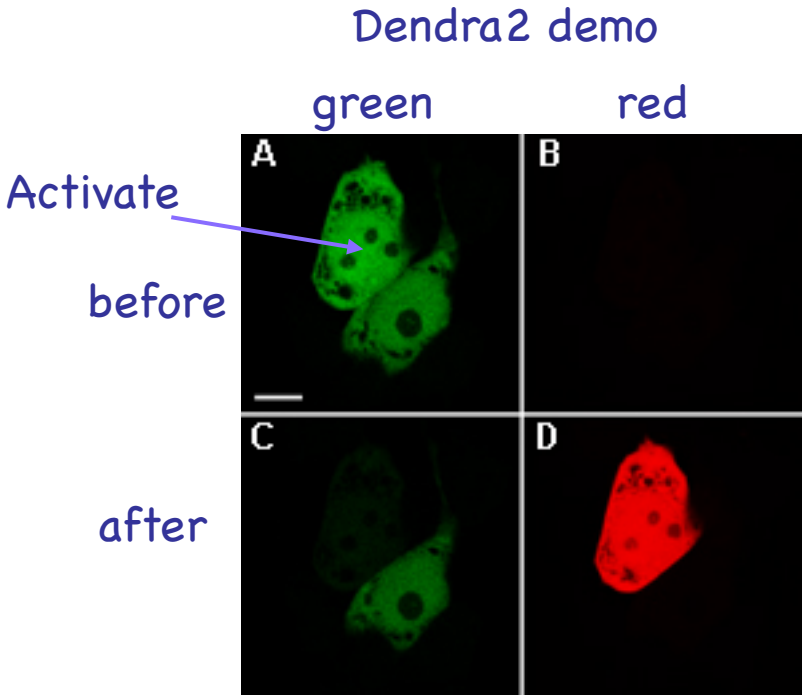
Activate a small area
Watch fluorescence *spread*

Photo-Activativatable Proteins

Off-On
- PA-GFP, PS-CFP

Color change
- Kaede, KikGR, Eos,
- Dendra (activatable by blue)

Reversibly Switchable
- asCP, KFP (tetrameric)
- Dronpa



Fluorescence Correlation Spectroscopy (FCS)

Speckle Analysis

FCS

Fluorescence Correlation Spectroscopy

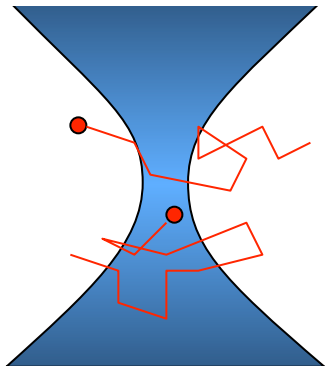
Small volume → only a few molecules → random fluctuations

- Study the *noise*
- Conclude about *random processes* at different *time scales*

Small excitation volume

Random molecular processes:

- Diffuse in and out
- Adopt different states
- Bind or react
- Photobleach
- ...



Study the auto-
(or cross-) correlation

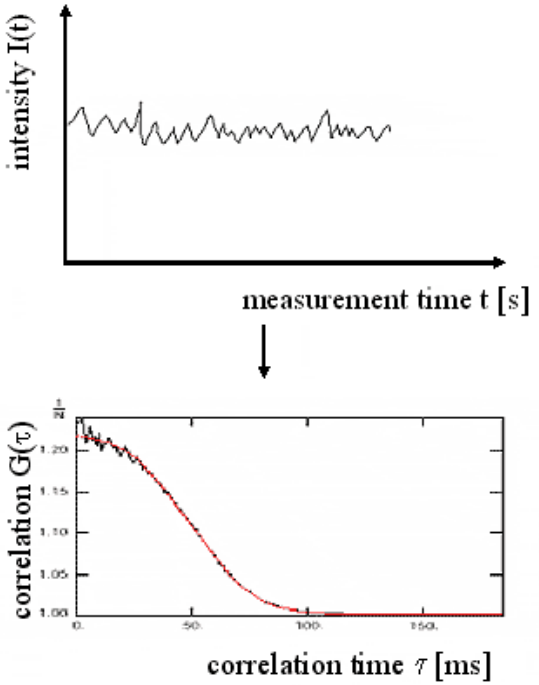


Image Correlation Spectroscopy

Image FCS

Sense the random fluctuations of fluorescence within an *image* or *image sequence*

Much slower than point FCS, but get whole area
Can see where you are & deal with motion
Can do spatio-temporal analysis

Image series
Measure time and space variations in intensity

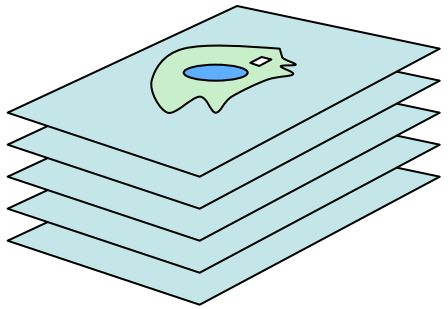
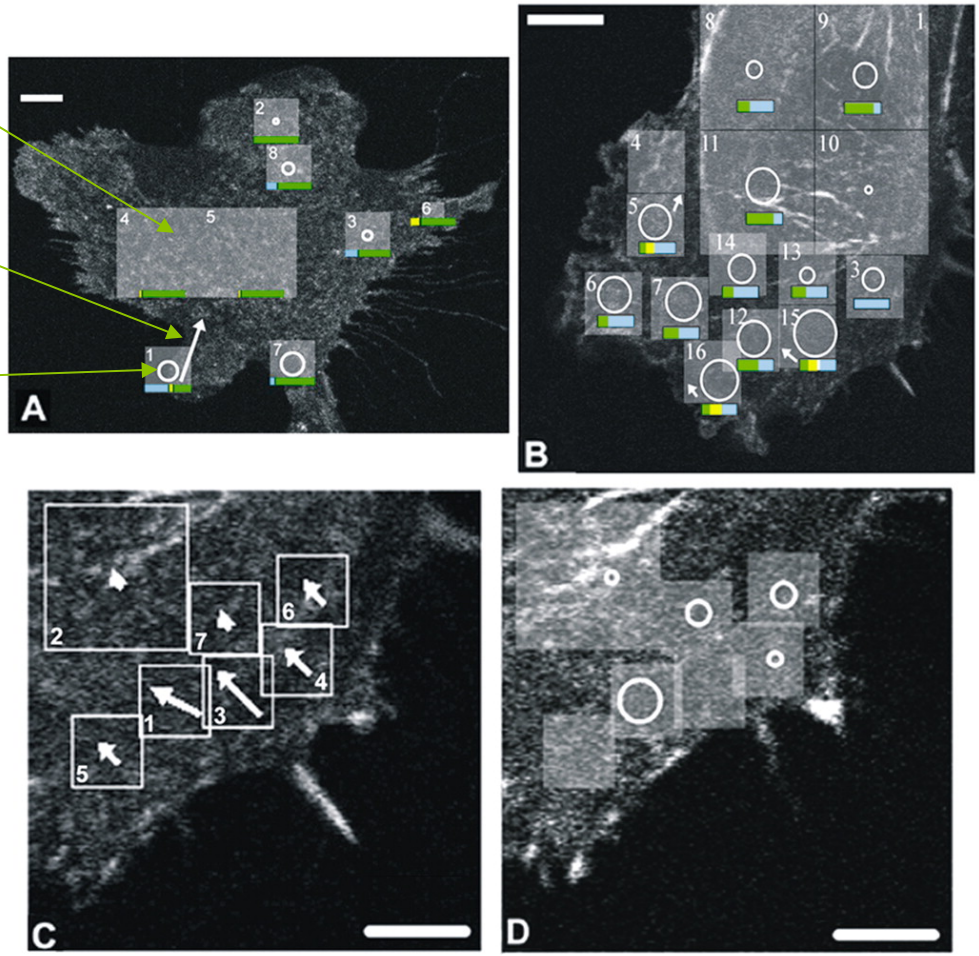


Image Correlation Spectroscopy

Areas analyzed

Flow vector

◯=10min diffusion length



Stationary fraction

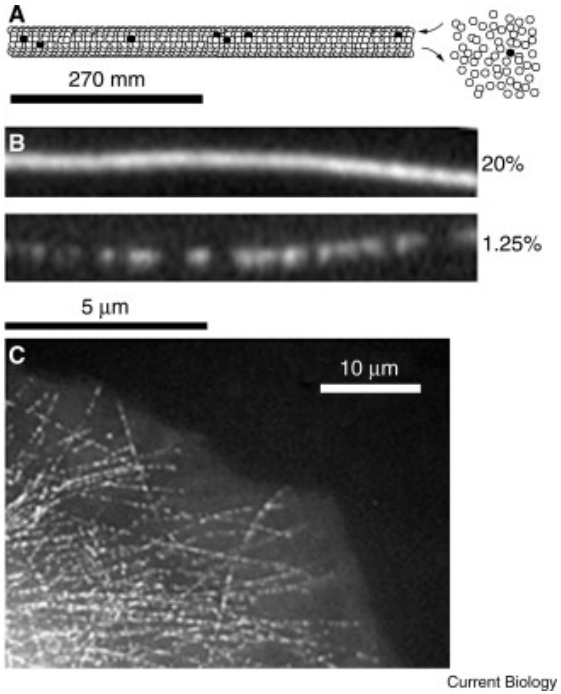
Flowing fraction

Diffusing fraction

Spatio-temporal autocorrelation

0 s 5 s 10 s 15 s

Fluorescece Speckle Microscopy FSM
(Clair Waterman-Storer)



% labeled tubulin

Analysis of:

Translocation of stable structure

Assembly of new molecules

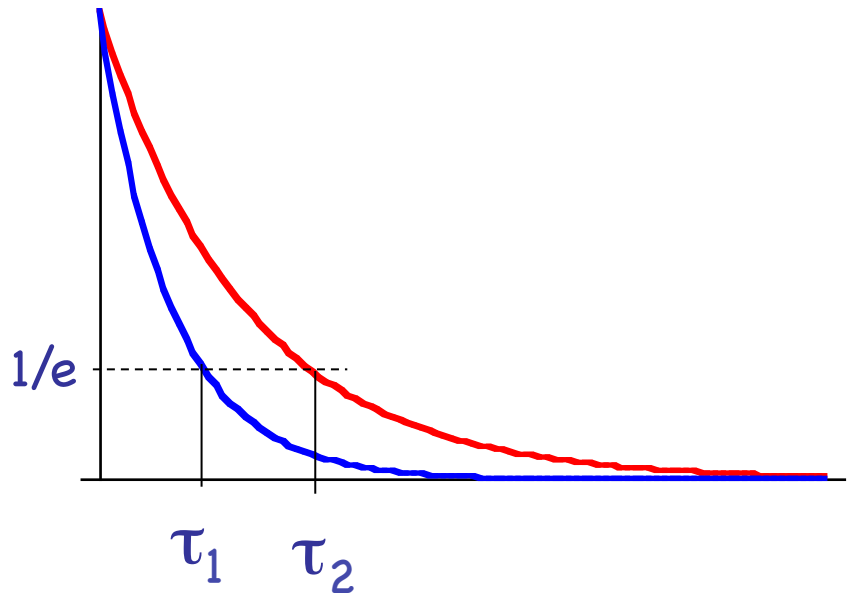
Fluorescence Life Time Imaging (FLIM)

FLIM

Fluorescence Lifetime Imaging

Measure the lifetime of the excited state

- Separate fluorophores with similar spectra
- Detect environmental parameters that affect lifetime (FRET, pH, ...)



FLIM

Measurement approaches

- Frequency domain
  - Modulated excitation
  - Lock-in detect emission phase

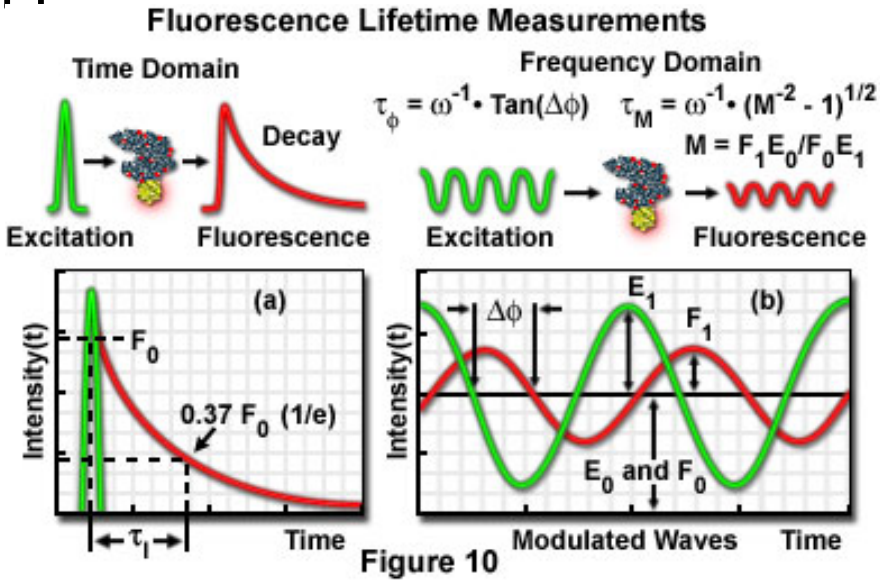
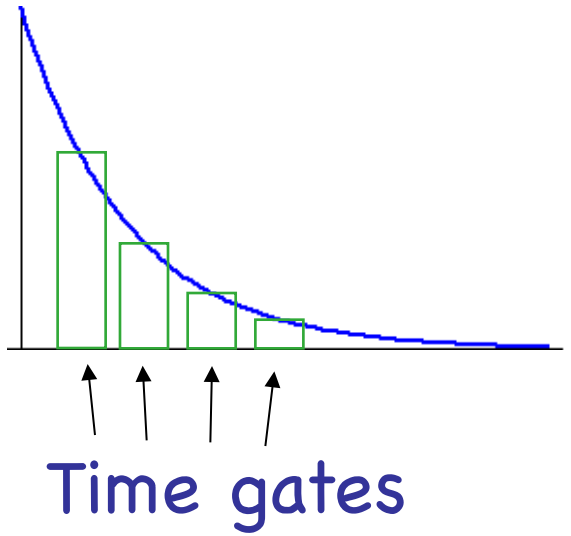
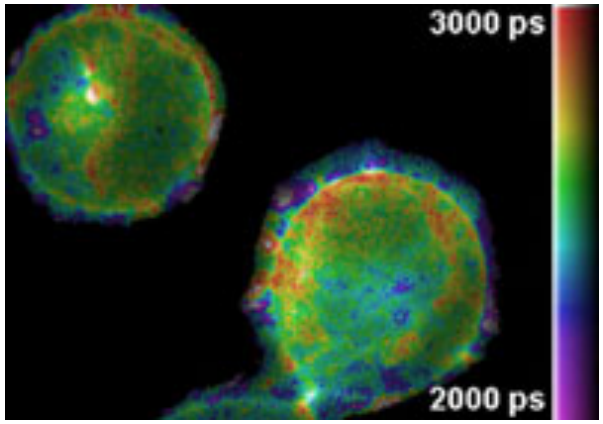


Figure 10

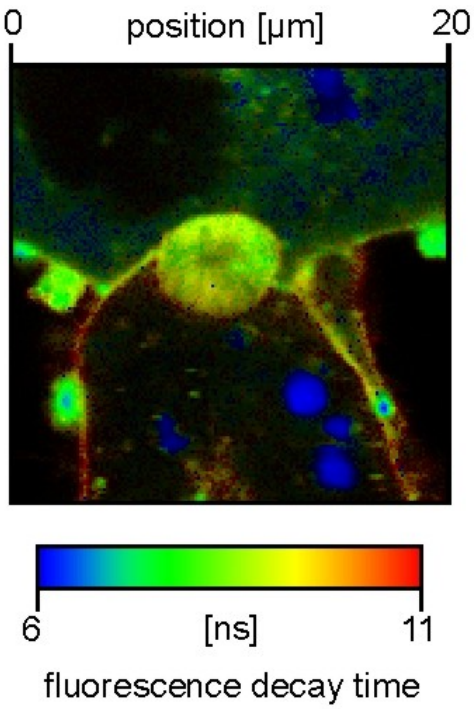
- Time domain (pulsed exc.)
  - Gated intensifier
    Photon inefficient
  - Time-correlated
    single photon counting
    Very efficient
    ≤ one photon per pulse ⇒ slow



FLIM
Examples



B-cells

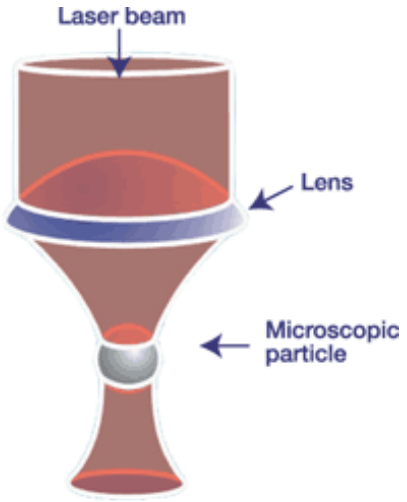


Hepatocyte membrane-stained
with NBD, which has a
hydrophobicity-dependent lifetime
(TCSPC, 3 minutes for 300x300 pixels)

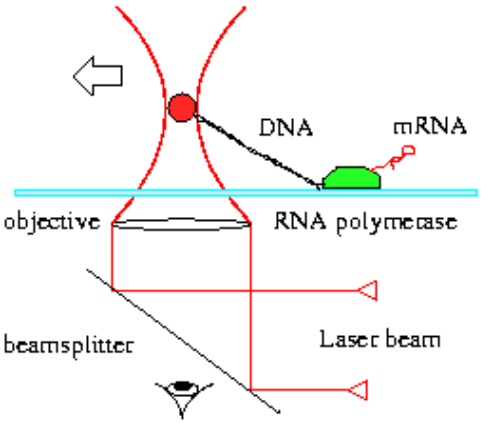
Optical Tweezers

Optical Tweezers

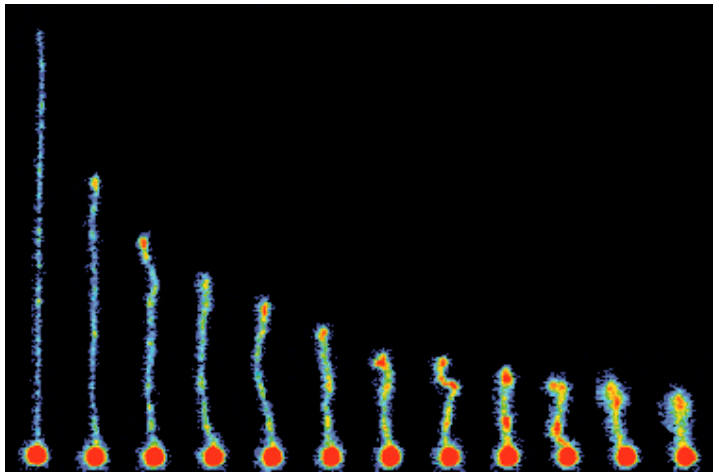
Mechanically manipulate the specimen with light



Why?



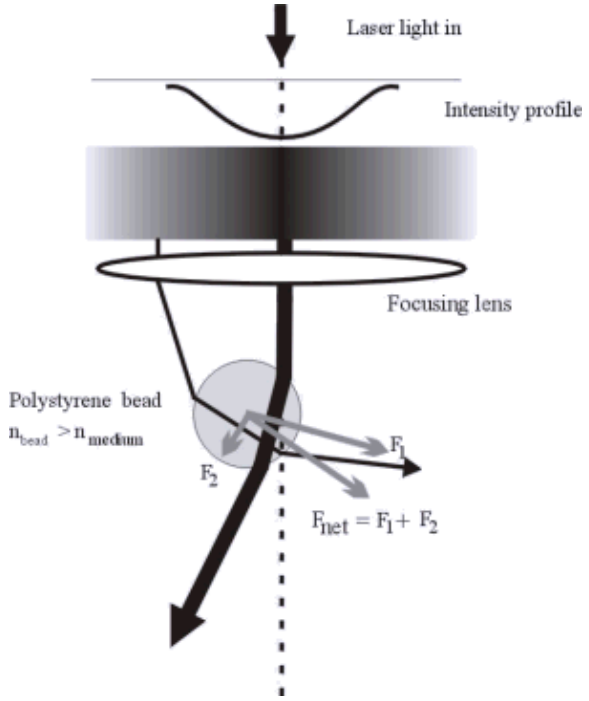
Measuring force and displacement of a single polymerase molecule



DNA recoil after stretching

Optical Tweezers
How does it work?

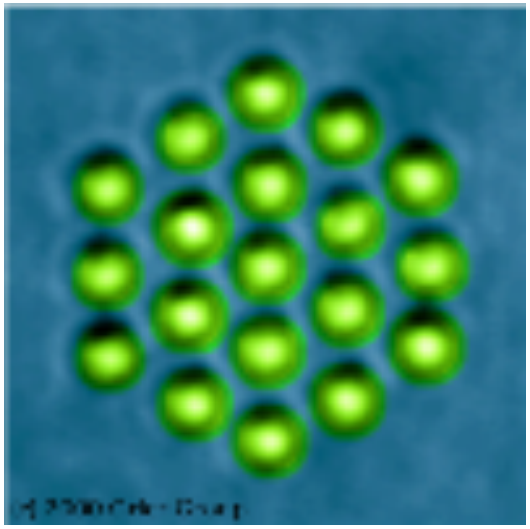
Photons carry momentum
Changing photon direction requires a force
More light refracted away from high intensity region
→ Force toward intensity maximum

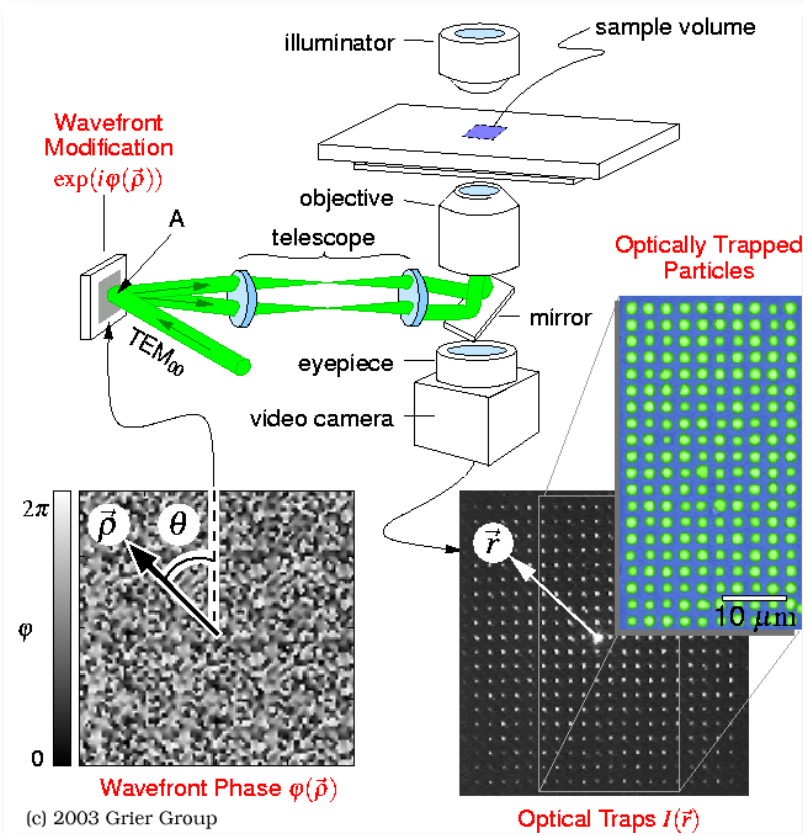


Alternative way to look at it:
Field energy is less in higher index
System energy depends on particle position
dE/dx = Force

Holographic Optical Tweezers

- Many traps at once
- Independently movable
- Made using a computer-controlled spatial light modulator in a pupil plane





Fiber Optics