MICRO-562

Biomicroscopy II

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Biomicroscopy-II Syllabus – (TENTATIVE)

Lecture 1	Course	Brief Review, Dark-Field
Lecture 2	Course	Phase Contrast & Polarization, Birefringence
Lecture 3	Course	DIC & Fluorescence Microscopy Techniques (FRET & TIRFM)
Lecture 4	Course	FRAP & Introduction to Confocal Microscopy
Lecture 5	Course	Advanced Microscopy-I
Lecture 6	Course	Advanced Microscopy-II
Lecture 7	TP	Practical: Intro to Wide-Field Microscopy
Lecture 8	TP	Practical: Transmission Techniques
Lecture 9	TP	Practical: Intro to Fluorescence
Lecture X	**EASTER**	
Lecture 10	TP	Practical: Intro to Confocal
Lecture 11	TP	Practical: Fluo/Conf
Lecture 12	TP	Practical: Fluo/Conf
Lecture 13	**EXAM**	
Lecture X	**HOLIDAY**	

Contrast enhancement techniques

- Higher contrast is easier to achieve with darker background

- Optical microscopy techniques that are used to enhance contrast for imaging transparent specimens:
  - **Stained specimen**
  - **Dark field microscopy**
  - **Phase contrast microscope**
  - **Polarization microscopy**
  - **Differential Interference Contrast (DIC) microscopy**
  - Fluorescence microscopy

Differential Interference Contrast Microscopy

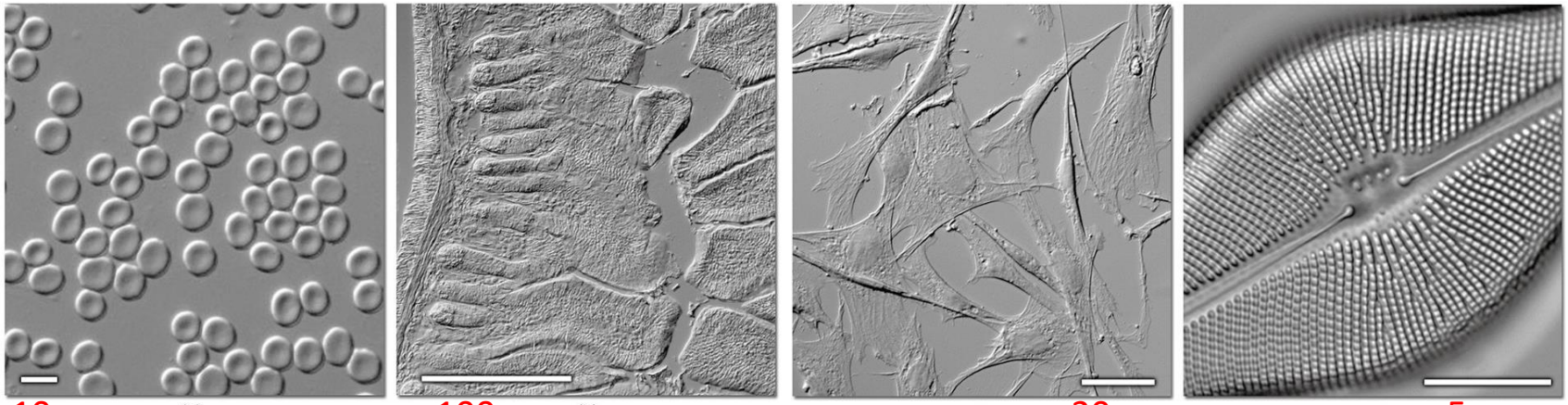
- We saw that **phase contrast microscopy converts an optical phase difference** in a specimen to an **intensity contrast** difference → phase objects (e.g. biological samples) become visible.
- Remember that optical phase difference is due to the **optical path difference between the two rays**:

  (i) Surrounding/background ray (S-wave) not interacting with the sample

  (ii) diffracting ray (D-wave) interacting with the sample

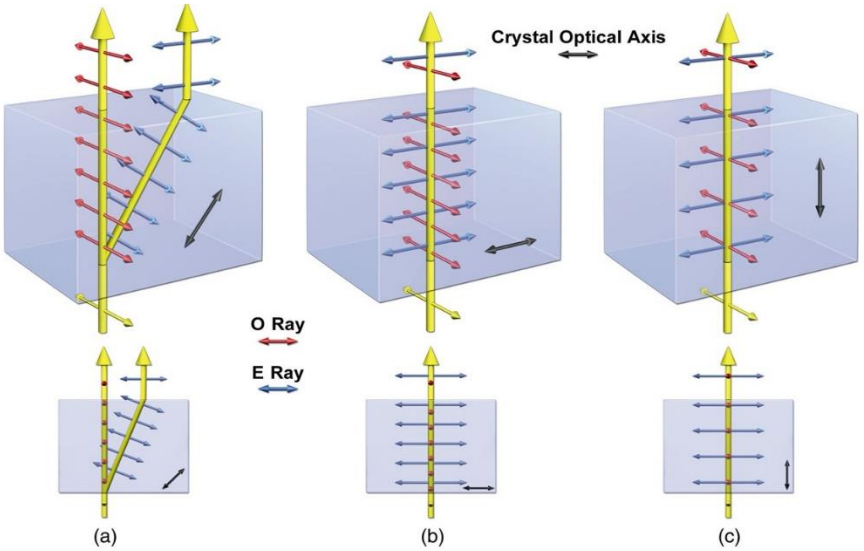
- Differential interference contrast (DIC) allows to view **GRADIENTS in optical path length**.
- Results in relief-like, shadow cast appearance → makes images appear deceptively 3D.

Widely used for applications demanding high resolution & contrast and inspection of cultured cells.



Human red blood cells | Mouse intestine section | Fibroblast cells | Diatom frustule

Reminder: Birefringent Crystals



Birefringent materials contain a single unique axis known as the crystal axis.

PANEL A) If incidence beam is **not perpendicular/parallel** to the crystal axis, light splits into two rays that follow separate paths with a **physical separation.** For this case:

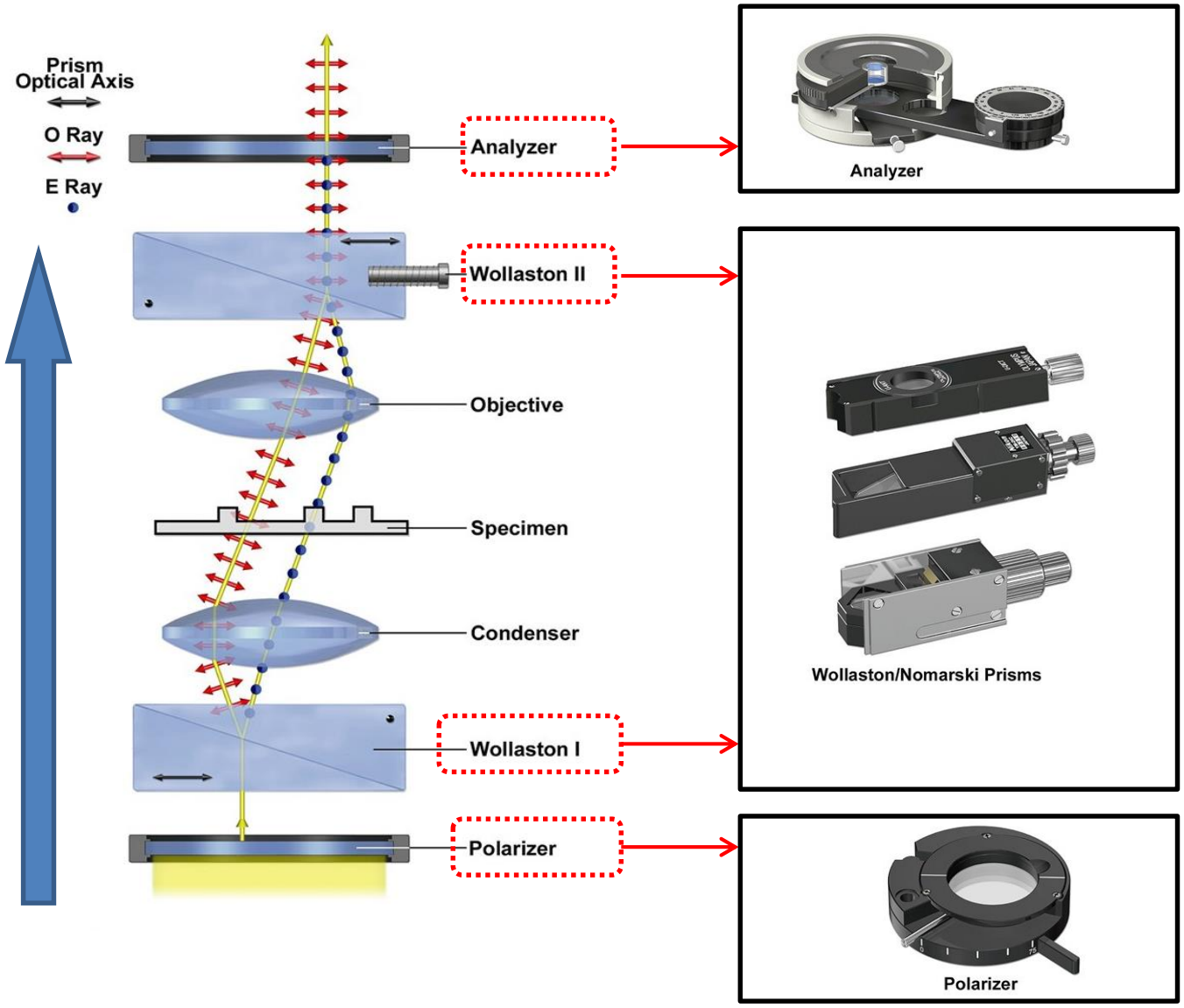
- Ordinary ray (**O-ray**) observes the regular law of refraction
- Extraordinary ray (**E-ray**) travels along a different path.
- For every incident ray entering the crystal, there is a pair of O-ray and E-ray that emerges, each of which is linearly polarized
- The E-field vectors of these two rays vibrate in mutually perpendicular planes.

PANEL B) If incident beam is **perpendicular to the crystal axis,** light splits into **O-Ray** and **E-Ray**, but their trajectories coincide:

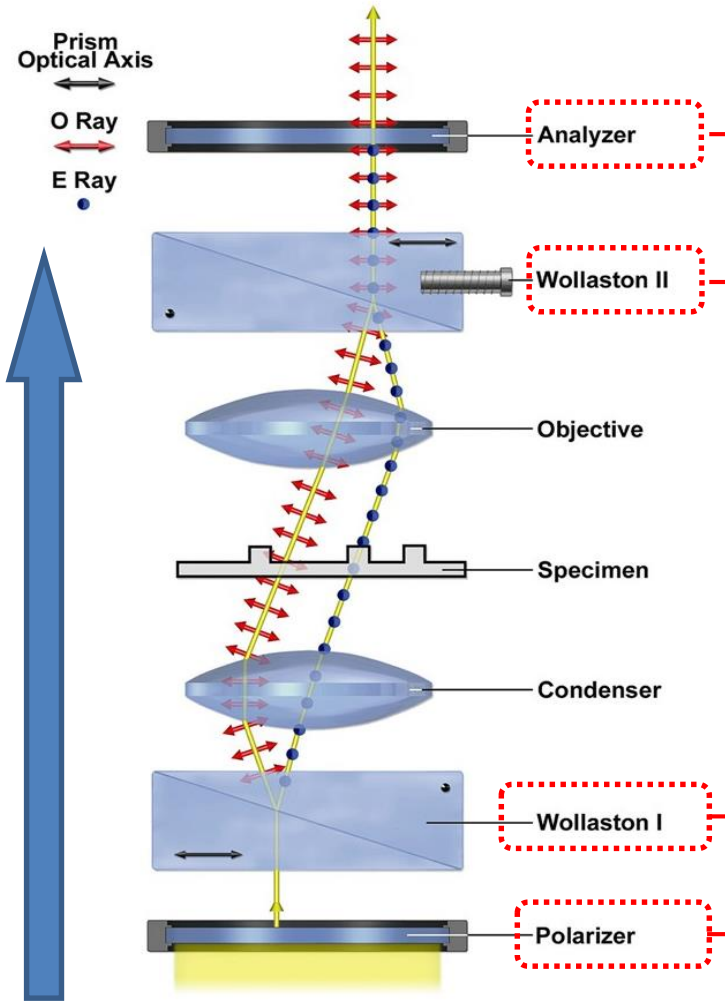
- **O-Ray** and **E-Ray** emerge at the **same location** on the crystal surface, **but** due to experience of different optical path lengths, they are **phase shifted**. Emerging light exits as **elliptically polarized**.
- This geometry pertains to most biological specimens that are examined in a polarizing microscope.

PANEL C) If incident beam is **parallel to the crystal axis, O-Ray** and **E-Ray** emerge at the **same location** on the crystal surface and with the **same phase**. Thus, light stays as **linearly polarized**.

DIC Equipment & Optics



DIC Equipment & Optics



4) An analyzer

- Analyzes rays of linearly & elliptically polarized light from the objective DIC prism.
- The analyzer has its vibration plane orientated vertically: top-to-bottom (i.e. it is ⊥ to the polarizer).
- Transmits light that is able to interfere & generate contrast image.

3) An objective DIC prism

- Mounted close to the rear aperture of the objective.
- Acts as a **beam combiner.**
- It is essential for interference & image formation.

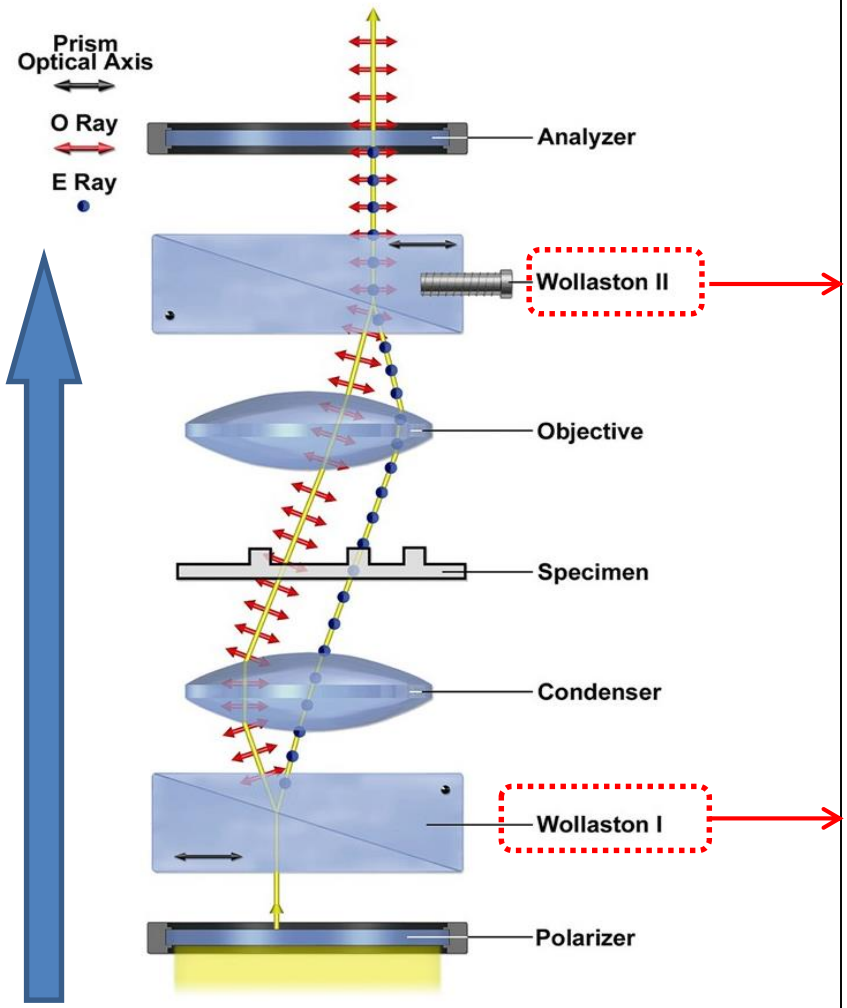
2) A condenser DIC prism

- Mounted close to the front aperture of the condenser.
- Acts as a **beam splitter.**
- Every incident ray (wave) of polarized light entering the prism splits into two rays (**O-Ray** & **E-Ray**) that function as the dual beams of the interference.

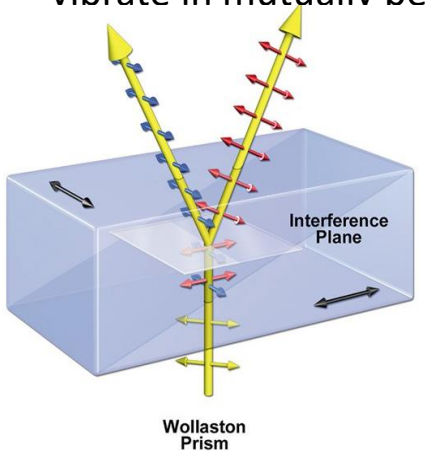
1) A polarizer

- In front of the condenser to produce a linearly polarized light.
- In this illustration the plane of vibration of the E vector is oriented horizontally (left-to-right).

DIC Prism



- DIC prism (a.k.a Wollaston prism) is a beamsplitter made of two wedge-shaped slabs of quartz.
- Because quartz is birefringent, with Wollaston I prism incident rays of linearly polarized light are **physically split or sheared into** two separate components as **O-Ray** and **E-Ray**.
- The shear axis (direction of shear) and the separation distance between the resultant O-Ray and E-Ray are same for all ray pairs across the face of the prism.
- The E vectors of the resultant O-Ray & E-Ray pairs vibrate in mutually perpendicular planes.



- *Shear distance (0.2-2μm) should be as small as possible or at least smaller than the spatial resolution of the objective.*
- *With a decreasing shear distance, the DIC resolution improves.*

➔ Every point in the specimen is sampled by pairs of beams that provide dual-beam interference in the image plane.

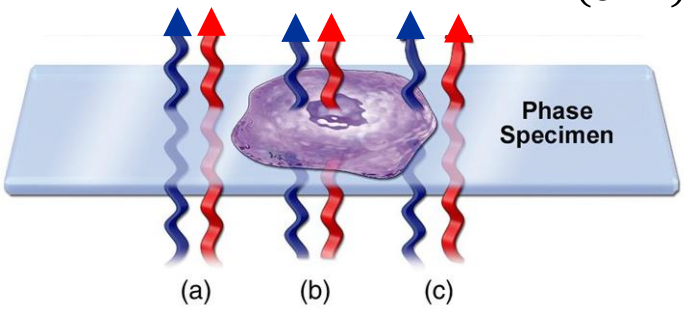
DIC

- In DIC, the specimen is sampled by **pairs of closely spaced rays** that are generated by a birefringent beamsplitter.

- When the members of a **ray pair traverse a phase object in a region with a gradient in the**
  - refractive index, or
  - Thickness, or
  - Both

  } *There will be an **optical path difference between the two rays** upon emergence from the object, relative to the background as follows:*

$$\Delta(OPL)_{12} = OPL_1 - OPL_2 = (t_1 \times n_1) - (t_2 \times n_2)$$

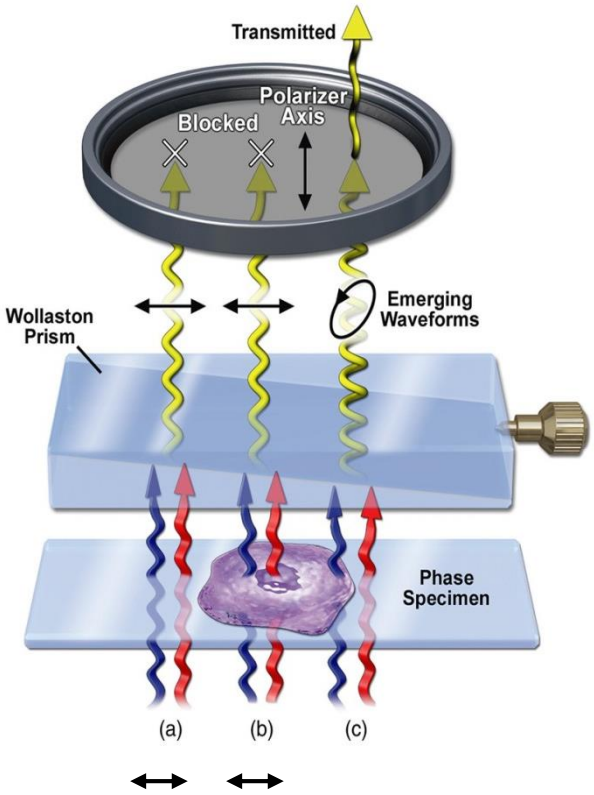


- This optical path difference is translated into a change in the amplitude.

- Since an optical path difference corresponds to a relative phase difference between the two rays, the presence of phase gradient is detected in DIC.

Formation of the DIC Image

➔ Every point in the specimen is sampled by pairs of beams that provide dual-beam interference.



(a) In the absence of a specimen or

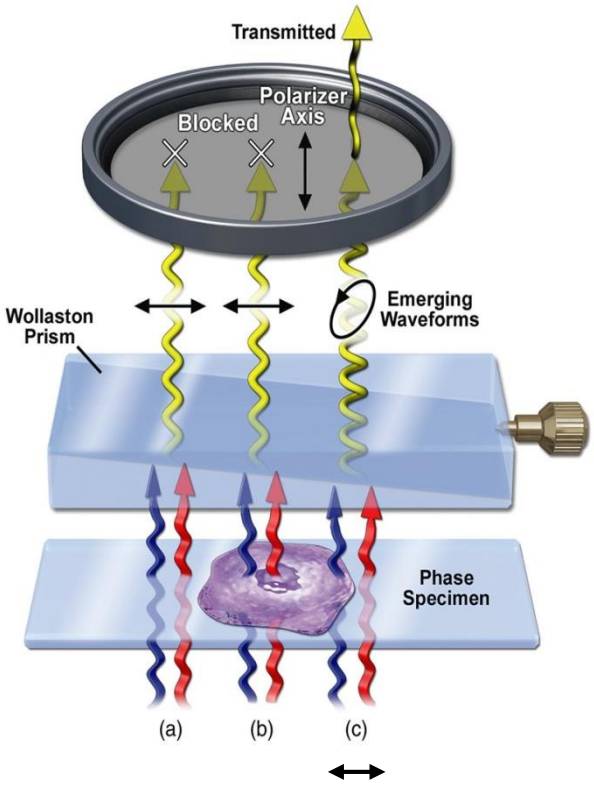
(b) both rays pairs pass the specimen*

No difference in the ΔO.P.L.

- Assume that the incident light is linearly polarized after passing through a polarizer and before hitting the sample.
- Recombination of these two waves will generate **linearly polarized light** whose E-field vibrates in the **same plane of the polarizer**.
- Therefore, the **resultant wave is blocked by the analyzer.**
- **<u>The image background looks black, i.e. *extinction.*</u>**

Formation of the DIC Image

➔ Every point in the specimen is sampled by pairs of beams that provide dual-beam interference.



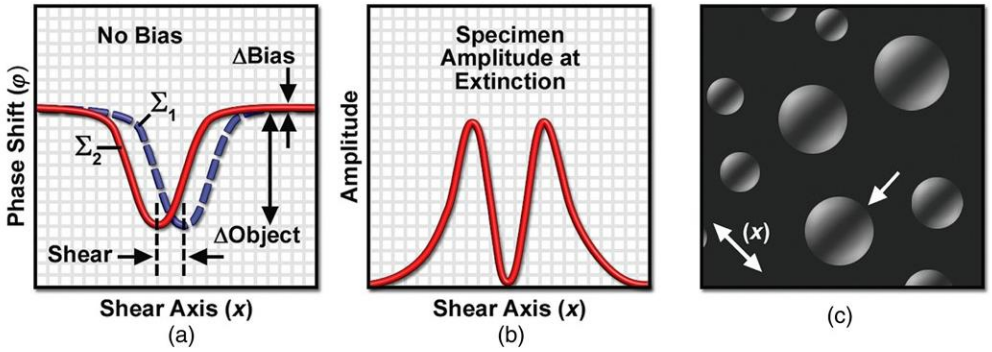
(c) If ray pairs encounter a phase gradient:

The two beams of the pair will have different ΔO.P.L and differentially shifted in phase.

- Again, we assume that the incident light is linearly polarized after passing through a polarizer and before hitting the sample.
- Recombination of these two waves generate **elliptical polarized light.**
- This wave partially passes through the analyzer, resulting a linearly polarized light with a finite amplitude.
- The phase gradient appears as bright (or at different tones of gray) against a black background.

Interpretation of the DIC Image

To better understand the importance of phase displacement between the two rays of the pair & the role of the objective DIC prism as a contrasting device, it is also useful to view the situation by **decomposing the rays in the image place** (i.e. after the analyzer) **into their corresponding O-Wave and E-Wave**:



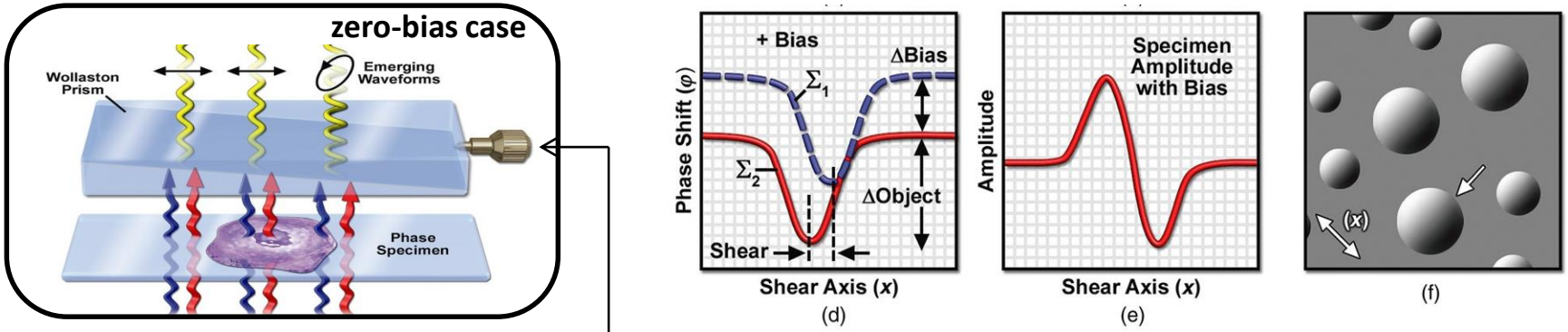
(a) Shows the phase shift of **O-wavefront** and **E-wavefront** (labelled Σ_1 and Σ_2, respectively) in the image plane. Each has a dip whose width represents the magnified object diameter and whose depth represents the amount of phase retardation. The x-axis represents the distance x across the object. This also is the direction of the shear induced by the prism (which is indicated by the double-headed white arrow shown in **(c)**).

(b) Shows the amplitude graph resulting from the interference and combination of **O-Ray** & **E-Ray**.

(c) The resultant image of the spherical objects (in agreement with the amplitude graph in **(b)** shows a dark central part with bright regions on both side. The **background** appears as **dark**.

Bias Retardation in DIC

In practice, the prism setting giving a total background extinction as "**black**" (i.e. no bias between E-wave & O-wave) is not used.



(d) **The objective prism is adjusted (moved) with a screw to introduce a phase displacement between the wavefronts of the O-Ray and E-Ray.**

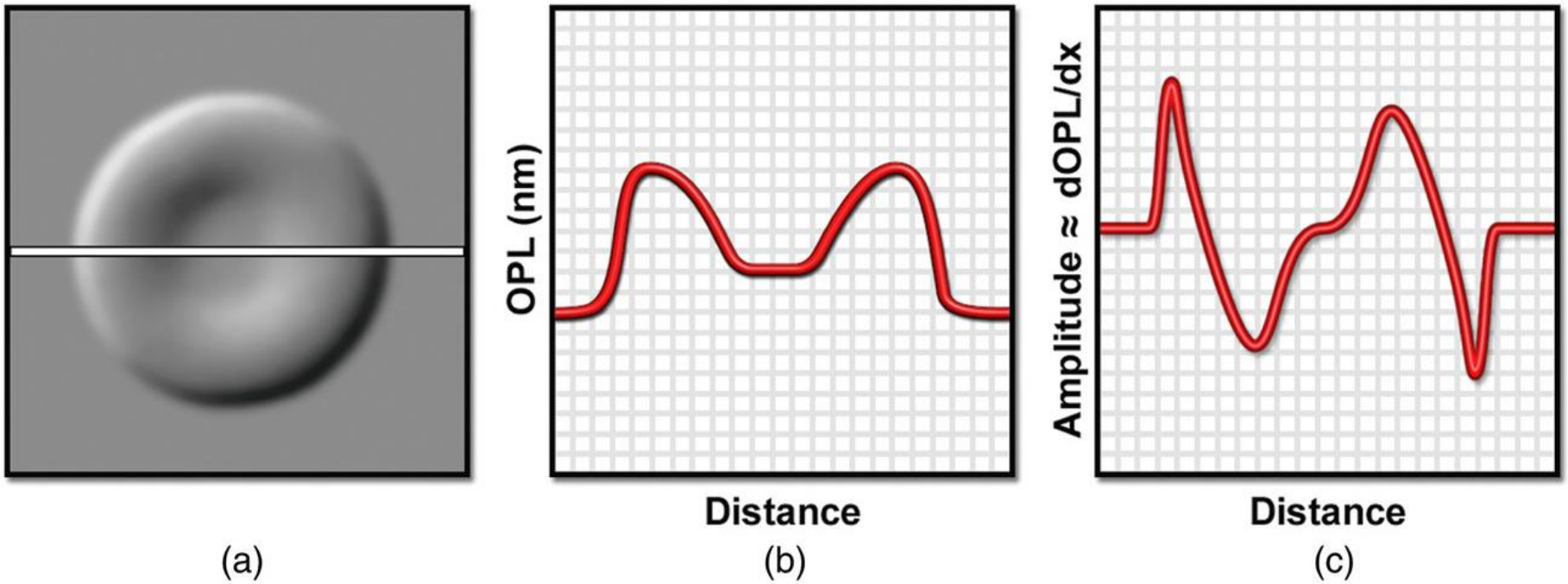
(e)-(f) Since background ray pairs are now differentially retarded and out of phase, they emerge from the objective prism as elliptically polarized waves and partially pass the analyzer, causing the background to look **"gray"**.

- Adding a bias retardation causes the object image to exhibit dark shadows and bright highlights (at the locations of phase gradient) against a gray background.

NOTES:

- Introducing a **bias retardation** makes objects easier to see because phase gradients in the specimen are represented by bright & dark patterns on a **gray background**.
- In DIC, the images exhibit a **relief-like appearance** with a shadow-cast effect as if the specimen is a 3D surface illuminated by a light source coming from a low-angle along the direction of the shear axis (indicated by the double-headed white arrow shown in (f)).
- Shadows & highlights indicate the sign & slope of the phase gradients, not necessarily high or low spots.

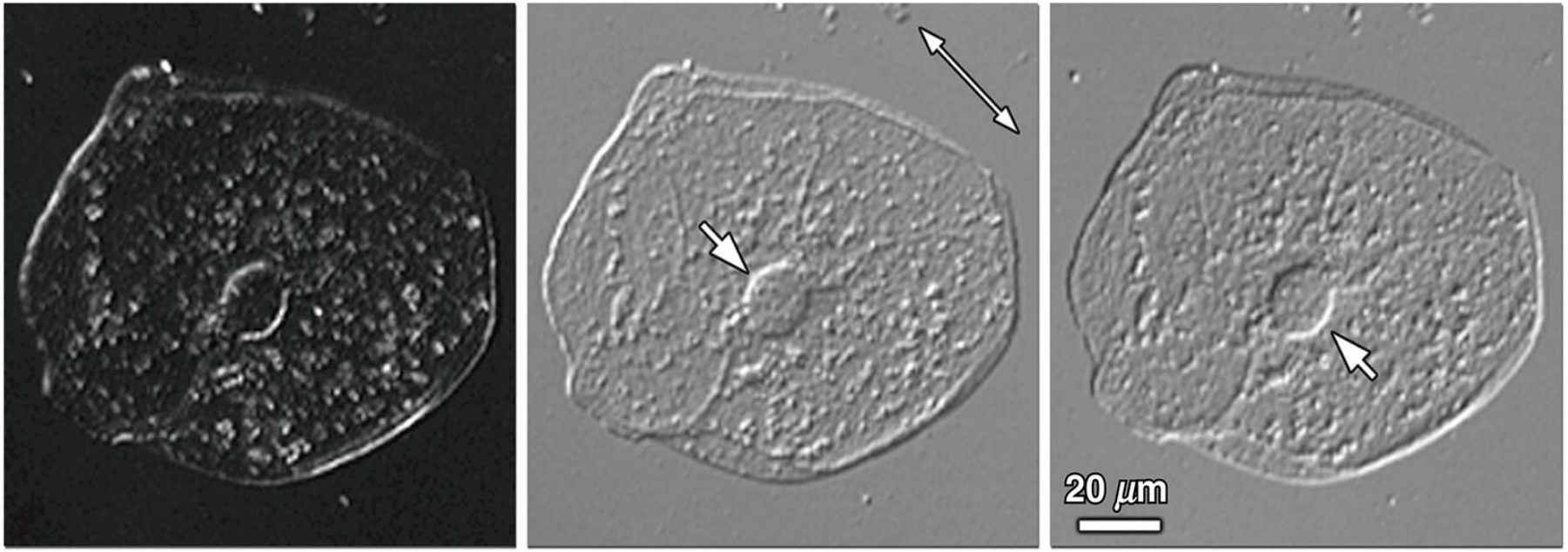
Formation of the DIC Image With Bias



(a) DIC image of a mammalian erythrocyte.
(b) Plot of the optical path length (OPL) across the cell diameter (indicated by the white line in panel **(a)**.
(c) Derivative of the optical path length curve shown in panel **(b)** added to a constant, gives the amplitude profile perceived by the DIC optics.

- Positive and negative slopes in panel **(a)** correspond to regions of higher and lower amplitude.
- Regions of the object exhibiting no change in slope (e.g., center of the cell) have the same amplitude as the background.

Interpretation of the DIC Images



Positive and negative bias retardation in DIC microscopy:

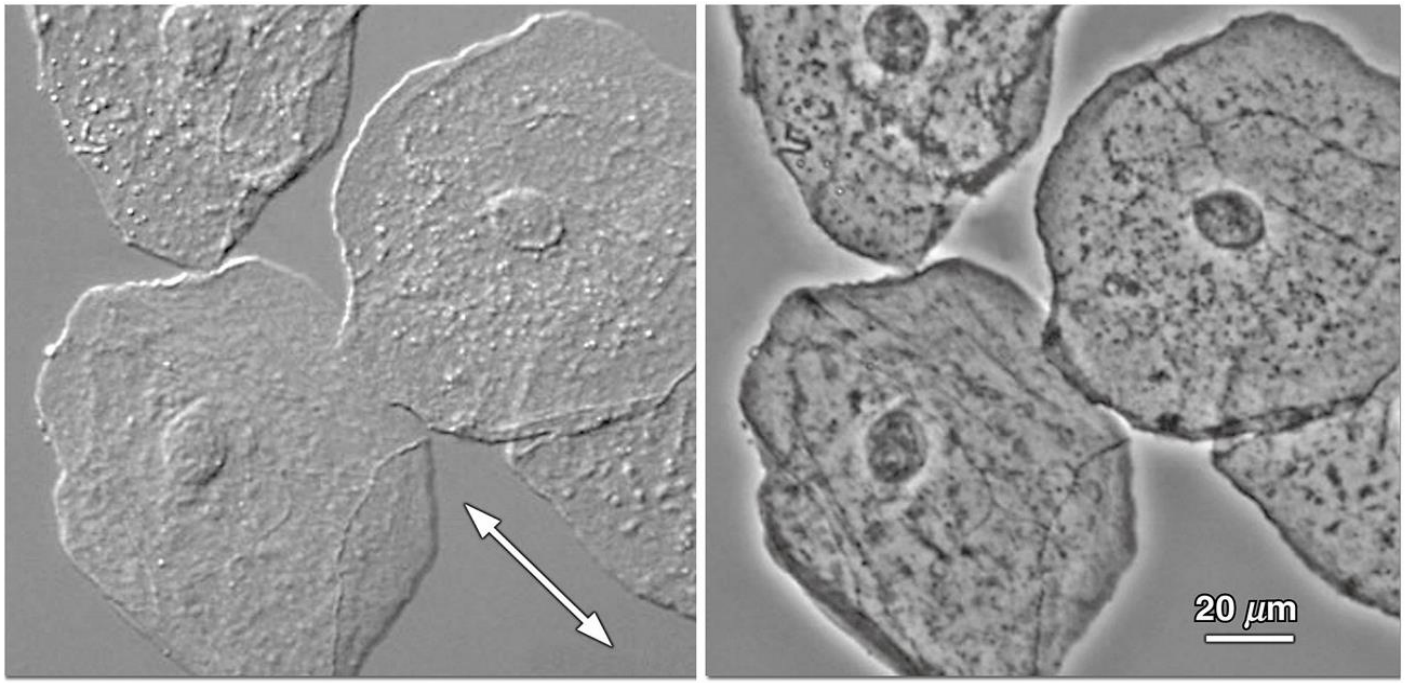
(a) When the DIC optical system is **set at extinction**, the field appears dark gray on a **black background** with bright highlights in regions having large refractive index and thickness gradients.

(b) Shifting the DIC prism in one direction (**positive bias**) lightens specimen features at one edge (white arrow pointing to the nucleus) while darkening the same features on the opposite edge.

(c) Shifting the DIC prism in the opposite direction (**negative bias**) reverses the intensity of features (white arrow).

The shear axis is indicated by the double-headed white arrow.

Comparison of DIC & Phase Contrast Images



Comparison of DIC and phase contrast images of buccal epithelial cells:

(a) **DIC:** The direction of the shear axis is shown by the white double-headed arrow in the micrograph. The cells appear as if illuminated by a grazing incident light source located in the upper left corner. Bright edges at the upper margins of the cell, the nucleus, and some of the small granular inclusions indicate these objects are phase-dense (have a higher refractive index) compared with their surrounding.

(b) **Phase contrast:** Positive phase contrast renders phase-dense objects as dark contrast features relative to the background. The cells themselves are surrounded by a bright phase *halo*, an artifact of the phase contrast optical system.

The information content (spatial resolution, detection sensitivity) of the two optical systems is similar.

Contrast in microscopy

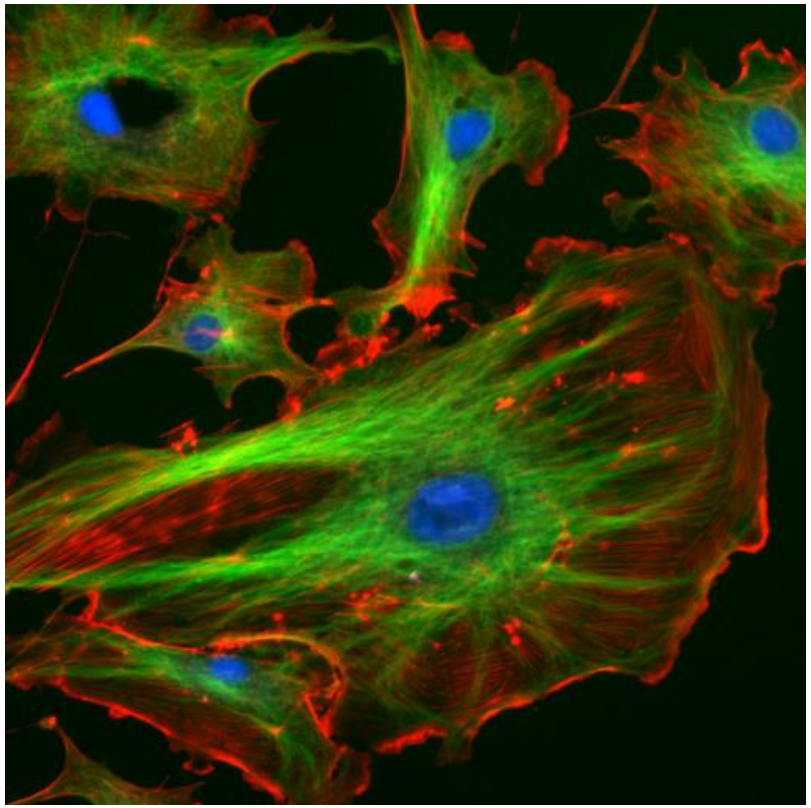
- For transparent specimens, adjust the contrast level:
  - **Bright field microscope** (low contrast)
  - **Stained specimen**
  - **Dark field microscopy**
  - **Phase contrast microscope**
  - **Polarization microscopy**
  - **Differential Interference Contrast (DIC) microscopy**
  - **Fluorescence microscopy**

Fluorescence microscopy: high contrast imaging technique

- **Fluorescence provides:**
  - High contrast
  - High specificity
  - (semi) quantitative monitoring

Implementation
Non-fluorescent molecules & entities are tagged with a fluorescent dye or fluorochrome in order to make them visible.

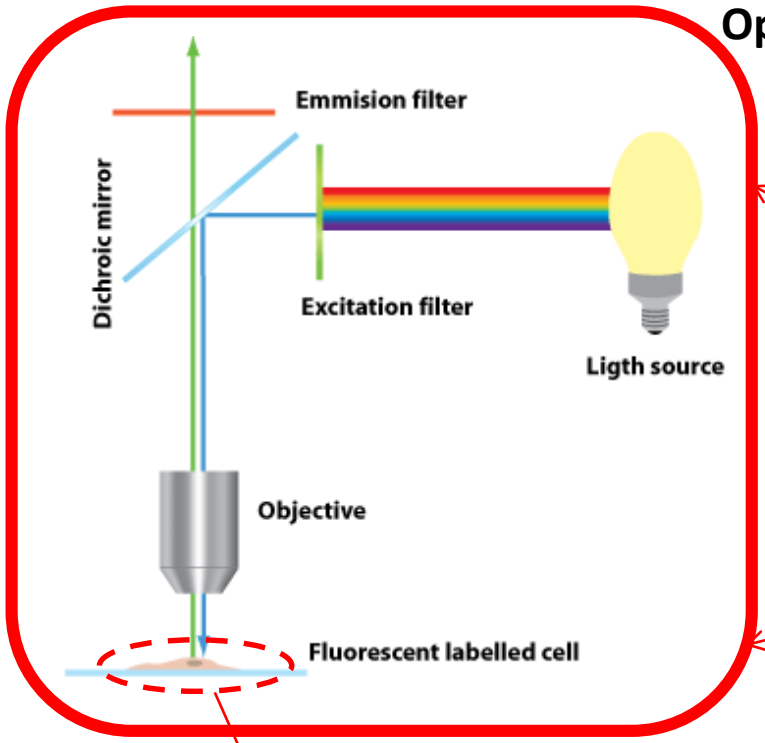
More on specificity:
- Specificity can be achieved at the molecular level.
- The amount, intracellular location, and movement of macromolecules, small metabolites, and ions can be studied by fluorescence.

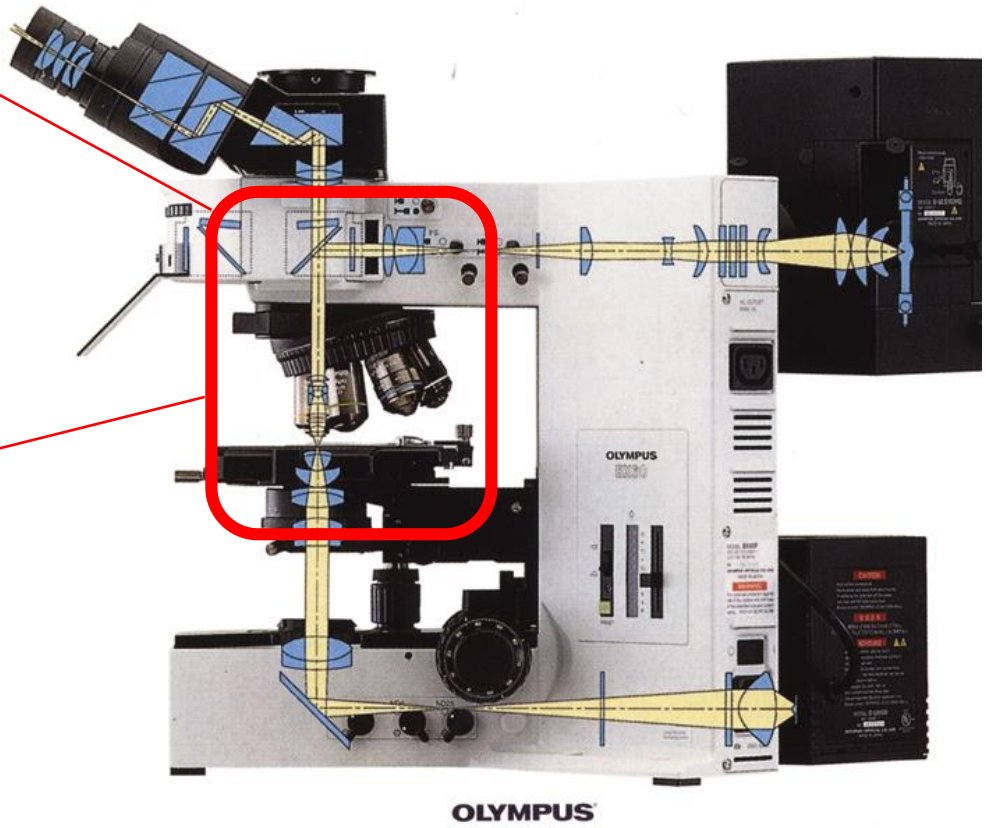


Bovine pulmonary artery **endothelial cells** under fluorescence microscopy (Wikipedia) :
- Nuclei are stained blue with DAPI
- Microtubules are labelled green by an antibody bound to FITC
- Actin filaments are labeled red with phalloidin bound to TRITC

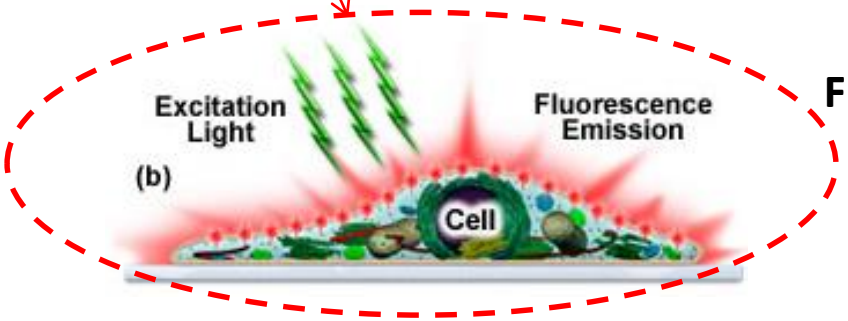
Fluorescence microscopy components

Optical hardware





Fluorescence labelling



A variety of fluorescence microscopy technique exists

- A strong feature of fluorescence microscopy is that the signals making up an image are “molecule-specific”.
- With the addition of time-lapse methods, it is possible to track time-dependent changes of molecules & dynamic molecular events.

A few examples of dynamic fluorescence imaging methods:

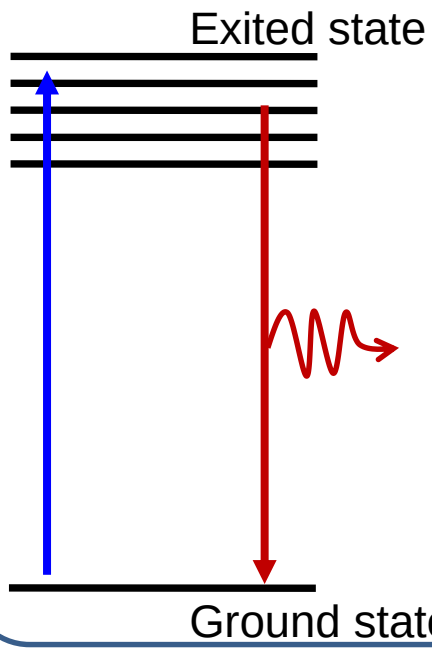
- FRET (Förster resonance energy transfer)
- TIRFM (Total internal reflection fluorescence microscopy)
- FRAP (Fluorescence recovery after photo bleaching)
- FLIM (Fluorescence lifetime imaging microscopy)
- FLIP (Fluorescence loss in photobleaching)
- FLAP (Fluorescence localization after photobleaching)
- FISH (Fluorescence in situ hybridization)
- FCS (Fluorescence correlation spectroscopy)

…

FRET (Förster Resonance Energy Transfer)

- a.k.a. Fluorescence Resonance Energy Transfer
- **The method is used** to study molecular proximity and interactions such as the identification of interacting proteins in multi-molecular complexes.
- **The principle** involves two closely spaced **donor and acceptor** fluorophores (**1-10 nm**). The excitation of the donor results in fluorescence, not of itself, but from the close by acceptor → **Transfer of energy from donor to acceptor.**

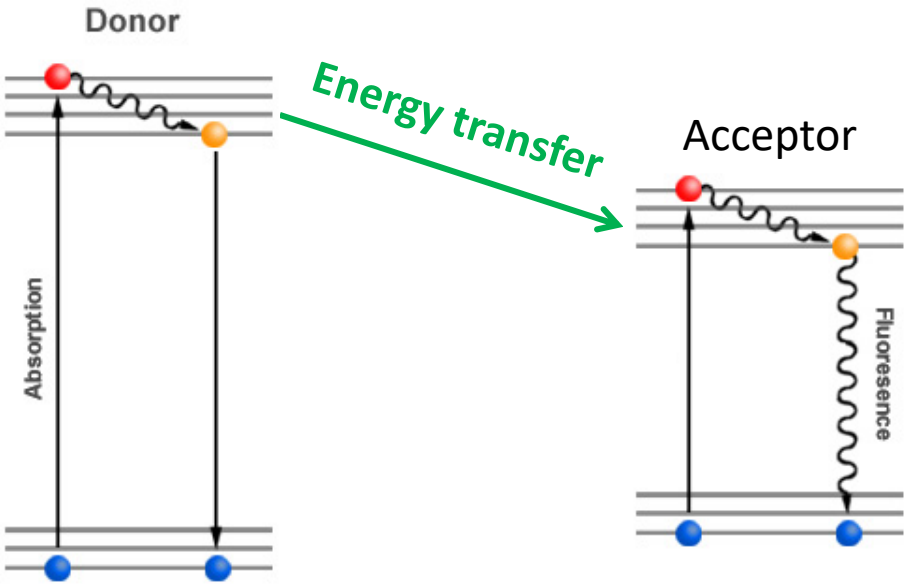
Classical Fluorescence:



FRET situation:

Excitation of the donor

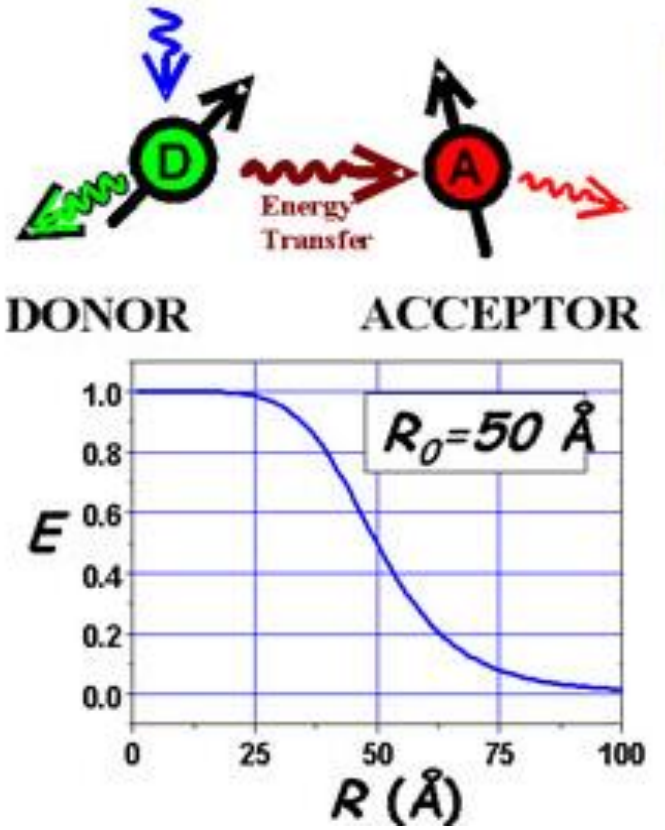
Emission comes from the close by (1-10 nm) acceptor



FRET

Some of the main requirements for FRET are:

1) fluorophore proximity
2) spectral overlap
3) dipole alignment
4) favourable environmental conditions



1) Proximity

- Energy transfer only occurs when donor & acceptor are within 1-6 nm (up to 10 nm) of each other.
- The proximity relationship is very strong → efficiency of transfer varies as the inverse 6th power of the distance → that is why spatial resolution of FRET is very high.

- The distance at which there is 50% probability of FRET transfer versus donor fluorescence is called Förster Radius (R_0).
- **EX:** For CFP and YFP R_0 is ~4-5 nm

Energy Transfer Efficiency

$$E = \frac{1}{1+(R/R_0)^6}$$

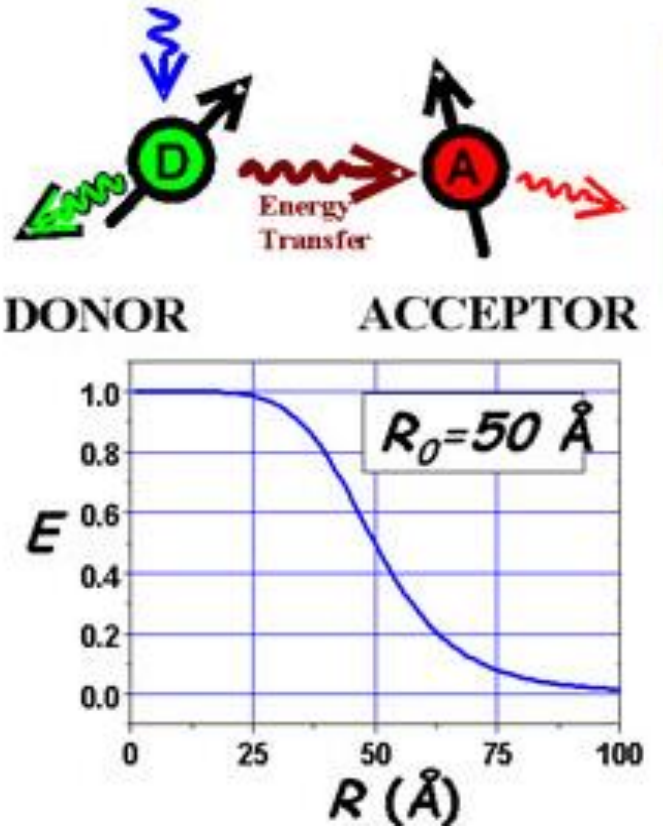
FRET: Typical Values of R_o

Donor	**Acceptor**	**R_o (Å)**
Fluorescein	Tetramethylrhodamine	55
IAEDANS	Fluorescein	46
EDANS	DABCYL	33
Fluorescein	Fluorescein	44
BODIPY FL	BODIPY FL	57
Fluorescein	QSY 7 dye	61
Cy3	Cy5	53
CFP	YFP	50

FRET

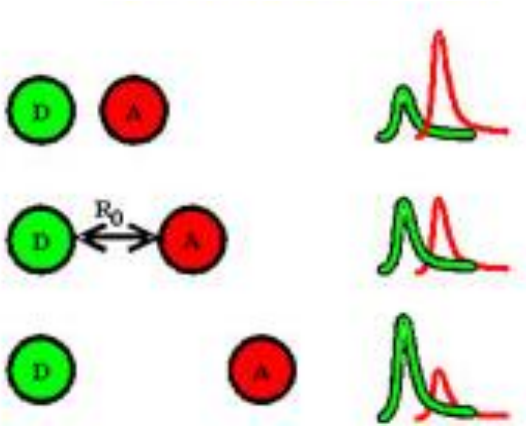
The main requirements for FRET are:

1) fluorophore proximity
2) spectral overlap
3) dipole alignment
4) favourable environmental conditions



1) Proximity → Precise Spatial Ruler

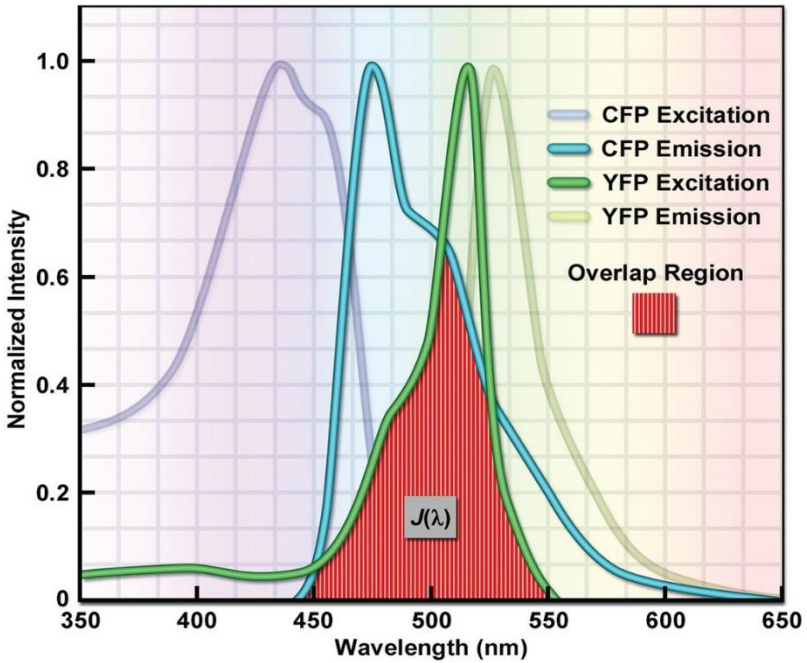
The proximity relationship is very strong → that is why spatial resolution is very high



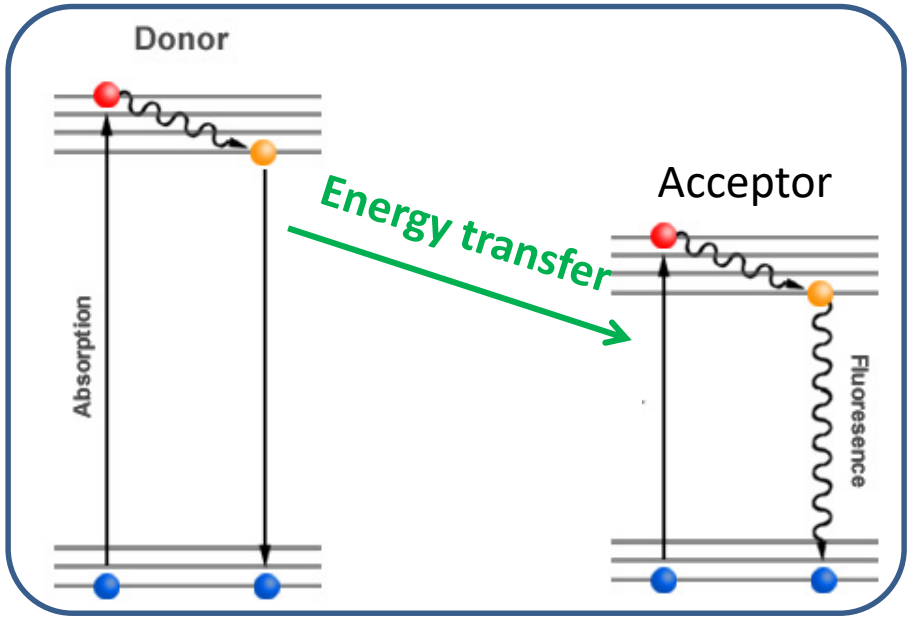
FRET

The main requirements for FRET are:

1) fluorophore proximity
2) spectral overlap
3) dipole alignment
4) favourable environmental conditions



Spectral overlap integral $J(\lambda)$ for the cyan (CFP) and yellow (YFP) fluorescent proteins is highlighted as a hatched region.



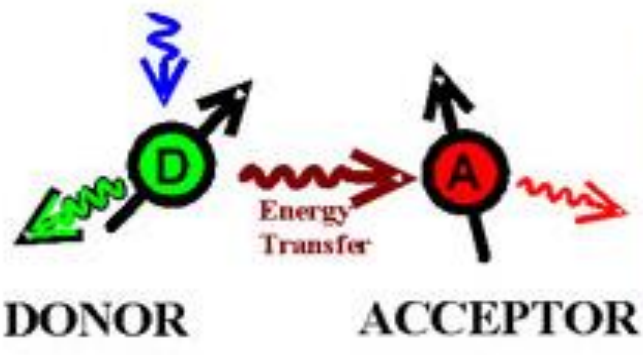
2) Spectral Overlap

- Energy transfer requires that the emission spectrum of donor overlaps with the excitation spectrum of the acceptor (see left figure).
- The amount of overlap, also called overlap integral determines FRET efficiency.
- More overlap gives stronger FRET.

FRET

The main requirements for FRET are:

1) fluorophore proximity
2) spectral overlap
3) **dipole alignment**
4) favourable environmental conditions



3) Dipole moment & alignment

- Excited fluorescent molecules behave as electrical dipoles where electron resonances in an excited molecule establish a state of electronic polarity and an associated dipole moment (i.e. product of the charges on either pole of the dipole and the distance separating them.)

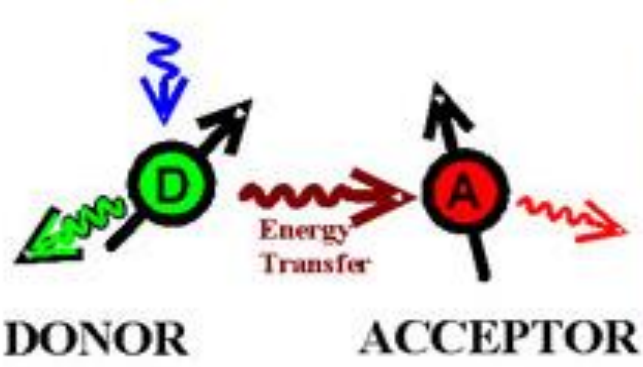
- FRET interaction occurs if the donor's electric field can induce electrons in the acceptor to oscillate & undergo transitions.

- FRET efficiency depends on dipole orientation and it is greatest when both dipoles are in line & parallel and least when they are perpendicular to each other.

FRET

The main requirements for FRET are:

1) fluorophore proximity
2) spectral overlap
3) dipole alignment
4) **favourable environmental conditions**



4) Environmental factors

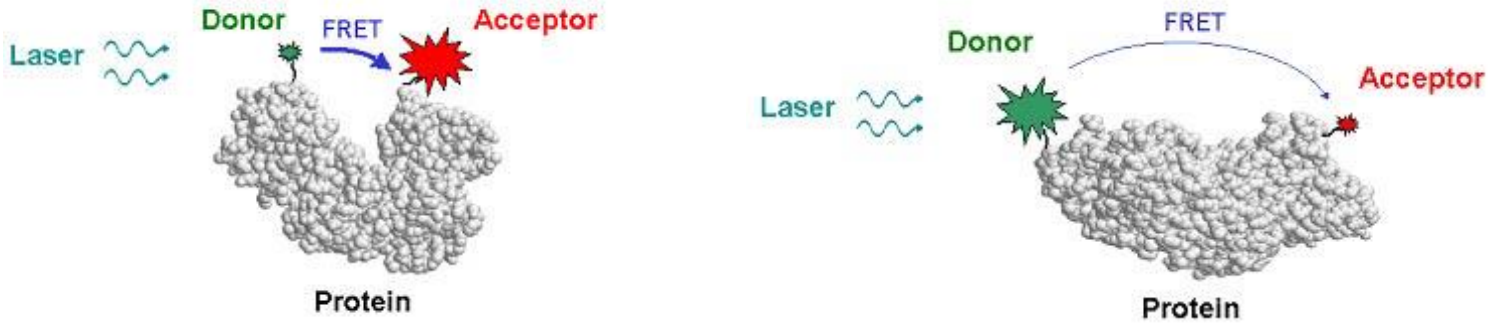
- FRET is sensitive to environmental conditions that affect dipole interactions, including solvent type, degree of hydration, ionic strength, pH and temperature among other factors.

Configurations of FRET

- **INTRA-MOLECULAR FRET:**

  - Donor-acceptor fluorophores are placed **on different regions of a single polypeptide** that undergoes conformational changes upon binding to substrate, causing the fluorophores involved in FRET to come together or move apart.

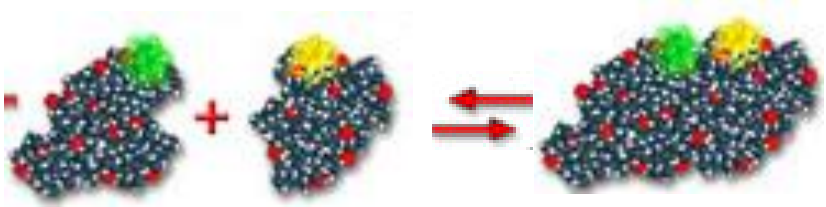
  - Such molecules are also called as "**biosensors**".



Different conformation gives → Different FRET signature

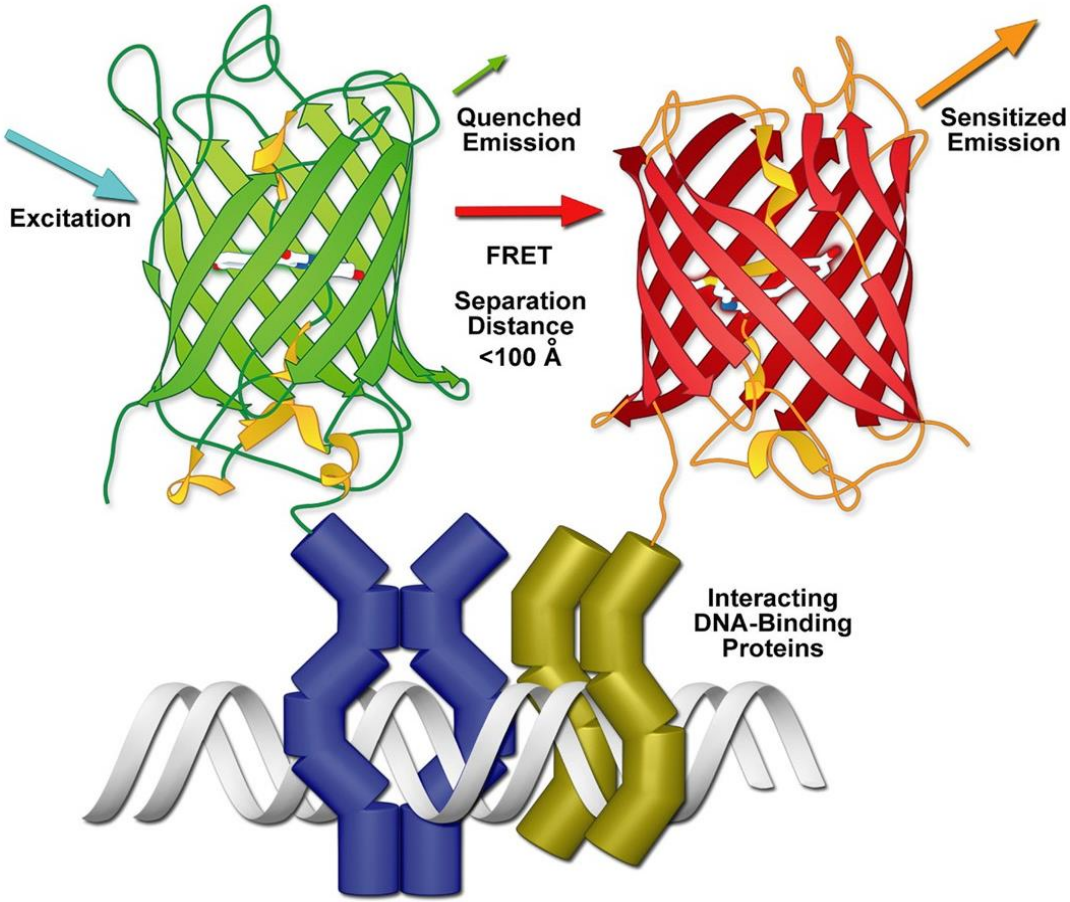
- **INTER-MOLECULAR FRET:**

  Donor-acceptor fluorophores are located **on two separate molecules that interact with each other**, and FRET indicates the proximity of the two receptors, and indirectly, the proximity of the two molecules.



Ligand-receptor interactions

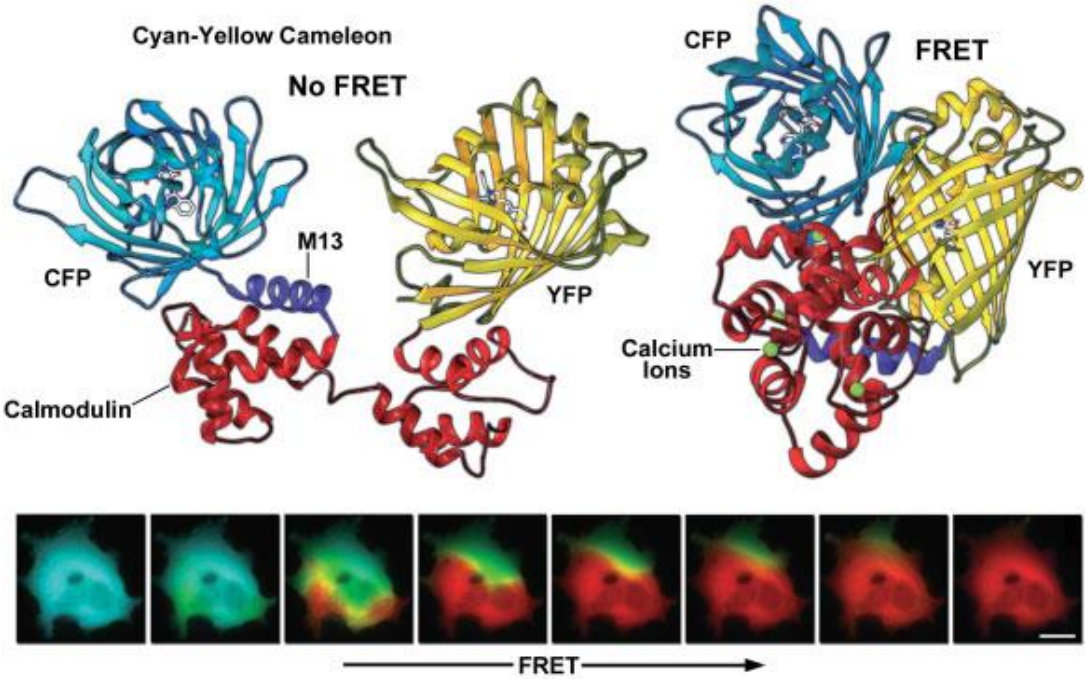
Applications of FRET in Biology – An Example



- **A pair of interacting nuclear proteins** attached to green and red fluorescent protein reporters are used for FRET.
- **For intermolecular FRET**, two interacting DNA-binding proteins of interest are joined to GFP and RFP reporters.
- When the proteins are bound to DNA, their interaction brings the reporters into close proximity, and they can then undergo FRET.

Applications of FRET in Biology – An Example

Calcium sensing by a cyan - yellow (CFP - YFP) cameleon:

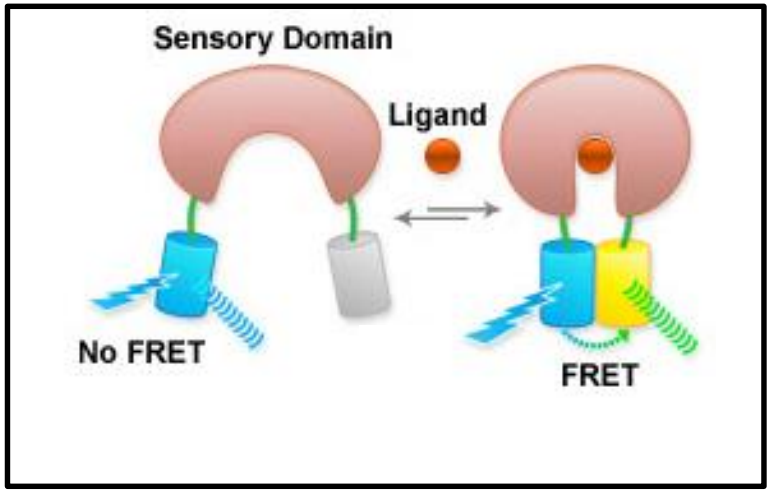


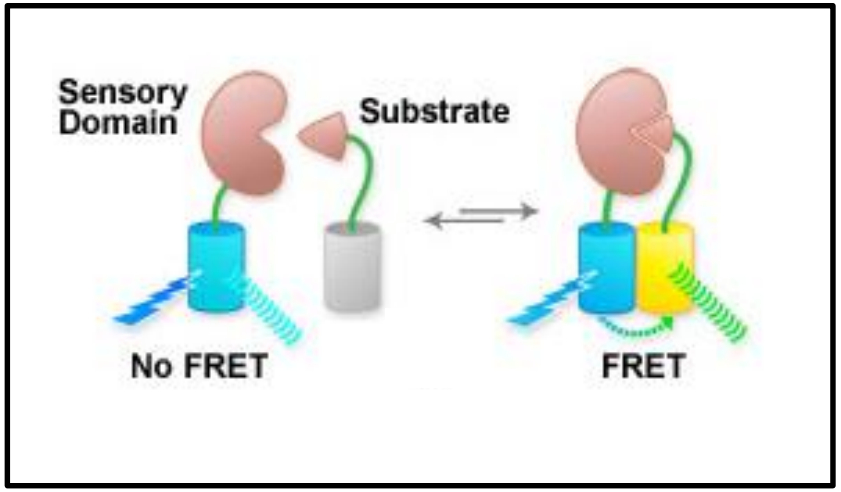
- **(Top left)** Molecular arrangement of CFP, YFP, calmodulin and M13 peptide in the chimeric biosensor called cameleon.
- **(Top right)** In environments containing calcium ion, calmodulin binds to ions, changes conformation, and wraps around the M13 peptide, creating a conformation suitable for FRET and allowing a shift in fluorescence emission from cyan to yellow-green.
- **(Bottom)** A series of images covering a time window of 2 seconds showing a calcium wave in HeLa cells expressing cameleon (**pseudocolored in a cyan to red range**) after addition of ionomycin to the culture medium. Bar = 20 μm.

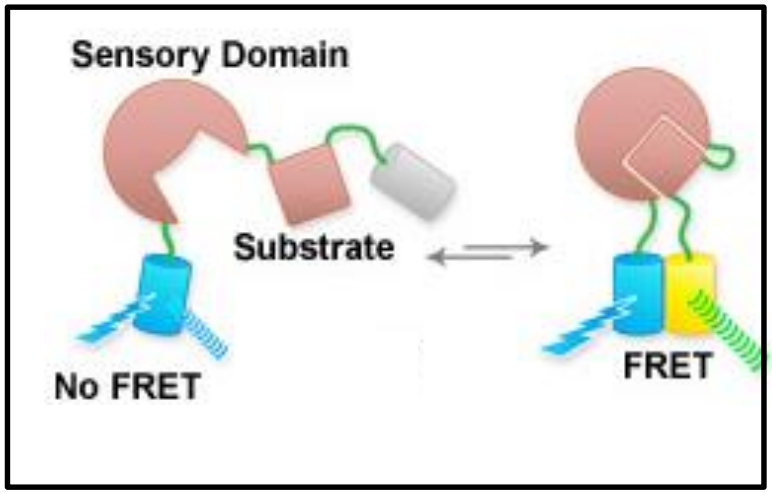
Applications of FRET in Biology - Examples

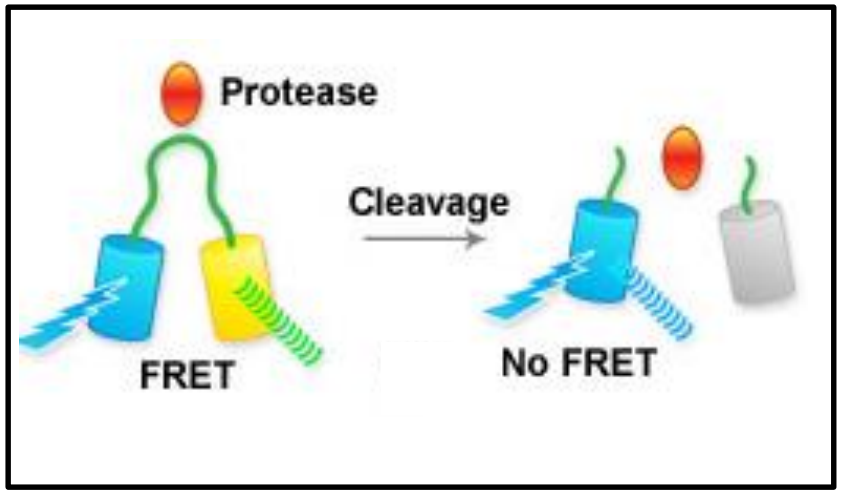
Some FRET assay examples:

Calcium Ion measurements, Protease activity, Protein tyrosine kinase activity, Phospholipase C activity, Protein kinase C activity, Membrane potential …









FRET in Biology

Advantage	Cheap to implement
	High resolution (1-10nm)
	Adaptable for live cells
	Real time
Disadvantage	Weak effect
	Location of fluorophores is critical
	pH and environment sensitive
	Free fluorophors can mask energy transfer

Variety of fluorescence microscopy technique exists

- A strong feature of fluorescence microscopy is that the signals making up an image are “molecule-specific”.
- With the addition of time-lapse methods, it is possible to track time-dependent changes of molecules & dynamic molecular events.

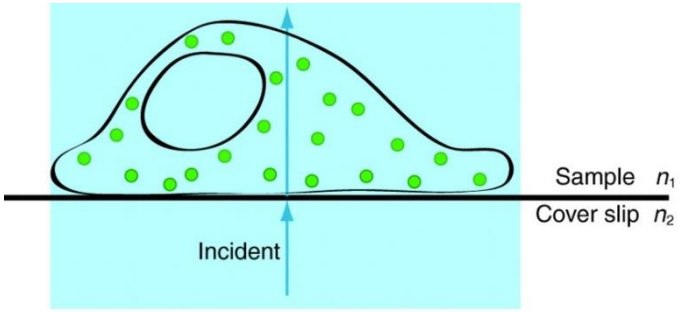
A few examples of dynamic fluorescence imaging methods:

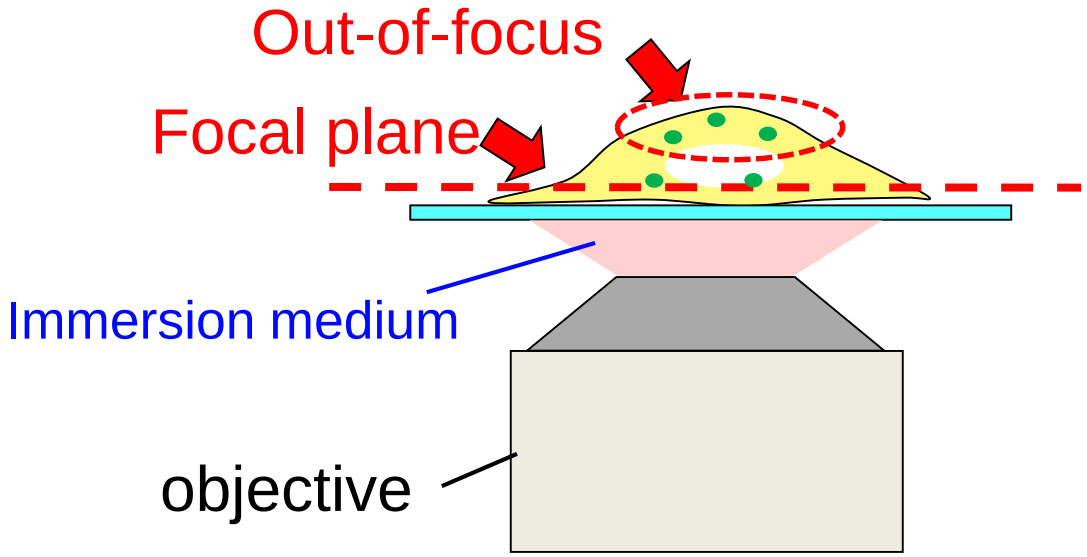
- **FRET (Förster resonance energy transfer)**
- TIRFM (Total internal reflection fluorescence microscopy)
- FRAP (Fluorescence recovery after photo bleaching)
- FLIM (Fluorescence lifetime imaging microscopy)
- FLIP (Fluorescence loss in photobleaching)
- FLAP (Fluorescence localization after photobleaching)
- FISH (Fluorescence in situ hybridization)
- FCS (Fluorescence correlation spectroscopy)

…

Limitations of conventional fluorescence microscopy

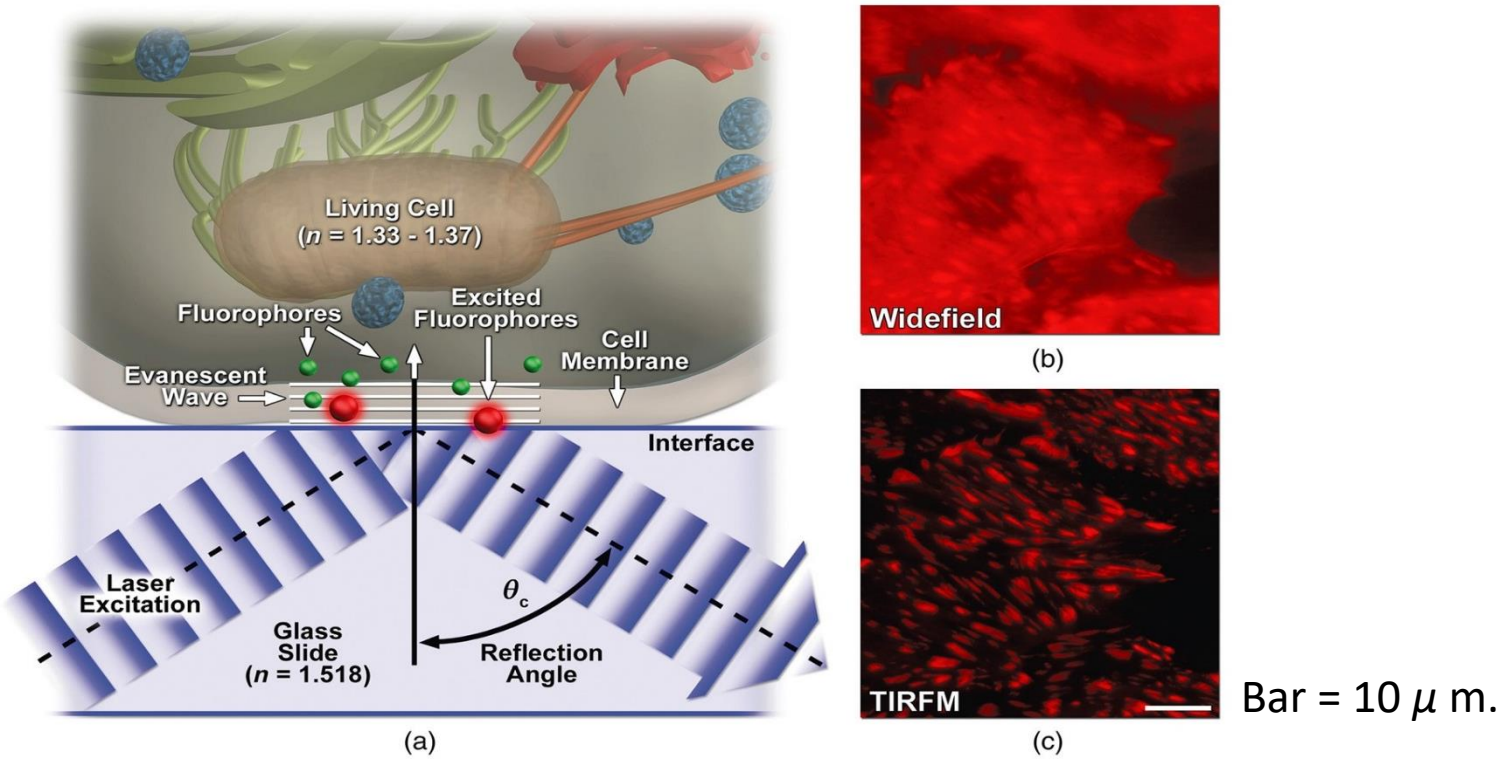
- In a standard fluorescence microscope, the excitation beam illuminates uniformly a wide field of the sample.
- If the sample is thick, fluorescence will be excited within the focal plane, but also within planes above and below the focus.
- Some of this fluorescence will be imaged onto the detector and will result in a defocused-looking image.





Total internal reflection fluorescence (TIRF)

- TIRF uses a specialized method of illumination to excite fluorescence in molecules that are located within the 100 nm of the surface of the coverslip to which specimens are attached.



(a) Cartoon of a cell on the surface of a coverslip labeled with fluorophores that are excited by the evanescent wave field from the reflected laser.
(b) Wide-field image of living cells expressing a red fluorescent protein fused to paxillin, a focal adhesion component.
(c) Same field of view in TIRF illumination. Note the higher contrast and S/N in the TIRF image.

TIRF is used to examine membrane proteins, cell signaling events, receptor clustering in membranes, focal adhesions, cytoskeletal components, and other membrane - associated molecules.

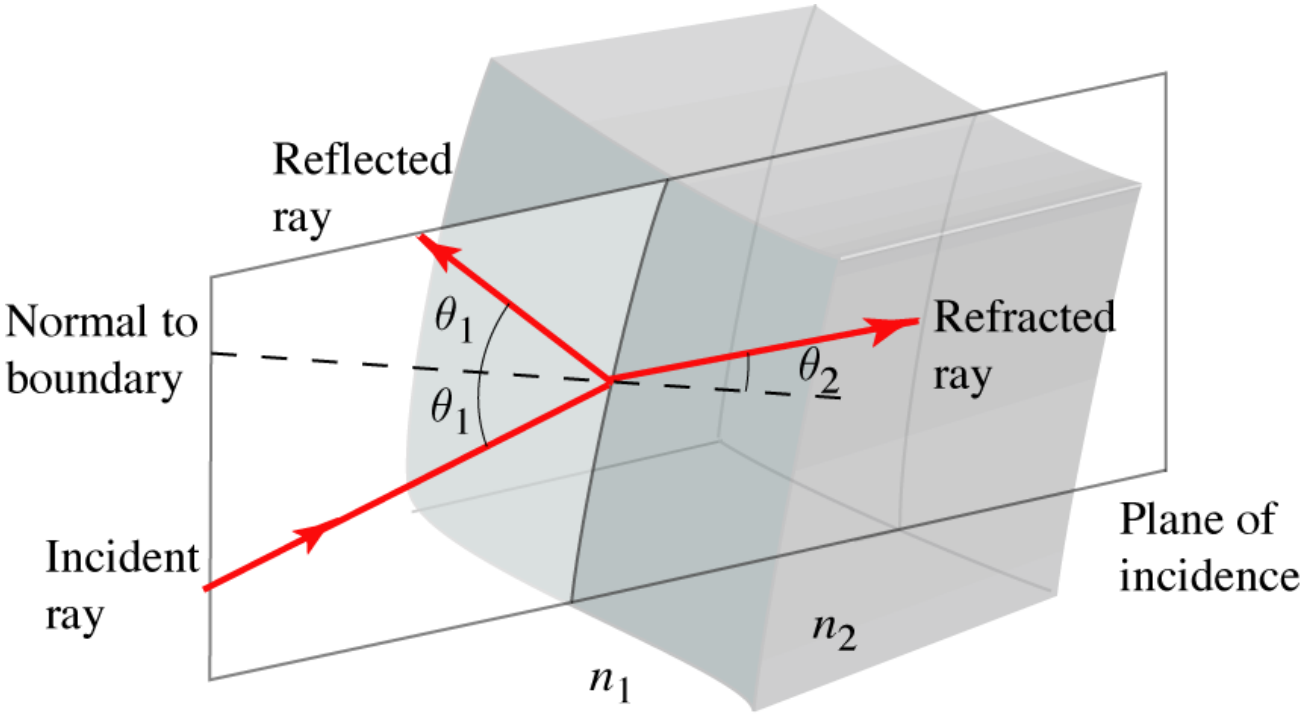
The Law of Refraction

Refraction occurs at a boundary between two different media:

1- The refracted ray lies in the plane of incidence (conservation of momentum ..)

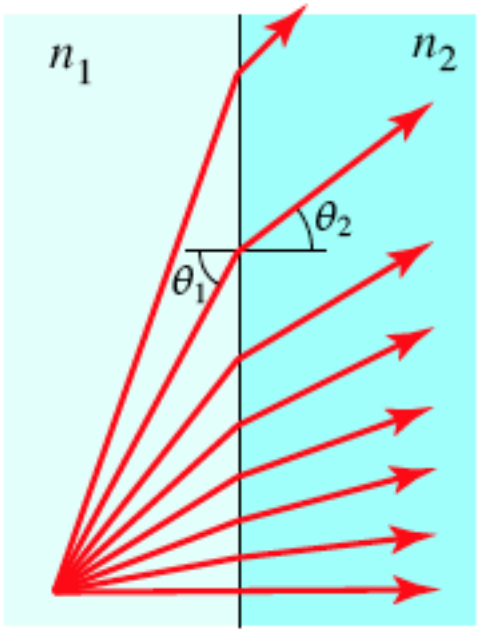
2- The angle of refraction is related to the angle of to the angle of incidence by Snell's Law

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



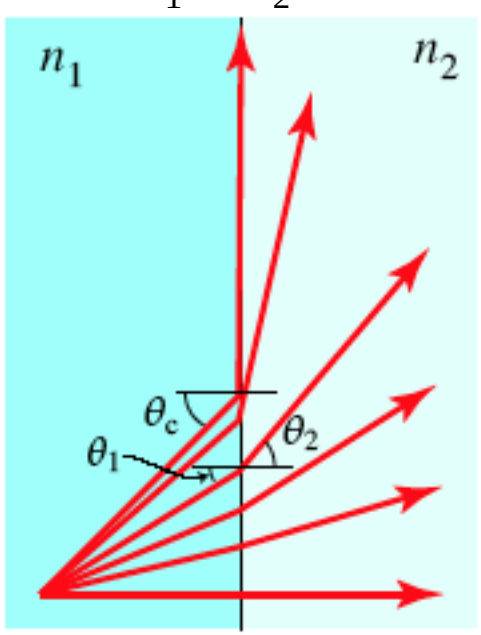
Planar boundary & application of Snell's law:

$n_1 < n_2$

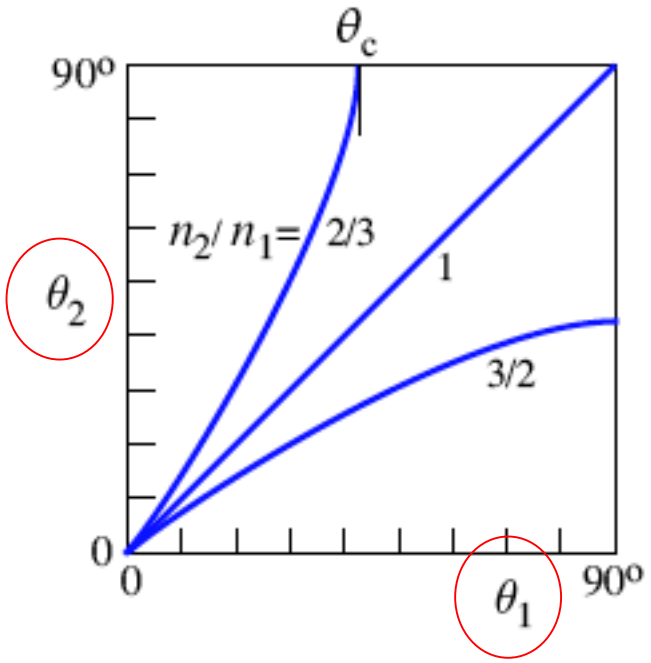


External refraction

$n_1 > n_2$



Internal refraction



Snell's Law:

$$\sin\theta_1 = (n_2/n_1)\sin\theta_2$$

Critical Incidence Angle:

$$\theta_c = \sin^{-1}\frac{n_2}{n_1} \qquad \theta_2 = \pi/2$$

Conditions required for TIR

The critical angle required for TIR is obtained from Snell ' s law:

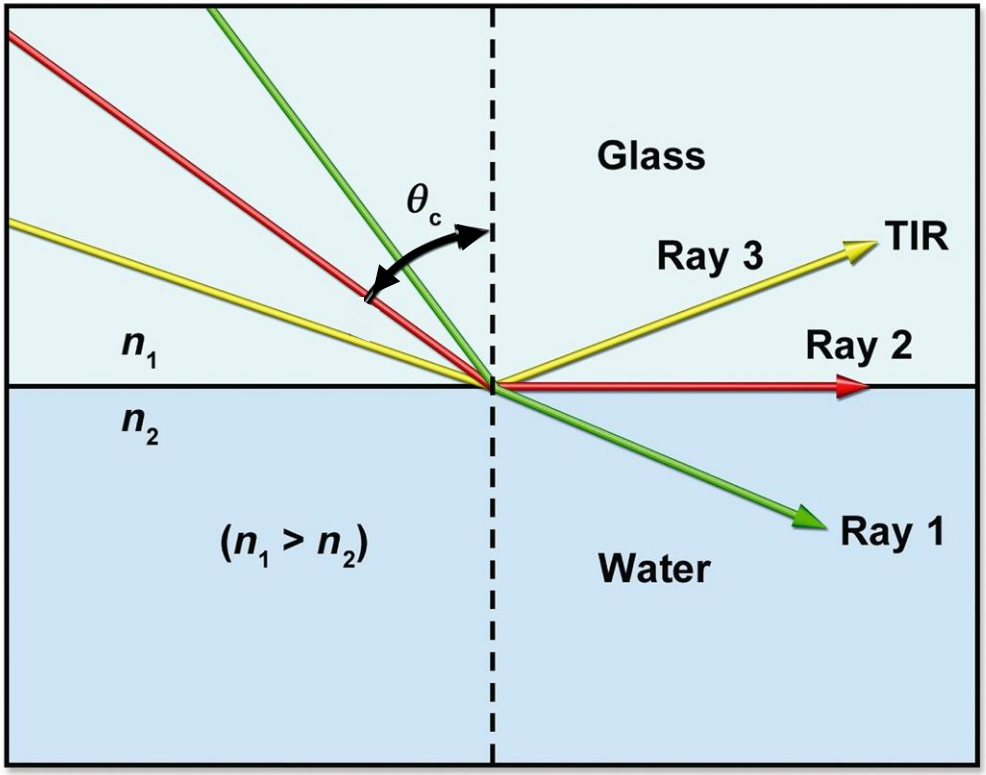
$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

If n_1, n_2 are known, then by setting the angle $\theta_2 = 90°$, the θ_c can be calculated as:

$$\theta_c = \sin^{-1}\frac{n_2}{n_1} \qquad (n_1 > n_2)$$

Example:
For the case of a cell (n = 1.35) attached to a glass coverslip (1.518), θ_c is 62°, and angles greater than this are required to obtain TIR.

Conditions required for TIR

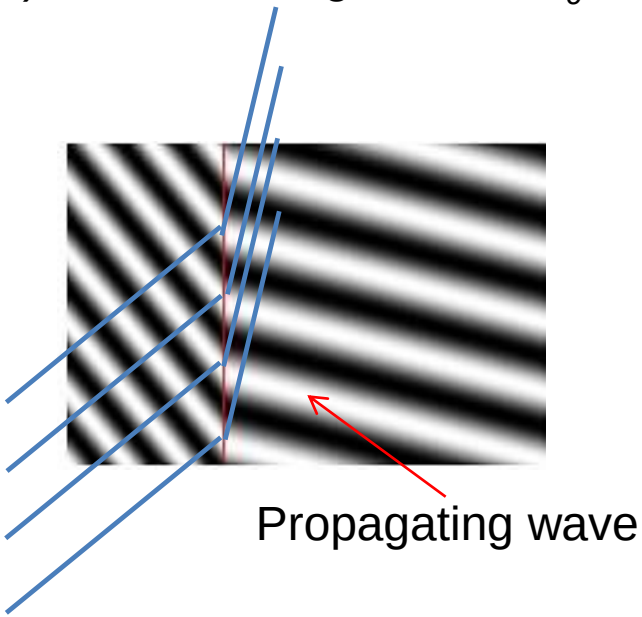


- TIR at the interface between two transparent media. is possible when beams of light encounter a medium of lower refractive index .

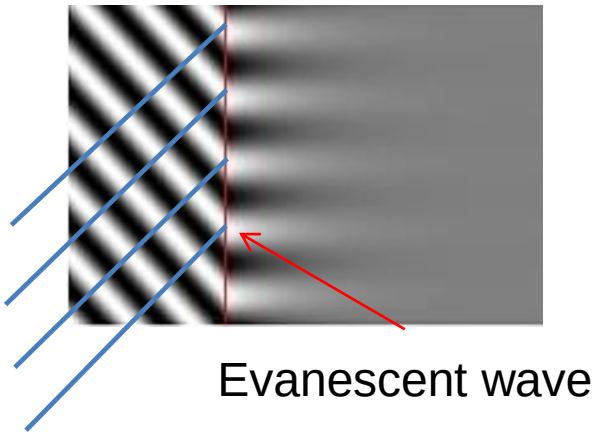
- Beams behave differently depending on the angle of incidence at the interface. Examples:
  - At angles less than θ_c, rays are refracted and enter the second medium **(Ray 1)**.
  - At the so-called critical angle θ_c, a ray is deflected at a 90° angle to the normal and travels along the interface **(Ray 2)**.
  - At angles greater than θ_c, rays experience total internal reflection **(Ray 3)**.

Evanescent wave in TIR

A) Incidence angle below θ_c



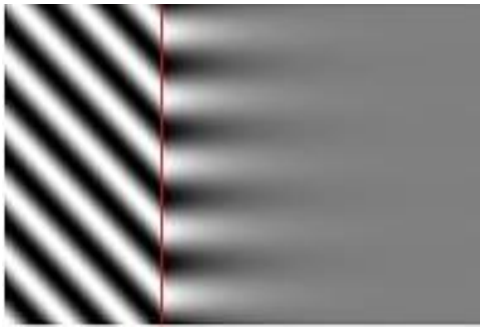
B) Incidence angle above θ_c



For B:

- The physics of wave reflection under TIR conditions from a dielectric material like glass requires that there is a “transmitted ” wave component, represented as a standing, evanescent wave at the *interface*, even though all wave energy is reflected.
- The electric field of this standing wave penetrates into the second medium over a short distance and it is called an ***evanescent wave***.

Penetration Depth in TIR



The intensity of ***evanescent wave*** decreases ***exponentially*** with increasing distance from the interface:

$$I = I_o e^{-\frac{z}{d}}$$

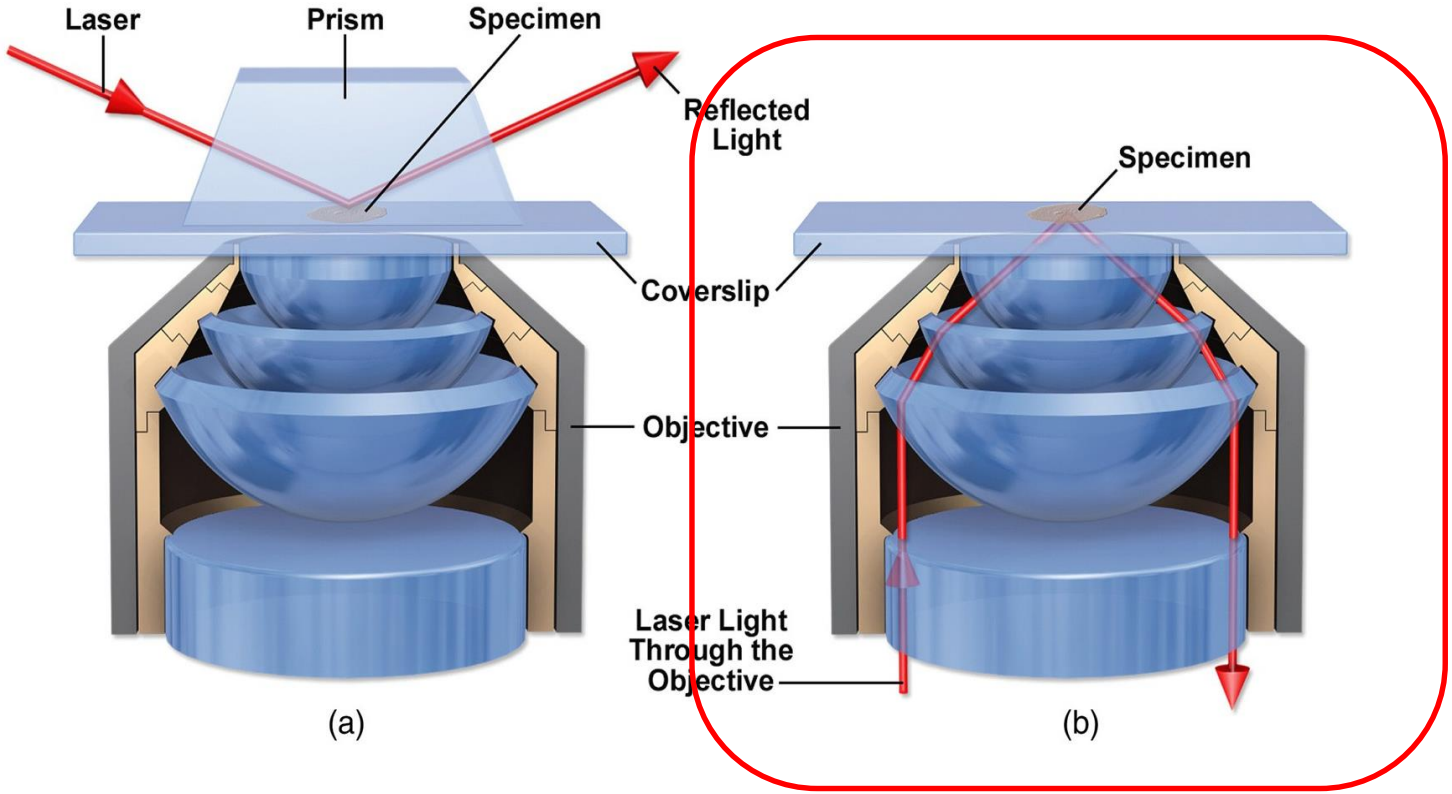
$$d = \frac{\lambda}{4\pi n_2 \sqrt{\left(\frac{n_1}{n_2}\right)^2 (sin\theta_i)^2 - 1}}$$

A smaller penetration depth into the second medium (thus better surface confinement) can be achieved with:

- Larger incidence angle
- Shorter wavelength
- Higher index contrast

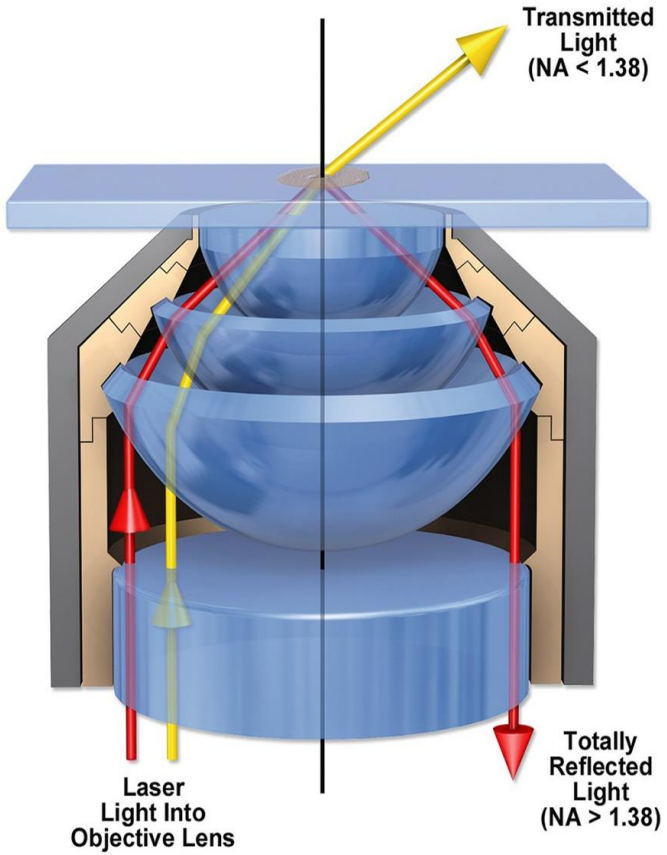
Implementation of TIRF on inverted microscopes

- Different implementation configurations exist for both inverted and upright microscopes.
- Below illustrate a few example configurations for inverted microscope.



Examples for modes of TIRF illumination.
(a) Prism TIRF, and (b) through - the - lens TIRF.

Implementation of TIRF on inverted microscopes with “through-the-lens”

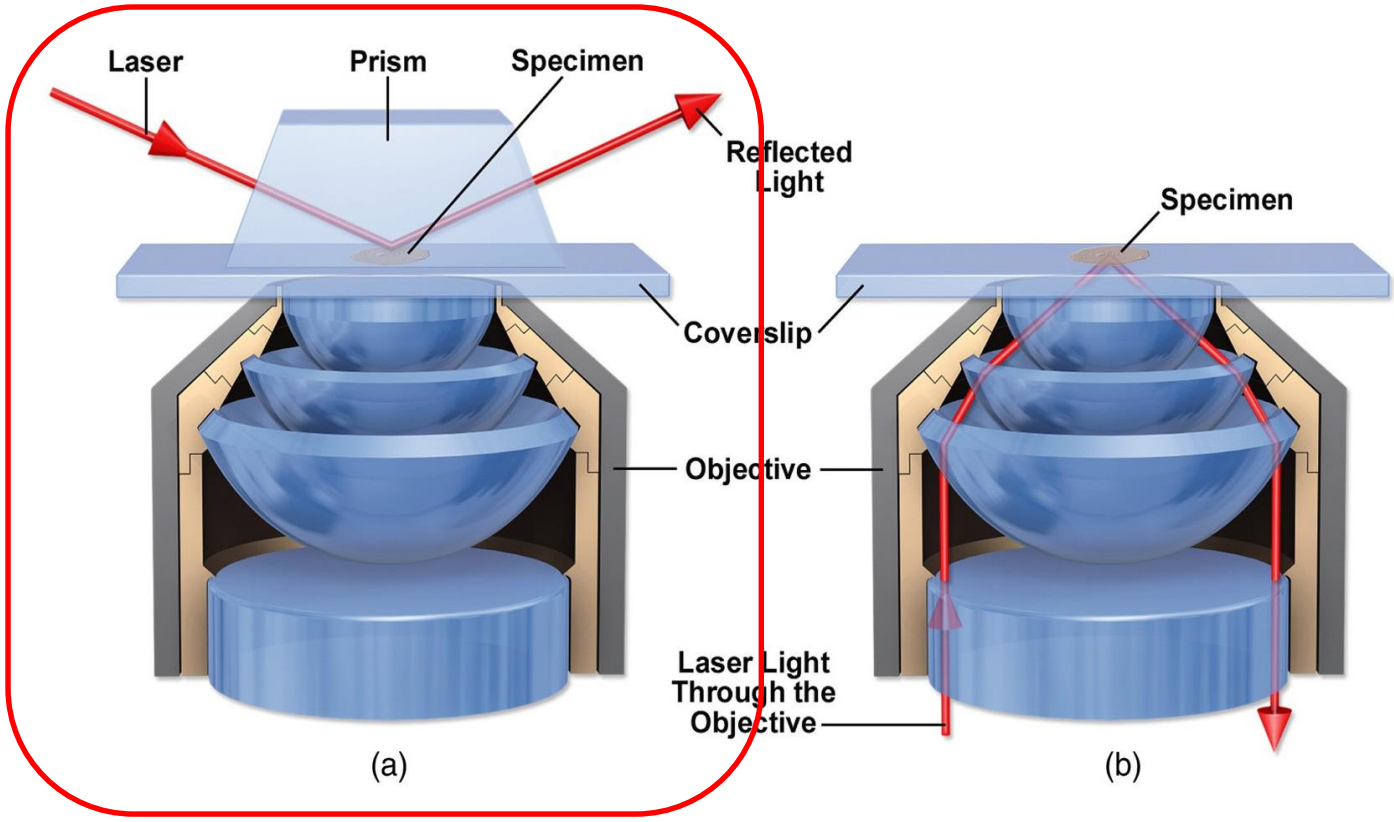


Adjustment of the critical angle for TIRF illumination:

- The laser beam is focused at the objective’ s rear aperture and then moved to one edge of the aperture.
- TIRF illumination is obtained when the angle of incidence at the specimen is greater than the critical angle.
- For a glass coverslip and living tissue culture cell, this angle is $\sim 62°$.

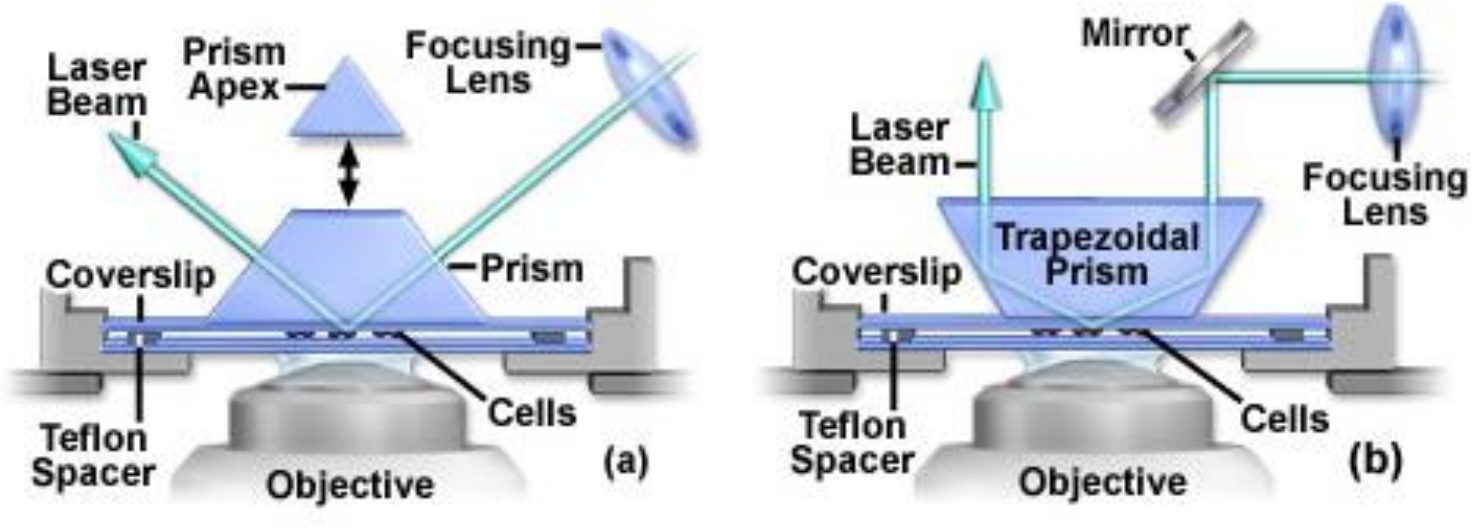
Implementation of TIRF on inverted microscopes

- Different implementation configurations exist for both inverted and upright microscopes.
- Below illustrate a few example configurations for inverted microscope.

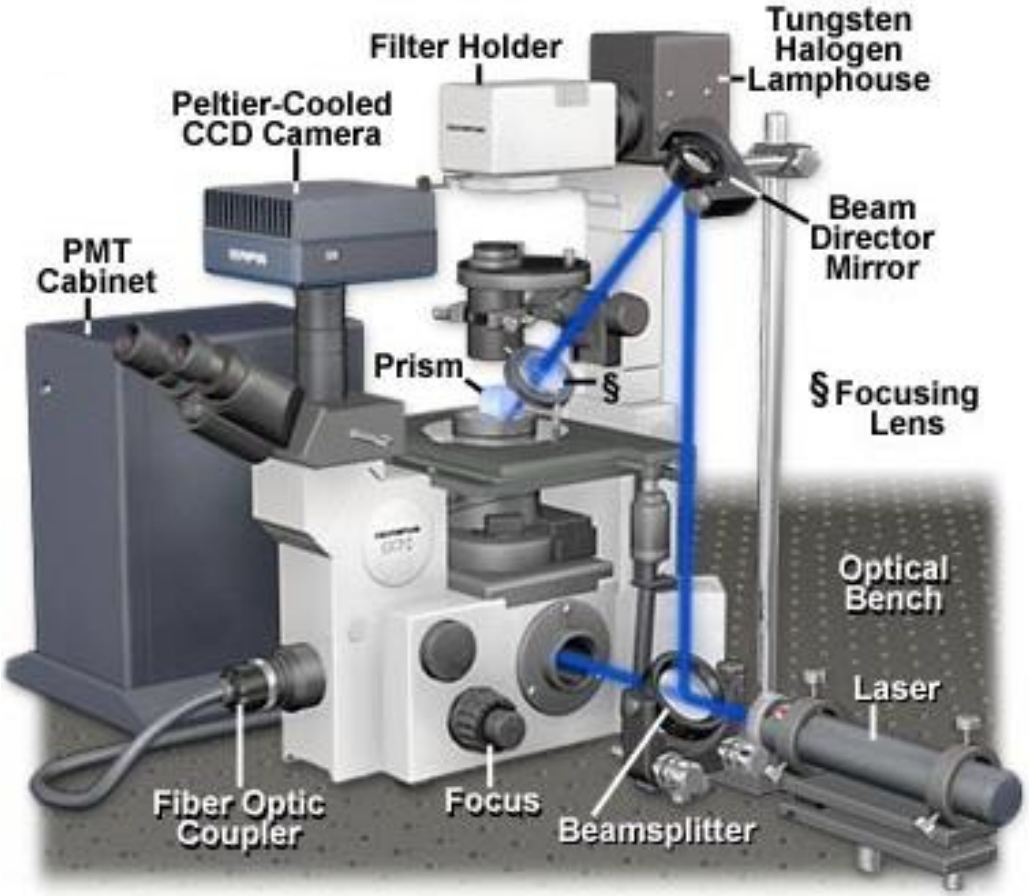


Implementation of TIRF on inverted microscopes with prism coupling

Illustration of two TIRM illumination examples with prism coupling on an inverted microscope.

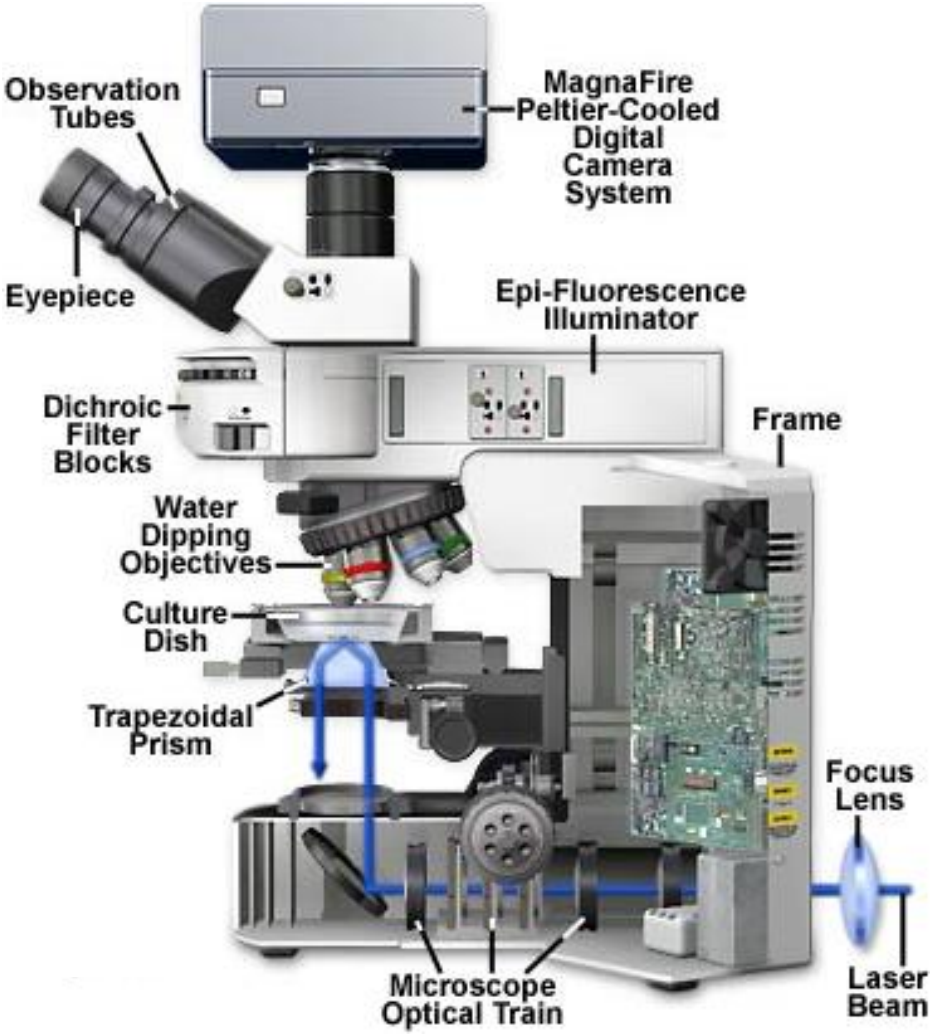


TIRF on inverted microscope



- This scheme illustrates one of the possible configurations for TIRF implementation on an inverted microscope.
- It uses prism coupling.

TIRF on upright microscope



- This scheme illustrates one of the possible configurations for TIRF implementation on an upright microscope.