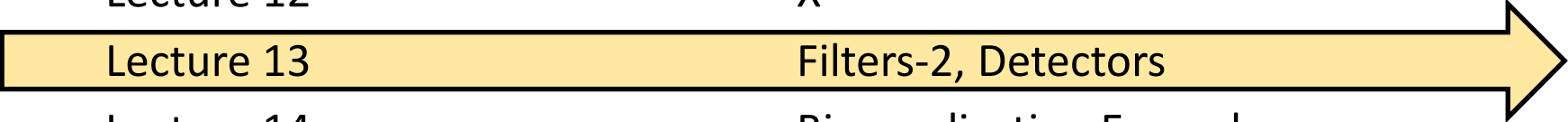


MICRO-561

Fundamentals of Biomicroscopy

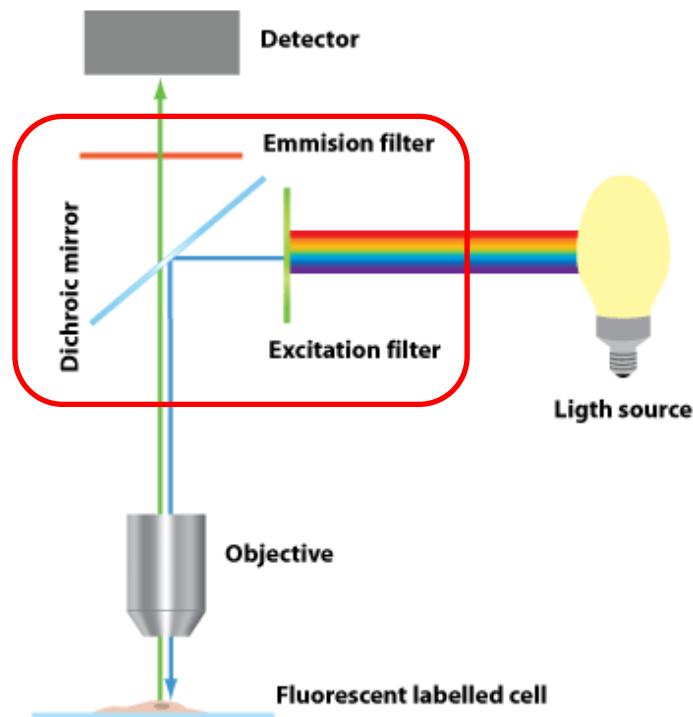
Syllabus (tentative)

Lecture 1	Introduction & Ray Optics-1
Lecture 2	Ray Optics-2 & Matrix Optics-1
Lecture 3	Matrix Optics-2
Lecture 4	Matrix Optics-3 & Microscopy Design-1
Lecture 5	Microscopy Design-2
Lecture 6	Microscopy Design-3 & Resolution -1
Lecture 7	Resolution-2
Lecture 8	Resolution-3
Lecture 9	Contrast & Fluorescence -1
Lecture 10	Fluorescence-2
Lecture 11	Sources, Filters-1
Lecture 12	X
Lecture 13	Filters-2, Detectors
Lecture 14	Bio-application Examples



Fluorescence Microscopy

- To understand fluorescence microscopy we need to be familiar with:
 - Basic principles of fluorescence
 - Properties of fluorescent dyes
 - Different kinds of fluorescence markers
 - **Important optical components**
 - Illumination sources
 - **Filters and filter sets**
 - Detectors
 - Their proper positioning in the optical train of the microscope



Filters in Microscopy

- Properties of the incident light on the sample are important for microscopy.
 - The power (intensity) of the incident light.
Example: high power can **photo-damage** delicate live cells
 - The color (energy) of photons.
Example: **UV photons** cells can kill live cells
- Properties of the incident light depend on the used illumination source.
- In practice, the color and the intensity of the light on the sample are controlled by external filters that are attached to the microscope (... in the optical train, filters are after the illumination source).

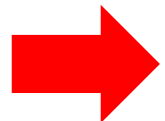
To properly use the filters in microscopy, it is crucial to understand their properties:

- Interpreting their spectra
- Selecting the best filter combination/set

→ This is particularly important for fluorescence microscopy because the spectra of the filters must MATCH to the excitation & emission spectra of the fluorescent markers.

Two main filter types are commonly used in microscopy:

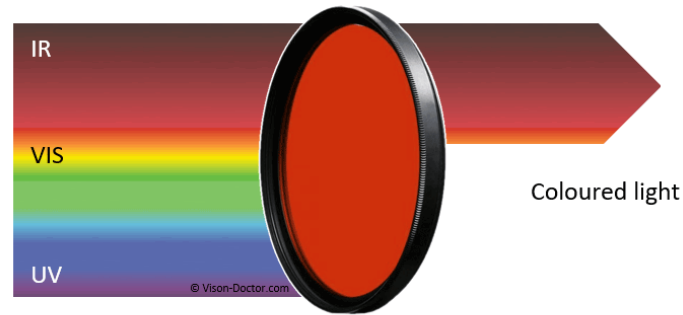
1) Neutral density filters



2) Spectral filters

Spectral Filters in Microscopy

Spectral filters can isolate specific colors or color (wavelength) bands



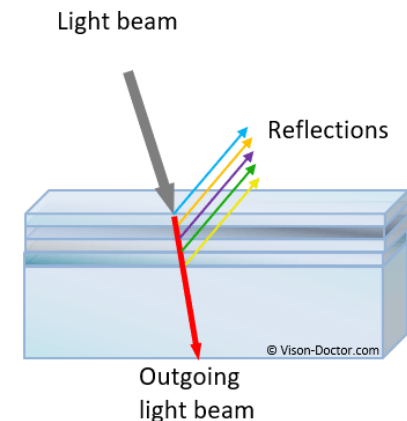
Colored-Glass Filters:

- Contain rare earth transition elements, which absorb non-transmitted wavelengths.
- Suitable for applications that don't require precise definition of transmitted wavelength.
- They are commonly used in wide-field microscopy to remove unnecessary UV or IR photons or give color to the images.
- As they are based on "absorption" they can get heated up & subject to alteration and even damage after prolonged use.

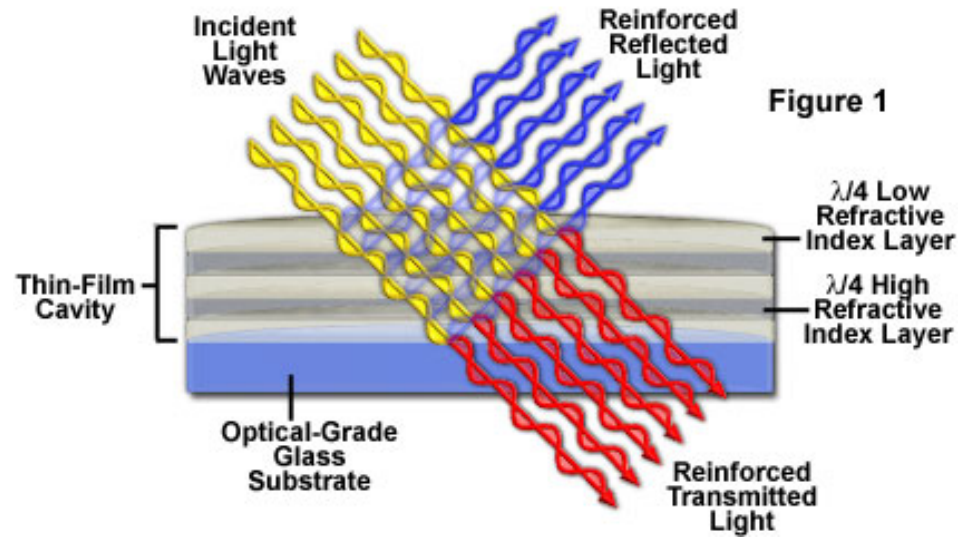
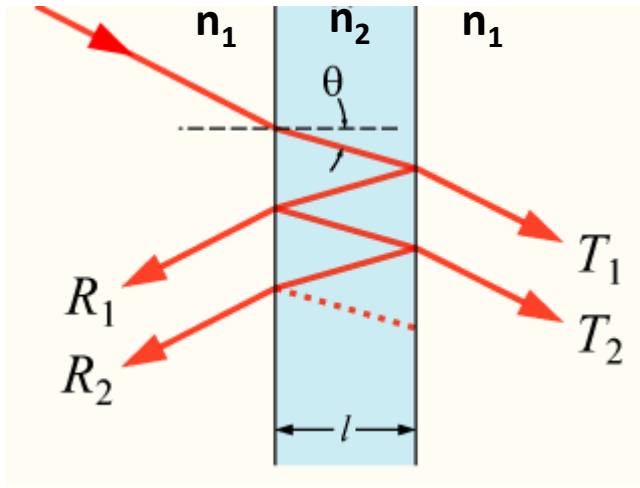


Interference Filters:

- Based on interference effects from optically transparent multi-layer dielectric stacks.
- Offer steeper transmission boundaries & cut-on & cut-off wavelengths → They offer precise definition of the transmitted spectrum.
- Frequently used in fluorescence microscopy.



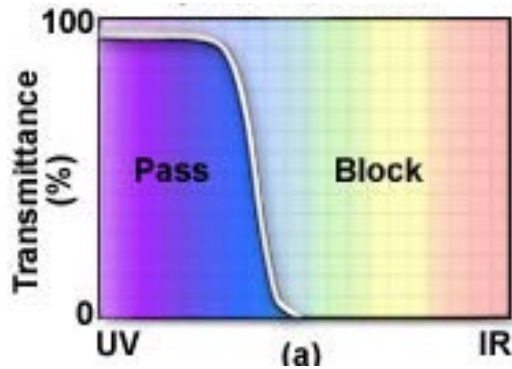
Interference Filters



- The interface between two materials of different refractive index (n_i) partially reflects incident light backward (R_i) & forward (T_i).
- Interference filters operate by selectively reinforcing and blocking the transmission/reflection of specific wavelengths through constructive & destructive interference.
- Total transmission and transmitted/reflected wavelengths depend on the thickness, refractive index and the design (stacking) of the layers.

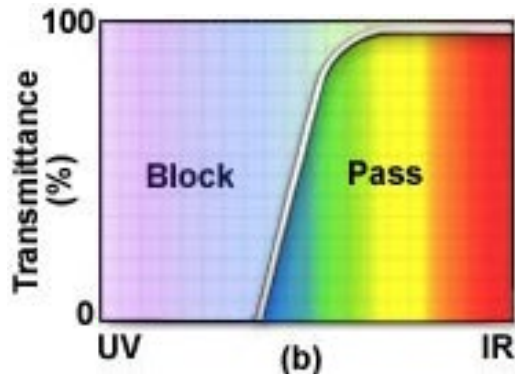
Spectral Filters in Microscopy

Spectral filters isolate specific colors or bands of wavelengths.



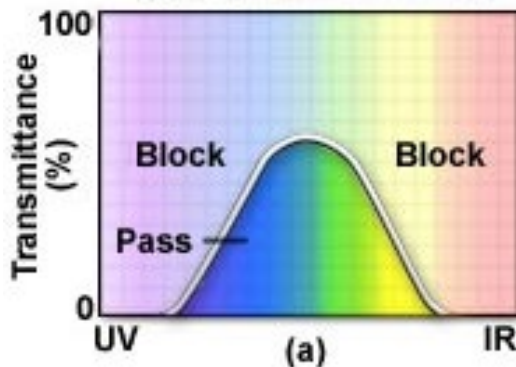
1) Short Pass Filters:

Transmits short wavelengths and block long ones



2) Long Pass Filters:

Transmits long wavelengths and block short ones

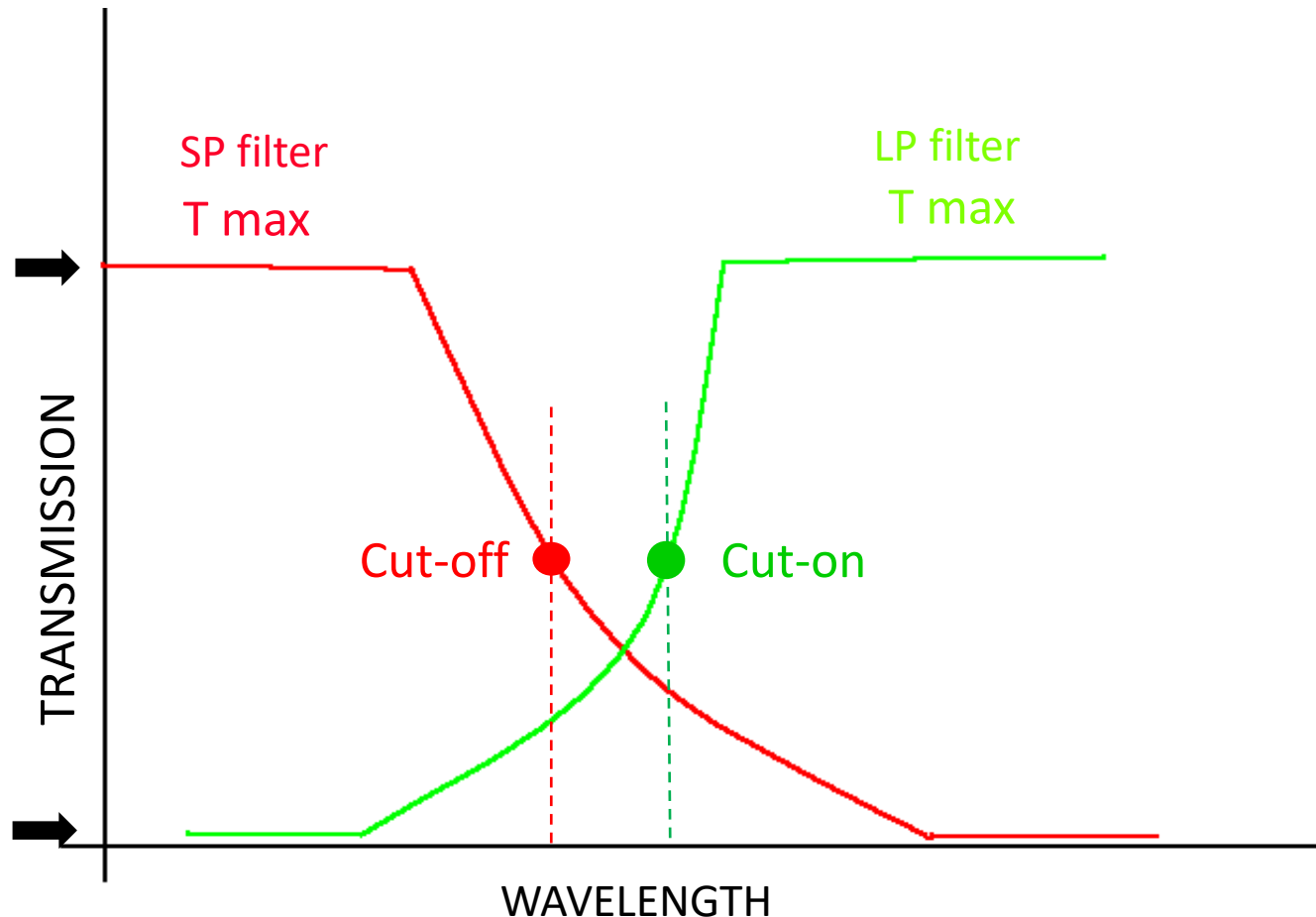


3) Band-Pass Filters:

Transmits a band of wavelength while blocking the wavelengths above and below the specified range of transmission.

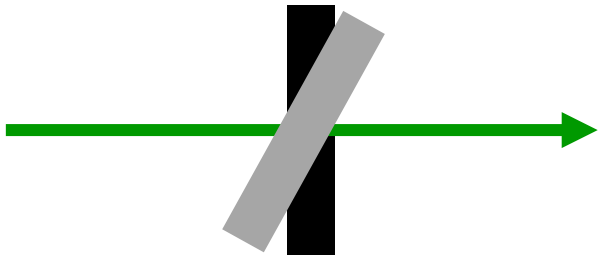
Short-Pass (SP) Filter & Long-Pass (LP) Filter

- Optical performance is defined in terms of
 - **Efficiency of transmission and blockage** (i.e. in terms of % of transmission, T_{\max})
 - **Steepness of the cut on/off boundary** between the adjacent transmitted and blocked domains.
- Here, cut on/off wavelength is defined by 50% of peak transmission.

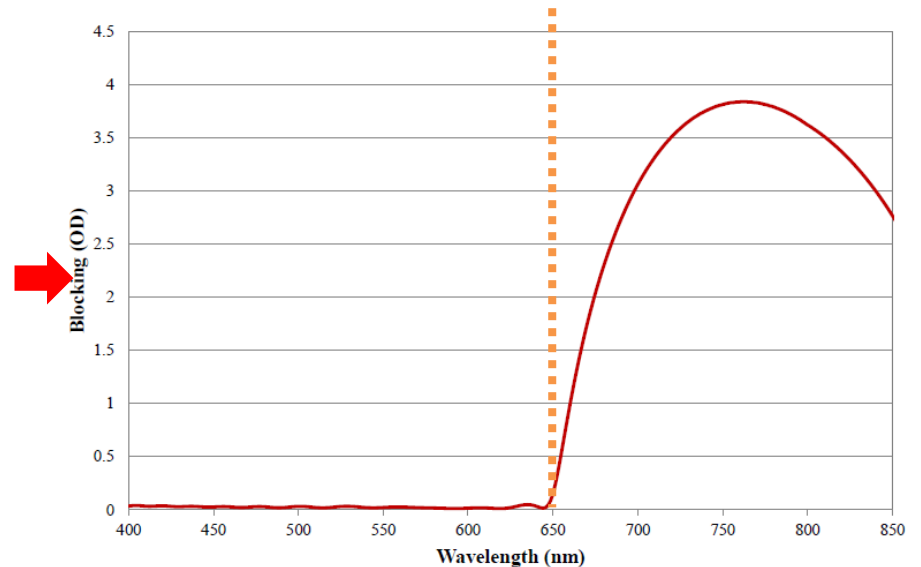
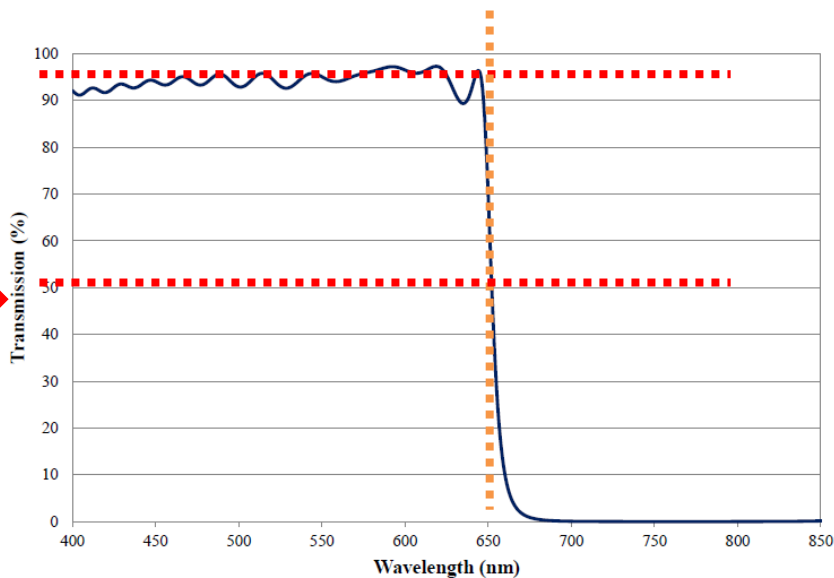
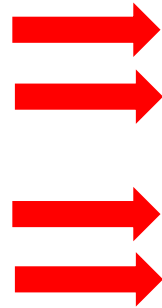


Example Spectrum for Short-Pass Filters

Specs and curves of SP650 from Edmund Optics



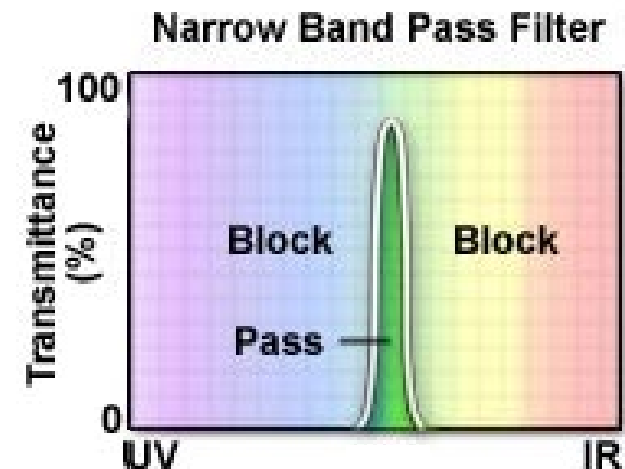
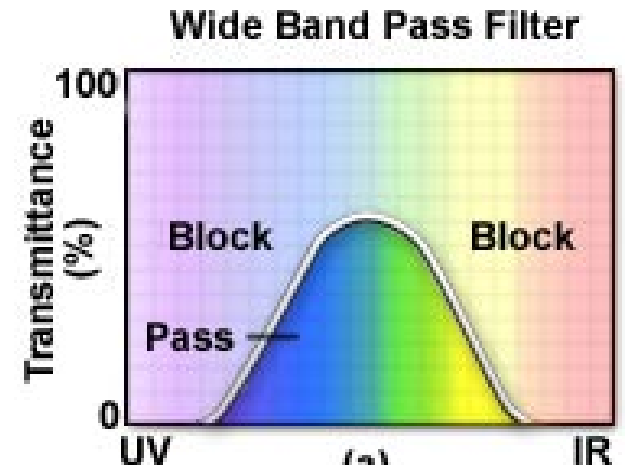
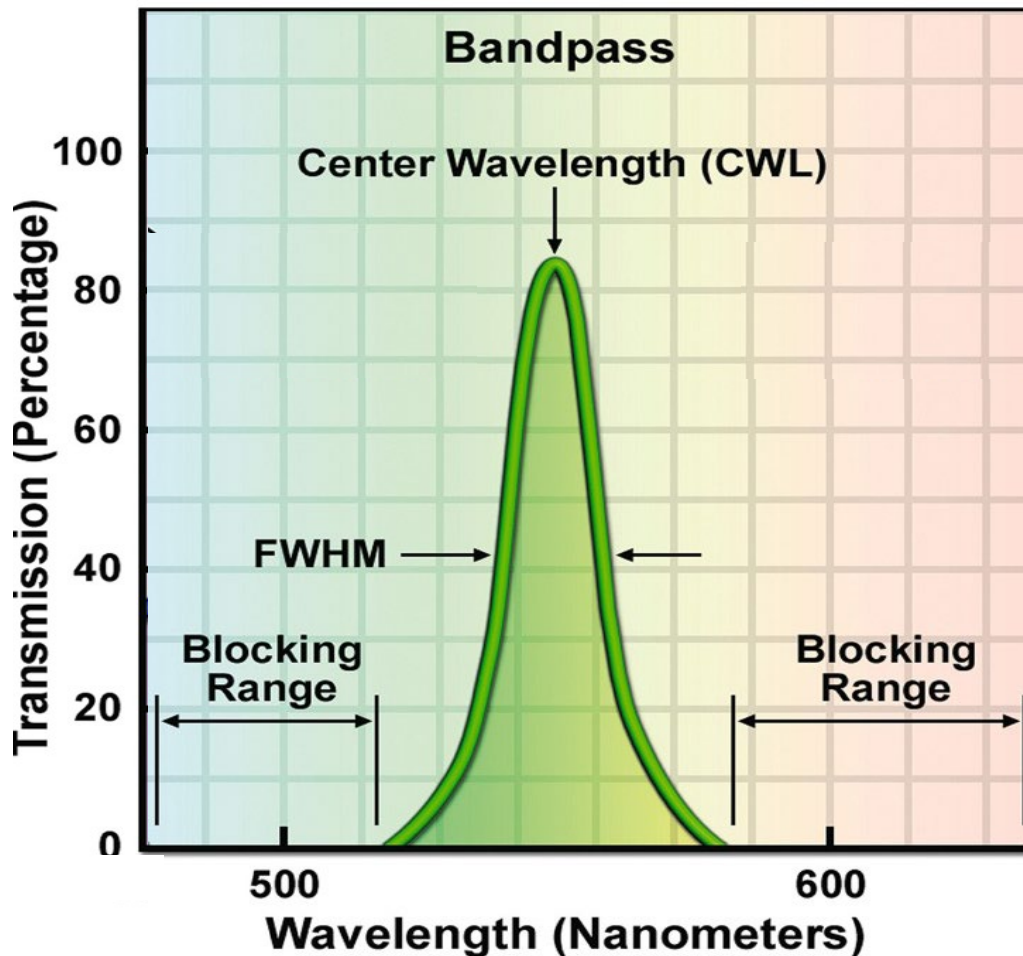
Type	Short-pass Filter
Wavelength Range (nm)	400 - 850
Transmission Wavelength (nm)	400 - 630
Transmission (%)	>85
Cut-Off Wavelength (nm)	650
Rejection Wavelength (nm)	675 - 850
Optical Density OD	≥2.0
Angle of Incidence (°)	0



Band-Pass (BP) Filters

BP filters are described by referring to the:

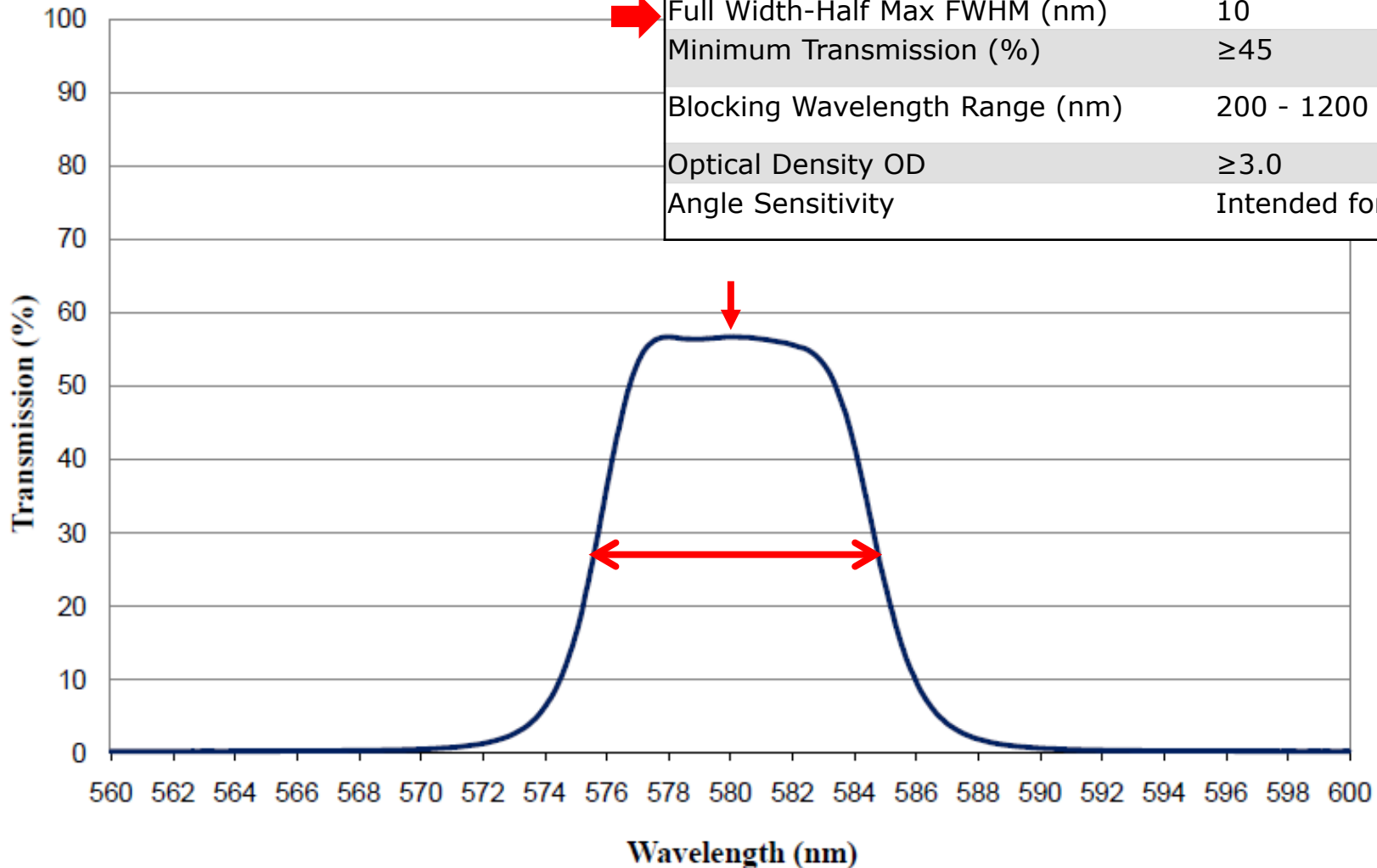
- Peak center wavelength (CWL)
- Full-width-half-maximum (FWHM), which is the range between the edges of the bandpass peak where the transmission is 50% of its maximum value



Example Spectrum for BP Filter

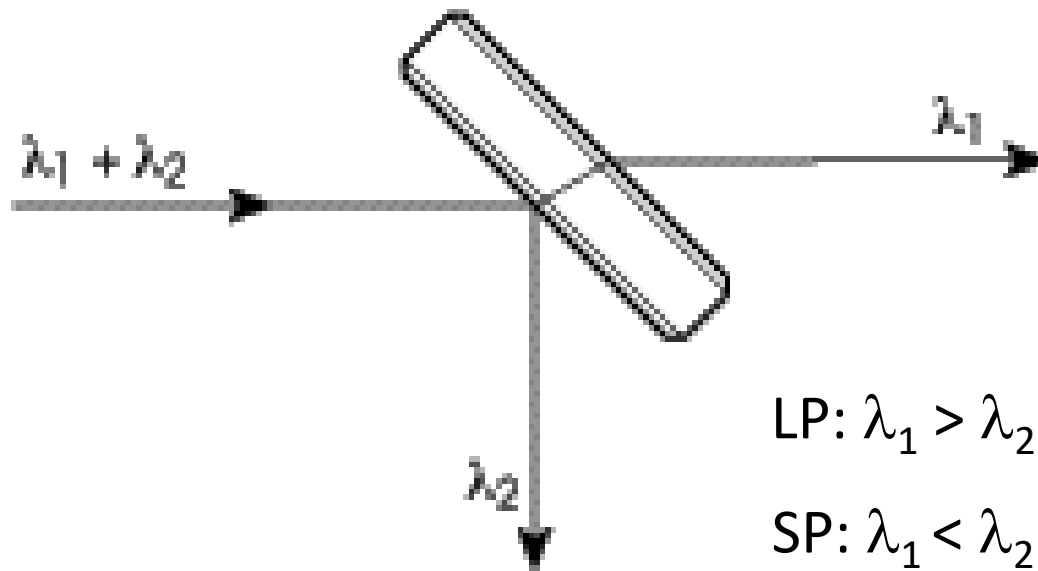
Specs and curves of BP580nm filter from Edmund Optics

Type	Bandpass Filter
Center Wavelength CWL (nm)	580
Mount Thickness (mm)	9.6
Full Width-Half Max FWHM (nm)	10
Minimum Transmission (%)	≥45
Blocking Wavelength Range (nm)	200 - 1200
Optical Density OD	≥3.0
Angle Sensitivity	Intended for Collimated Input



Dichroic Mirror (DM)

- *Di-* is Greek for two, and *-chroic* is Greek for color → “bicolored”
- Spectrally separates (or combines) light by transmitting and reflecting photons as a function of wavelength
- DM is a LP or SP interference filter that is designed to be used at **45°**

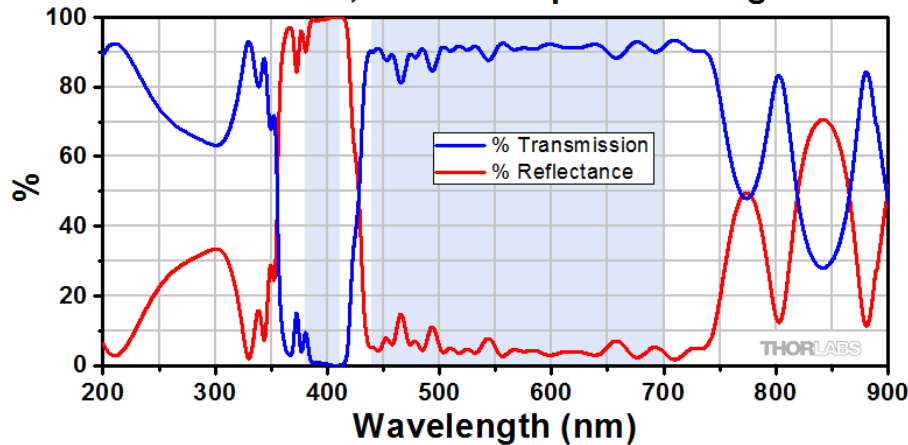


- **Long-pass (LP) dichroic mirrors** dichroic mirrors are highly **transmissive** above the cutoff wavelength and highly reflective below it.
- **Short-pass (SP) dichroic mirrors** are also available which are highly **reflective** above the cutoff wavelength and highly transmissive below it.

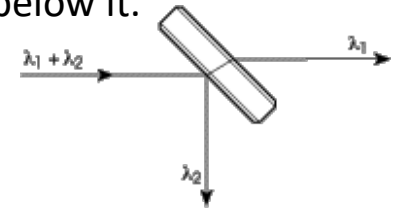
Example Spectra for “Dichroic” Mirror

- Data show additional transmission/reflection bands & sharp oscillations at the spectrum edges. Such features are typical for interference filters.

DMLP425, 45° AOI Unpolarized Light

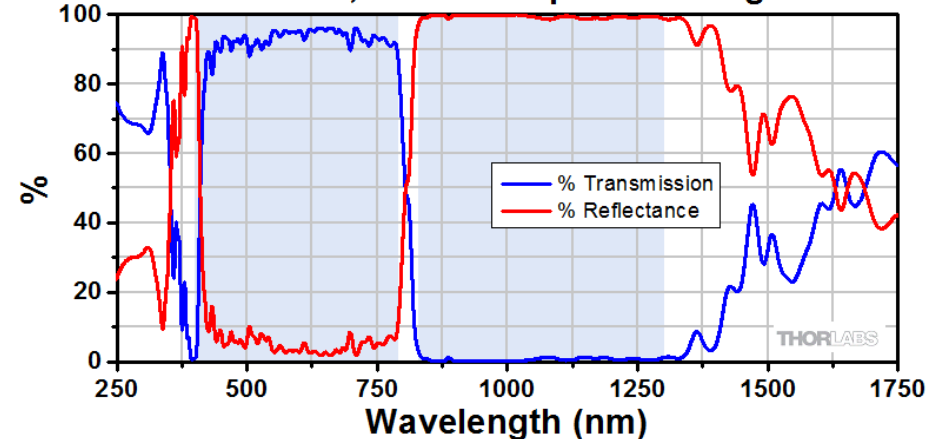


- LP dichroic mirrors (DMLP) are highly transmissive above the cutoff wavelength and highly reflective below it.



- It operates at 45°
- For DMLP425, cut-off wavelength is 425 nm

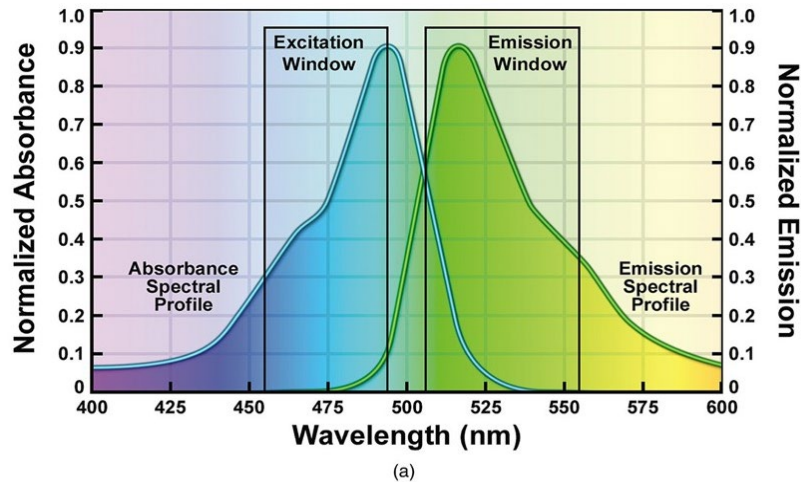
DMSP805, 45° AOI Unpolarized Light



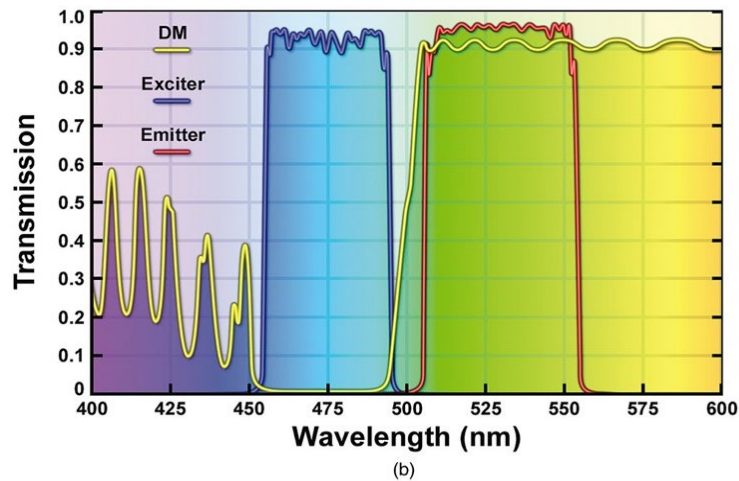
- SP dichroic mirrors (DMSP) are highly reflective above the cutoff wavelength and highly transmissive below it.
- It operates at 45°
- For DMSP805, cut-off wavelength is 805 nm

An Example for Choosing a Filter Set

(a) Fluorescein absorption & emission spectra:



(b) Fluorescein filter set spectra:



Notes:

- Fluorescein absorption & emission spectra overlap. Therefore, to maintain a distinct separation, the transmission profiles of the filters are chosen not to exactly centered at the excitation & emission maxima.
- Boundaries between the transmitted & reflected wavelengths are designed to be as steep as possible with the filters.
- Rapid spikes (termed as “ringing”) on the sides are typical for dichroic mirrors – this is due to the thin film interference effect.

1) Exciter - BP Filter:

- Transmits mainly the absorption region

2) Emitter - BP Filter:

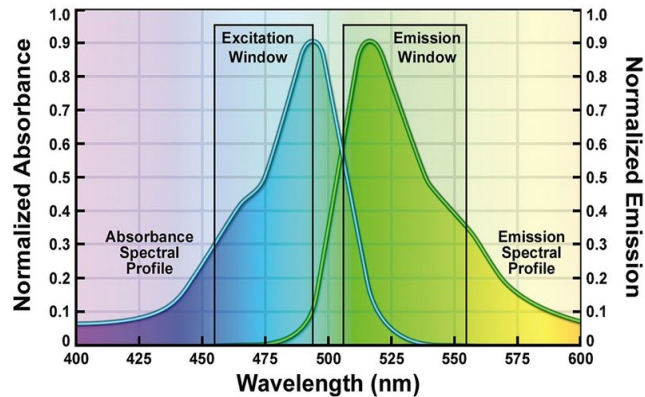
- Transmits mainly the emission region

3) Dichroic Mirror - DM

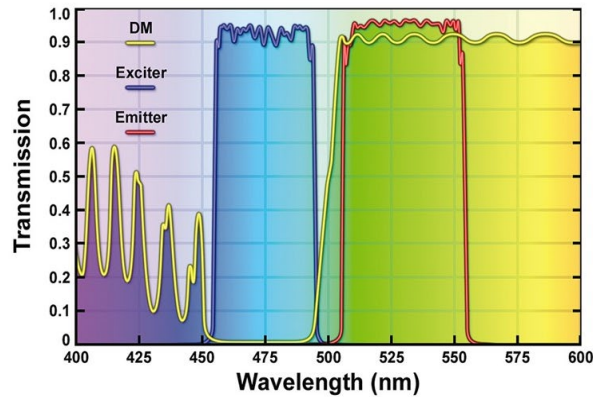
- Transmits most of the emission region

- Reflects most of the excitation region

An Example for Choosing a Filter Set



(a)



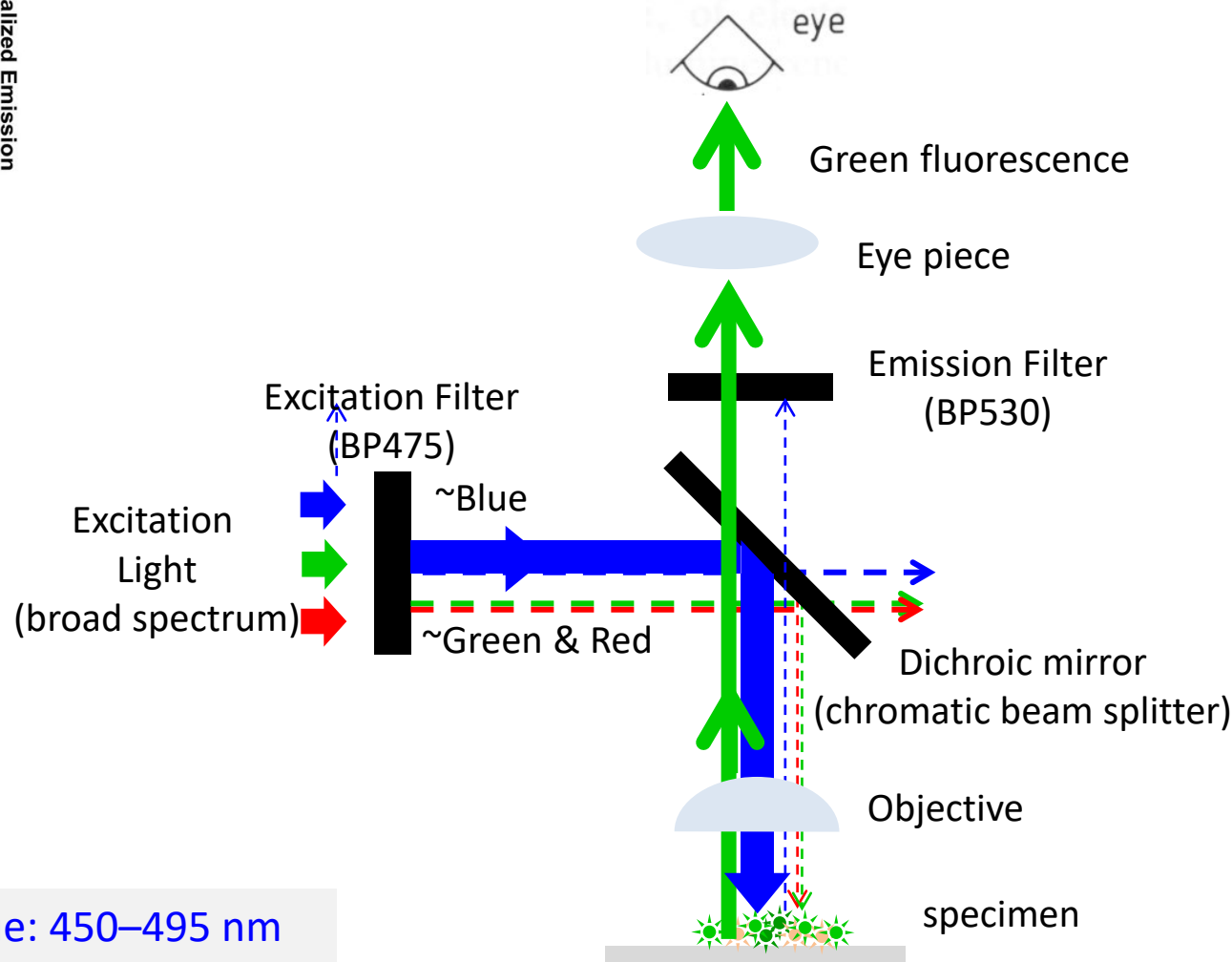
(b)

1) Excitation - BP Filter:
BP475, ~40nm FWHM

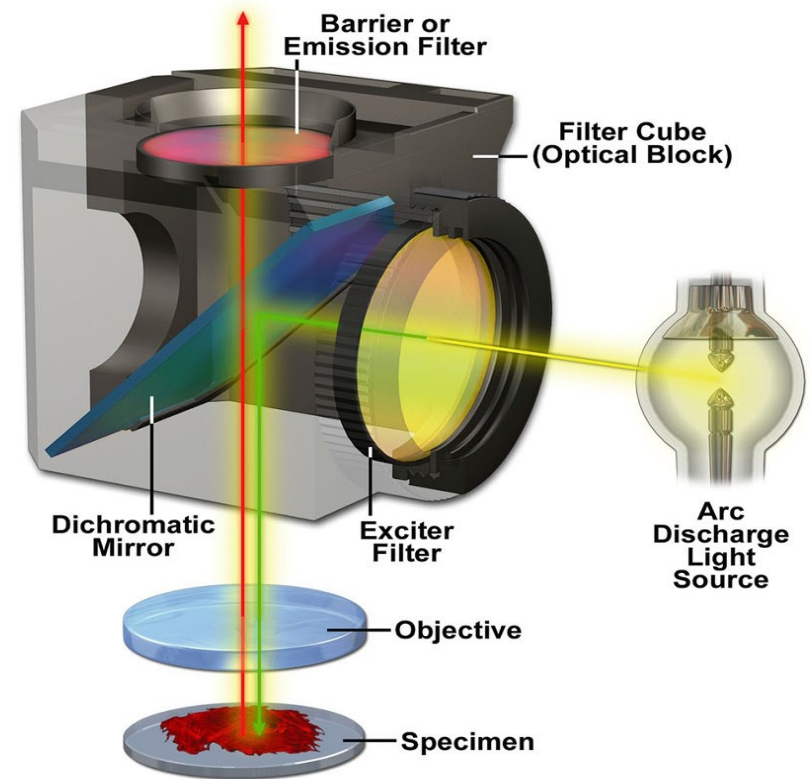
2) Emission - BP Filter:
BP530, ~50nm FWHM

3) Dichroic Mirror: LP500

Blue: 450–495 nm
Green: 495–570 nm
Red: 620–750 nm



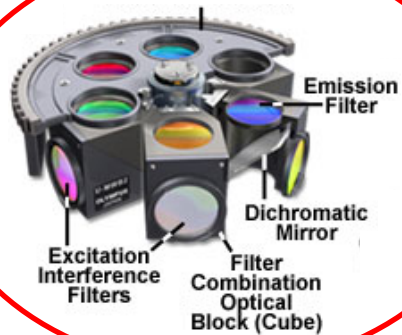
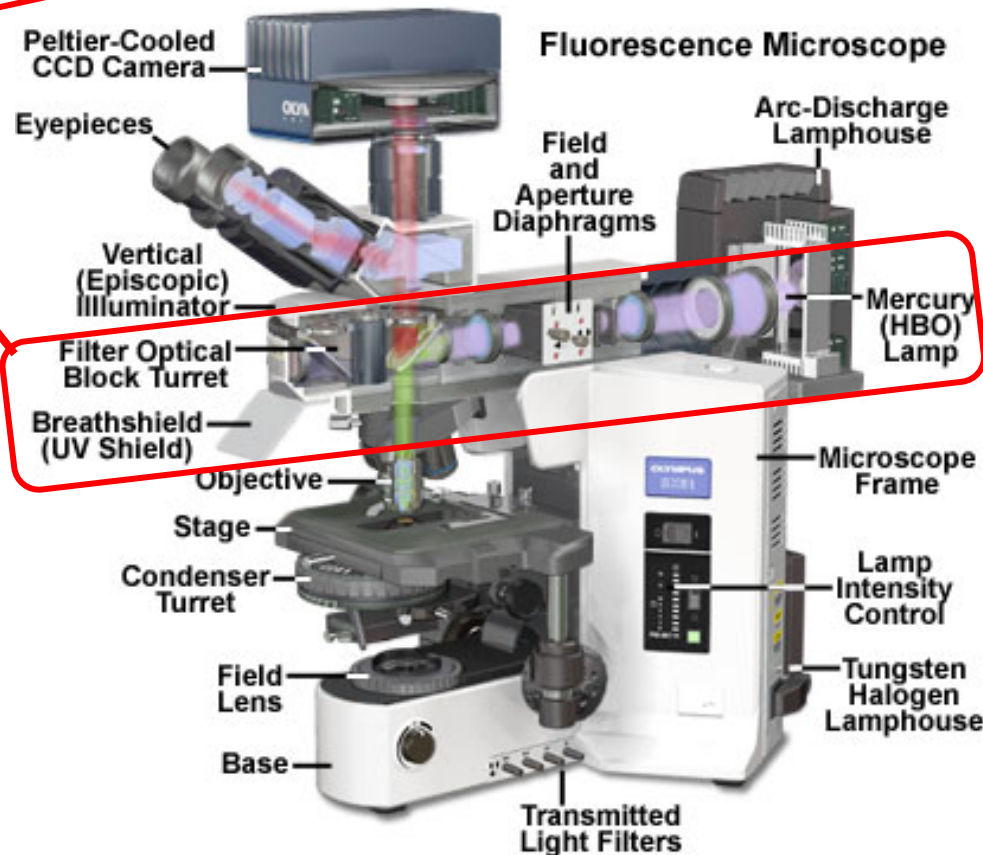
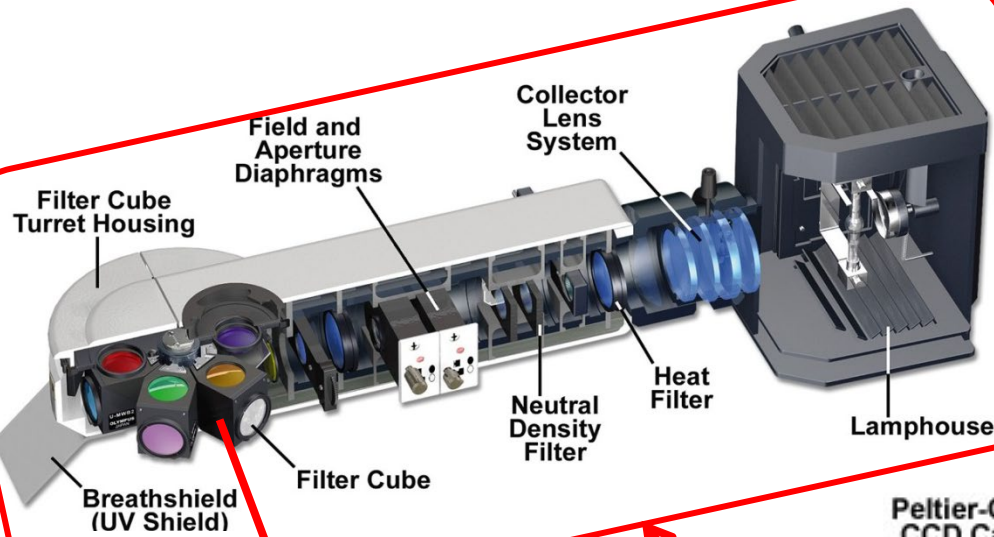
Arrangement of a Filter Cube



Arrangement of filters in a fluorescence filter cube.

- The diagram shows the orientation of filters for an epi-illuminator for an upright microscope.
- The excitation beam (**indicated by yellow line**) passes through exciter filter, reflected by the dichroic mirror and directed towards specimen (**green line**).
- Excitation light back-reflected or scattered at the specimen are again reflected by the dichroic mirror back towards the light source. Additionally, any excitation light that has managed to pass through the dichroic mirror is further blocked by the emission (barrier) filter.
- The emitted fluorescence signal (**red line**) passes through the dichroic mirror and emission filter to the eye or camera.

Epi-illuminator: Filter Turret & Cubes



An Example for Multi-Color Imaging

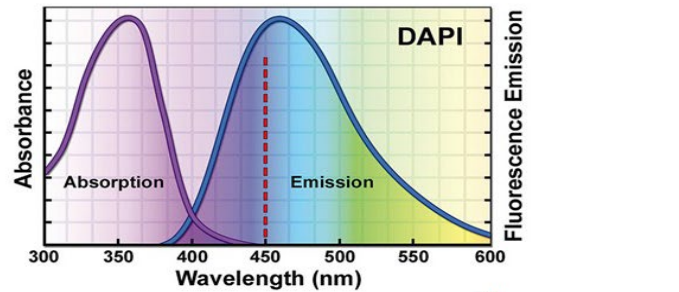
The absorption & emission spectra of:

(a) DAPI

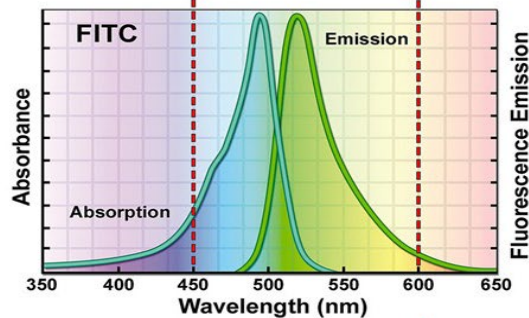
(b) Fluorescein isothiocyanate (FITC)

(c) Tetramethyl rhodamine (TRITC)

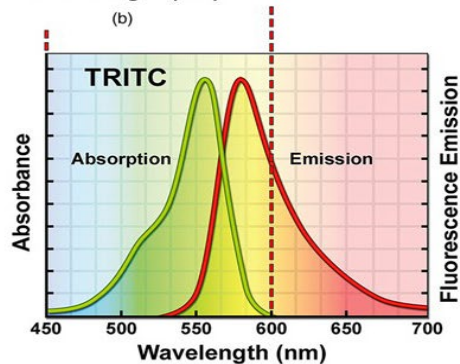
- Although the emission spectra of these 3 dyes partially overlap, it is possible to examine each dye separately using suitable filter sets.



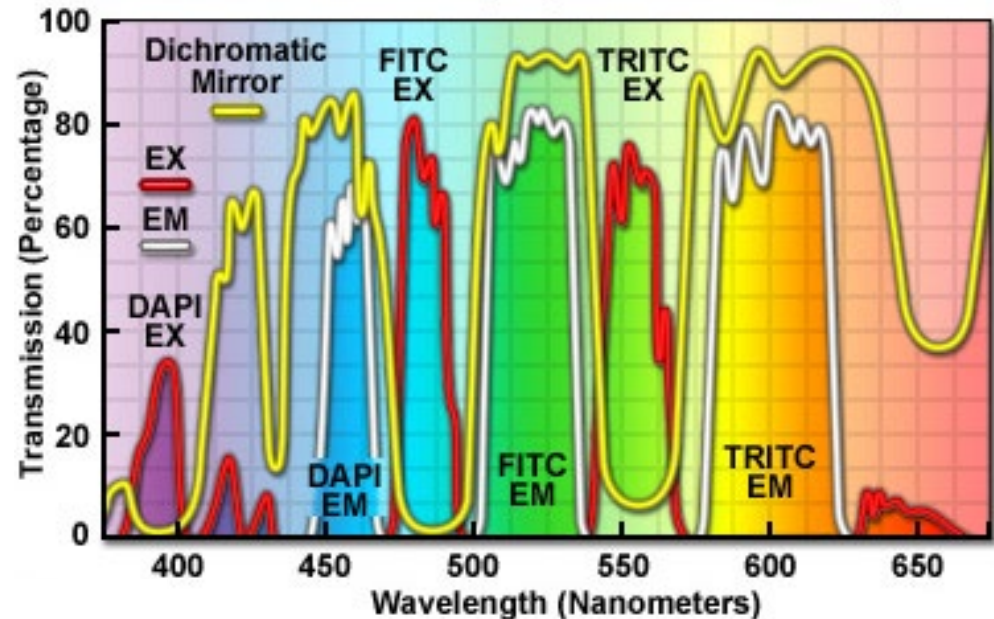
(a)



(b)



(c)



Triple Excitation Filter Block for DAPI-FITC-TRITC from Nikon:

Wavelengths of Excitation Filters (EX): 1) 385-400 nm (BP with 393 CWL), 2) 475-490 nm (BP with 483 CWL), 3) 545-565 nm (BP with 555 CWL)

Wavelengths of Dichromatic Mirrors (DM): 1) 435-470 nm (DM), 2) 500-540 nm (DM), 3) 570-645 nm (DM)

Wavelengths of Emission Filters (EM): 1) 450-465 nm (BP with 458 CWL), 2) 505-535 nm (BP with 520 CWL), 3) 580-620 nm (BP with 600 CWL)

Example for multi-color imaging

- Multi-colored fluorescence image of a culture of bovine pulmonary artery endothelial cells obtained by **DAPI-FITC-TRITC bandpass emission (triple band excitation) set**.

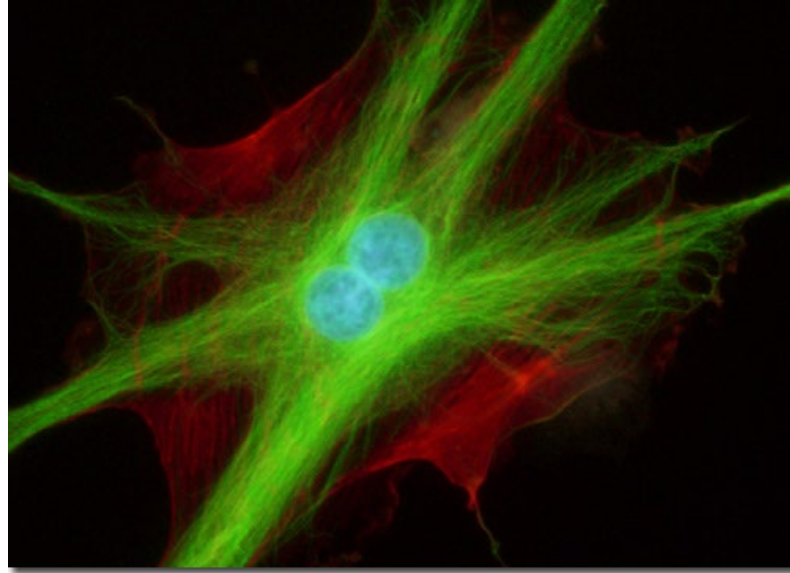


Image and notes are from Nikon web-site.

The labellings are as follows:

- immunofluorescently labeled with primary anti-bovine ***alpha-tubulin*** mouse monoclonal antibodies followed by goat anti-mouse Fab fragments **conjugated to BODIPY FL (green)**.
- simultaneously stained with **Texas Red (red)**, which target the **cytoskeletal actin network**
- simultaneously stained with **DAPI (blue)**, which target **DNA in the nucleus**.

The absorption maximum and the emission maximum occurs at:

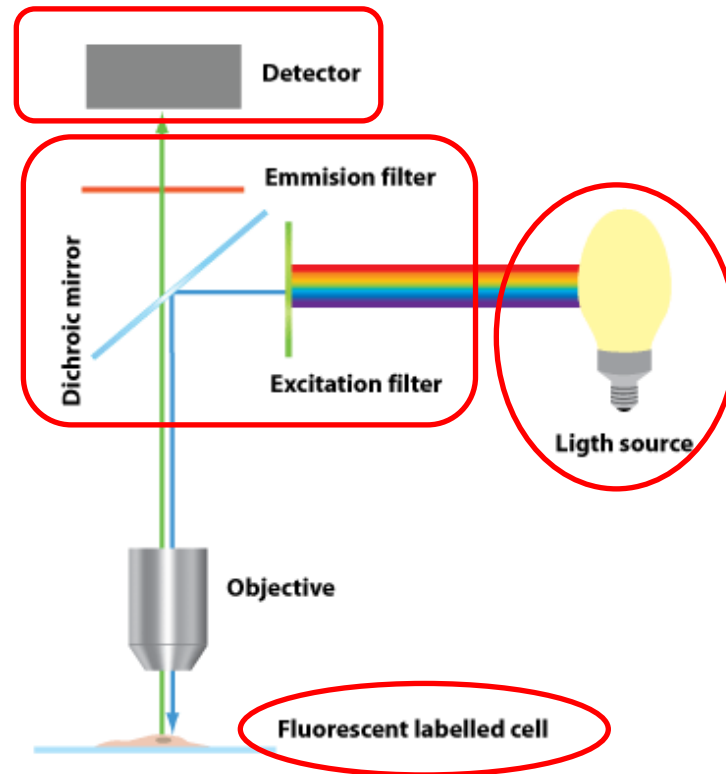
- 505 nm and 513 nm for BODIPY FL (green)
- 595 nm and 620 nm for Texas Red (red)
- 358 nm and 461 nm for DAPI (blue)

- Note the presence of high signal levels from DAPI and BODIPY FL, but the relatively low signal exhibited by Texas Red because this filter combination does not efficiently gather signal at longer red wavelengths).

Outline: Detectors in Microscopy

- To understand fluorescence microscopy we need to be familiar with:
 - Basic principles of fluorescence
 - Properties of fluorescent dyes
 - Different kinds of fluorescence markers
 - Important optical components
 - Illumination sources
 - Filters and filter sets
 - ➔ - **Detectors**
 - Their proper positioning in the optical train of the microscope

➔ Different detector types used in microscopy



Detector Types

- **Eye**

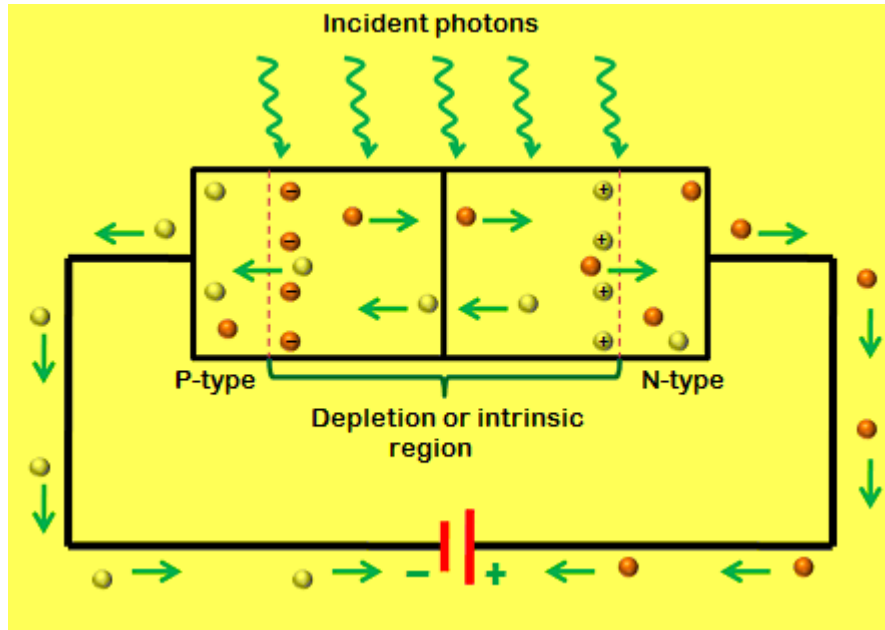


- **Photodiode**

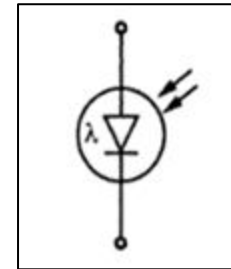
- single element → no spatial information
- Limited sensitivity & time resolution



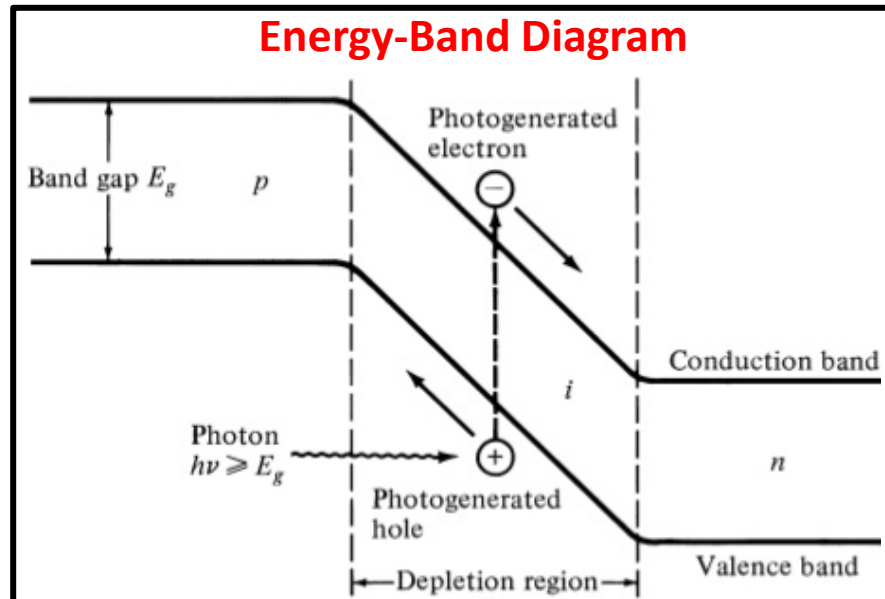
Photo-diode & Photo-current



- **Photo-diode** is an semiconductor p-n junction device



Circuit symbol of photodiode

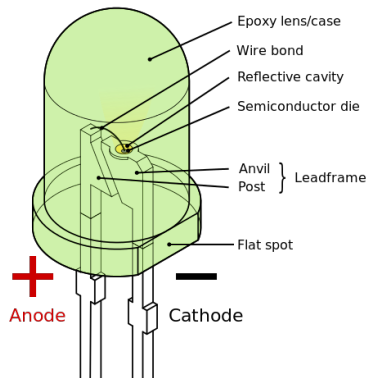
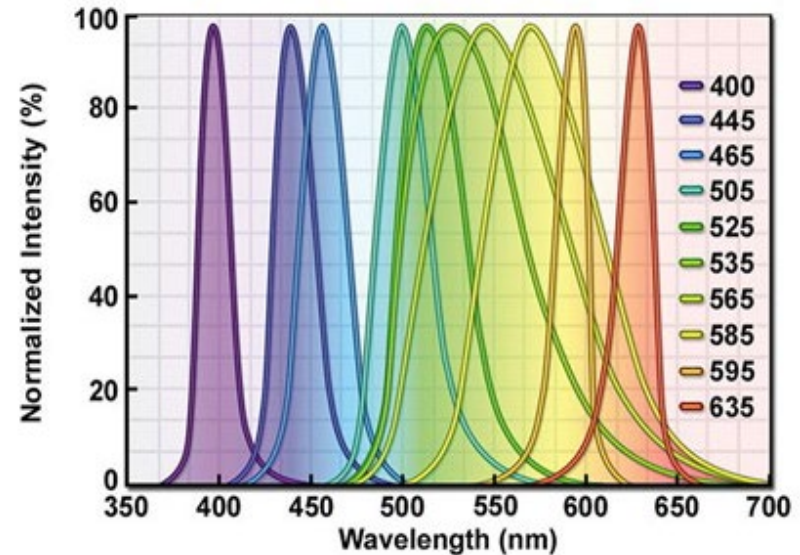
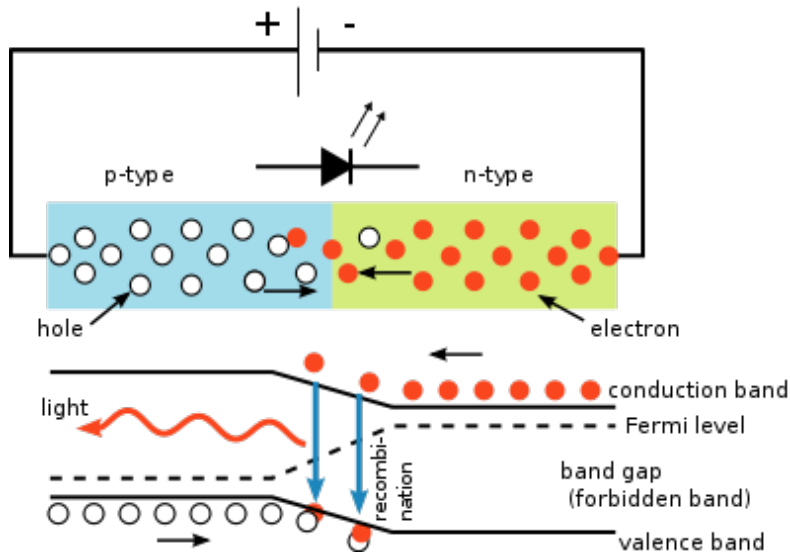


- **Photo-current** is generated when the incident photon energy is above the band-gap.
- **Cut-off wavelength** (λ_c) depends on the band gap energy of the p-n-junction material.

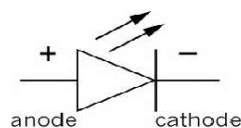
$$\Rightarrow \lambda_c = \frac{hc}{E_g} = \frac{1.24}{E_g (eV)} \mu\text{m}$$

Recall: Light Emitting Diodes (LEDs)

- LED is an **semiconductor p-n junction device**
- Applied electric bias results in current and the **recombination of circulating electrons and holes in the depletion zone** leads to the light generation
- The emitted light has a **relatively narrow 20-50 nm bandwidth**
- **The wavelength (color) of the light emission is dependent on the energy band-gap size (at the p-n junction)**

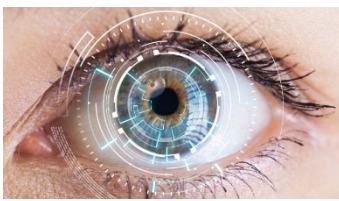


Circuit symbol of LED



Detector Types

● Eye



● Photodiode

- single element → no spatial information
- Limited sensitivity & time resolution



● PMT: PhotoMultiplier Tube

- single element → no spatial information
- very high time resolution
- used for laser scanning confocal microscopy



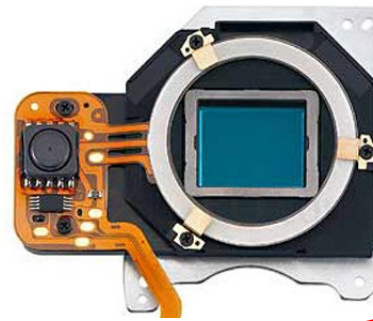
● APD: Avalanche PhotoDiode

- very sensitive to low intensity lights
- pixels can be arranged in 2D arrays → it offers spatial information & can be used in 2D imaging



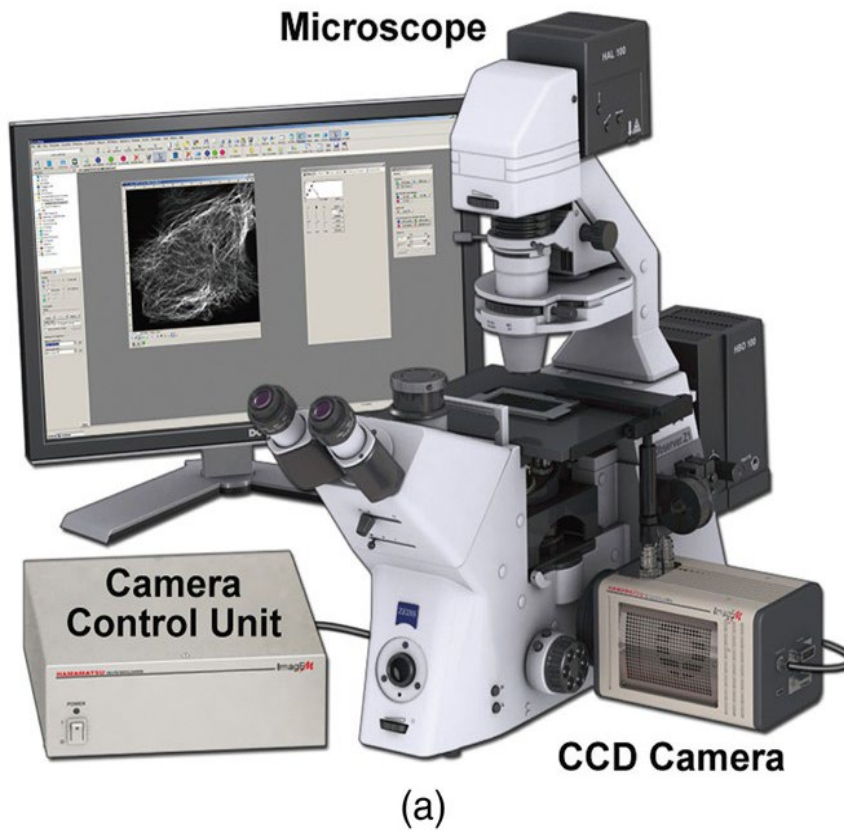
● CCD, EMCCD & CMOS cameras

- 2D pixelated → offers spatial information (2D imaging)
- Limited time resolution
- Sensitive with high quantum efficiency



→ most commonly used in biomicroscopy

Digital Imaging System



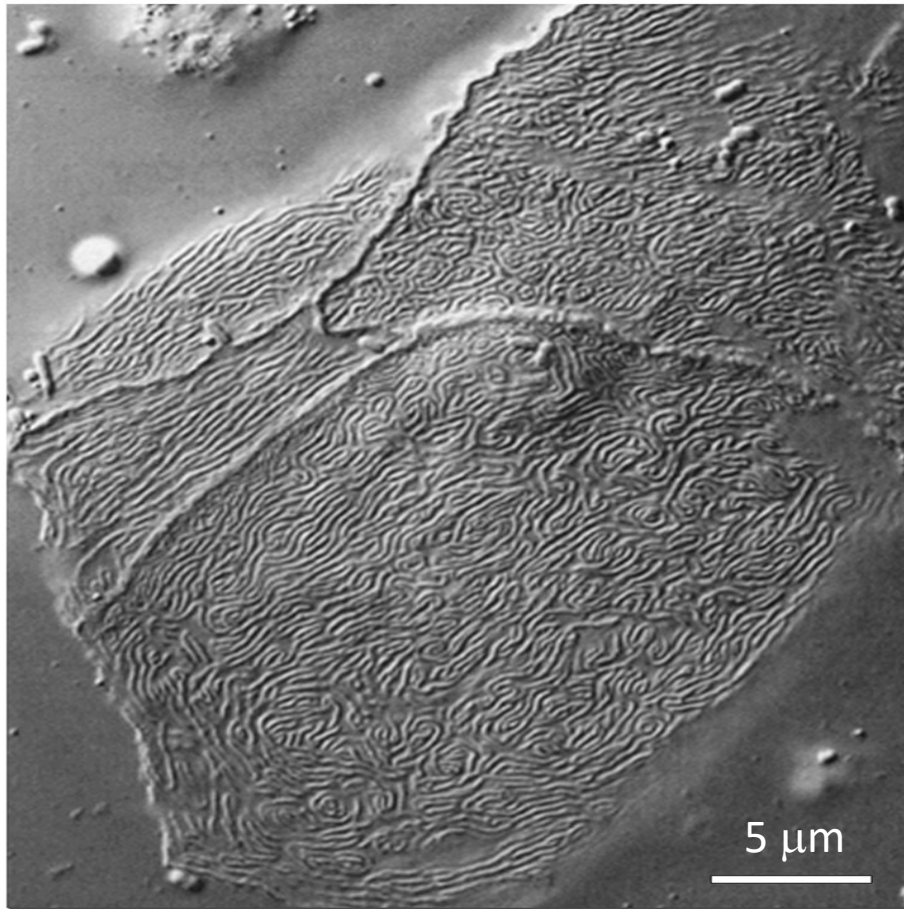
(b)

The combination of microscope and digital camera, together with a computer, camera control unit & imaging software, defines what is called a **digital imaging system**:

(a) Camera is mounted on the microscope. Camera control unit connected to the camera communicates with a host computer.

(b) Multiple options available ... cooled CCD, color camera, EMCCDs, scientific CMOS camera

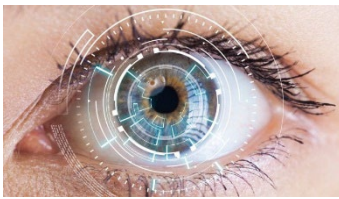
Imaging in Microscopy



- DIC (differential interference contrast) microscope image of the surface of an epithelial cell recorded with a **1.4 megapixel CCD having a pixel size of 6.8 μm at 100x magnification with 1.3 NA objective.**
 - Scale bar is 5 μm.
 - The spacing between the ridges is ~400 nm.
- At an illumination peak wavelength of ~550 nm, diffraction limited spot at the camera is ~25 μm. Therefore the full optical resolution is retained with this camera choice.

Detector Types

● Eye



● Photodiode

- single element → no spatial information
- Limited sensitivity & time resolution



● PMT: PhotoMultiplier Tube

- single element → no spatial information
- very high time resolution
- used for laser scanning confocal microscopy



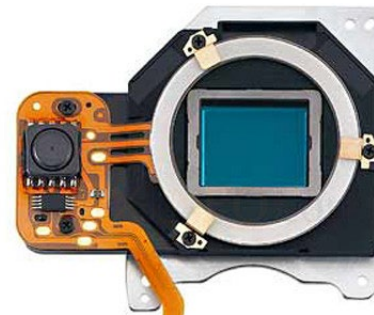
● APD: Avalanche PhotoDiode

- very sensitive to low intensity lights
- pixels can be arranged in 2D arrays → it offers spatial information & can be used in 2D imaging



● CCD, EMCCD & CMOS cameras

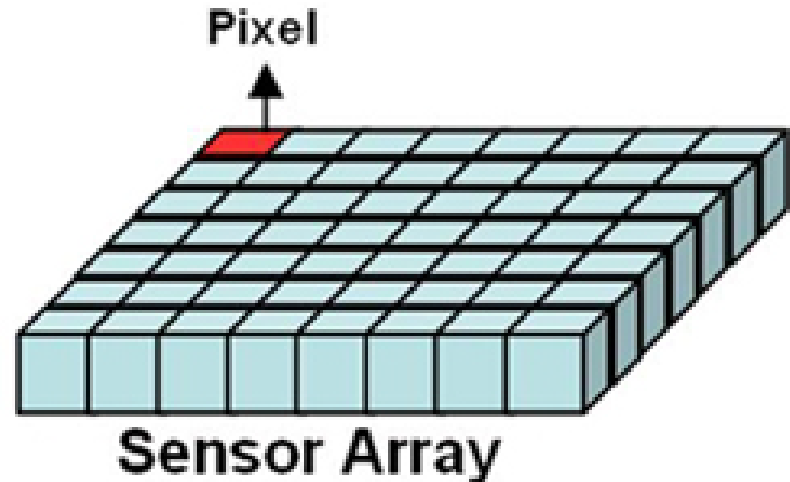
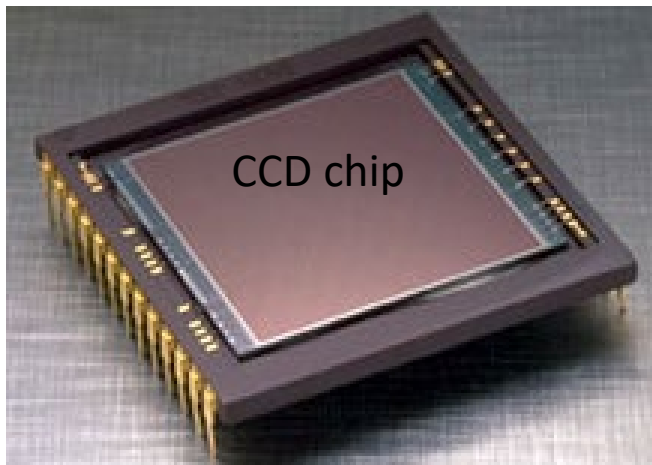
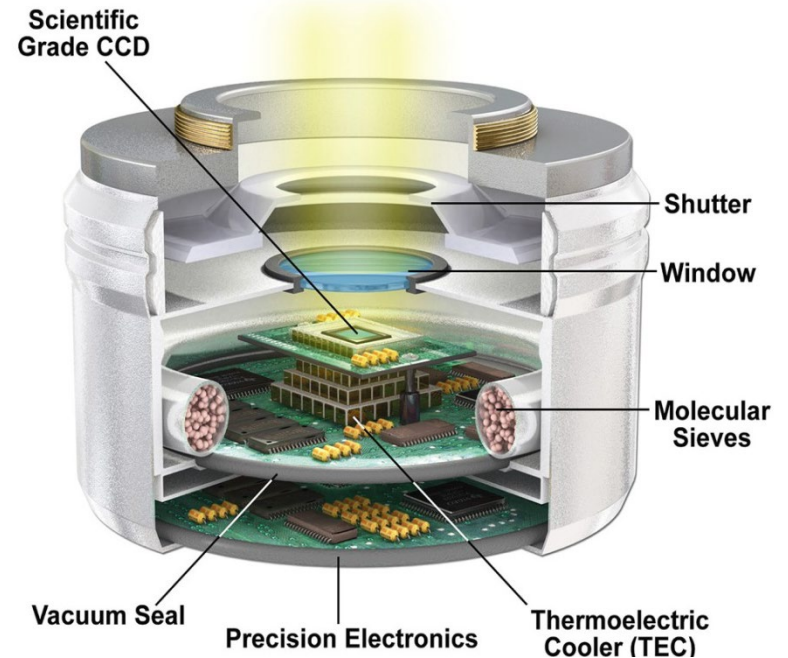
- 2D pixelated → offers spatial information (2D imaging)
- Limited time resolution
- Sensitive with high quantum efficiency



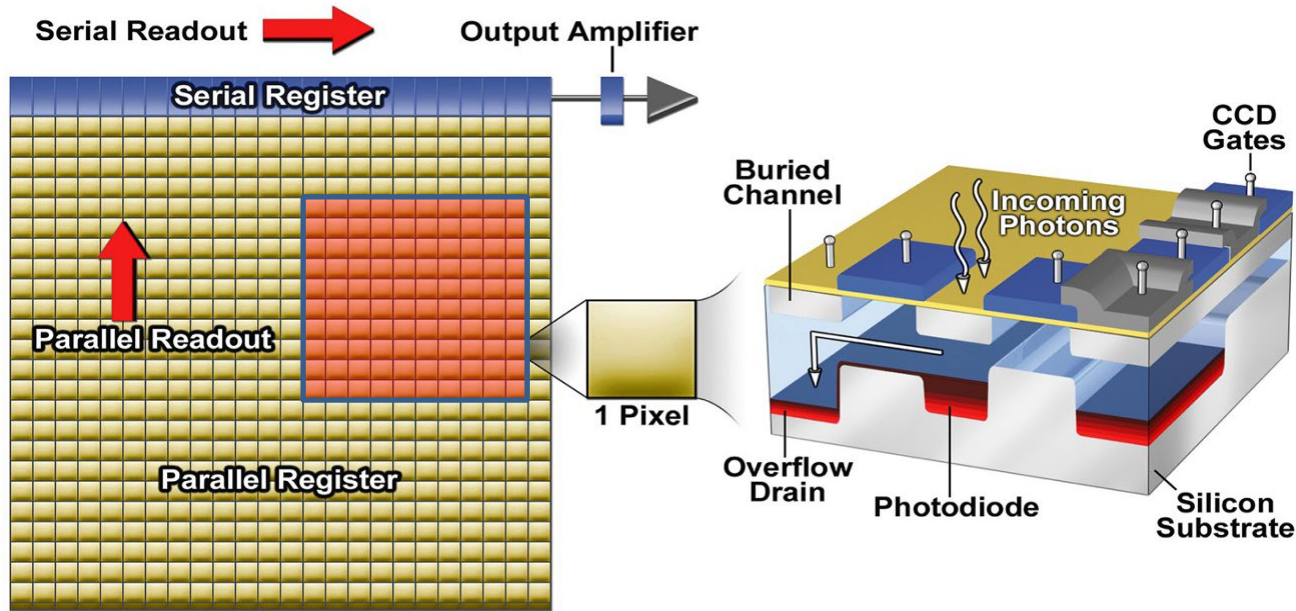
→ most commonly used in biomicroscopy

CCD → Charge-Coupled Device

- Invented in 1970 at Bell Labs (U.S.)
- It is a **silicon chip** structured as a 2D array of photosensitive pixels.
- It converts incident light into an electrical signal
- About the device architecture:
 - The chip is mounted in a hermetically sealed chamber filled with dry N₂ or vacuum.
 - In the front side, there is a transparent window allowing incident light to enter
 - At the back side, there is a TEC to reduce thermal noise
 - The back end also contains electronic components such as pre-amplifier, ADC & circuit for readout



CCD camera contains thousands of pixels

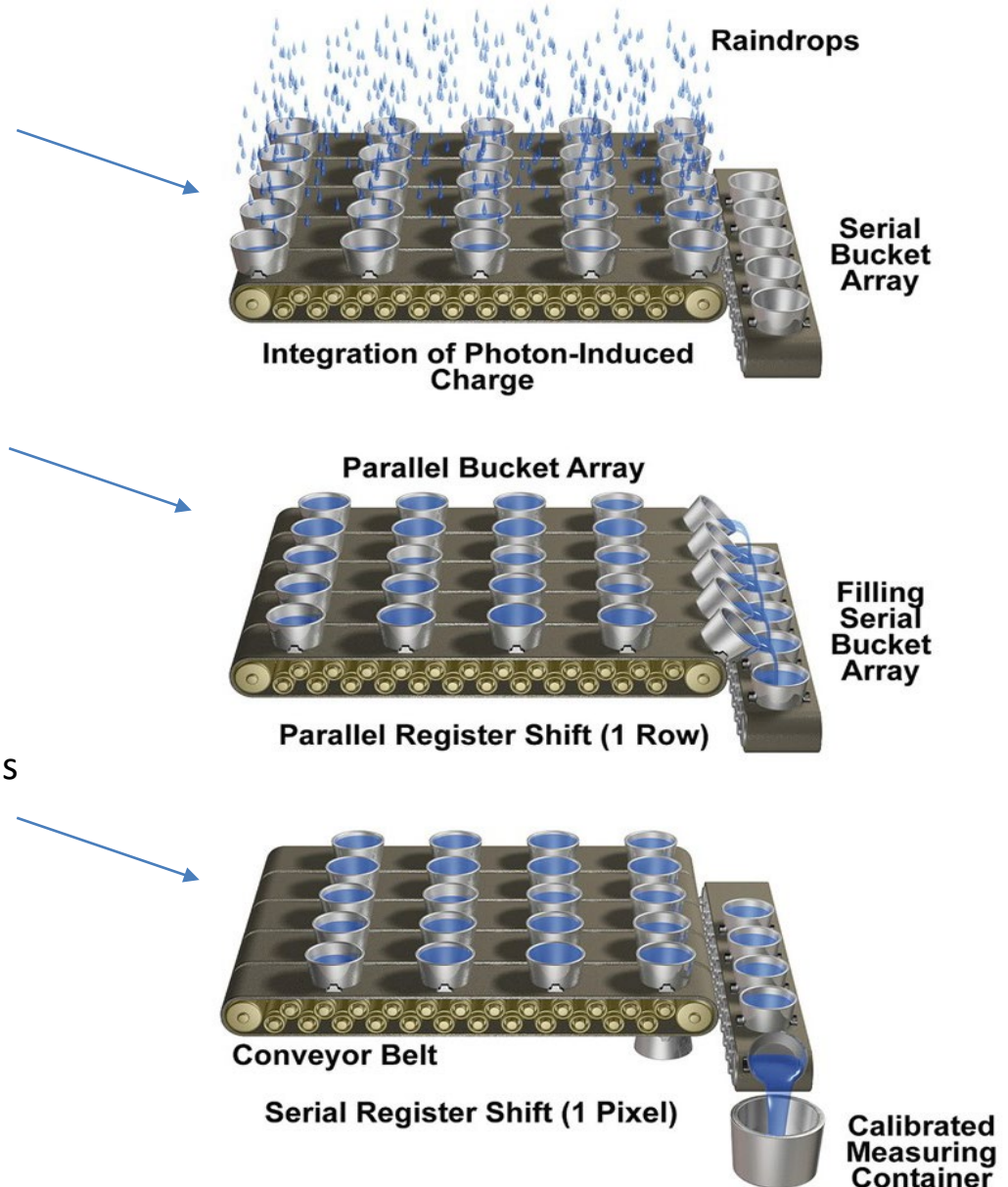


Sequence of events:

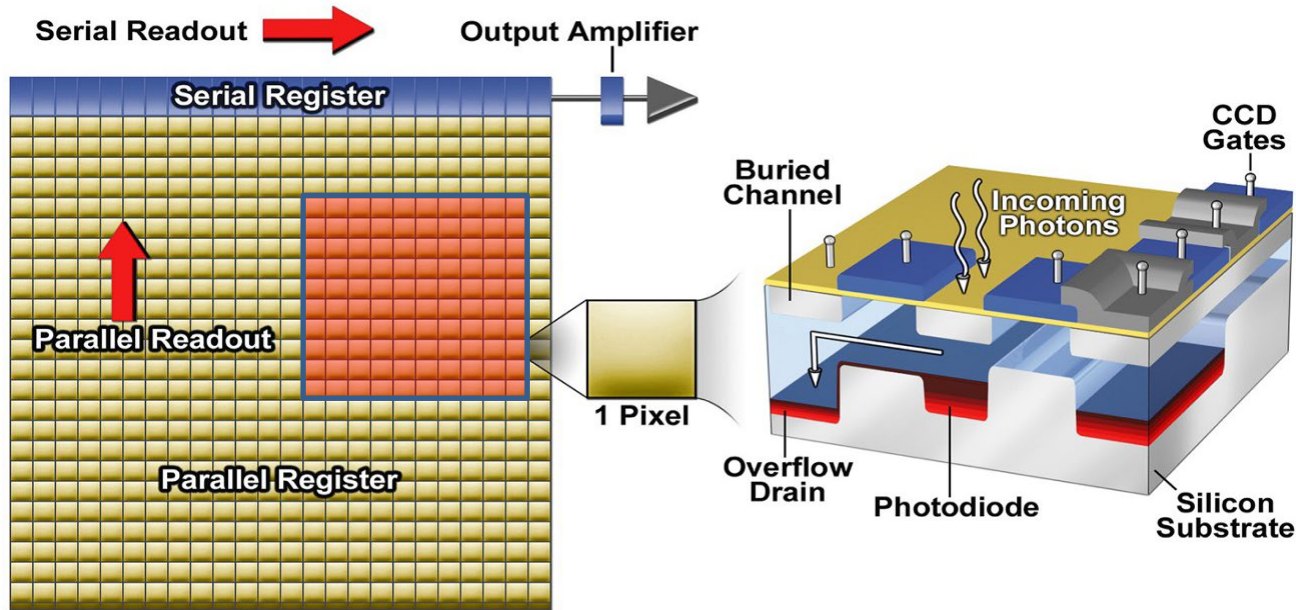
- The camera shutter opens & the pixels (indicated as yellow boxes in the **parallel register** region) accumulate photo-electrons. The number of photo-electrons is depended on the incident light intensity.
- The shutter closes, and pixel content moves one row at a time by voltages applied to the strips on the CCD.
- Each row at the end of the parallel register is transferred to a special row of pixels, called **serial register**.

Bucket Brigade Rainfall Analogy

- Rain intensity may vary from place to place (similar to the photo-electron numbers vary from pixel to pixel)
- Collection time = integration time
- **Parallel buckets** on a conveyor belt transported stepwise to a row of empty **serial buckets**
- Serial buckets move on a second conveyor oriented perpendicularly to the first
- Accumulated rain water in each bucket is transferred sequentially into a calibrated measuring container (= CCD output amplifier)
- Process is repeated until all parallel buckets are shifted to the serials



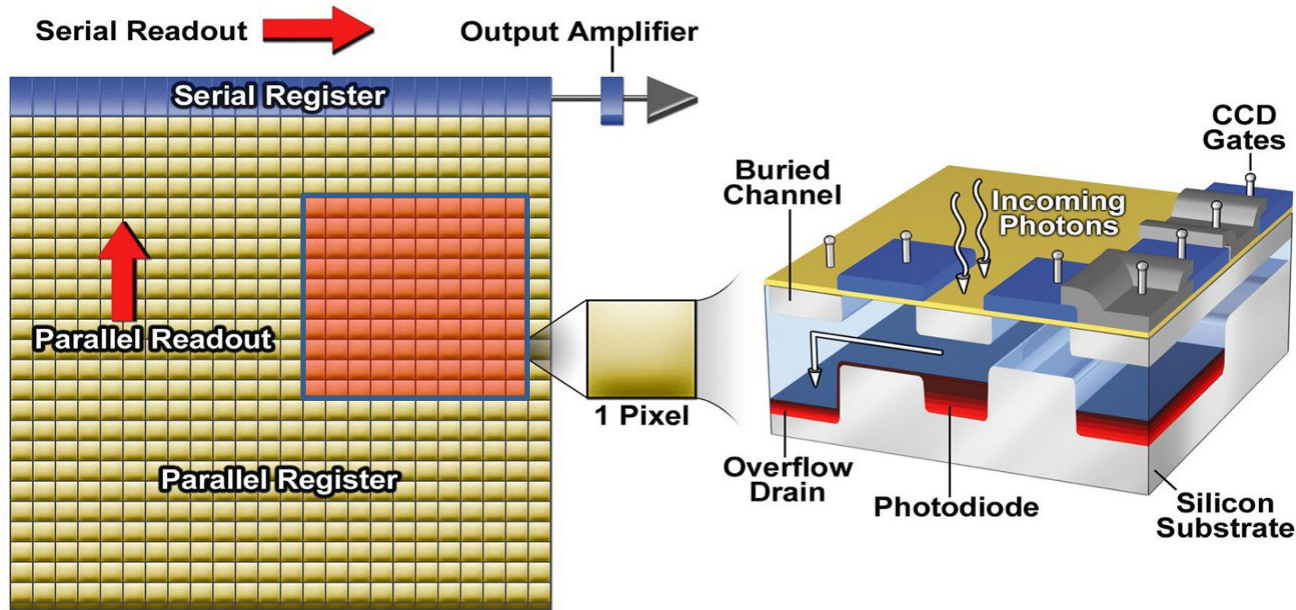
CCD camera contains thousands of pixels



Sequence of events:

- The camera shutter opens & the pixels (indicated as yellow boxes in the **parallel register** region) accumulate photo-electrons. The number of photo-electrons is depended on the incident light intensity.
- The shutter closes, and pixel content moves one row at a time by voltages applied to the strips on the CCD.
- Each row at the end of the parallel register is transferred to a special row of pixels, called **serial register**.
- **Pixels are transferred one pixel at a time down the serial register with an on-chip pre-amplifier, which boosts the electronic signal & generates an analog voltage.**
- An A/D converter assigns a digital code for each pixel depending on the signal amplitude (light intensity).
- Pixel values are stored in a frame buffer in the computer.
- *The process repeats until all 1000+ rows of pixels of the parallel register are emptied.*

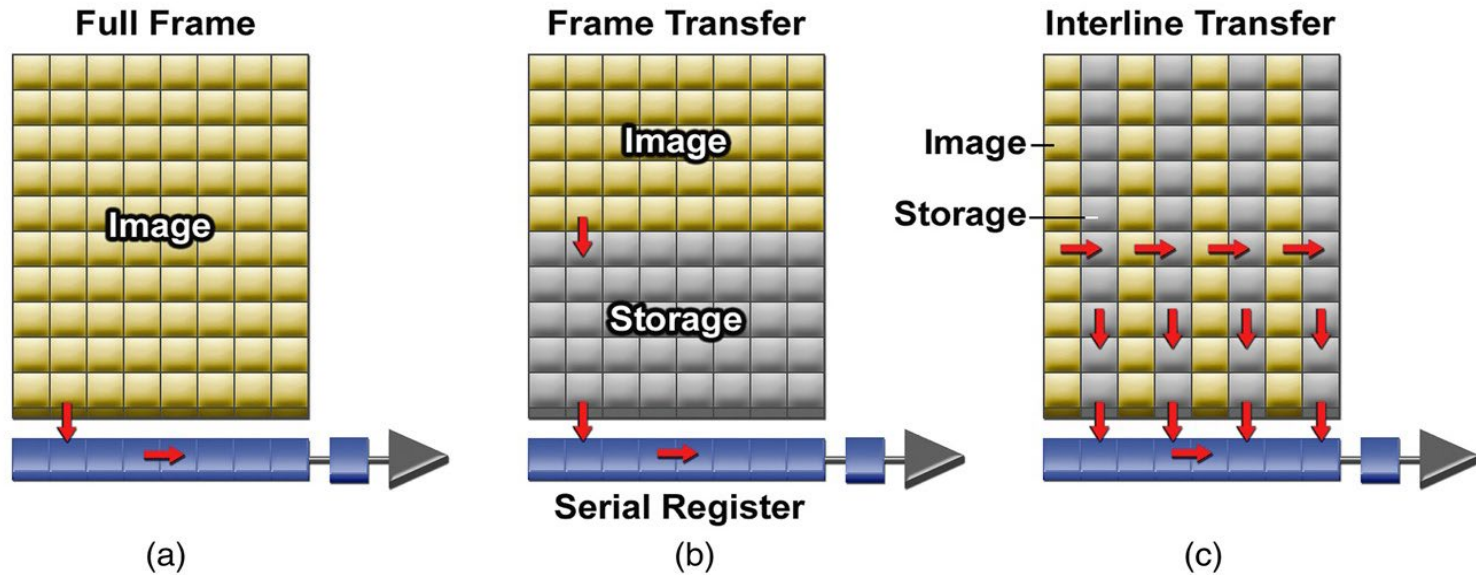
CCD camera contains thousands of pixels



Sequence of events:

- The camera shutter opens & the pixels (indicated as yellow boxes in the **parallel register** region) accumulate photo-electrons. The number of photo-electrons is depended on the incident light intensity.
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Types of CCD architectures

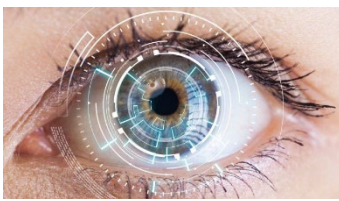


(a) Full Frame CCD (b) Frame-Transfer CCD (c) Interline Transfer CCD.

- Full Frame CCD was the original design used for biological imaging.
 - It requires a shutter.
 - The **fastest frame rates are limited by the electro-mechanical shutter.**
- Frame-transfer and interline transfer are more suitable for applications requiring to capture **fast dynamics**.
 - In both cases **no shutter** is required.
 - Part of the pixels are reserved for storage & read-out. Therefore, **light exposure & read-out** can happen **simultaneously**.
 - CCD cameras with interline transfer architecture provide high temporal resolution at close to video rates!

Detector Types

● Eye



● Photodiode

- single element → no spatial information
- Limited sensitivity & time resolution



● PMT: PhotoMultiplier Tube

- single element → no spatial information
- very high time resolution
- used for laser scanning confocal microscopy



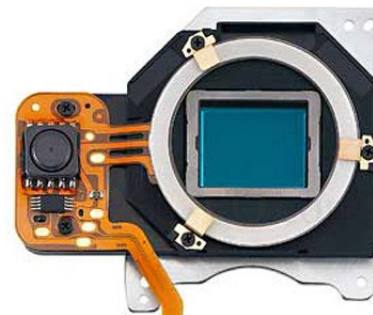
● APD: Avalanche PhotoDiode

- very sensitive to low intensity lights
- pixels can be arranged in 2D arrays → it offers spatial information & can be used in 2D imaging



● CCD, EMCCD & CMOS cameras

- 2D pixelated → offers spatial information (2D imaging)
- Limited time resolution
- Sensitive with high quantum efficiency

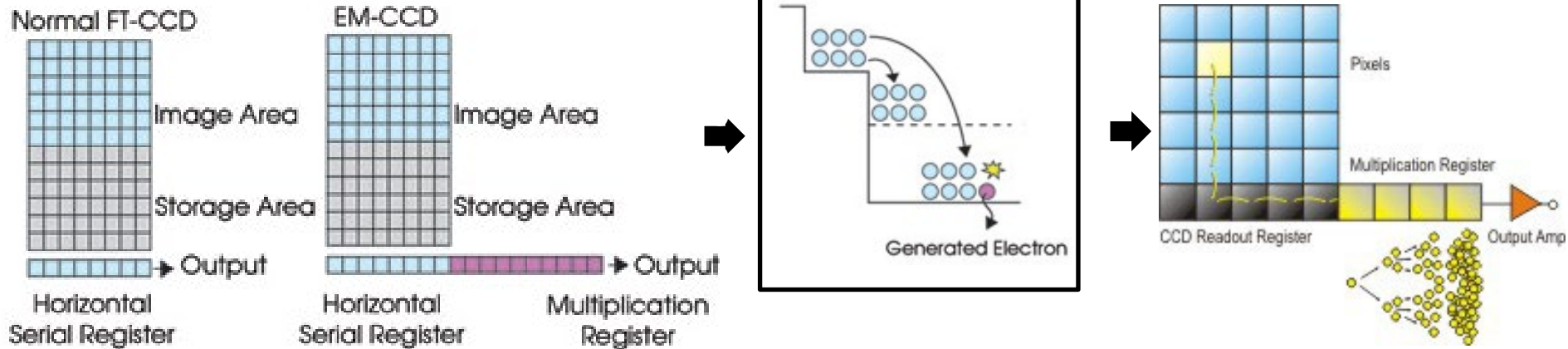


→ most commonly used in biomicroscopy

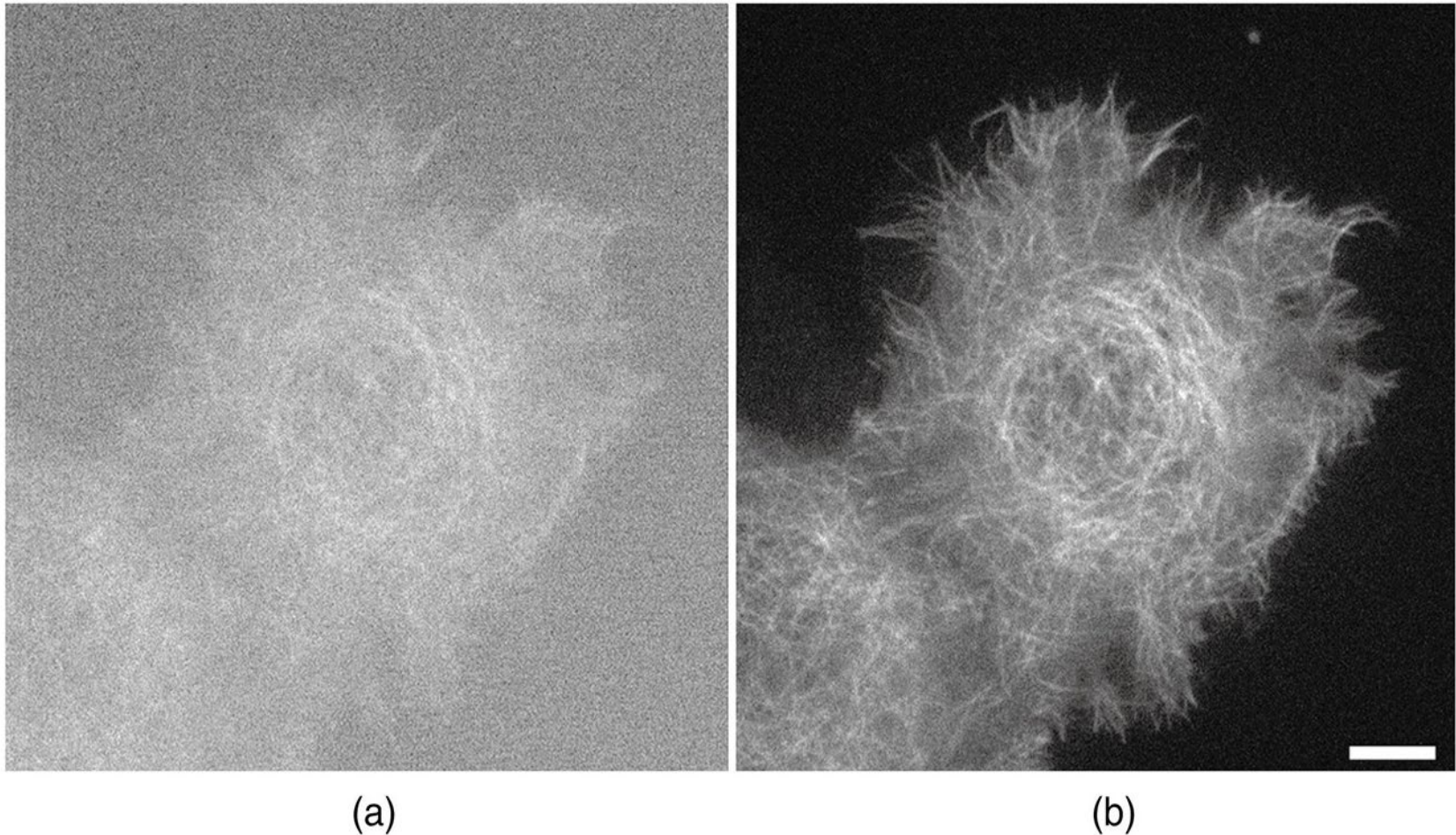
EMCCD- Electron Multiplying CCD

- It has an additional on-chip **Electron Multiplication register** ('gain register' between the usual serial shift register and the output amplifier)
- **EM gain amplifies the signal**
 - One can work in low-light conditions and acquire images that are otherwise not possible to obtain with a standard CCD camera.
- **EM gain also reduces the required exposure time**
 - Minimize problems with photo-bleaching & prolong cell viability

CCD in "frame transfer" architecture:



EMCCD in bio-microscopy

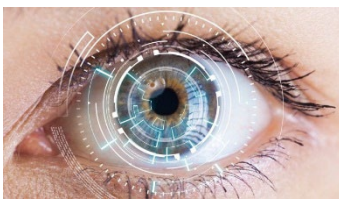


[Figure 17.11](#)

Low light-level imaging of the microtubule-associated protein, tau, fused to green fluorescent protein. In panel a, the extended multiplication register on the EMCCD was turned off to simulate imaging with a standard cooled scientific CCD. (b) Turning on the EM gain to a setting of 50% dramatically reduces noise and enhances visibility and definition of dim structures. Bar = 10 μm .

Detector Types

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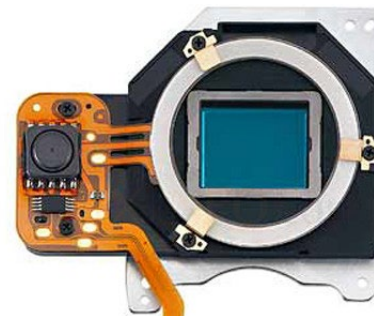
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● CCD, EMCCD & CMOS cameras

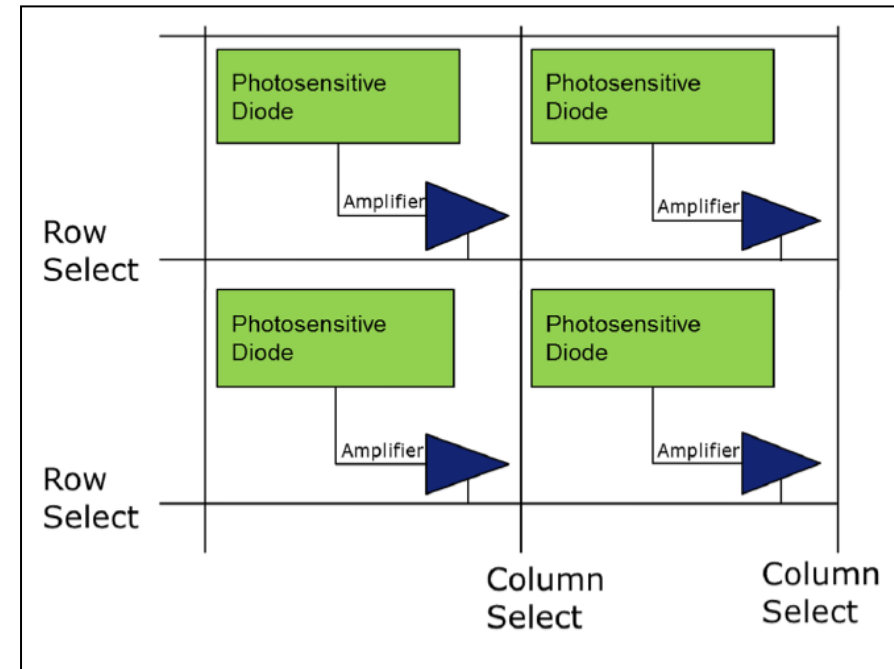
- 2D pixelated → offers spatial information (2D imaging)
- Limited time resolution
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→ most commonly used in biomicroscopy

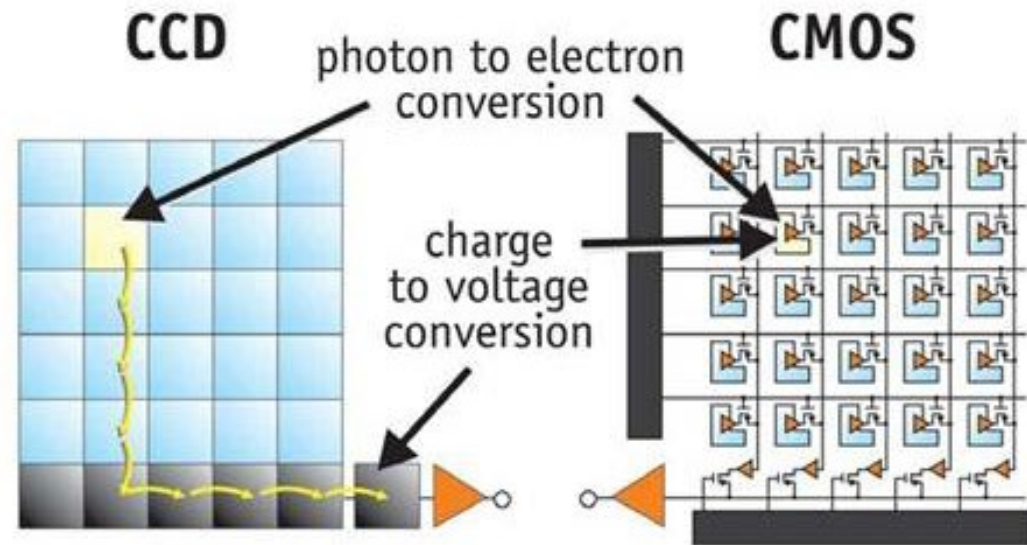
CMOS – Complementary Metal-Oxide-Semiconductor

- CMOS technology uses an array of light sensitive **pixels** to collect full area image.
- CMOS technology differs from CCD by completing all **digitization at the pixel point**:
 - Each pixel has its own amplifier → no need to transfer pixel content thus **faster** imaging is possible
 - CMOS sensors require **less power** than CCD (perfect choice for phone camera sensors)
 - It is relatively **low-cost**.



- **Disadvantages:**
 - Small pixels so low dynamic range
 - Higher noise level
 - Lower QE

• With recent progress, **scientific CMOS cameras** are offering improved performance



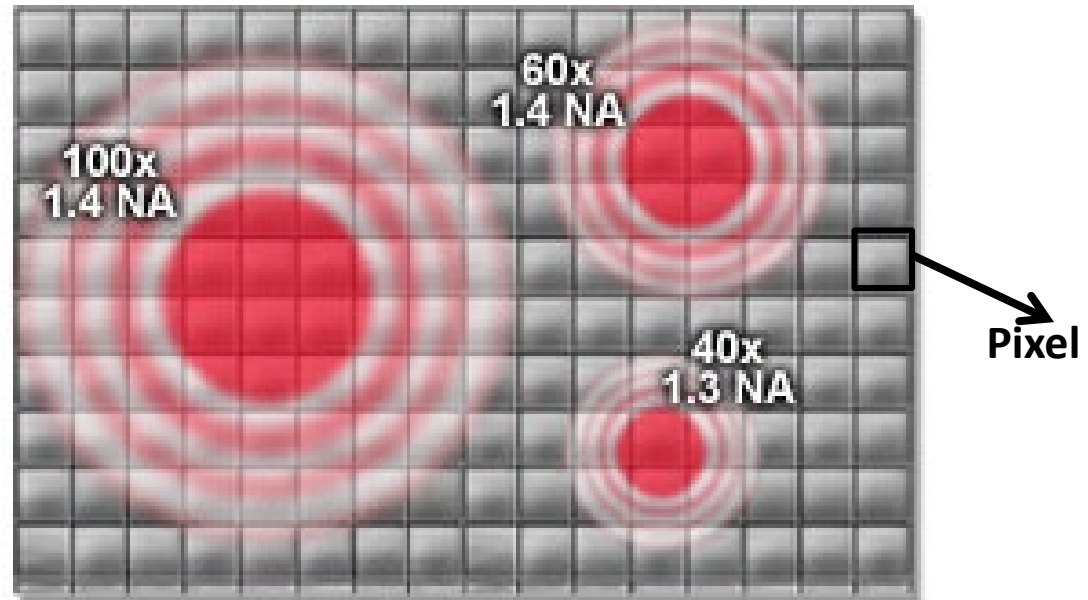
Basic characteristics of imaging cameras



- Spatial resolution
- Spectral bandwidth & quantum efficiency
- Noise sources
- Signal to Noise Ratio
- Temporal resolution
- Sub-array option
- Binning option
- Dynamic range

Spatial resolution of cameras

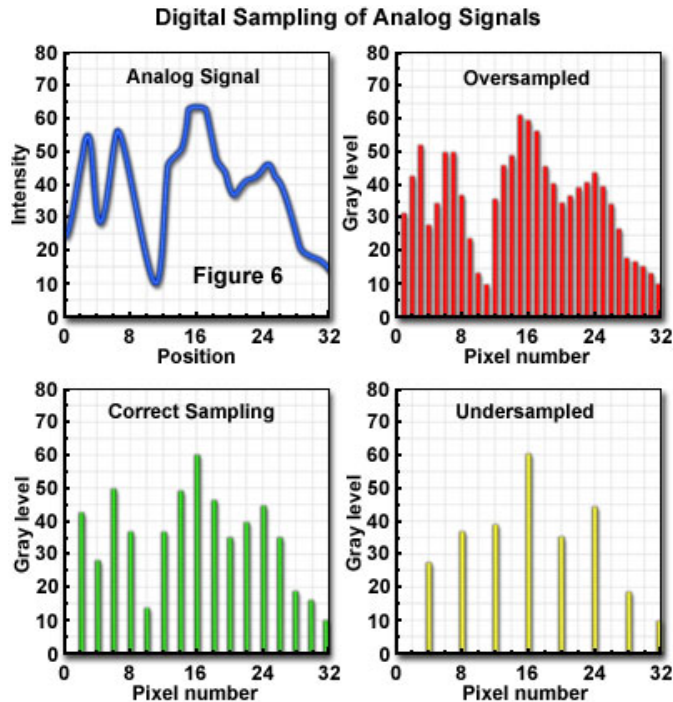
- To retain fully the resolving power of the microscope, correct camera choice depends on:
 - the physical size of the pixels on the detector
 - the magnification needed for the application
 - the NA of the objective used in the microscope
 - the wavelength of light used for imaging



Camera Sensor CCD Pixel Array:

- The size of the “**magnified airy disk**” on the CCD surface must be larger than the size of the CCD pixel.
- According to the **Nyquist sampling theorem**, preservation of the spatial resolution of the optics requires that **the magnified air disk radius should be covered by a minimum of 2 adjacent pixels on the CCD.**

Digital sampling



Nyquist–Shannon sampling theorem¹

If a function $x(t)$ contains no frequencies higher than B hertz, it can be determined by giving its ordinates at a series of points spaced with $1/(2B)$ seconds apart.

¹ Wikipedia: http://en.wikipedia.org/wiki/Nyquist%E2%80%93Shannon_sampling_theorem

Spatial Resolution: Dimensions of Magnified Airy Image & Pixels

Example

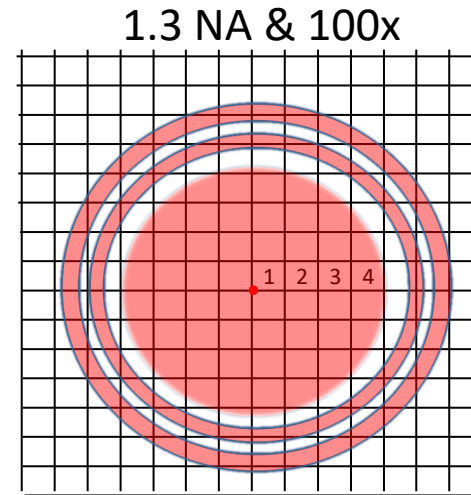
- Consider a CCD chip with $6.45 \mu\text{m}$ pixel size
- Use an objective with 1.3 NA & 100X
- If imaging is done at 550 nm, the radius of magnified airy disk on the CCD is:

$$1.22 \times \frac{0.55}{2 \times 1.3} \times 100 \mu\text{m} \approx 26 \mu\text{m}$$

- There are ~ 4 pixels per diffraction spot radius:

$$\text{Number of pixels} \frac{26}{6.45} \sim 4$$

- **Thus, system resolution is very good!**



Example

- Consider the same CCD chip with $6.45 \mu\text{m}$ pixel size
- This time, use 1.3 NA & 40X objective
- If imaging is done at 550 nm, the radius of magnified airy disk on the CCD is reduced to:

$$1.22 \times \frac{0.55}{2 \times 1.3} \times 40 \mu\text{m} \approx 10 \mu\text{m}$$

- There are < 2 pixels per diffraction spot radius

$$\text{Number of pixels} \frac{10}{6.45} \sim 1.6$$

- **Thus, system resolution is not good!**

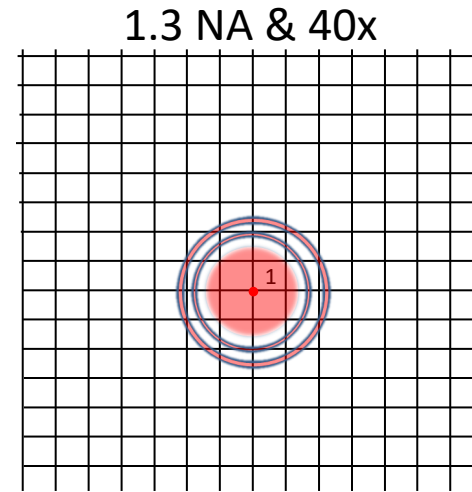
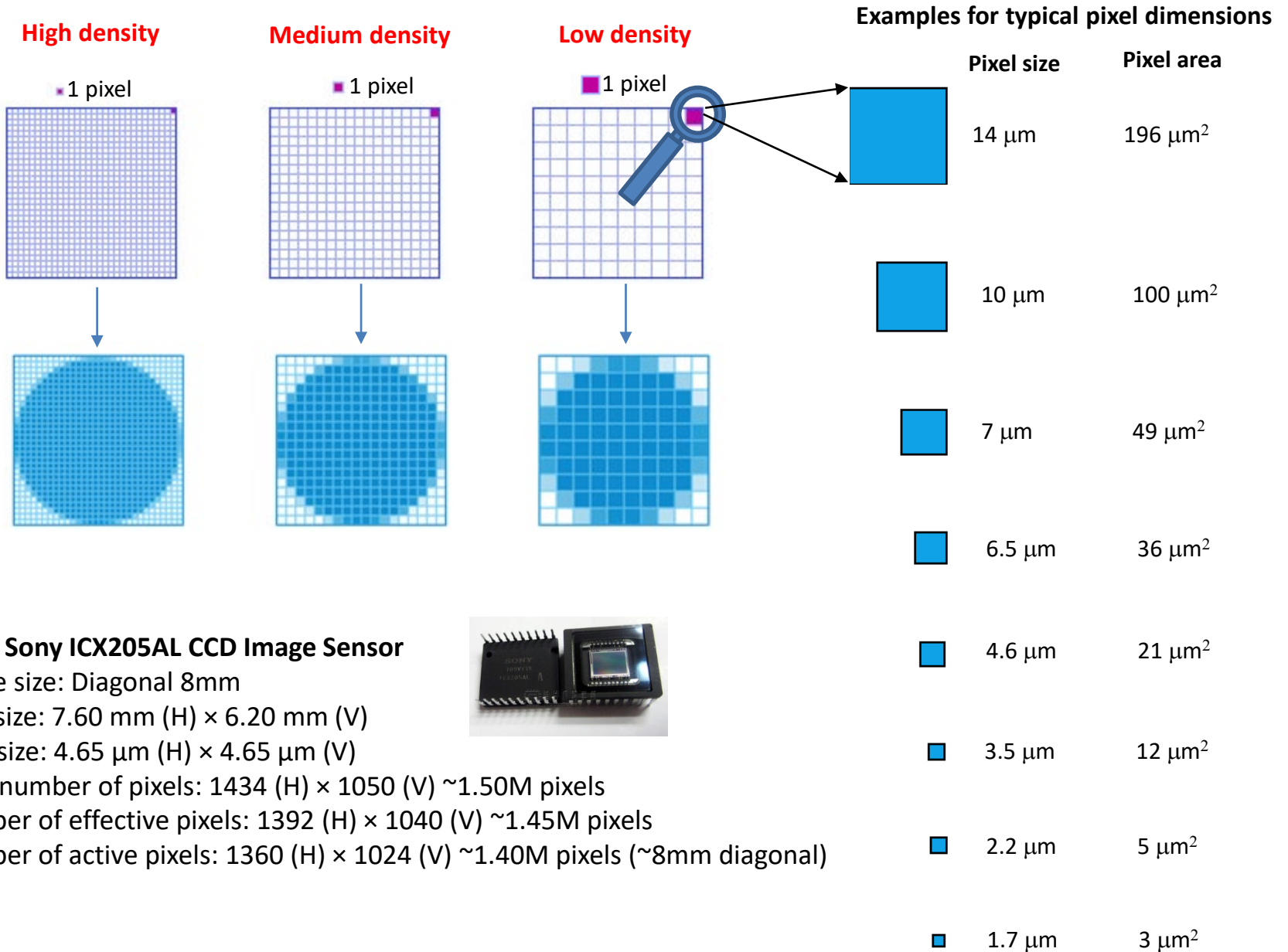


Image sensor size and the number of pixels



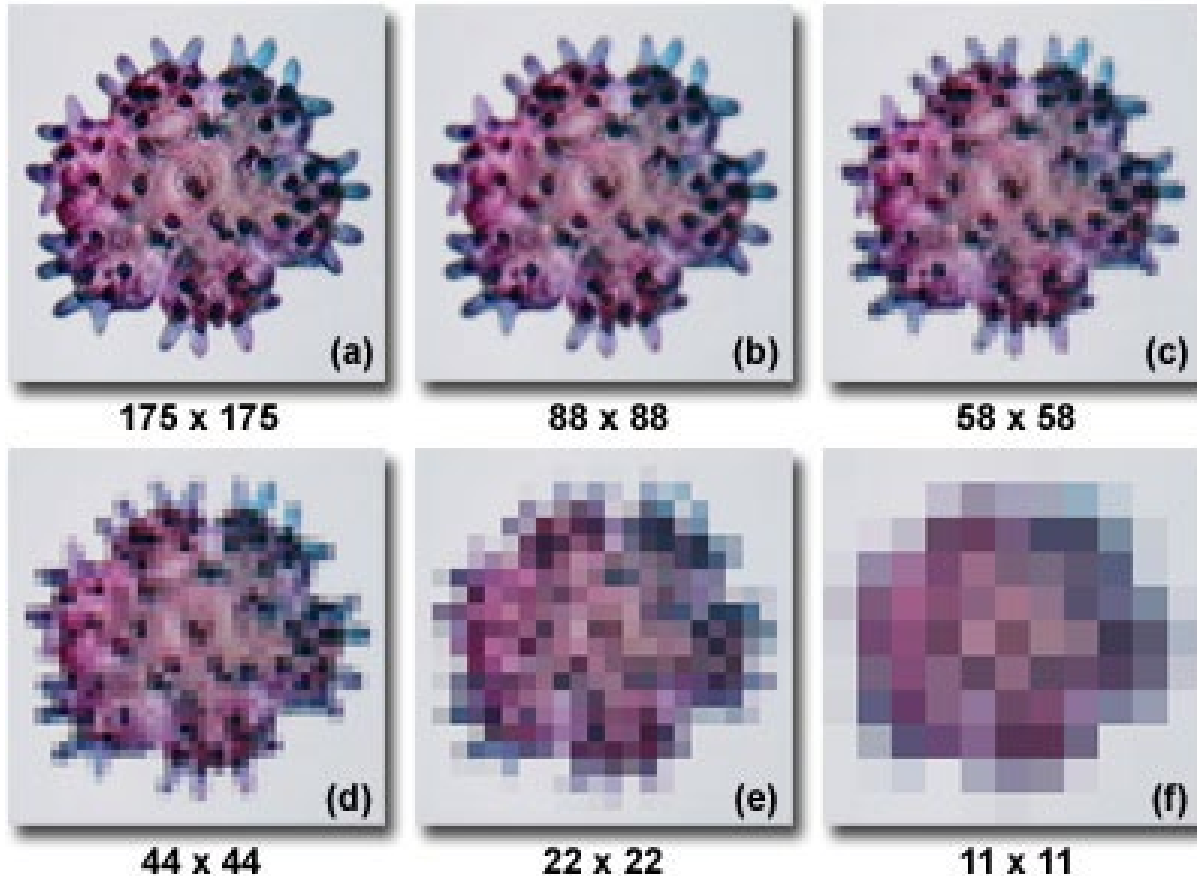
Example: Sony ICX205AL CCD Image Sensor

- Image size: Diagonal 8mm
- Chip size: 7.60 mm (H) \times 6.20 mm (V)
- Pixel size: 4.65 μm (H) \times 4.65 μm (V)
- Total number of pixels: 1434 (H) \times 1050 (V) \sim 1.50M pixels
- Number of effective pixels: 1392 (H) \times 1040 (V) \sim 1.45M pixels
- Number of active pixels: 1360 (H) \times 1024 (V) \sim 1.40M pixels (\sim 8mm diagonal)



Pixel Size & Resolution

Spatial Resolution Effect on Pixelation in Digital Images



For the same imaging area (and sample):

Larger pixel size, thus less pixel numbers, leads to less sampling frequency