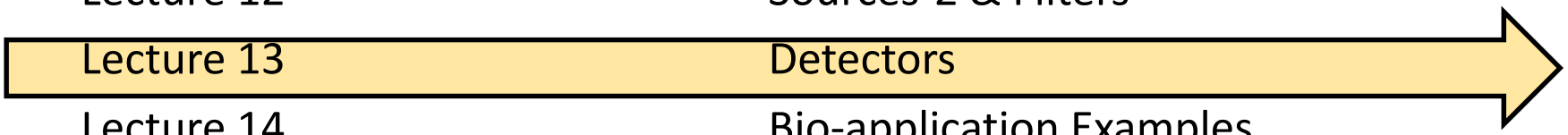
MICRO-561

Biomicroscopy I

Syllabus (tentative)

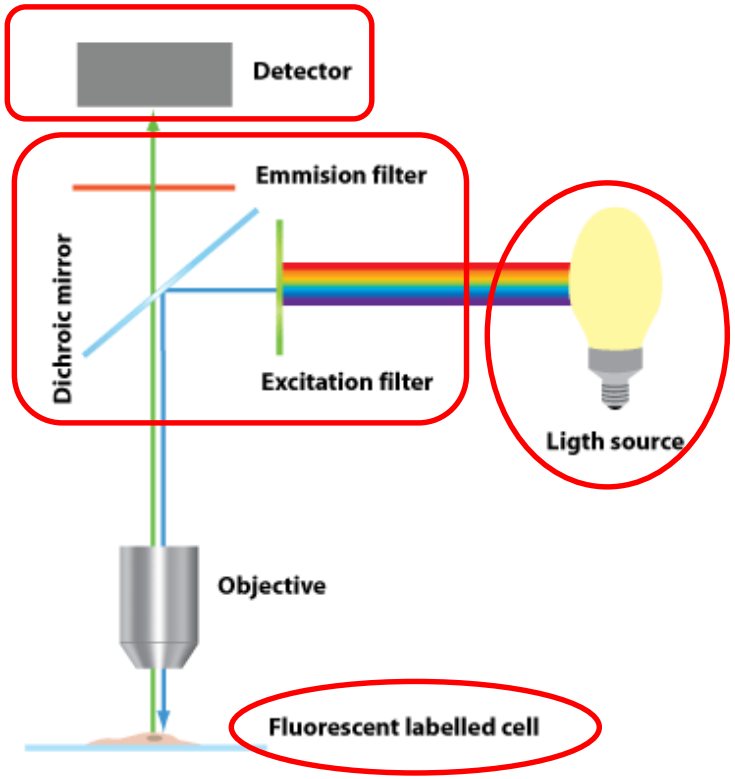
Lecture 1	Introduction & Ray Optics-1
Lecture 2	Ray Optics-2 & Matrix Optics-1
Lecture 3	Matrix Optics-2
Lecture 4	Matrix Optics-3 & Microscopy Design-1
Lecture 5	Microscopy Design-2
Lecture 6	Microscopy Design-3 & Resolution -1
Lecture 7	Resolution-2
Lecture 8	Resolution-3
Lecture 9	Resolution-4, Contrast-1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



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

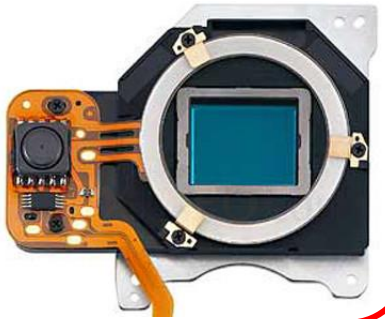
- **Eye**

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

- **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

(a)

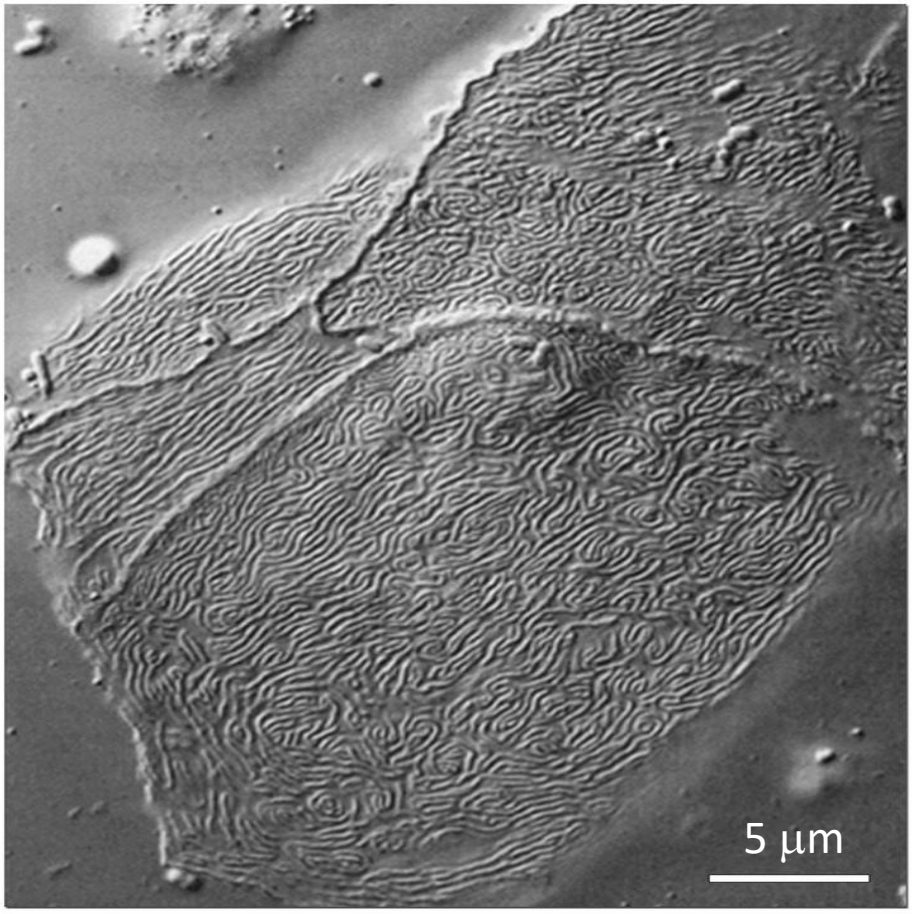
(b)

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.

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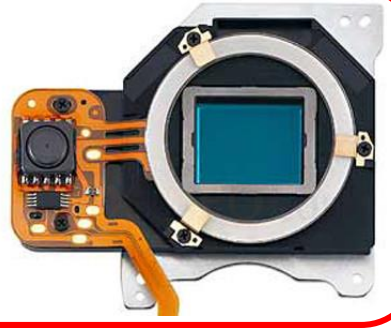
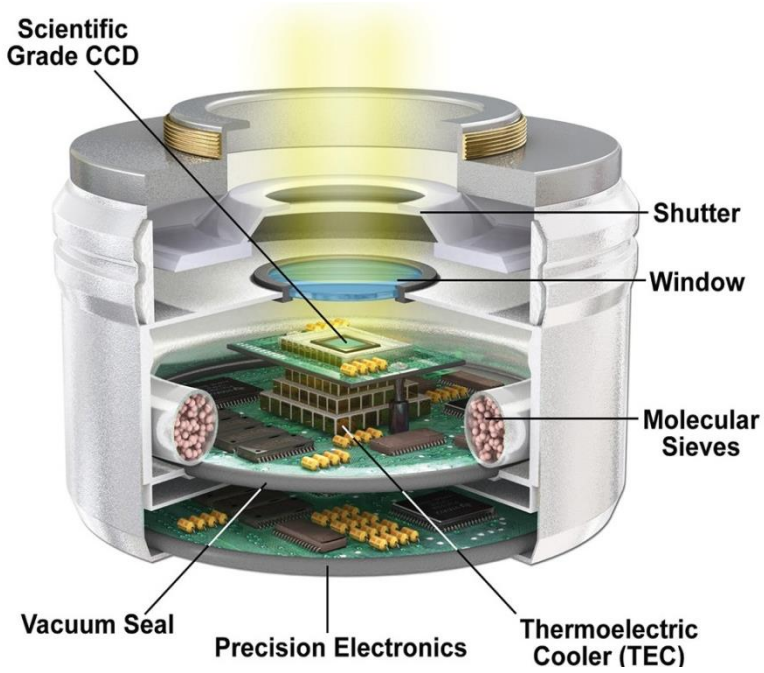
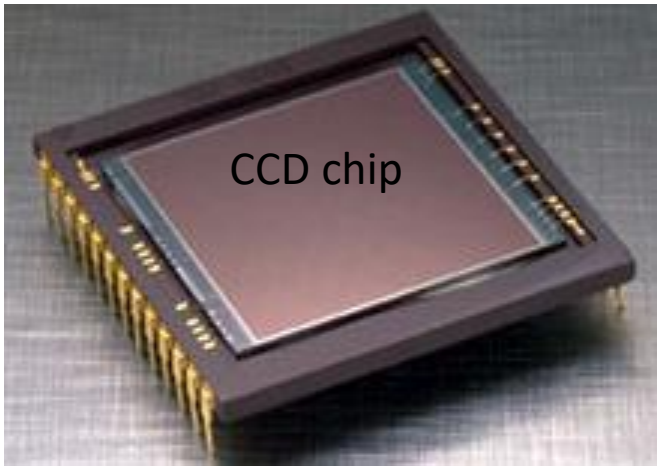


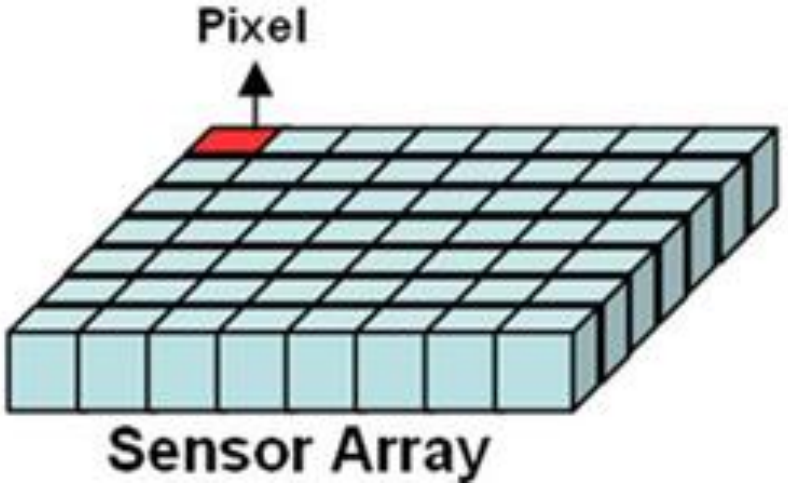
→ *most commonly used in biomicroscopy*

CCD → Charge-Coupled Device

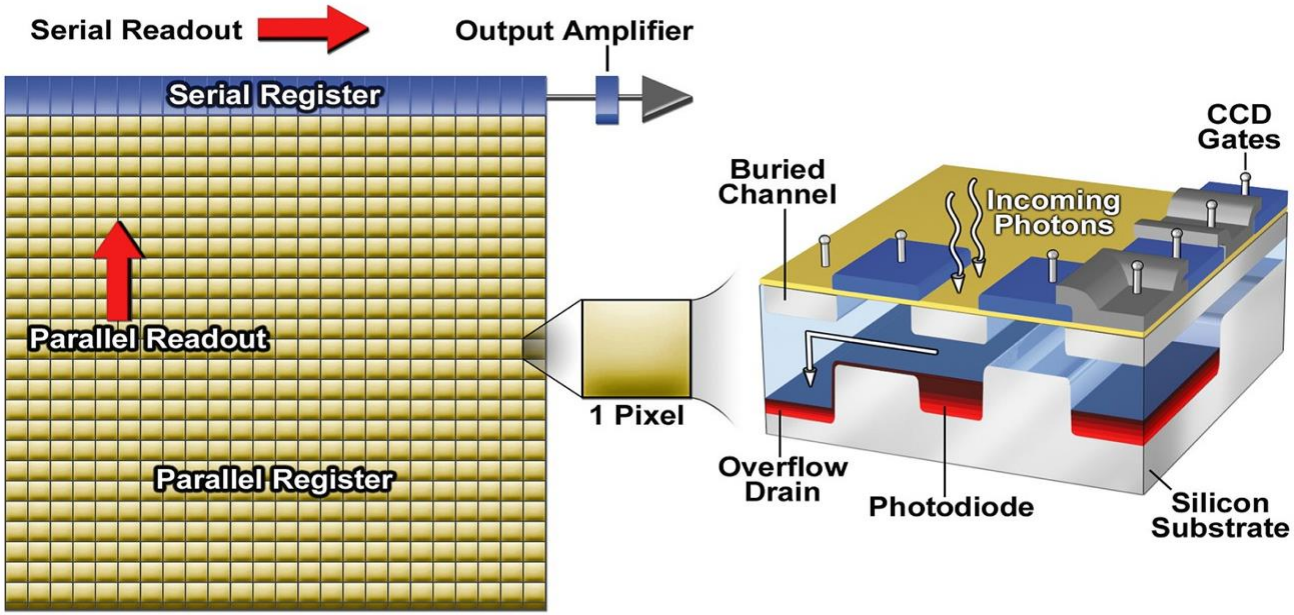
- Invented in 1970 at Bell Labs (U.S.)
- It is a **silicon chip** structured as an 2D array of photosensitive pixels.
- It converts incident light into an electrical signal
- About the device architecture:
  - The chip is mounted in a hermetically sealed chamber filled with dry N_2 or under vacuum.
  - In the front, there is a transparent window allowing incident light to enter
  - At the back, there is TEC to reduce thermal noise
  - The back end also contains several electronic components such as pre-amplifier, ADC, circuits for readouts







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 raw at a time by voltages applied to the strips on the CCD.
- Each raw 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)

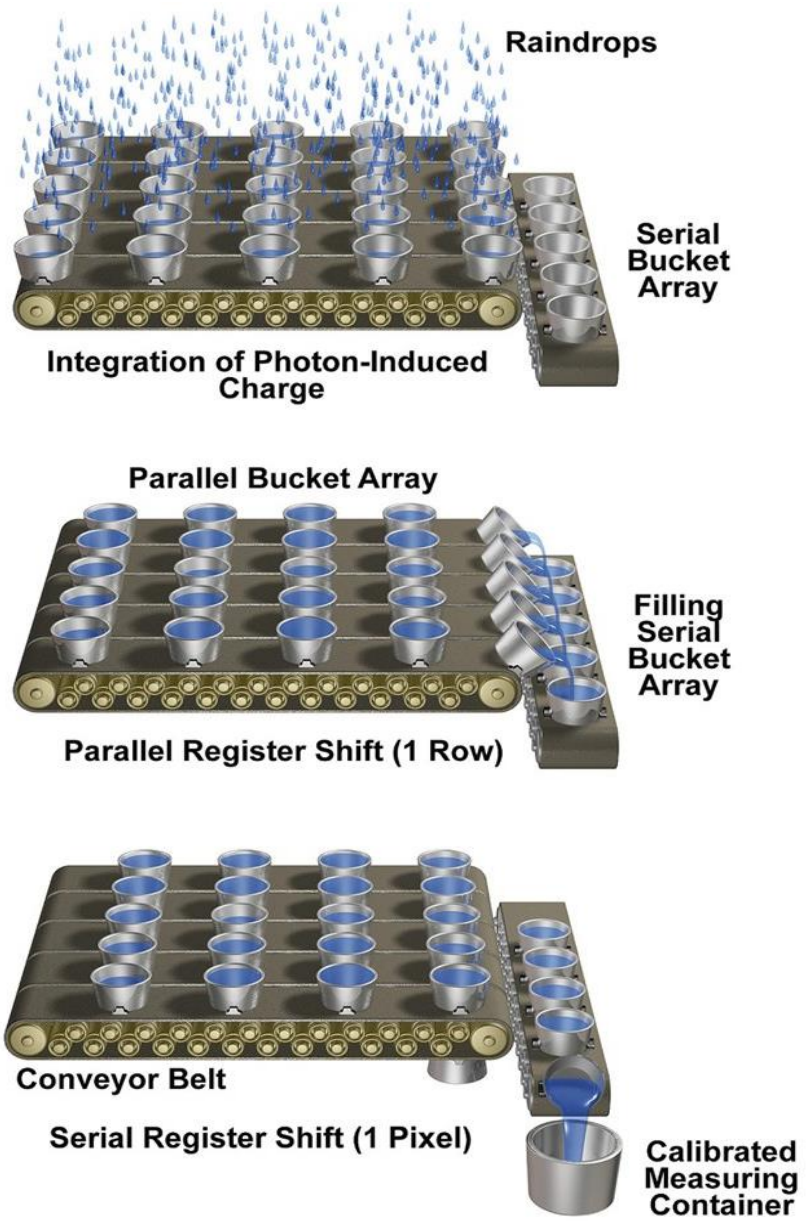
- Collection time = integration time

- **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



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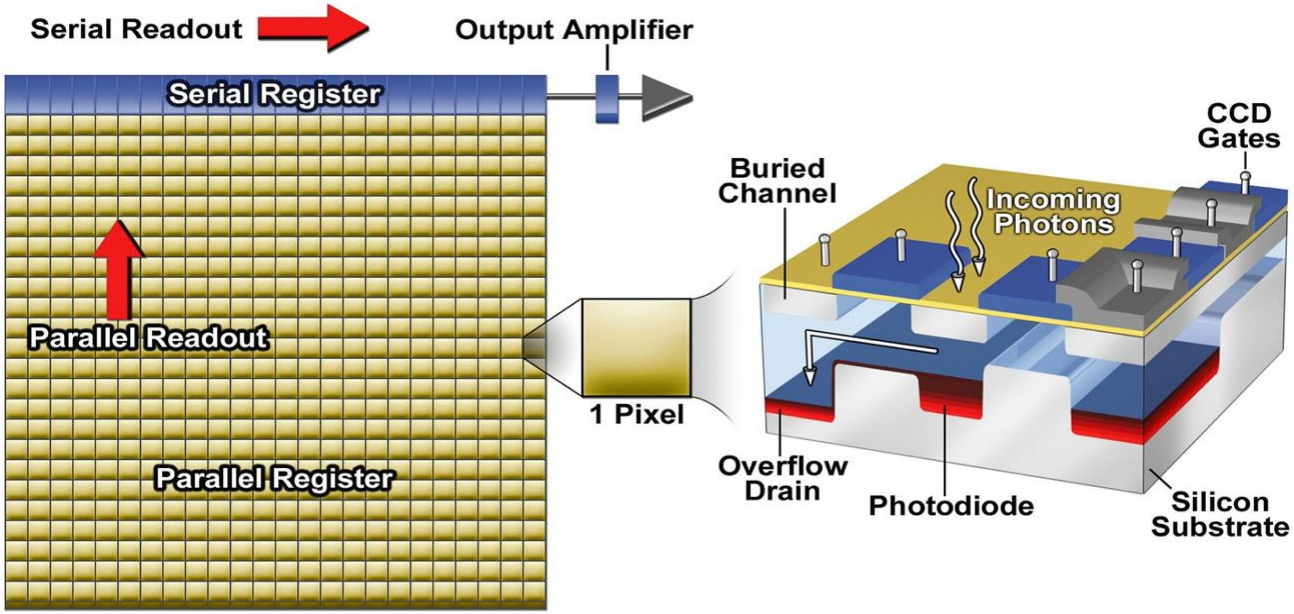

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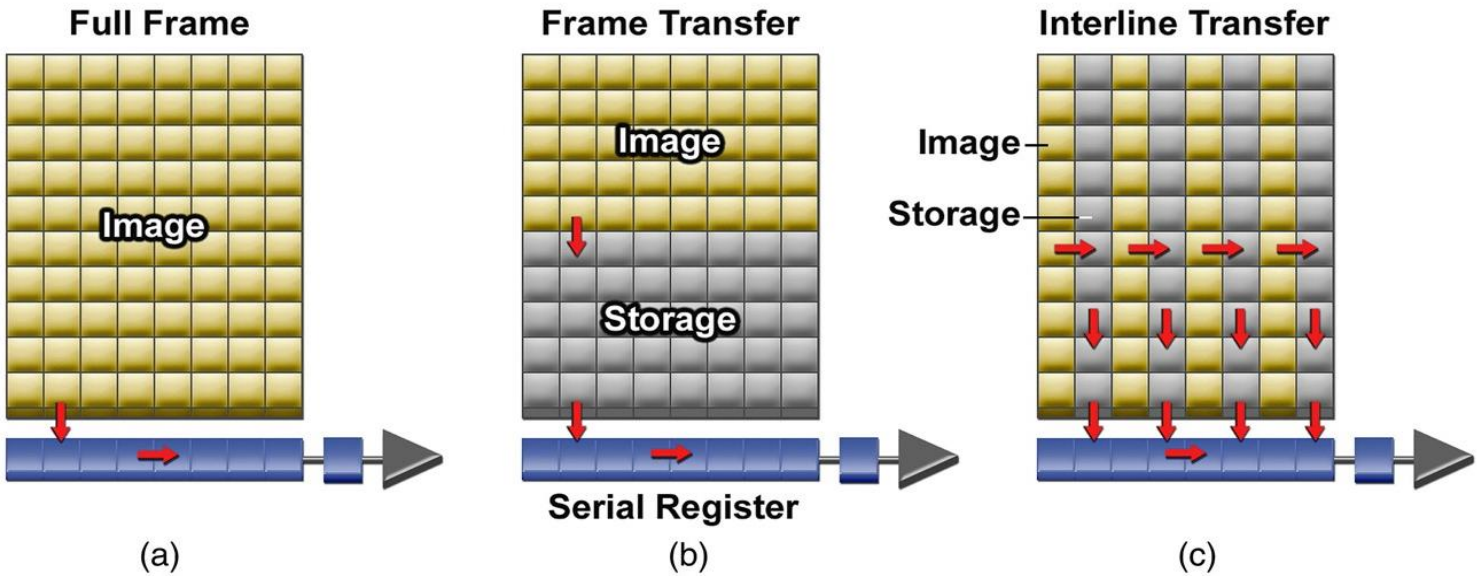
- Each raw at the end of the parallel register is transferred to a special row of pixels, called **serial register**.
- **Pixels are transferred one pixel at a time down the serial register with an on-chip pre-amplifier, which boosts the electronic signal & generates an analog voltage.**
- An A/D converter 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.*

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!

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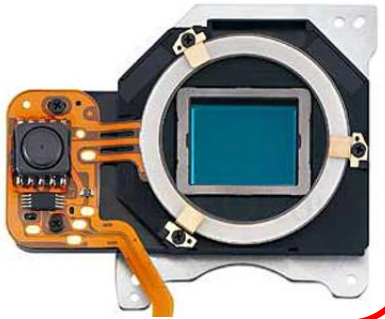


→ *most commonly used in biomicroscopy*

EMCCD- Electron Multiplying CCD

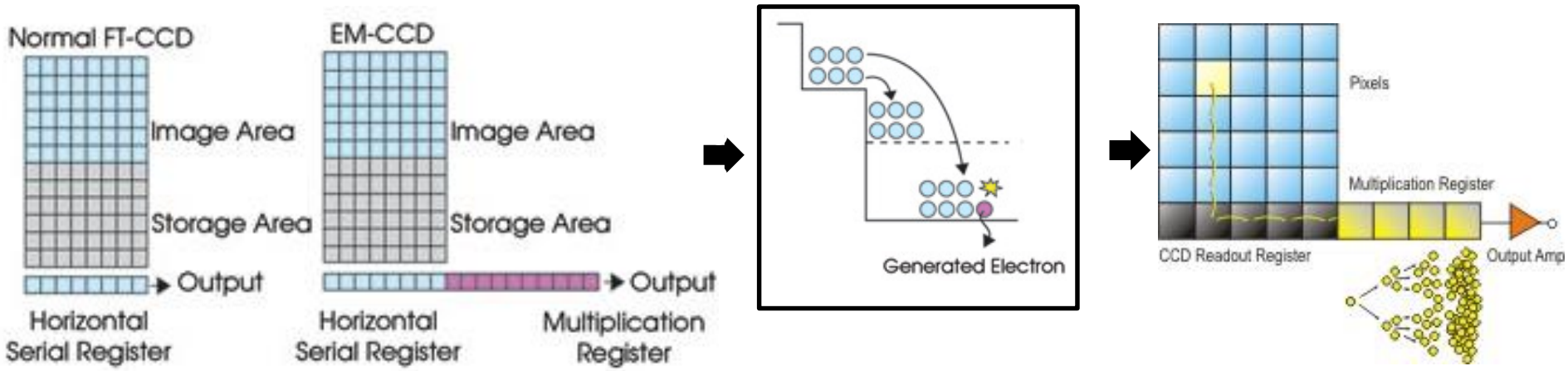
- It has an additional on-chip **Electron Multiplication register** (‘gain register’ between the usual serial shift register and the output amplifier)
- **EM gain amplifies the signal**

→ One can work in low-light conditions and acquire images that are otherwise not possible to obtain with a standard CCD camera.

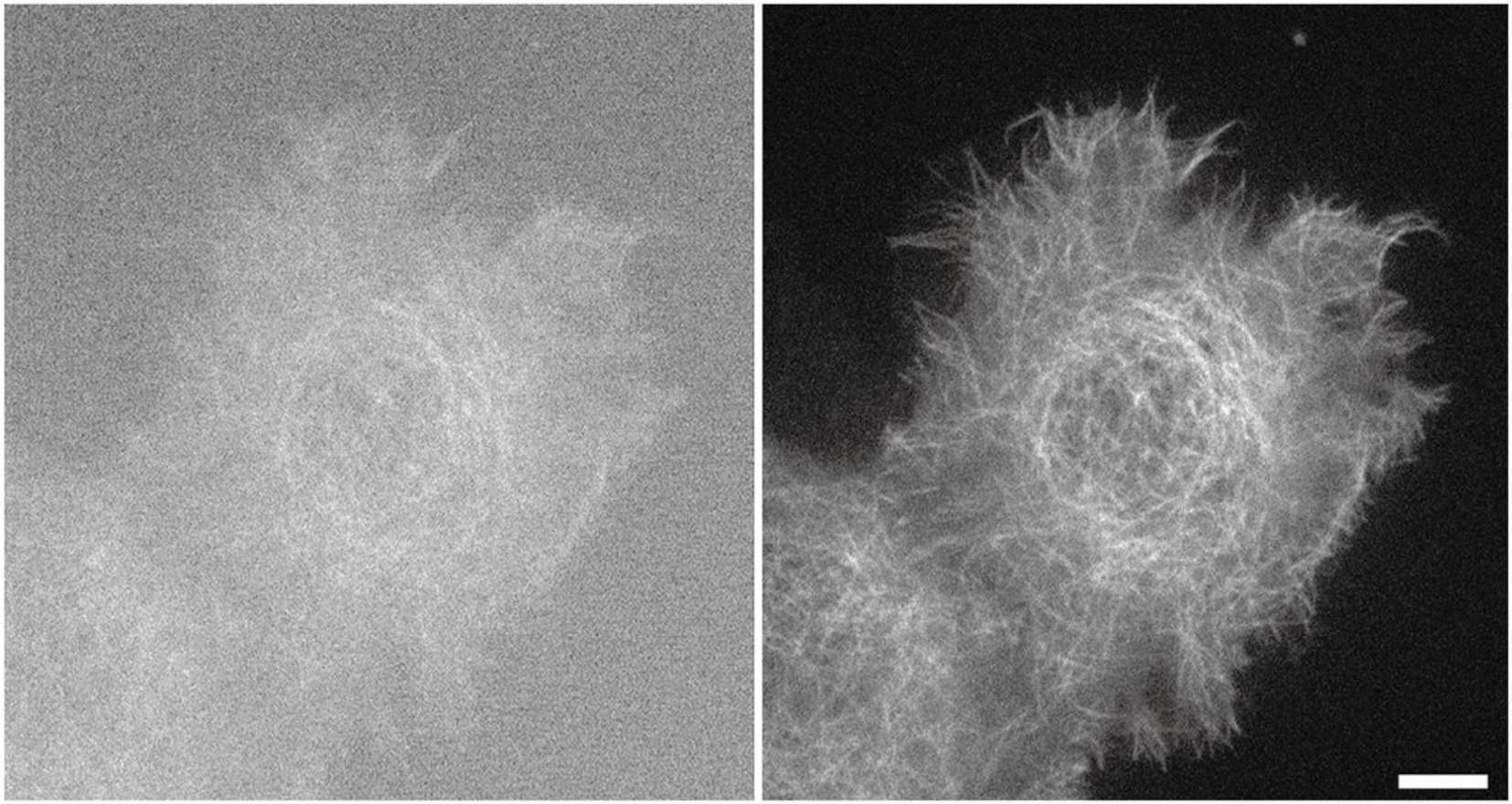
- **EM gain also reduces the required exposure time**

→ Minimize problems with photo-bleaching & prolong cell viability

CCD in “frame transfer” architecture:



EMCCD in bio-microscopy



(a) (b)

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.

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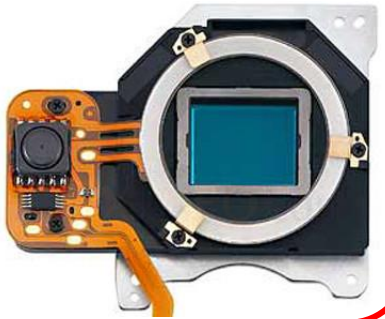

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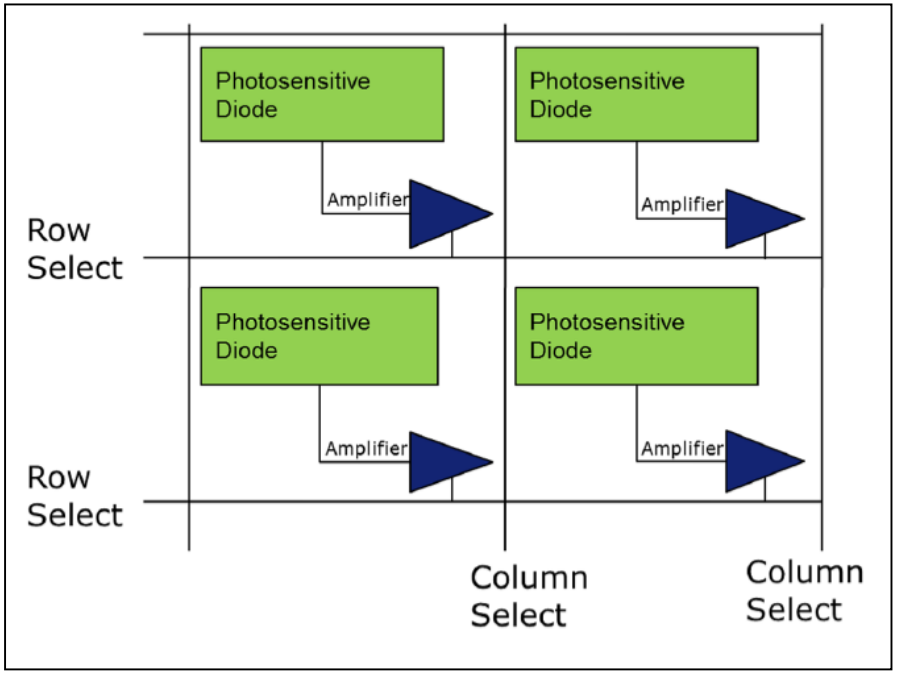
CMOS – Complementary Metal-Oxide-Semiconductor

• 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**:

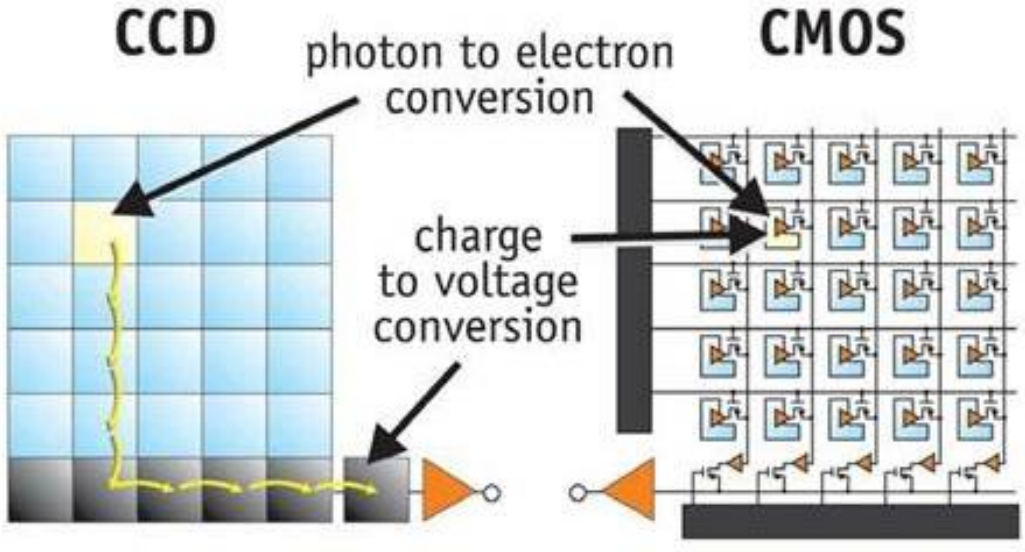
- Each pixel has its own amplifier → no need to transfer pixel content thus **faster** imaging is possible
- CMOS sensors require **less power** than CCD (perfect choice for phone camera sensors)
- It is relatively **low-cost.**



• **Disadvantages:**

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

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



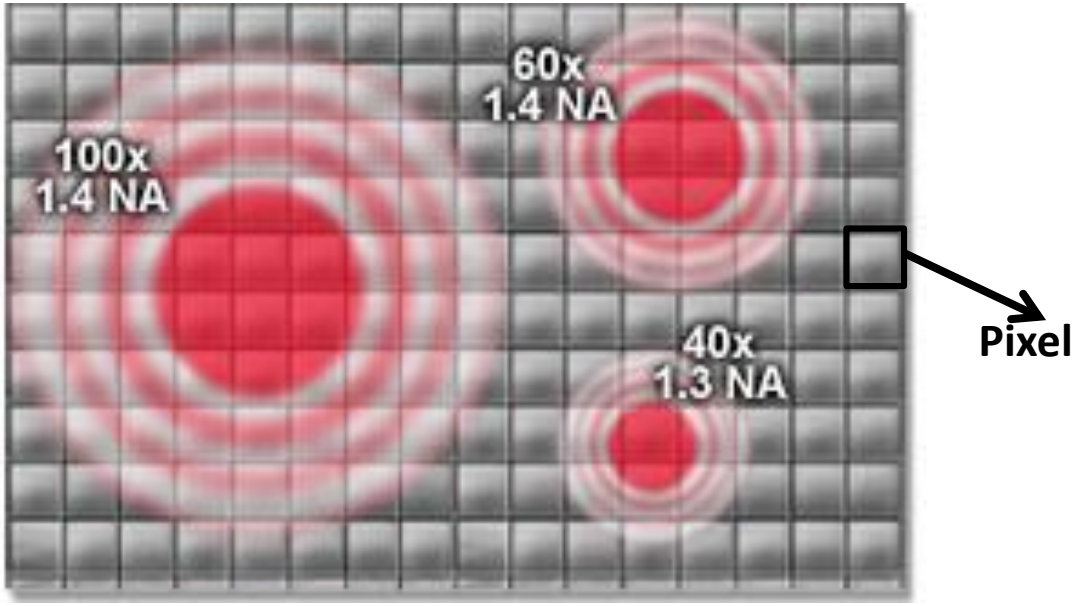
Basic characteristics of imaging cameras

- 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

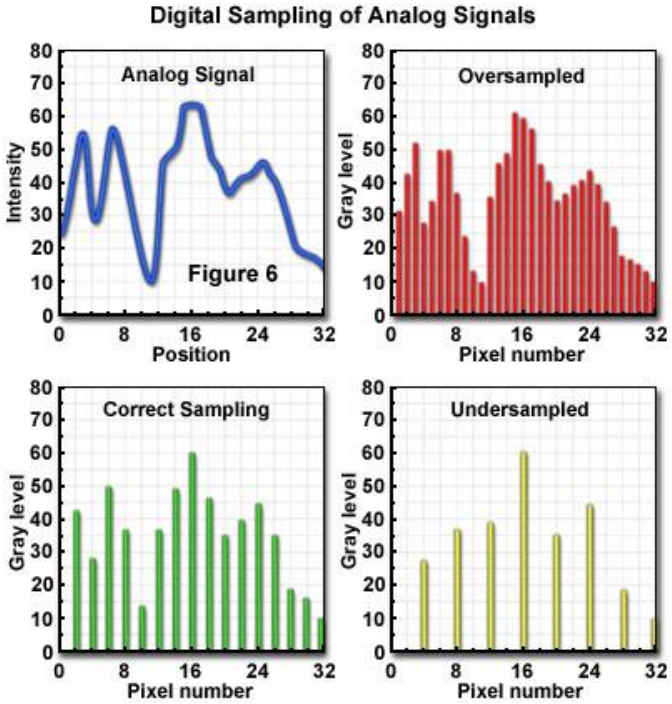
- To retain fully the resolving power of the microscope, correct camera choice depends on:
  - the physical size of the pixels on the detector
  - the magnification needed for the application
  - the NA of the objective used in the microscope
  - the wavelength of light used for imaging

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 theorem[1]

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

In other words, a bandlimited function can be perfectly reconstructed from an infinite sequence of samples if the bandlimit, B, is no greater than ½ the sampling rate (samples per second).

[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 μm 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:

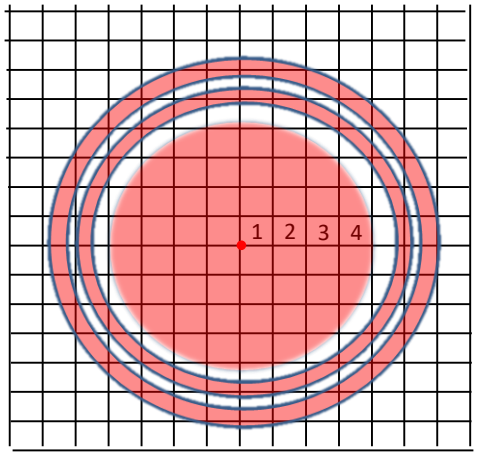
$$1.22 \times \frac{0.55}{2 \times 1.3} \times 100\ \mu m \approx 26\ \mu m$$

- There are ~4 pixels per diffraction spot radius:

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

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

1.3 NA & 100x



Example

- Consider the same CCD chip with 6.45 μm 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:

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

- There are <2 pixels per diffraction spot radius

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

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

1.3 NA & 40x

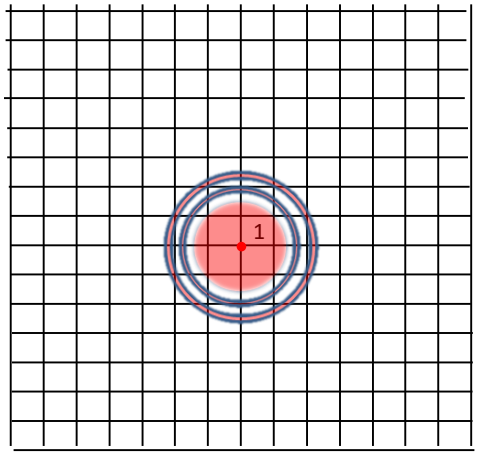
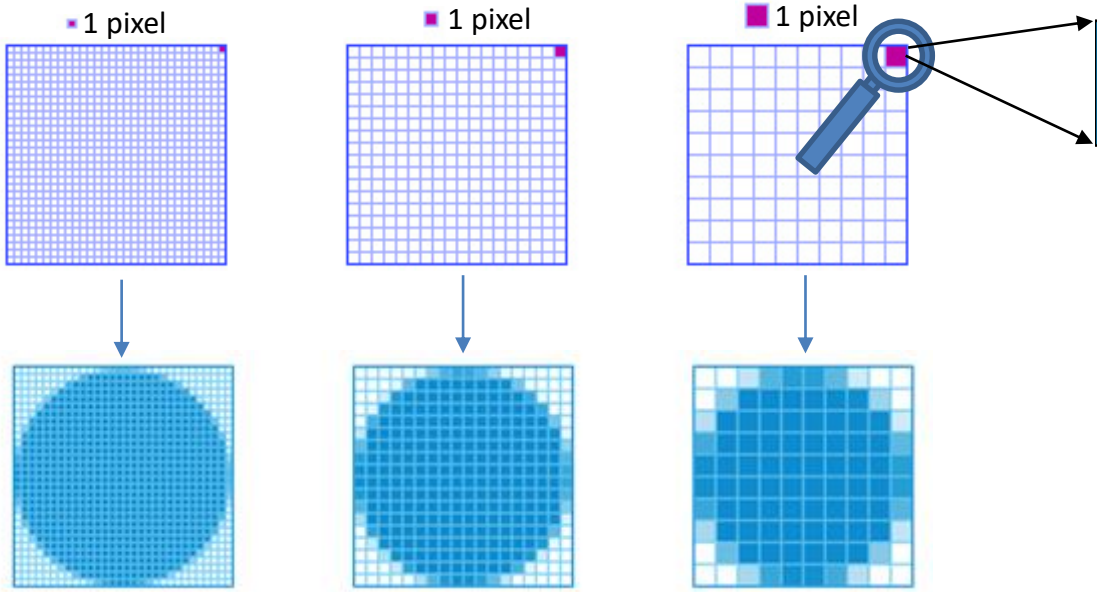


Image sensor size and the number of pixels



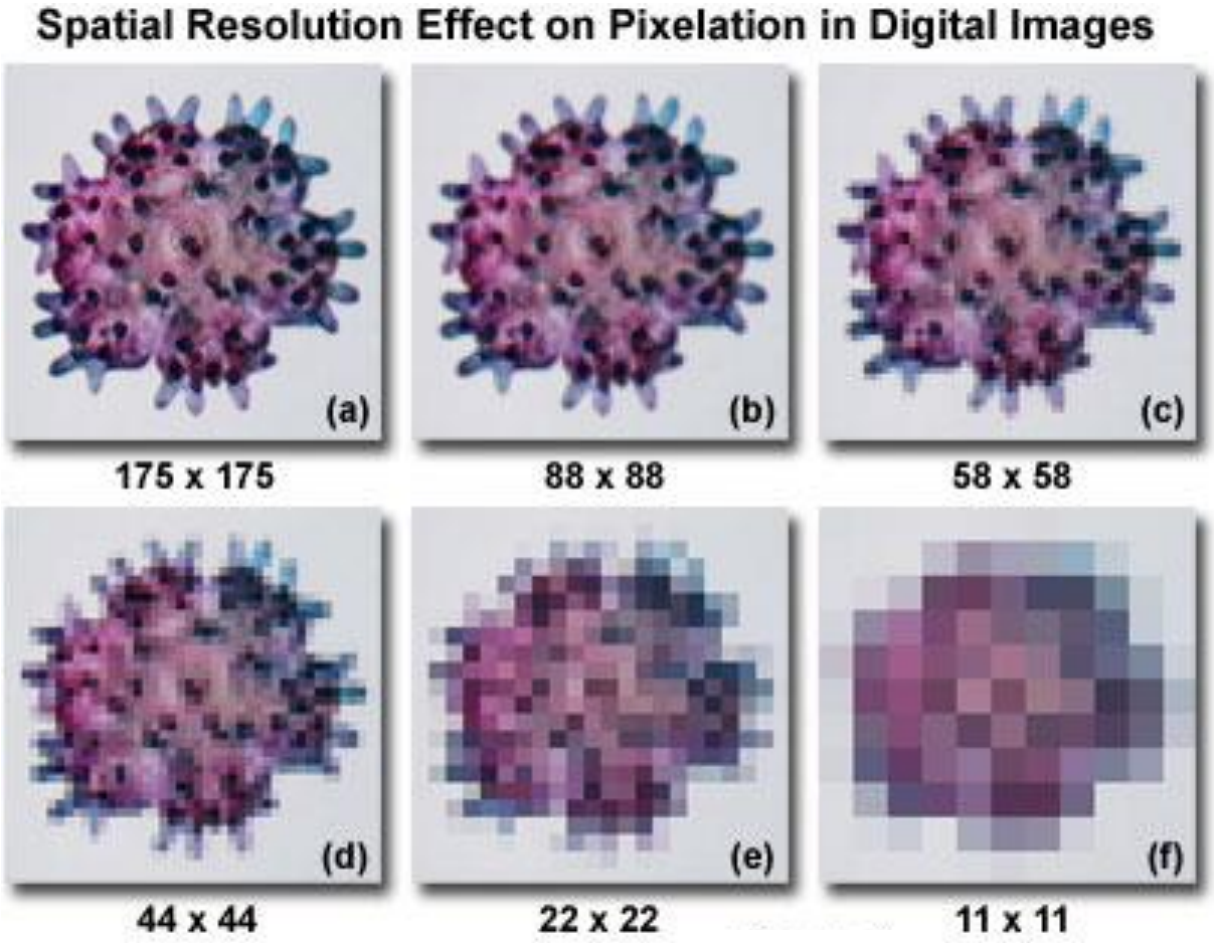
Examples for typical pixel dimensions

Pixel size	Pixel area
14 μm	196 μm^2
10 μm	100 μm^2
7 μm	49 μm^2
6.5 μm	36 μm^2
4.6 μm	21 μm^2
3.5 μm	12 μm^2
2.2 μm	5 μm^2
1.7 μm	3 μm^2

Example: Sony ICX205AL CCD Image Sensor

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

Pixel Size & Resolution



For the same imaging area (and sample):

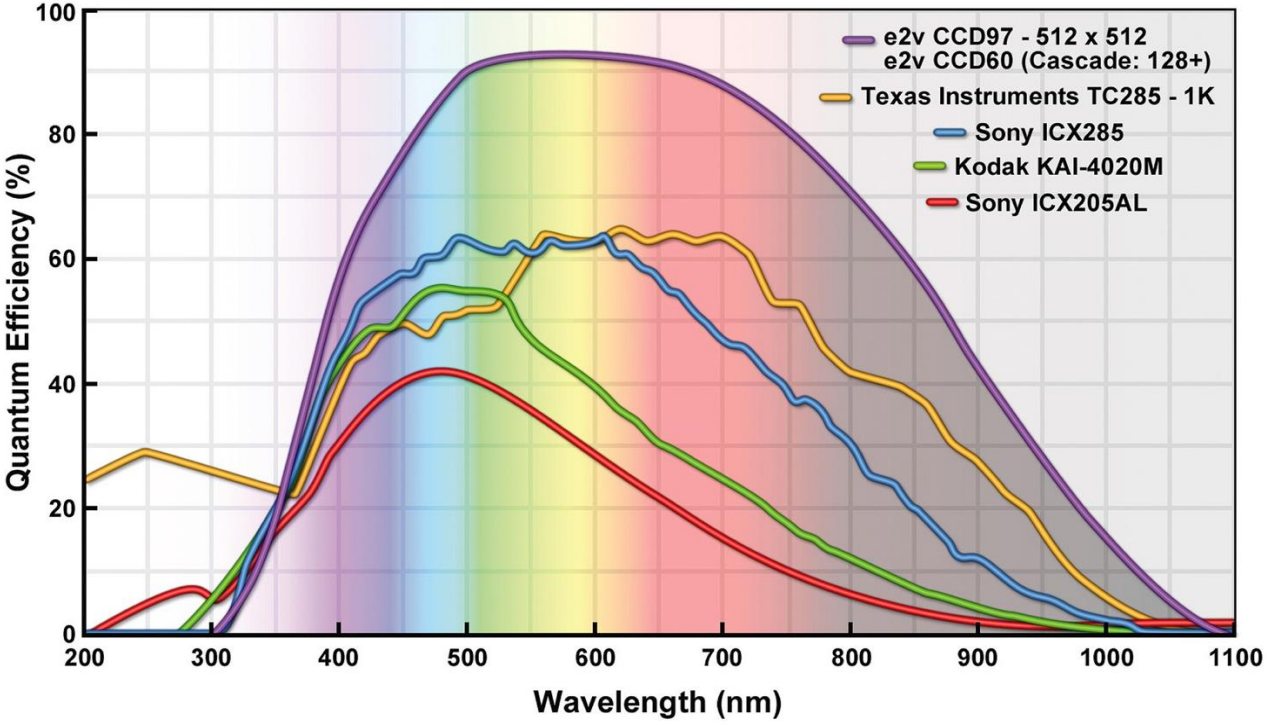
Larger pixel size, thus less pixel numbers, leads to less sampling frequency

Basic Camera Characteristics

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

Spectral bandwidth & quantum efficiency

- Spectral bandwidth refers to the wavelength range that the camera can detect light
- Quantum efficiency refers to the efficiency of photon-to-electron conversion in the camera

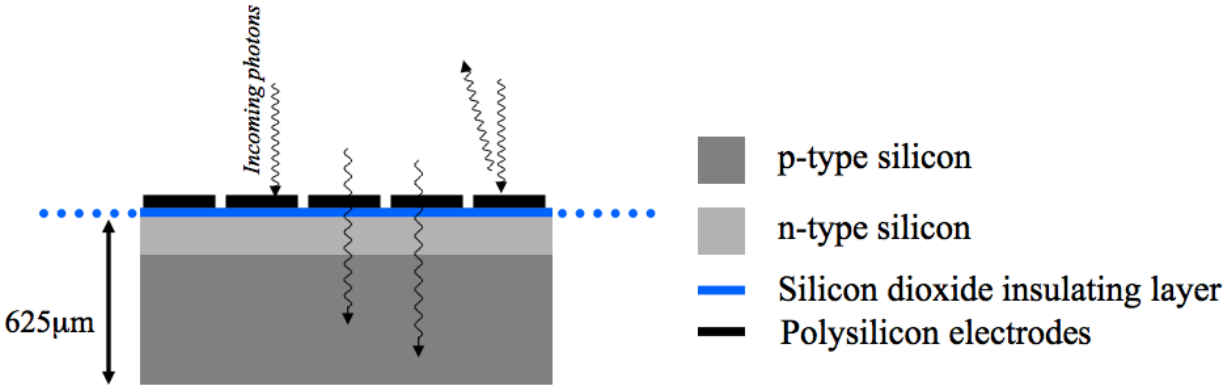


Wavelength distribution of the quantum efficiency for some popular CCD cameras

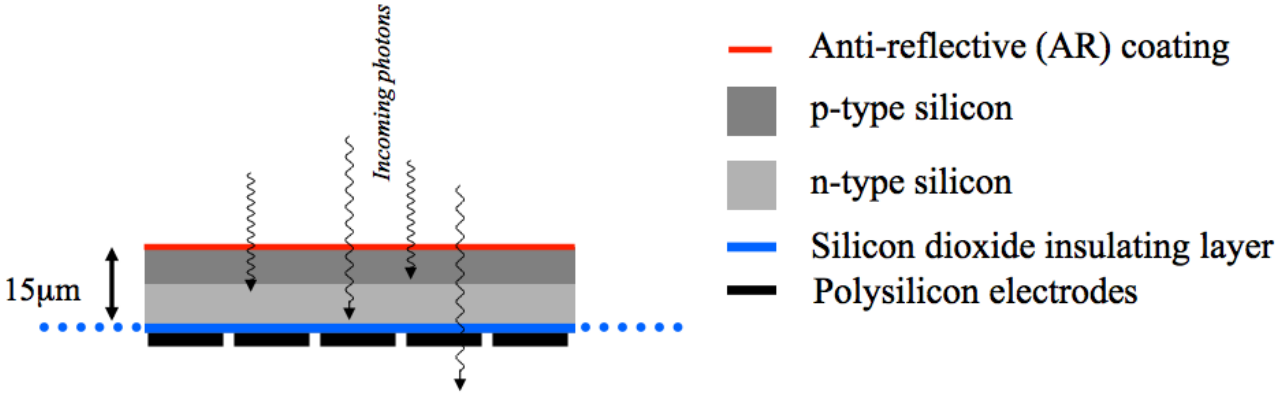
- Standard front-illuminated CCDs have a peak QE of 40-50% **(red curve)**
- New sensors are extending QE to 60% for 400-1100 nm range with peak sensitivity at 550-800 nm **(blue & yellow curves)**
- With back-illuminated CCDs, QE can be >80% but they are more expensive (**purple curve**)

Improving Quantum Efficiency

Front-side illumination: light needs to pass through several layers before reaching the silicon
→ **high light loss**

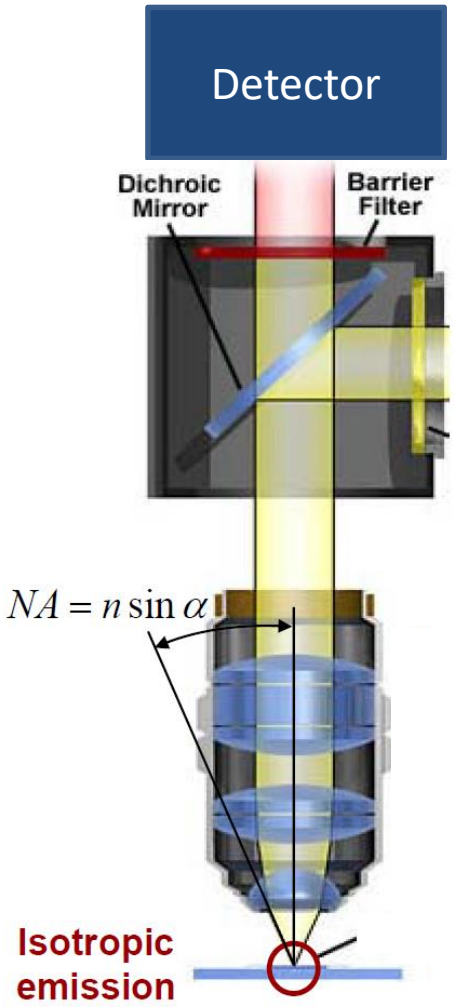


Back-illuminated sensor orientates the wiring behind the photocathode, which improves the chance of an input photon being captured → **increases QE from 60% to more than 90%**



Fluorescence Collection Efficiency

- Fluorescent molecules will result in photon generation depending on molecules QE.
- But, not all of these photons can reach to the detector.
- Some of the major loss factors are as follows:



- Detector's limited QE also contributes to the photon loss.

- Absorption by optical components contributes to the photon loss:

$$\left.\begin{aligned} T_{objective} &\approx 80\% \\ T_{tube\ lens} &\approx 95\% \\ T_{cover\ slip} &\approx 90\% \\ T_{dichroic} &\approx 90\% \\ T_{filter} &\approx 60\% \\ T_{lenses} &\approx 90\% \end{aligned}\right\} \quad T_{optics} \approx 30\%$$

- A major loss happens by the objective lens due to its limited collection angle, α.

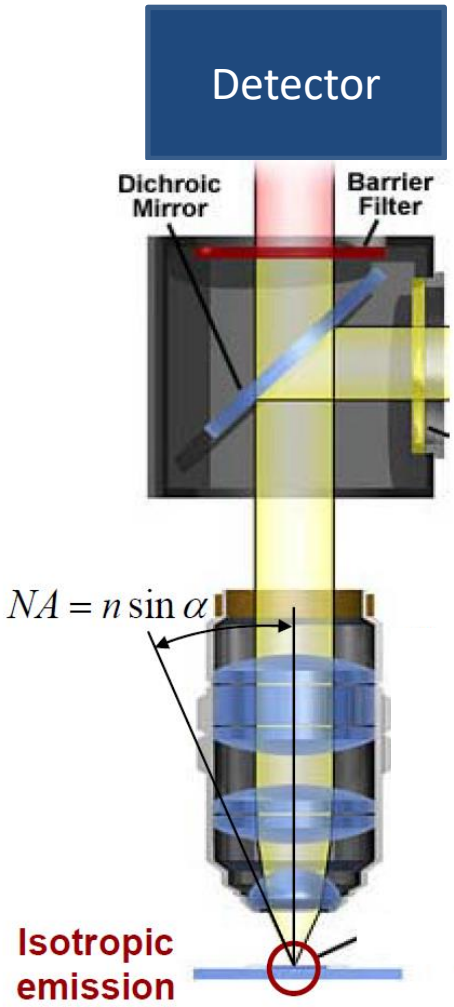
Collection efficiency CEF

$$P_{\text{collection}} = \frac{1}{4\pi}\int_0^{\alpha} \sin\theta \, \mathrm{d}\theta \int_{-\pi}^{+\pi} \mathrm{d}\varphi = \frac{1}{2}(1-\cos\alpha)$$

Example: **CEF** for 1.2 NA water immersion objective is ~28%

Fluorescence Collection Efficiency

Example: With a 1.2 NA water immersion objective and a 60% QE detector QE:
Total collection yield can be as low as: $28\% \times 30\% \times 60\% \sim \mathbf{5}\%$



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Noise sources in cameras

1. Photon or Shot Noise (Fundamental)

- A statistical uncertainty that is always observed when discrete quanta, such as photons, are measured within a finite time or space.
- It depends on signal level **as square route of the signal** (Poisson distributed).

$$N_{shot} = \sqrt{N_{photon}}$$

N_s : shot noise
N_{ph} : number of photons

2. Dark Current (or Thermal Noise)

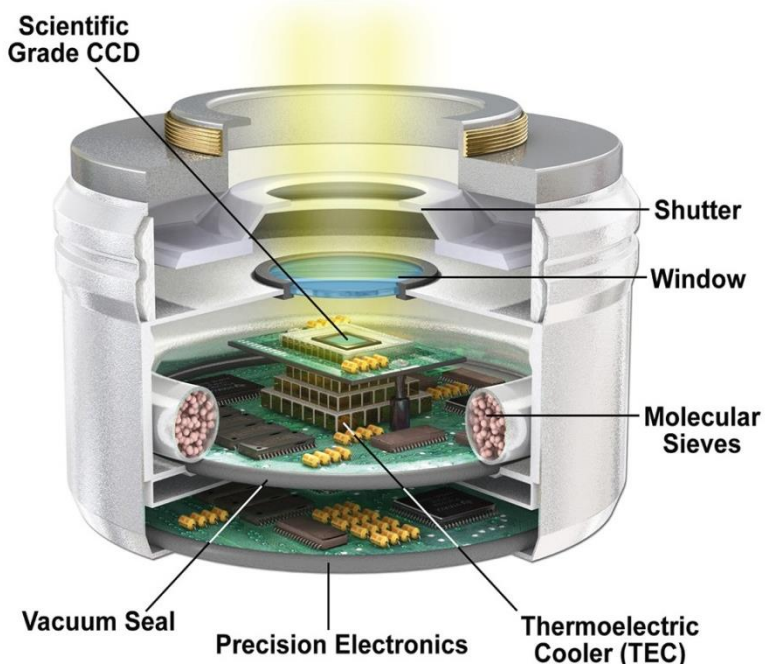
- Originates from heat and cosmic sources: electrons are freed by these sources rather than by the photons from the imaged sample.
- It depends on the exposure time.
- It is less important than the other noise sources & can be reduced to very small value by **thermoelectric cooling**.

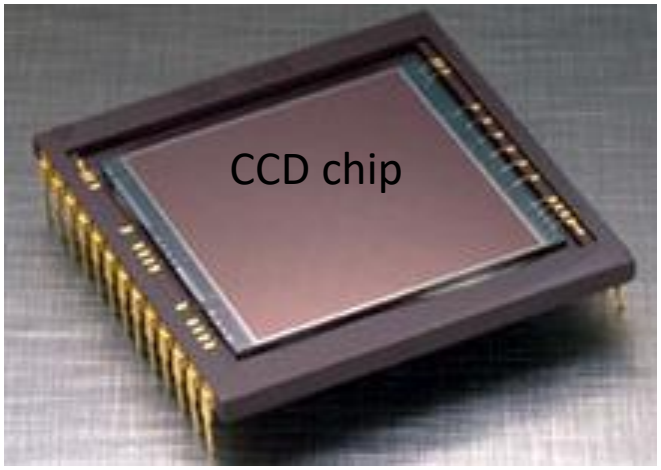
3. Read Noise

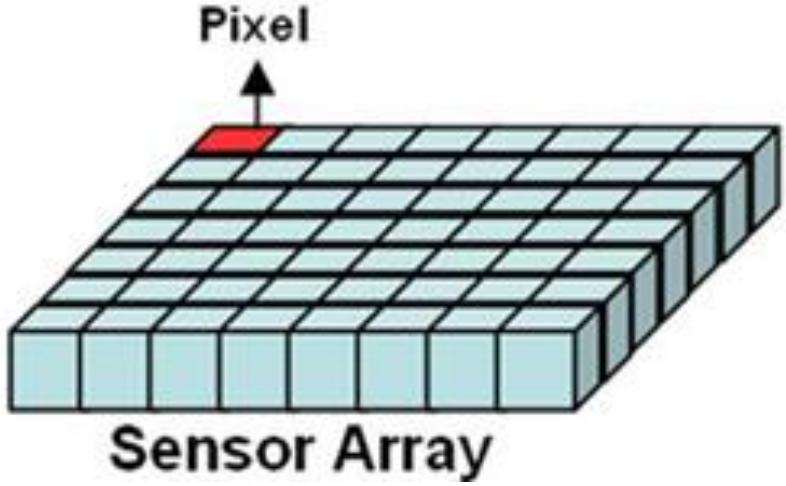
- Originates mainly from the amplification of pixel photo-electron counts in the on-chip amplifier where electron counts induce a voltage, which is then carried to the ADC.
- *Manufacturer give the value of this noise in electrons, e^-.*

Recall: Charge-Coupled Device

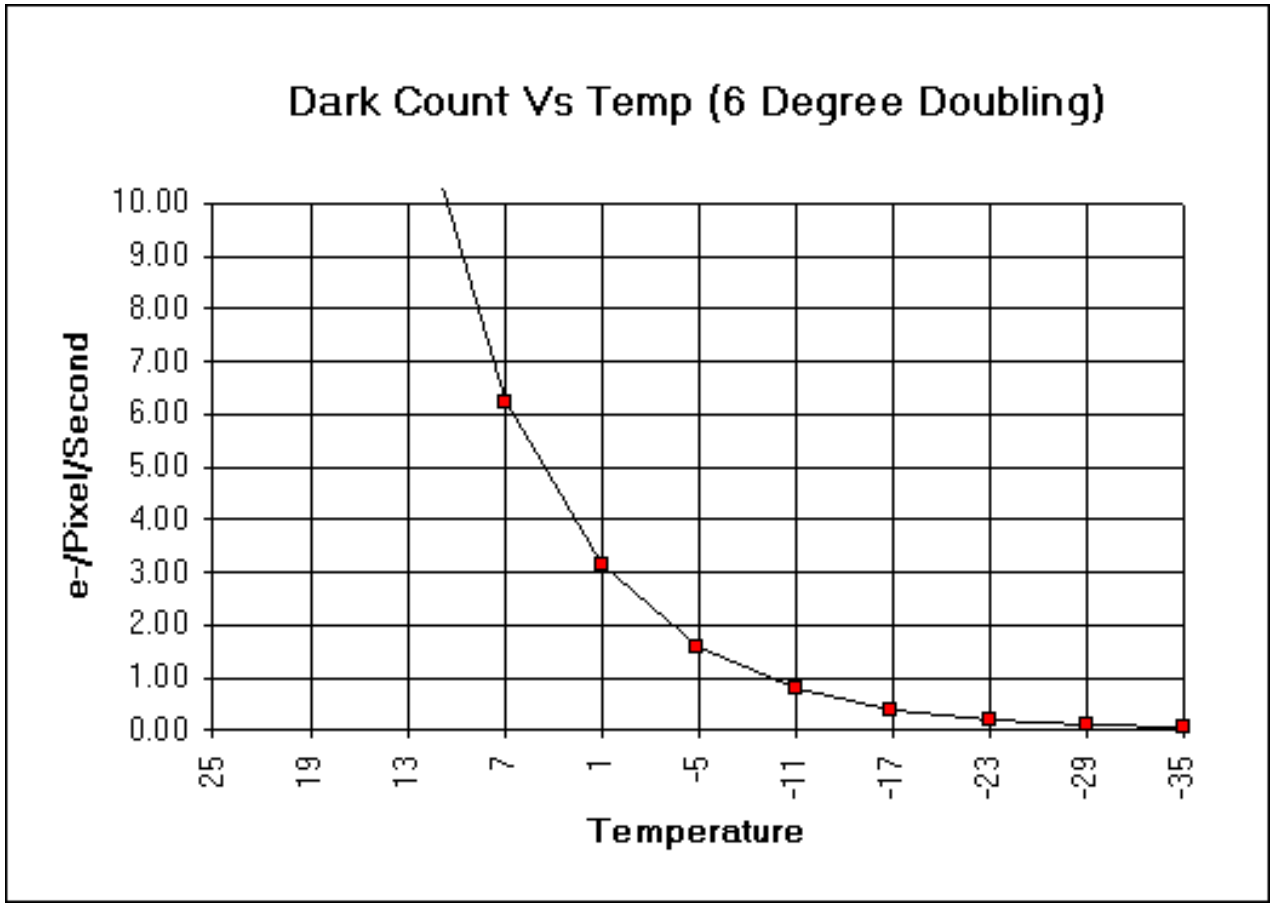
- Invented in 1970 at Bell Labs (U.S.)
- It is a **silicon chip** structured as an 2D array of photosensitive pixels.
- It converts incident light into an electrical signal
- About the device architecture:
  - The chip is mounted in a hermetically sealed chamber filled with dry N_2 or under vacuum.
  - In the front, there is a transparent window allowing incident light to enter
  - **At the back, there is TEC to reduce thermal noise**
  - The back end also contains several electronic components such as pre-amplifier, ADC, circuits for readouts







Dark current: dark counts



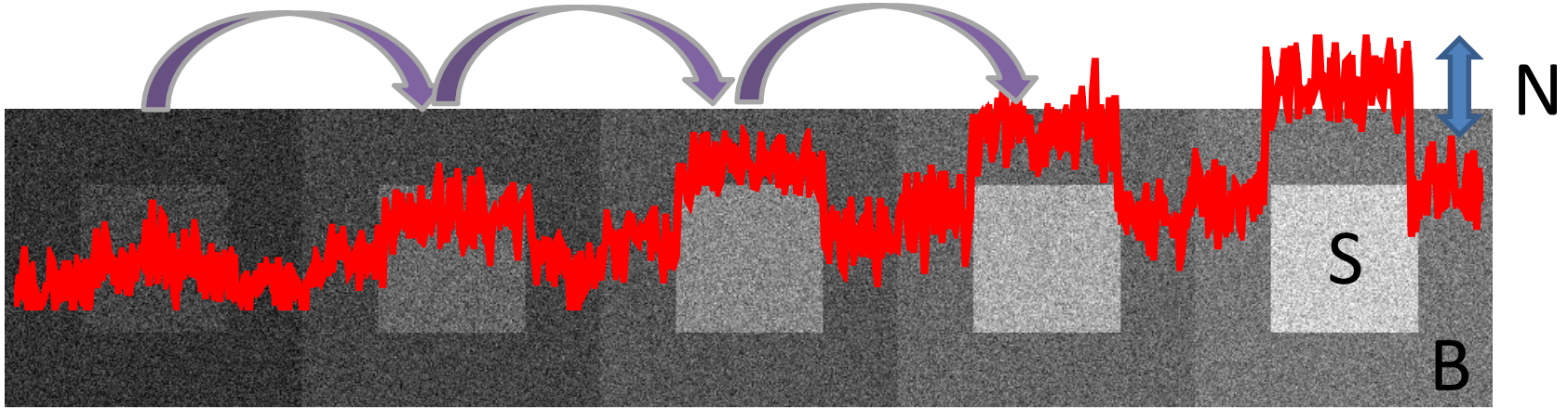
Cooling methods:

Liquid Nitrogen

Thermal Electric

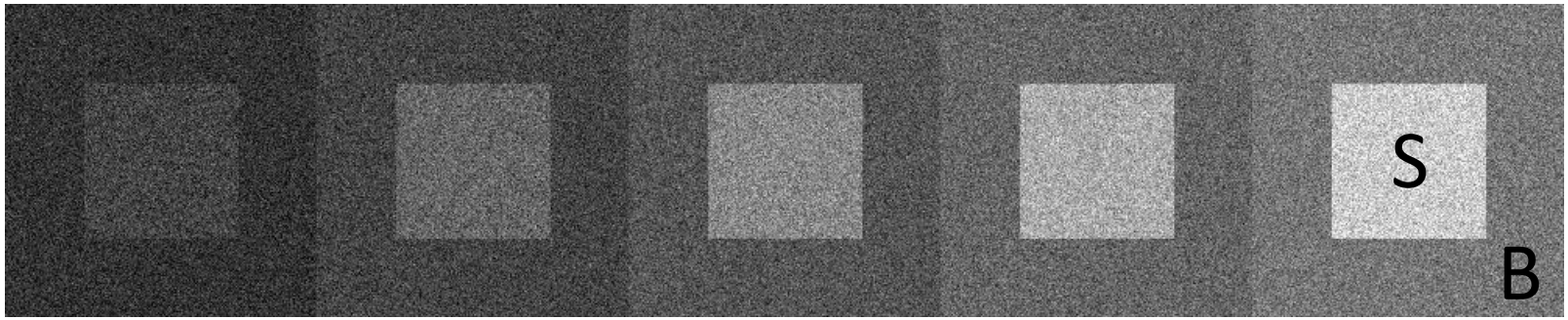
Thermal Electric
in ultrahigh vacuum

Signal and Noise



Increasing of the Signal/Noise (SNR- Signal to Noise Ratio), increases the contrast …

Signal and Noise



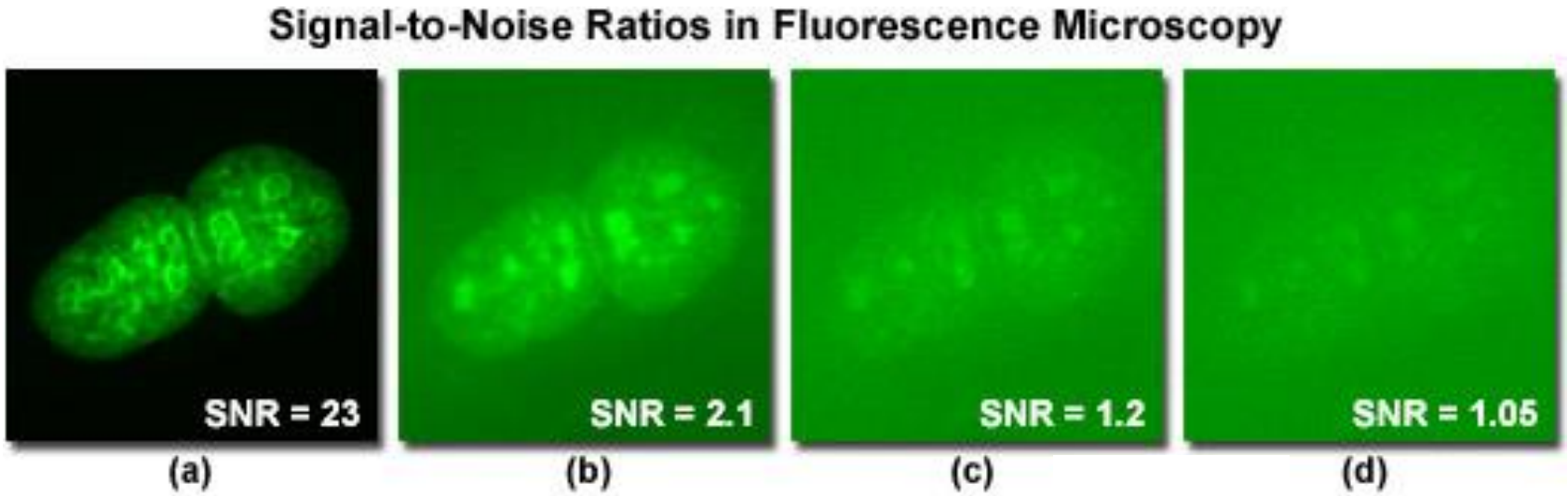
$$N_{tot} = \sqrt{N_s^2 + N_d^2}$$

$$N_s = \sqrt{N_{ph}}$$

N_{tot} : total noise
N_d : detector noise
N_s : shot noise
N_{ph} : number of photons

Signal-to-Noise Ratio (SNR or S/N Ratio)

- Qualitatively, SNR describes the clarity & visibility of the objects in an image.



The specimen is an adherent culture of opossum kidney proximal tubule epithelial cells (**OK** cell line) stained with SYTOX Green to image the nuclei.

(a) At high SNR, a pair of interphase nuclei is imaged with sharp contrast and good definition of the fine details against a black background.
As the SNR decreases:
(b & c) the definition and the contrast of the nuclei decrease until they almost completely blend into the noisy background
(d) as the SNR approaches unity, the imaging visibility is compromised.

Basic Camera Characteristics

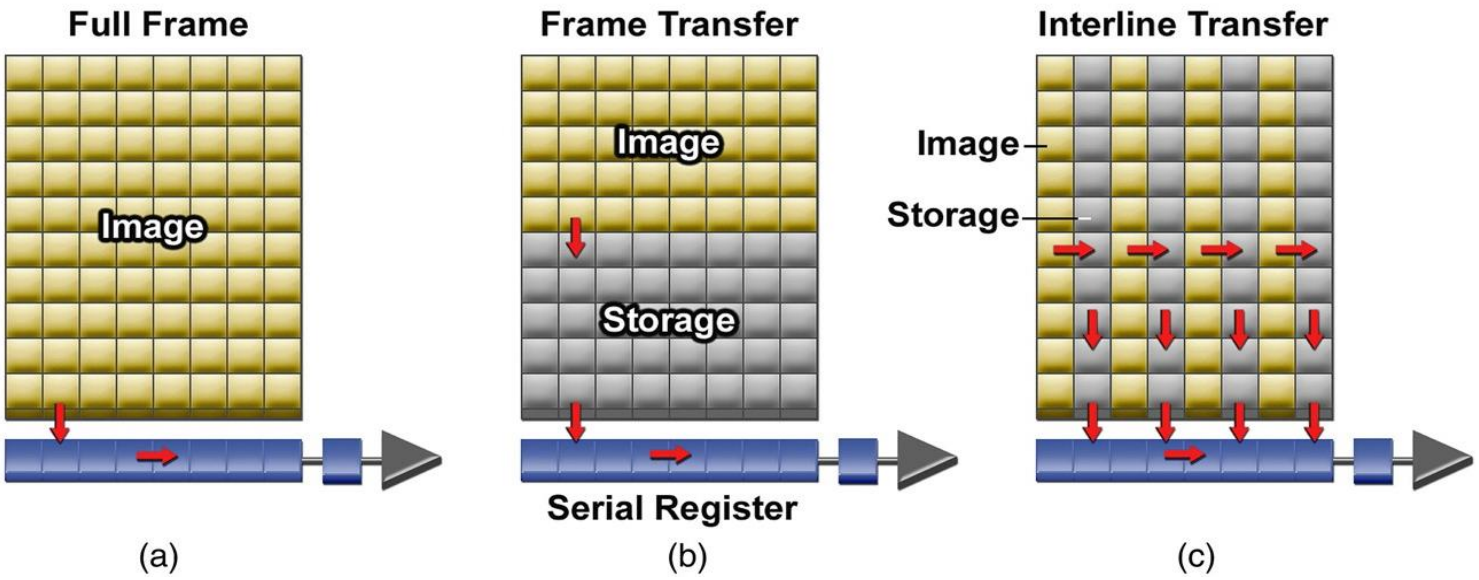
- Spatial resolution
- Spectral bandwidth & quantum efficiency
- Noise sources
- Signal to Noise Ratio
- Temporal resolution
- Sub-array option
- Binning option
- Dynamic range

Temporal Resolution

- It is the ability of the camera to resolve events at different points in time.

As a reference point: Time resolution used for movies is usually 24 to 48 frames per second (frames/s).

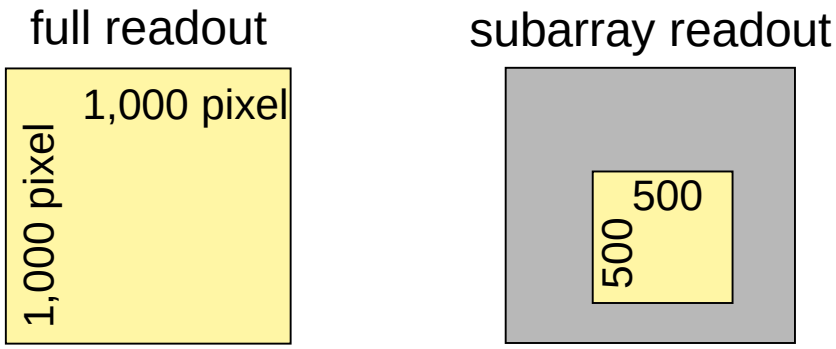
- New CCD architectures have been developed to improve temporal resolution
  - Full frame was the original design.
  - Alternative architectures include frame transfer & interline transfer



Sub-Arraying Option

Sub-array readout:

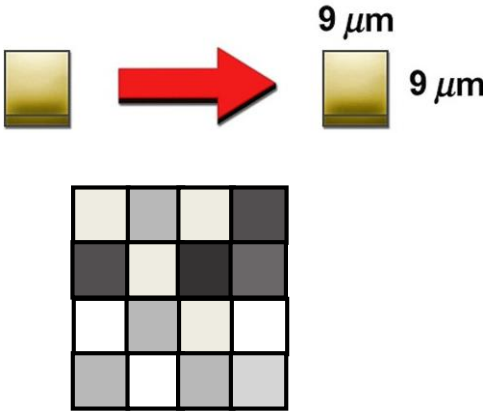
- It is possible to define a small subset of pixels on the CCD corresponding only a portion of the full image area for acquisition & display on the monitor.
- **Sub-array read-out is faster** because not in-use pixels are not processed by the ADC and are discarded.
- With sub-arraying option, rates of several hundred frames per second can be obtained.
- Image files (particularly for time-lapse acquisition in live cell imaging applications) are also smaller & more manageable.



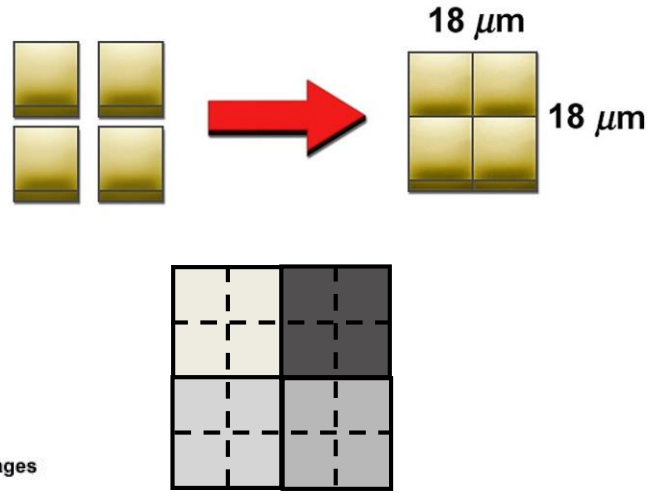
Binning Option

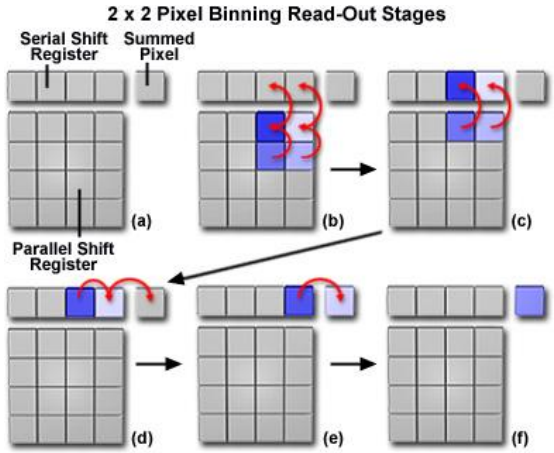
- Binning is the combination or pooling together of photo-electrons of adjacent pixels on the CCD to form **electronic super-pixels**.
- For example, a 2x2 super-pixel contains the combined photo-electron content of 4 physical pixels. But, this is processed by the camera and amplifier as a single pixel.

1x binning



2x binning





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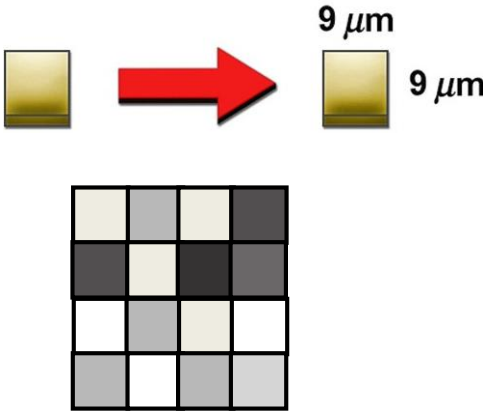
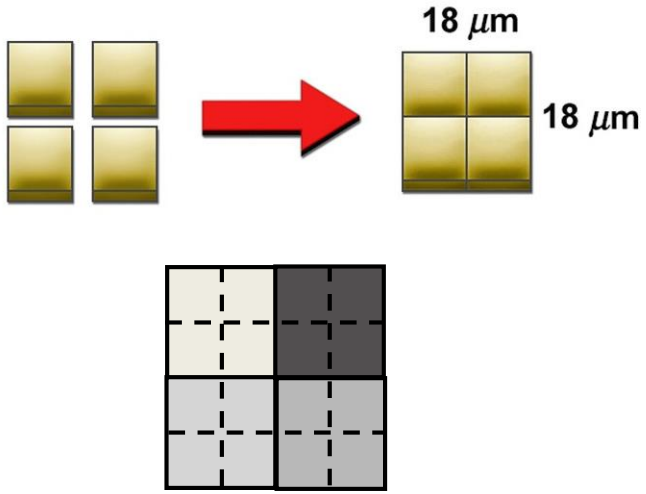


2x binning



- Sensitivity improves → shorter exposure time is required to obtain the same brightness (a major benefit for live-cell imaging)
- For the same exposure time, S/N ratio improves
- Faster acquisition time of the image (i.e. faster read-out)
- Smaller size of image files on the computer
- BUT binning degrades spatial resolution with increasing “effective pixel size” (less number of pixels per diffraction limited spot radius)

Basic Camera Characteristics

- Spatial resolution
- Spectral bandwidth & quantum efficiency
- Noise sources
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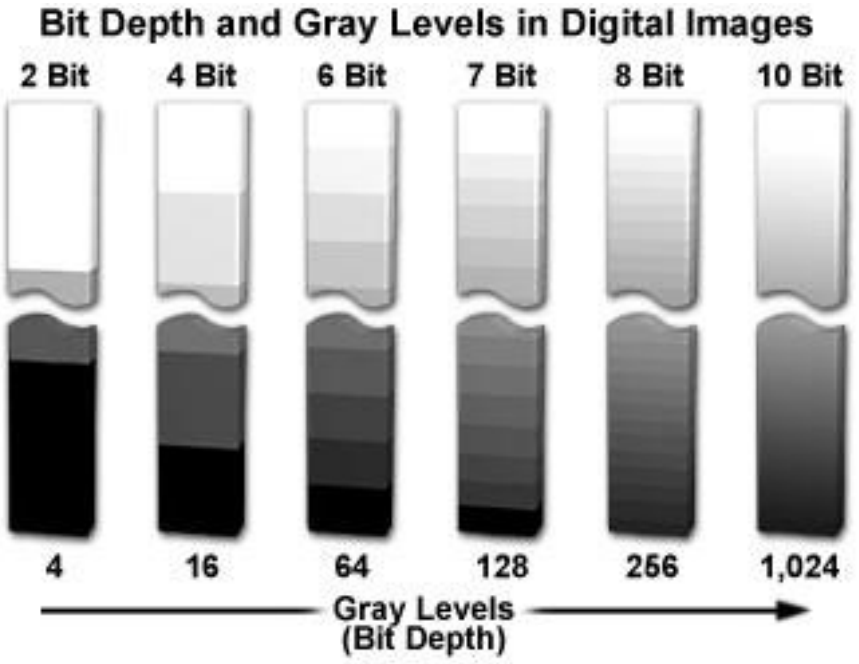
- Dynamic range

Dynamic Range

- The number of resolvable steps of light intensity, described as gray-level steps ranging from black to white, is called dynamic range (DR).
- **DR is used to describe the potential number of gray-level steps capable of being recorded by a camera.**
- The bit gives the number of steps as 2^X
  Ex: 8, 10, 12 bits → 2^8=256, 2^{10}=1024, 2^{12}=4096 gray levels



Choosing a camera for high imaging performance

Basic characteristics of cameras:

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

In real applications, it is hard to optimize all of these simultaneously.

Example:

- To obtain a time sequence of a live fluorescence specimen, it may be necessary to reduce the total exposure time to avid photobleaching & phototoxicity.
- This can be accomplished by:

  - Exposing the specimen less often to light **but at the expense of** lower temporal resolution.

  - Binning the image **but at the expense of** lower spatial resolution.

  - Applying a higher gain in the camera settings **but at the expense of** reduced S/N.

How to choose a camera?

- There are different types of imaging cameras available.
- Choosing a suitable camera is not an easy task.
- It depends on the application (and sometimes performance & budget trade-off limits the choices).

CCD cameras have been the standard for general microscopy applications for many years and will continue to be the main choice for a variety of applications from color imaging and fixed sample fluorescence to 'long-term imaging applications.

EMCCD cameras offer a better solution when imaging at very low light levels with speed, for example single molecule fluorescence applications.

CMOS cameras offer the advantage of high-speed operation.

Scientific CMOS is a new addition for high-speed microscopy applications (up to 300 fps). It also offers sensitivity, a larger field of view and lower noise.

Example Specifications: Different Cameras

	CCD Sony Interline	EM CCD	sCMOS
Sensor Format	1.4 MP	1 MP (max.)	5.5 MP
Pixel Size / µm	6.45	8-24	6.5
Frame Rate	12 fps @ 20 MHz	> 30 fps	100 fps
Read Noise/	4-8 e^-	Negligible < 1 e^-	1 e^- @ 30 fps 1.4 e^- @ 30 fps
QE	60 %	65 %- 90 %	57 %
Dynamic Range	3.000:1	8.500:1	25.000:1
Dark current	0.0003 e/pix/sec	0.001 e/pix/sec	0.07 e/pix/sec