Principles in Light Microscopy

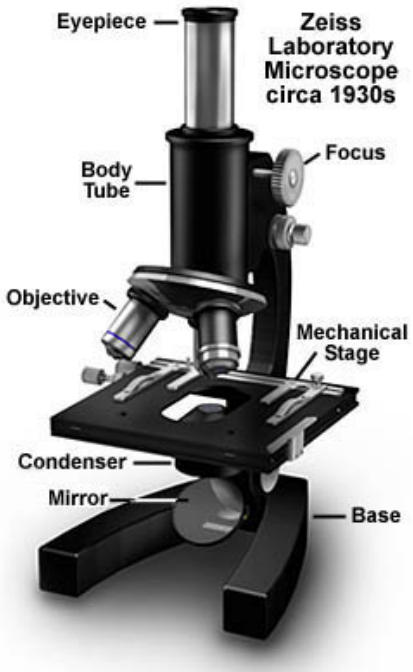


Figure 6

Lecture I. Technical Introduction
Lecture II. Applications

Microscopy Techniques

http://www.mcb.ucdavis.edu/faculty-labs/kaplan/

- Visible Light Microscopy: http://www.micro.magnet.fsu.edu/index.html
  - Optical microscopy (diascopic)
    - Illumination
    - Objective Lenses
    - Optical Aberrations
    - Contrast optics
  - Fluorescence microscopy (episcopic)
    - Excitation/Emission
    - Reflected Light; epifluorescence
    - Detectors
    - Confocal illumination
- Electron Microscopy
  - Cryo E.M.
- Atomic Force Microscopy

Diffracted Light and Resolution

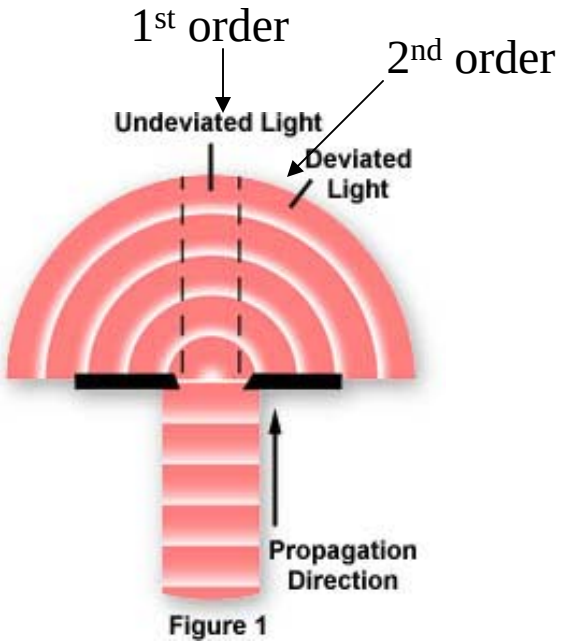


Figure 1

- Light passes unhindered and deviated (diffracted) through specimens.

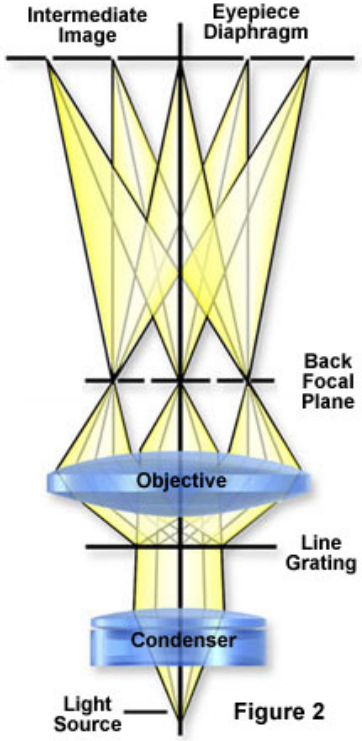


Figure 2

•The light is projected by the objective across the image plane.

•Destructive and constructive interference results in bright and dark areas.

d=1.22(λ/2NA)
d space between particles to be resolved
λ is wavelength of illumination light
NA is numerical aperture of lens (200nm for light microscopes)

•The greater number of higher diffracted orders admitted, the smaller the details that can be resolved.

Visible Light Microscopy: Kohler Illumination I

- Light must be uniform in intensity
  - Filament is focused on back focal plane of objective
- Light is focused with the field lens and reflected into the field diaphragm
  - Field diaphragm controls the width of the light beam
  - Centered and just outside the field of view: too closed reduces resolution (glare)
- Substage condenser is the most critical adjustment to be made
  - Centered and focused
  - Cone of light determines the numerical aperture

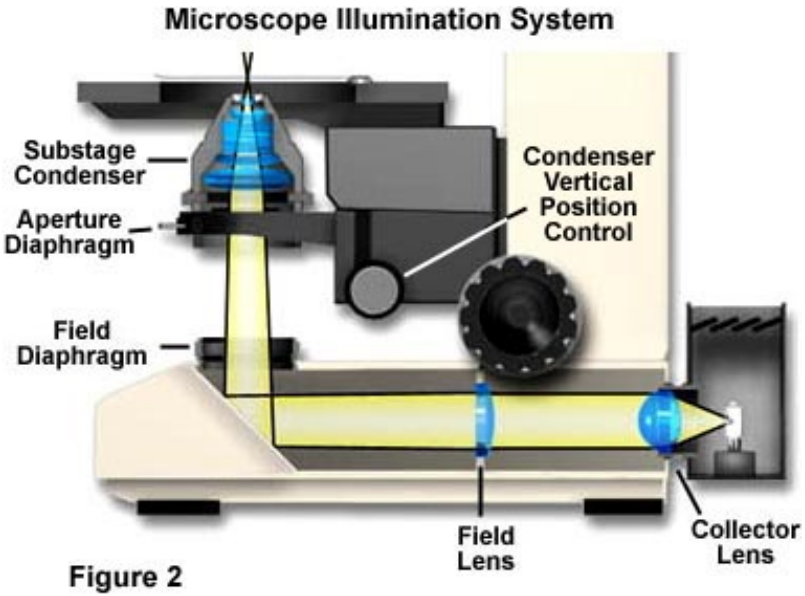


Figure 2

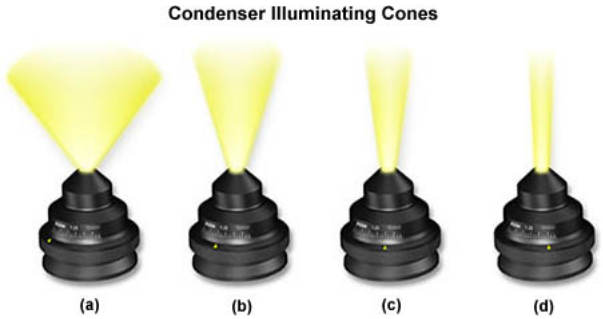


Figure 3

Visible Light Microscopy
Kohler Illumination II

- Even illumination on sample and eliminates imperfections in light path from being focused on sample.
- Conjugate Illuminating Planes
  - Lamp filament
  - Condenser aperture
  - Back focal plane of objective
  - Eyepoint of the eyepiece
- Conjugate image forming planes
  - Field diaphragm
  - Focused specimen
  - Intermediate image plane (eyepiece)
  - Retina or detector
- Can use conjugate planes to locate source of visual imperfections

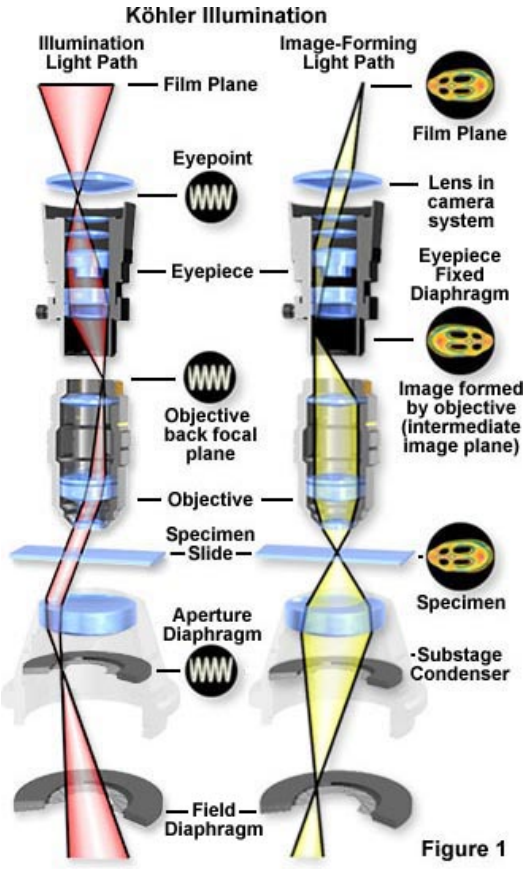
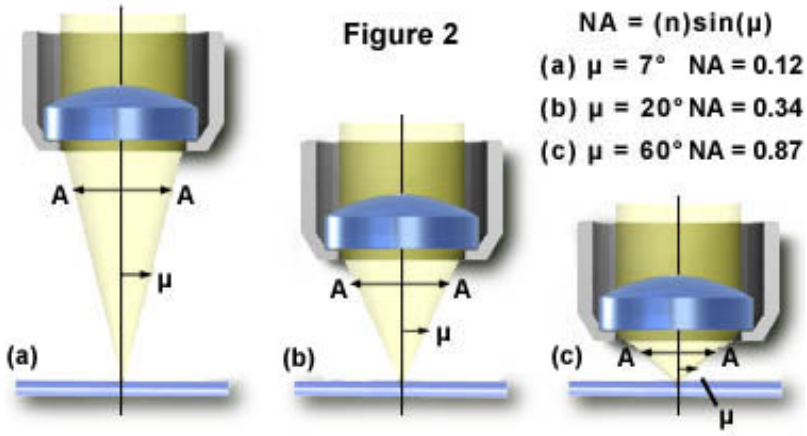


Figure 1

Visible Light Microscopy: Objectives: numerical aperture

- NA=ability of lens to gather light and resolve detail at a fixed distance from object.
  - Dependent on ability of lens to capture diffracted light rays.
- n=Refractive index is limiting (air=1.0, oil=1.51)
  - Do not mix mediums when using a lens
- Theoretical resolution depends on NA and the wavelength of light. NA=n·sin(μ)
  - Shorter wavelengths=higher resolution.
  - Resolution limit for green light (NA=1.4, 100X) is 0.2 μm.
    - R=0.61λ/NA



http://www.micro.magnet.fsu.edu/primer/java/microscopy/immersion/index.html

Visible Light Microscopy
Objectives: Specifications and Identification

- Older lenses need to match oculars, now lenses are infinity-corrected.
- Information on objective barrel:
  - Linear magnification
  - Numerical aperture
  - Optical corrections
    - Achromatic: color (red/blue) corrected.
    - Fluorite: optical aberration corrected
    - Apochromatic: color (red, green, blue and spherical aberration corrected
  - Microscope tube length
  - Coverglass thickness (0.17mm)
  - Immersion medium (air, water, oil)

Figure 1

Visual Light Microscopy
Contrast Optics:

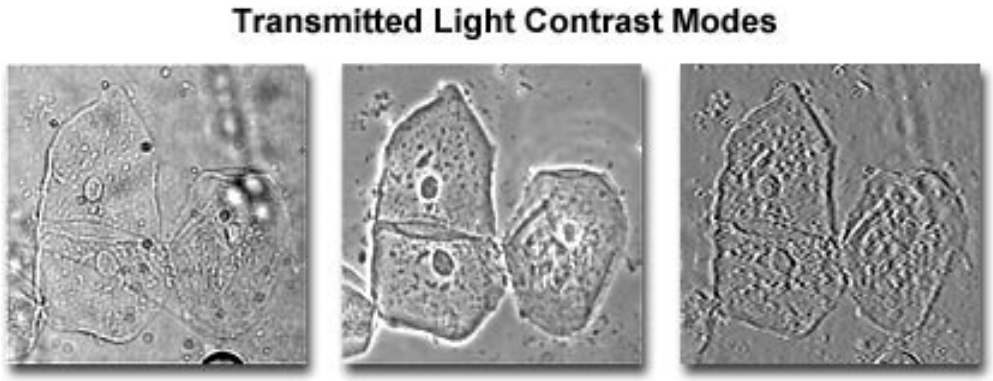


Figure 2

Brightfield Phase Hoffman modulation Contrast

- Contrast is the difference in light intensity between the image and the adjacent background relative to the overall background intensity.
- Objects fall into three categories:
  - Amplitude (absorb light partially or completely; naturally colored or stained)
  - Phase (do not absorb light; most cells)
  - Reflected (do not pass light; thick samples)

Visual Light Microscopy
Contrast Optics: Phase

- Phase specimens diffract light because of their refractive index or thickness (or both) causing light to lag behind approximately ¼ wavelength and arrives at image plane “out of step/phase” but with no change in intensity.
- Speed up direct light by ¼ step, resulting in a ½ wavelength. This results in destructive interference, ie. Darkness at edges of refractive sample.

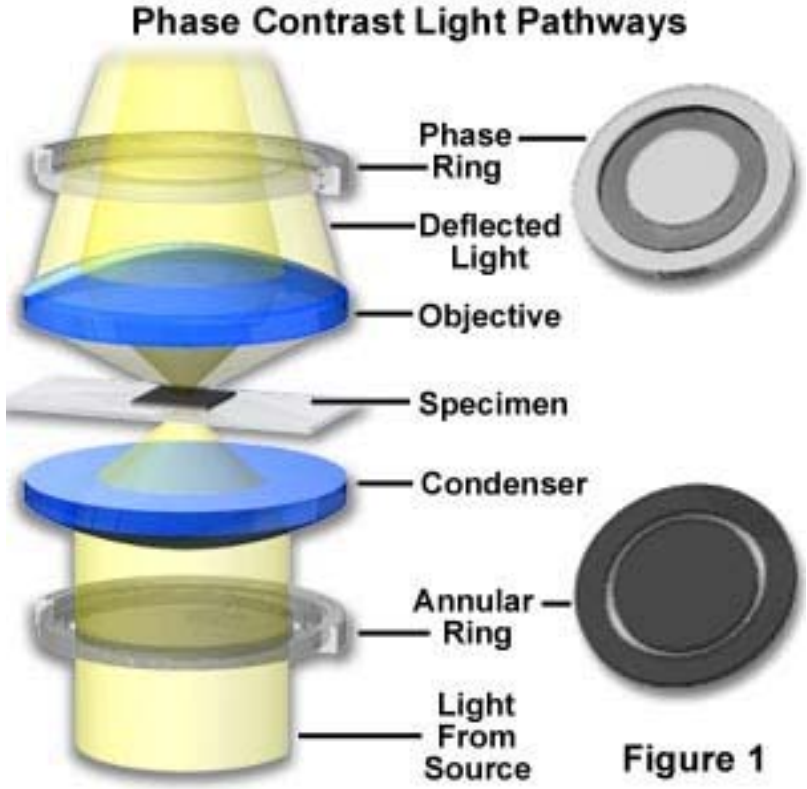


Figure 1

Visual Light Microscopy
Contrast Optics: DIC

- Plane polarized light is split into two rays (Wollaston prism I).
- Rays pass through condenser and travel parallel through specimen.
- The thickness/refractive index of the specimen changes the wave path of the two rays.
- Rays are focused on the rear focal plane of the objective where they are recombined by a second prism.
- The optical path differences lead to interference when the beams are recombined by a second polarizer.
- Interference gives rise to “shadows” and a pseudo-three dimensional appearance.

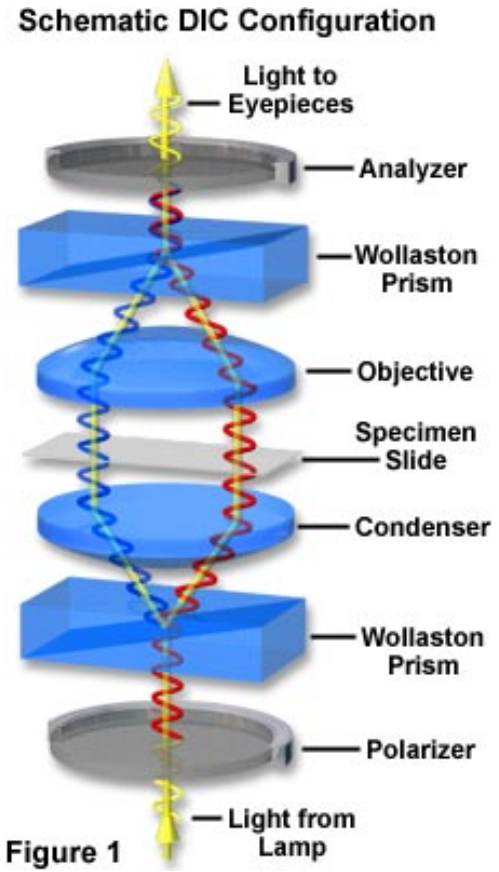


Figure 1

Kohler Illumination: Reflected Light used in epifluorescence microscopy

- Objective serves as both condenser and collector; no need to adjust the NA when changing objectives.
- Aperture diaphragm controls the angle of light reaching the specimen (60-95% open; sample dependent).
- Focus on Field diaphragm and center light source.

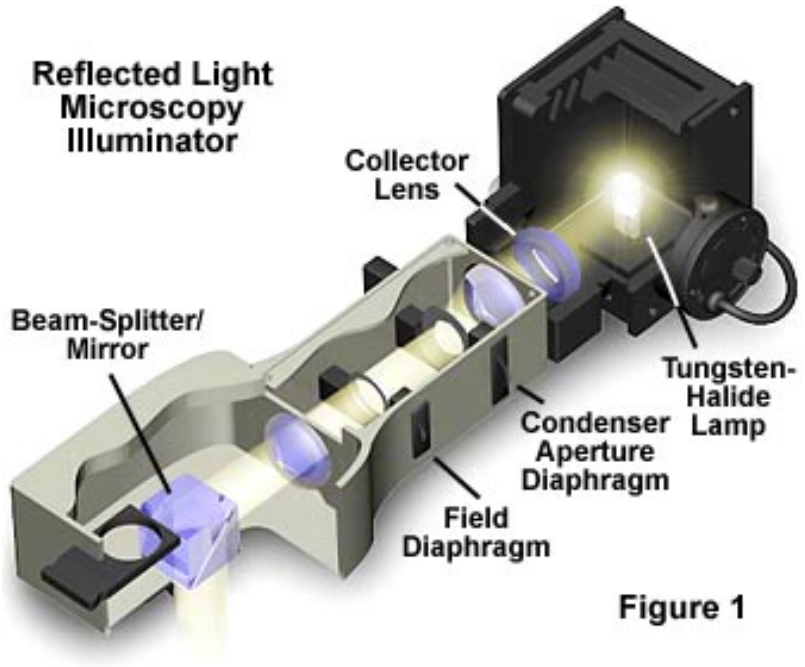


Figure 1

Fluorescence Microscopy: Excitation/Emission

- Goal is to illuminate specimen with an excitation wavelength, to capture emitted light and block reflected light.
- Fluorochromes have a peak excitation and a peak emission but often overlap.
- Fading of fluorescence:
  - Quenching
    - Transfer of energy to other acceptor molecules
    - Oxidizing agents, salts, heavy metals
  - Dependent on oxygen in sample
    - Use oxygen scavengers in mounting medium (1% n-propylgallate and others)

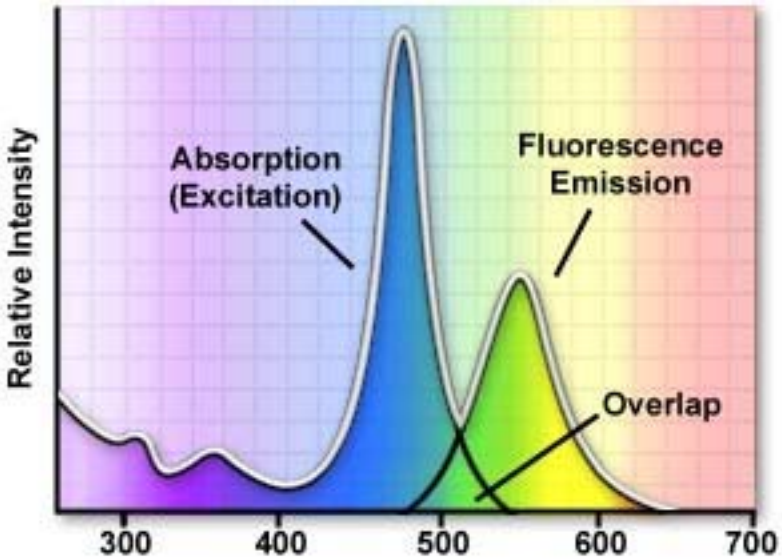


Figure 2

Fluorescence Microscopy: Filter Cubes

- Excitation filters
  - Permit only selected wavelengths of light through to the specimen
- Barrier Filters (emission)
  - Block/absorb excitation wavelengths and permit only selected emission wavelengths to pass toward the detector.
- Dichroic Filters (mirror)
  - Reflect excitation wavelengths and pass emission wavelengths

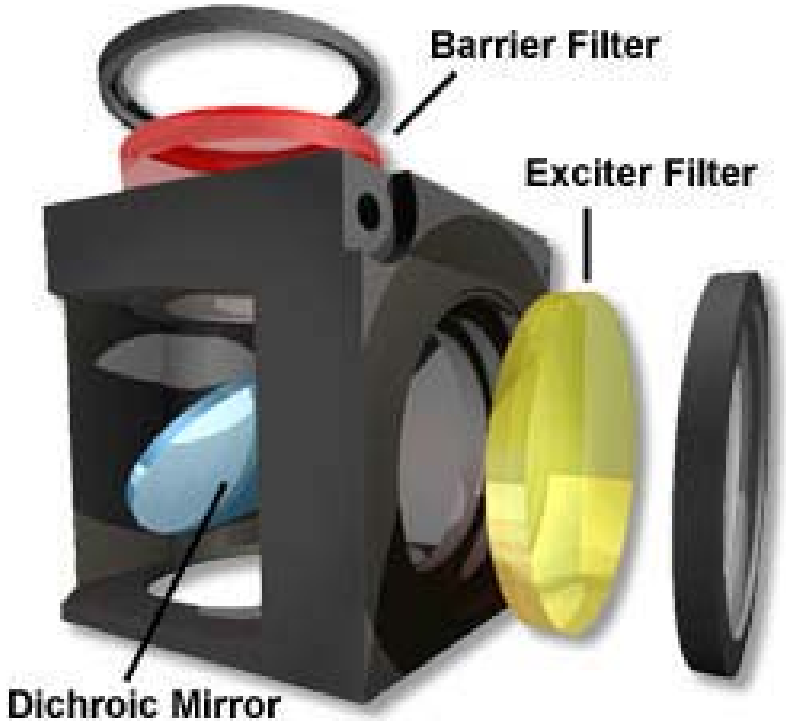


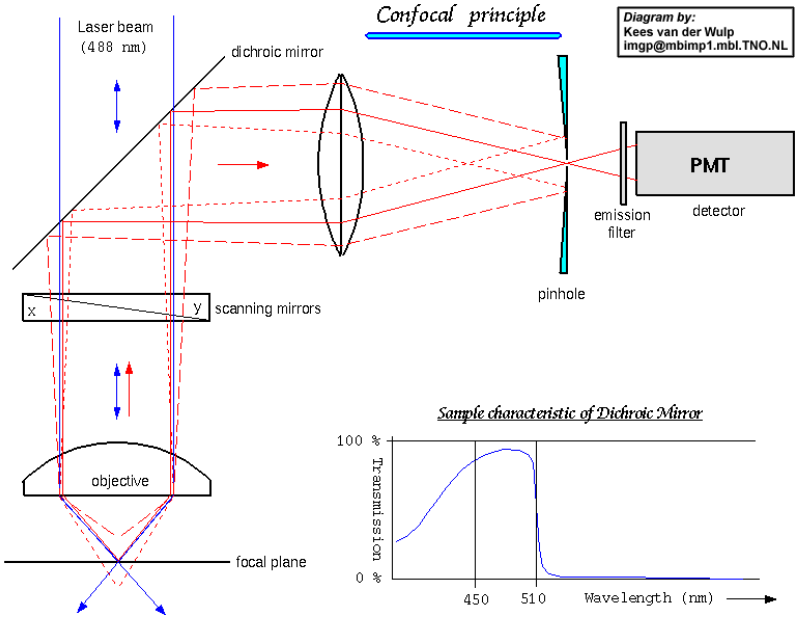
Figure 1

Fluorescent microscopy issues

- Fluorescence markers
  - Antibodies
    - Specificity
    - Availability
  - GFP variants:
    - Functional fusion
- Specimen preparation:
  - Fixation conditions
  - Thick samples
  - Photo-damage of living cells
- Detection: (single molecule visualization)
  - Fast acquisition
  - Sensitivity

Fluorescence Microscopy: Confocal

- Laser light and filters are used to excite fluorophores.
- Light is raster-scanned using galvanomotor controlled mirrors.
- Reflected and fluorescent light is captured by objective.
- A confocal aperture (pinhole) in front of the detector obstructs light from out of focus parts of the specimen.
- Light is detected by PMT.
- Good for thick specimens where there are large amounts of out-of-focus information.



Fluorescence Microscopy: PMT Detectors

- PMT-photomultiplier tube
  - Cannot resolve spatial information
  - Respond to changes in input light fluxes
  - Amplify signals
  - Extremely fast recording times
    - Important for scanning mode of a confocal
  - Low noise
  - Large dynamic range
  - Only detect about 1/3 of the incident light.

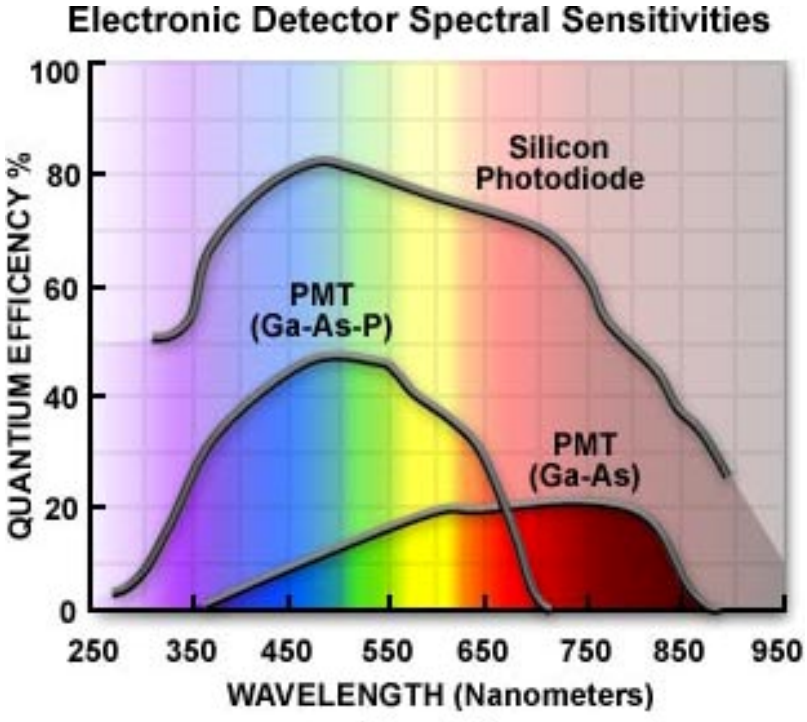


Figure 3

Fluorescence Microscopy: CCD Detectors

- Area detectors, solid state detectors, charge-couple device (CCD)
  - Matrix of photodiodes
  - Stores and transfers light information
  - High efficiency
  - Parameters:
    - Spectral issues
    - Acquisition time
    - Noise (dark)

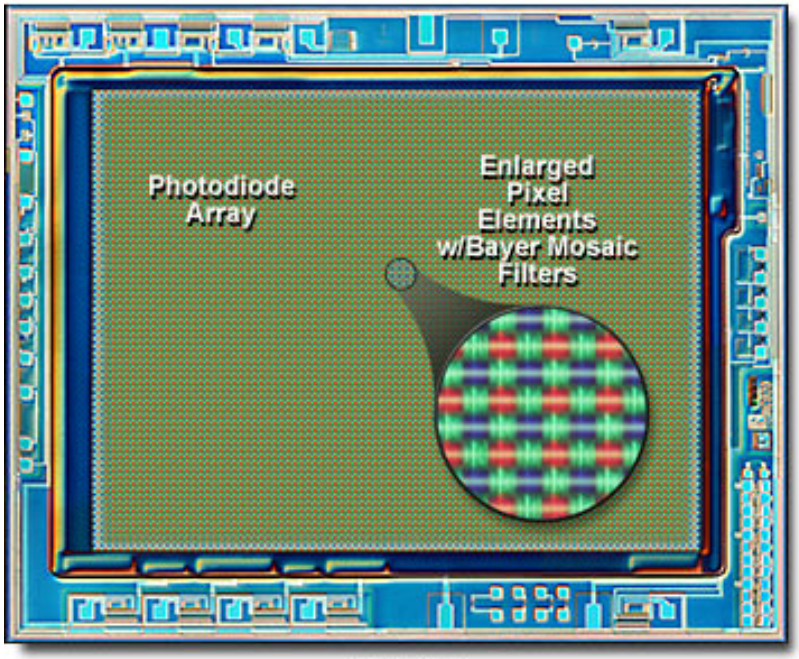


Figure 2

Fluorescence Microscopy: CCD Detectors

- Each Pixel: 2 to 4 wells/pixel:
  - Sensitivity
    - Maximum number of electrons/well determines upper limit of dynamic range
    - Electrons in well before photons is the low end of the dynamic range (T^{o}C dependent)
  - Speed
    - Transfer of electrons to the edge of the chip accomplished before the next intergration
      - Parallel transfer to one row at a time (clock speed limited)
      - Serial transfer to amplifier

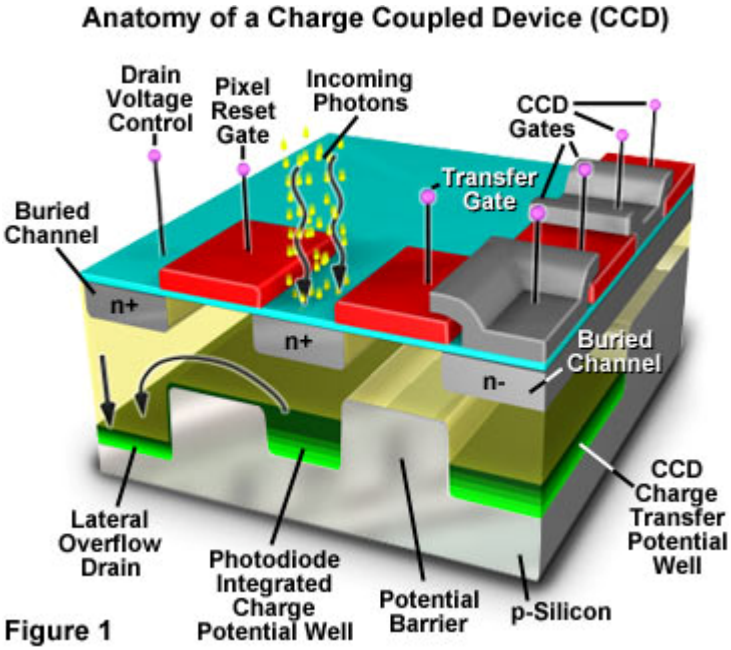


Figure 1

Fluorescence Microscopy: CCD Detectors

- CCD characteristics:
  - Dynamic range
  - Quantum efficiency
  - Signal:Noise
  - Linearity
- CCD formats
  - Shutters:
    - Bright images, fast acquisition
  - Clocking:
    - Gates/pixel-
      - Density of wells/chip
      - Readout speed
      - Efficiency of electron transfer
  - Frame architecture
    - Full frame
    - Interline (fast)

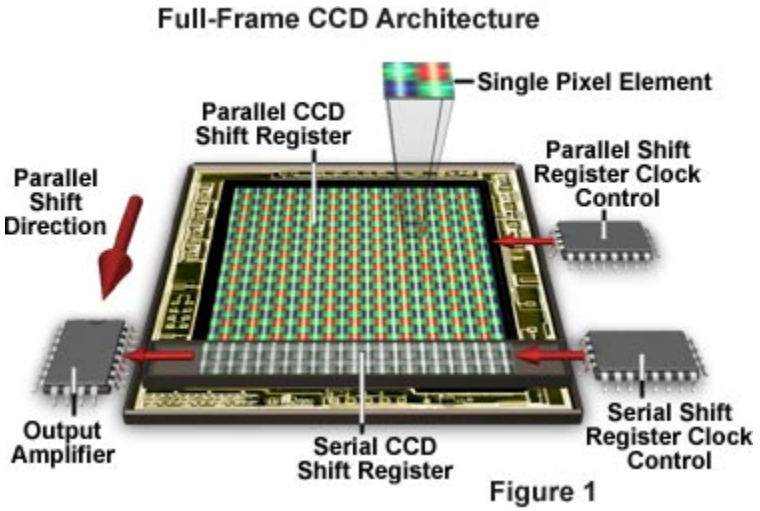


Figure 1

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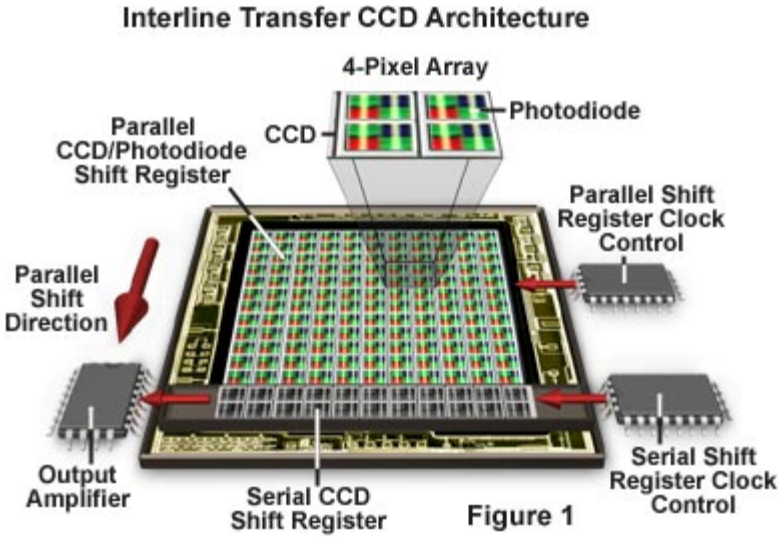
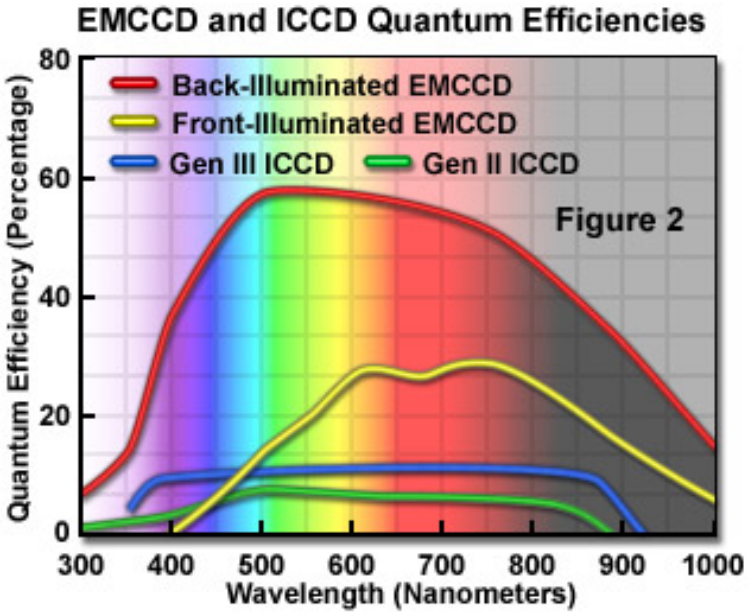
  - Shutters:
    - Bright images, fast acquisition
  - Clocking:
    - Gates/pixel-
      - Density of wells/chip
      - Readout speed
      - Efficiency of electron transfer
  - Frame architecture
    - Full frame (cheaper)
    - Interline (fast, less sensitive)
  - Electron multiplying or charged multiplying CCD
    - Extended serial register amplifies “independently” of read noise



Fluorescence Microscopy: CCD Detectors

- Companies:
  - Roper Scientific, Inc. (Photometrics)
  - SI Photonics
  - Chips
    - Sony
    - Kodak
- Speed vs. sensitivity
- Price vs. function
- Test drive models



Fluorescence Microscopy: Spinning-disc confocal

- Nipkow spinning disk (very fast)
  - Pinholes (20,000) pass light into multiple beams
  - Micro-lenses helps to capture light from sample.
- Laser illumination
- No raster-scanning allows CCD
- Low light intensity
  - Prevents photo-bleaching
  - Reduces photo-toxicity

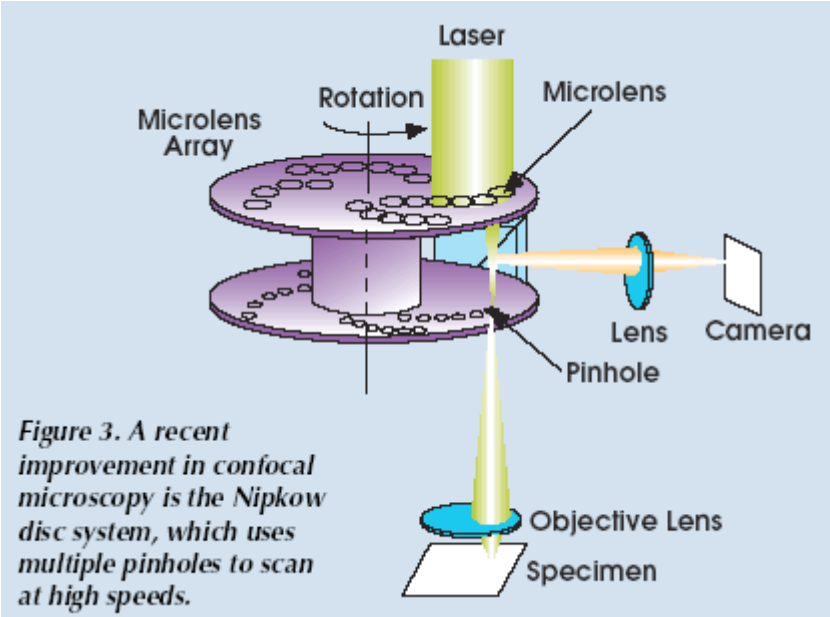
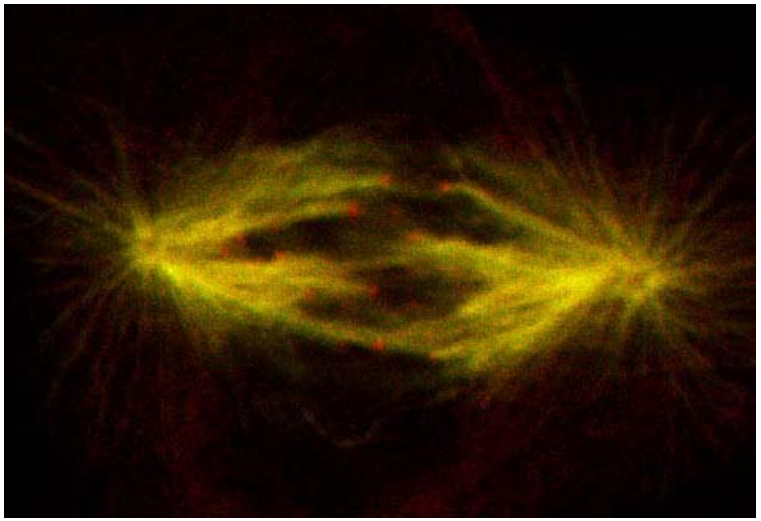


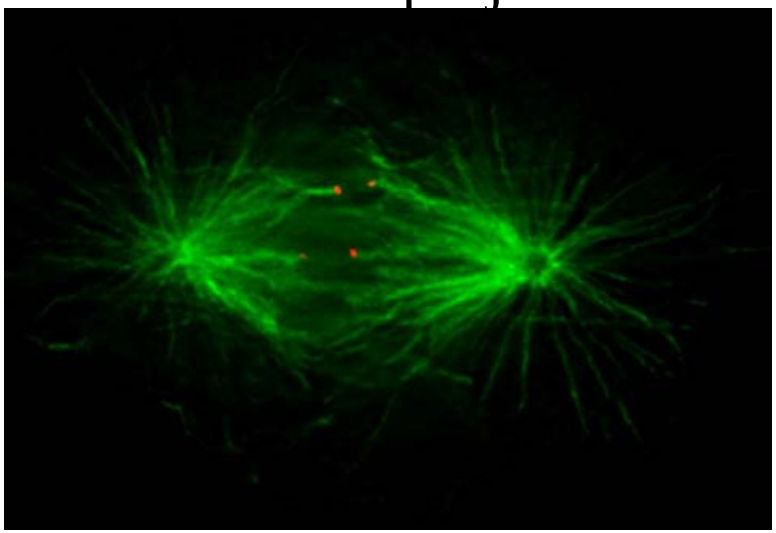
Figure 3. A recent improvement in confocal microscopy is the Nipkow disc system, which uses multiple pinholes to scan at high speeds.

Fluorescence Microscopy: Deconvolution

- Hg-lamp illumination
  - Good for live imaging
  - Minimal bleaching
  - Standard filter cubes (340nm-700nm)
- Fast acquisition CCD
  - 10X increased linear range compared to confocal
- Deconvolution algorithm
  - Removes out of focus light
  - High resolution observed (<0.2μm)
- Motorized stage
  - Collect optical sections (0.2μm).



Confocal projection



Deconvolved projection

Total Internal Reflection Fluorescence Microscopy

- Laser light focused by mirror into prism
- Angle of light adjusted to generate the evanescent wave
- Wide field can also be used
- Fluorescence emitted is collected by CCD
- Excellent for live-cell imaging
  - Reduces photo damage
  - Eliminates out-of-focus excitation of fluorophores

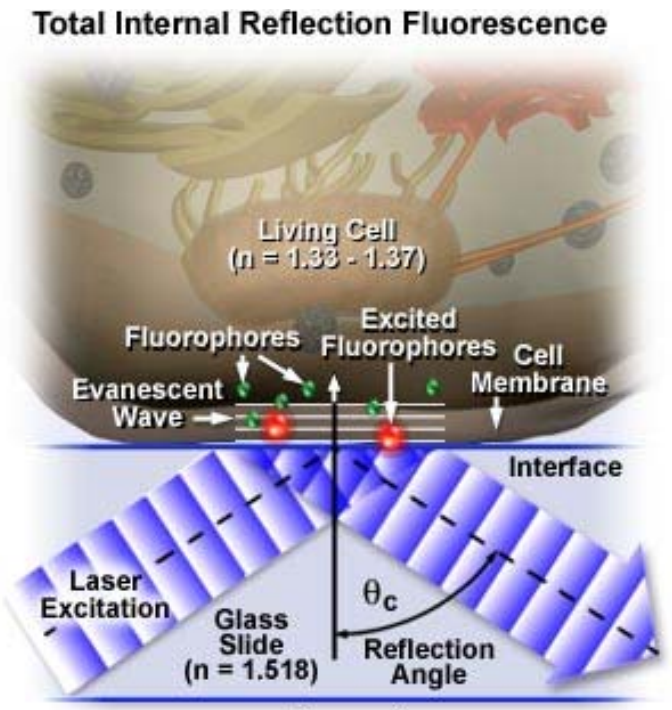
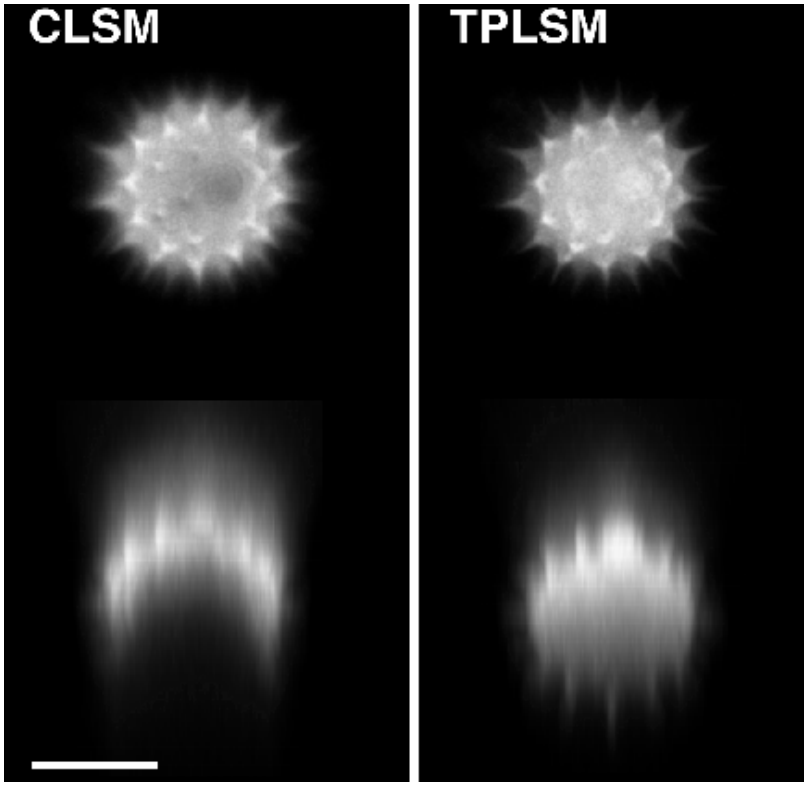


Figure 1

Fluorescence Microscopy: Two Photon Confocal

- Chromophore is excited by two lower-energy photons (infrared).
  - Low energy reduces photobleaching and phototoxicity
- Nonlinear behavior of the incident light intensity.
  - Only dye molecules very near focus of beam are excited
  - Low levels of heating prevents sample damage, allowing live imaging.



Summary

- Kohler illumination is critical for good microscopy.
  - Formation of several conjugate planes of light so that the filament is focused at the field diaphragm and the sample is focused by the ocular. Separates imperfections in bulb and lens so that they are not in focus with the sample.
- Choose objective to match application
  - Correction for color and spherical nature of lenses
- Contrast Optics
  - Phase
  - DIC
  - Hoffman contrast
- Fluorescence Microscopy
  - Filter sets critical for optimum signal/noise ratio
  - Confocal vs. deconvolution
  - Two photon