Principles in Light Microscopy
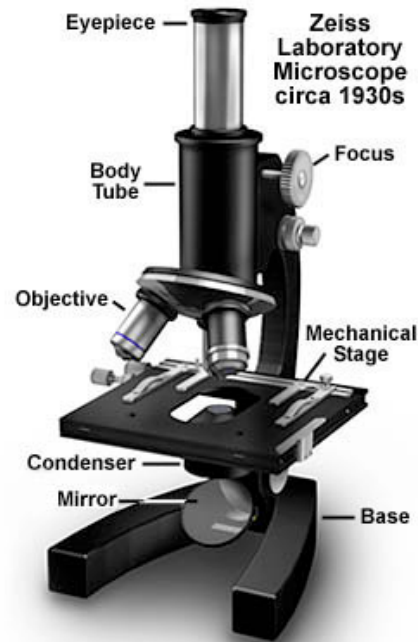

Lecture I. Technical Introduction
Lecture II. Applications

Microscopy Techniques

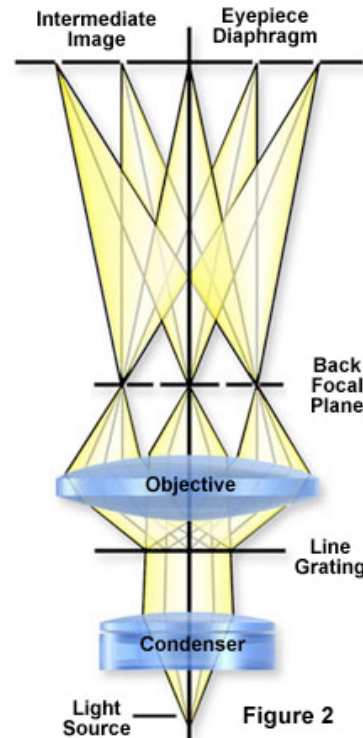http://www.mcb.ucdavis.edu/faculty-labs/kaplan/

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

Diffracted Light and Resolution

1st order

2nd order

Undeviated Light

Deviated Light

Intermediate Image

Eyepiece Diaphragm

Back Focal Plane

Objective

Line Grating

Condenser

Light Source

Figure 2

Propagation Direction

Figure 1

$d=1.22(\lambda/2NA)$

d space between particles to be resolved

λ is wavelength of illumination light

NA is numerical aperture of lens (200nm for light microscopes)
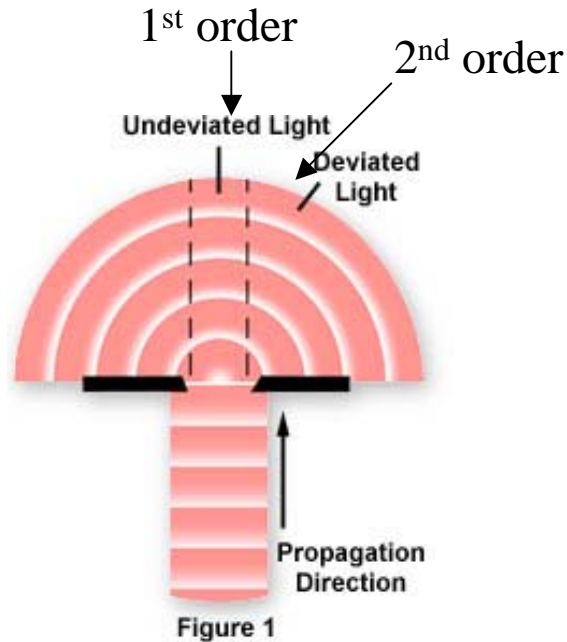
- Light passes unhindered and deviated (diffracted) through specimens.

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

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

- 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 open, scattered light degrades image
- Substage condenser is the most critical adjustment to be made
  - Centered and focused
  - Cone of light determines the numerical aperture
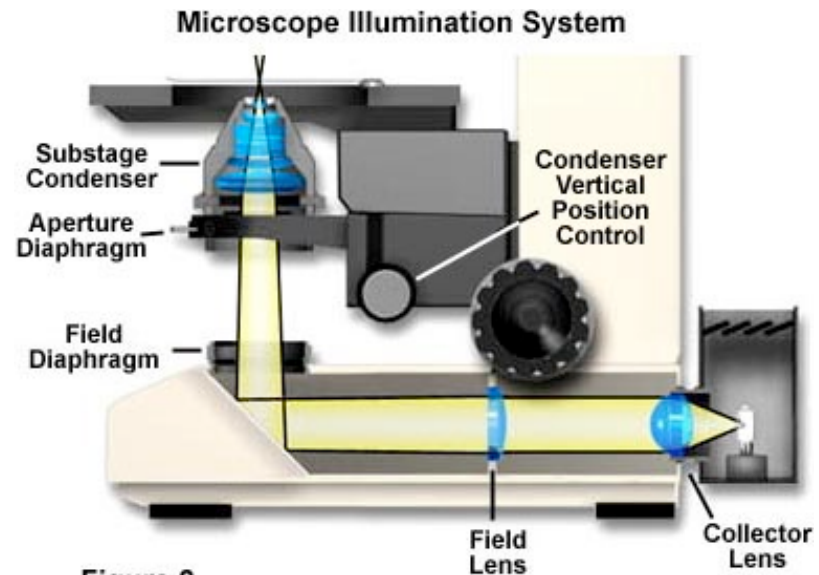

Microscope Illumination System

Substage Condenser
Aperture Diaphragm
Field Diaphragm
Condenser Vertical Position Control
Field Lens
Collector Lens

Figure 2

Condenser Illuminating Cones
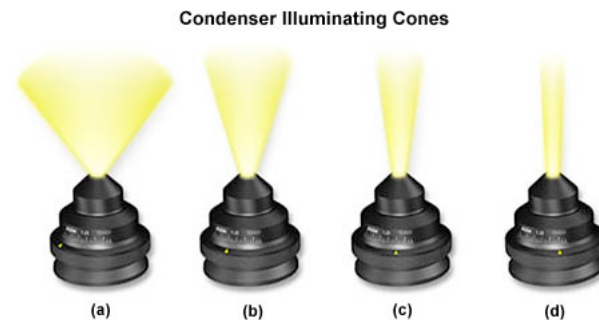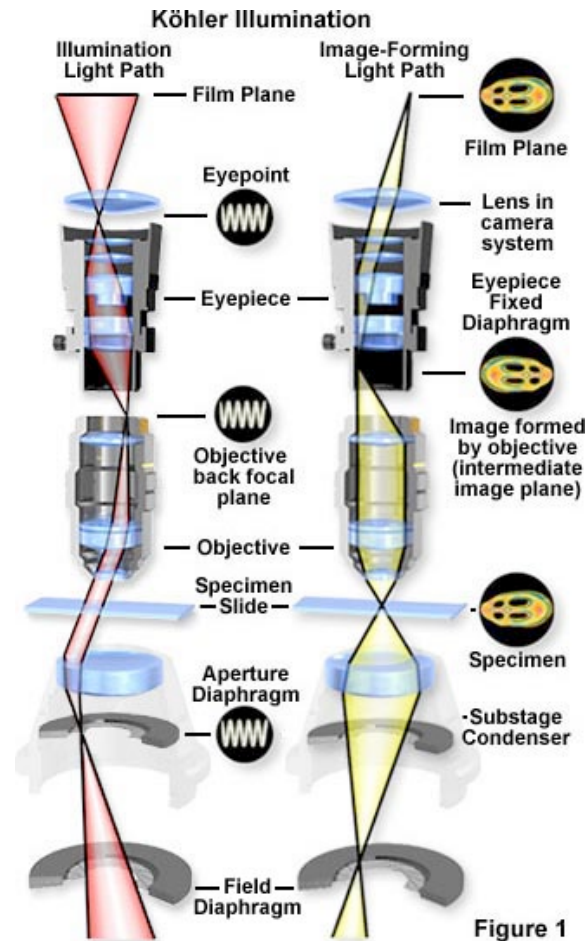
(a)   (b)   (c)   (d)

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

Köhler Illumination

Illumination Light Path | Image-Forming Light Path
- Film Plane
- Eyepoint
- Eyepiece
- Objective back focal plane
- Objective
- Specimen Slide
- Aperture Diaphragm
- Field Diaphragm

Film Plane
Lens in camera system
Eyepiece Fixed Diaphragm
Image formed by objective (intermediate image plane)
Specimen
Substage Condenser

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

Figure 2

NA = (n)sin(μ)
(a) μ = 7°   NA = 0.12
(b) μ = 20° NA = 0.34
(c) μ = 60° NA = 0.87
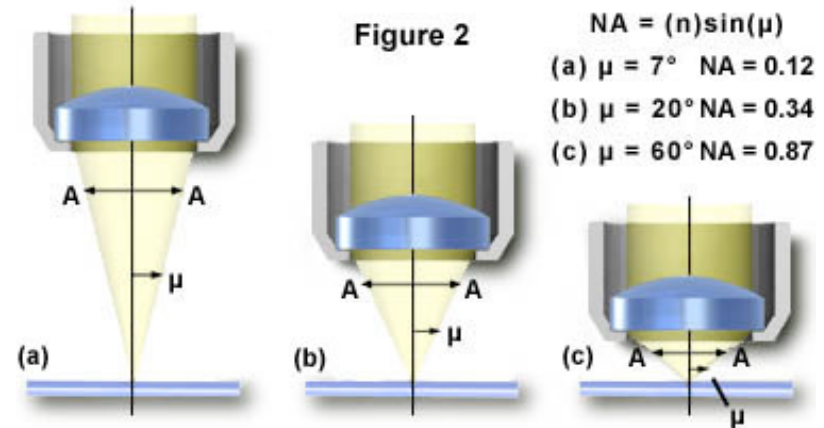
Visible Light Microscopy
Objectives: Specifications and Identification

- 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)

60x Plan Apochromat Objective

Screw Thread

Manufacturer

Flat-Field Correction

Aberration Correction

Numerical Aperture

Linear Magnification

Nippon JAPAN
Plan Apo
60X/1.40 Oil
DIC H
∞/0.17 WD 0.21

Immersion Medium

Specialized Optical Properties

Tube Length

Working Distance

Cover Slip Thickness

Color Code

Spring-Loaded Front Lens

Figure 1

Visual Light Microscopy Contrast Optics:

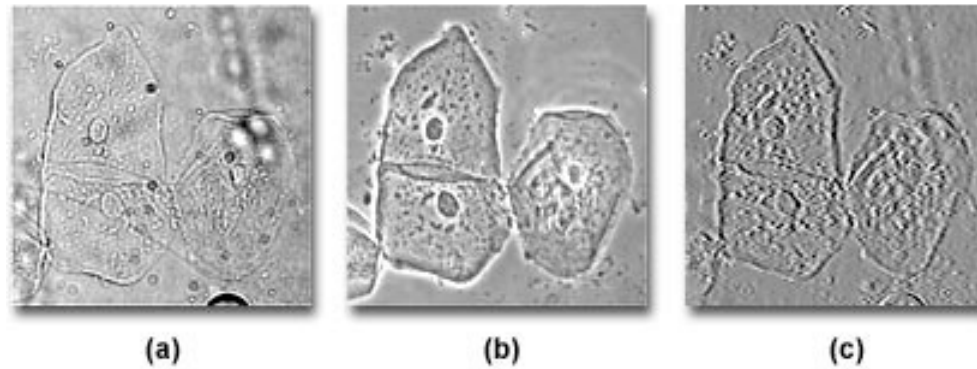**Transmitted Light Contrast Modes**
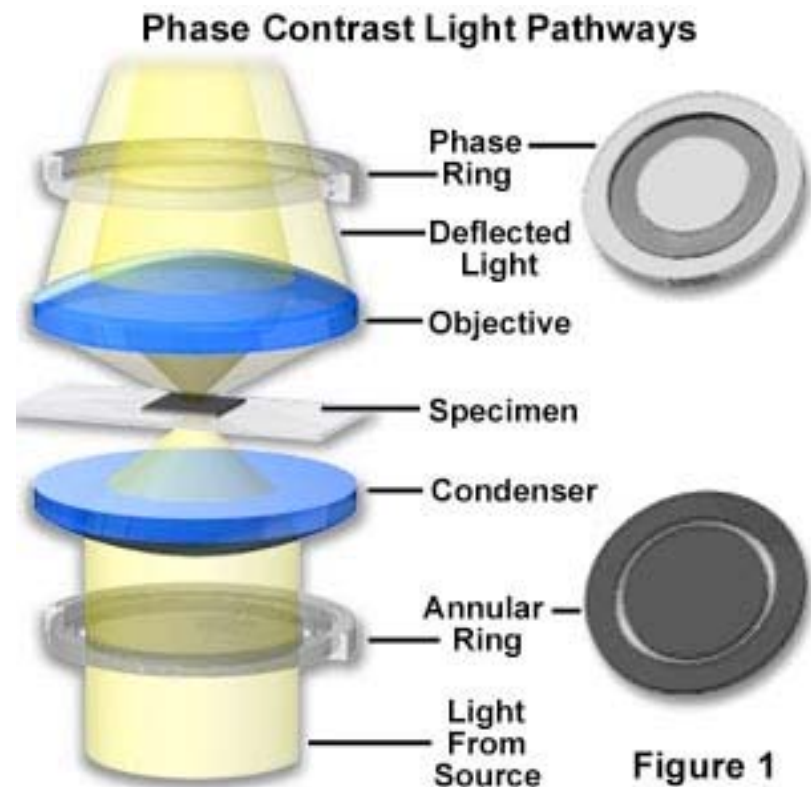

(a) (b) (c)

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)
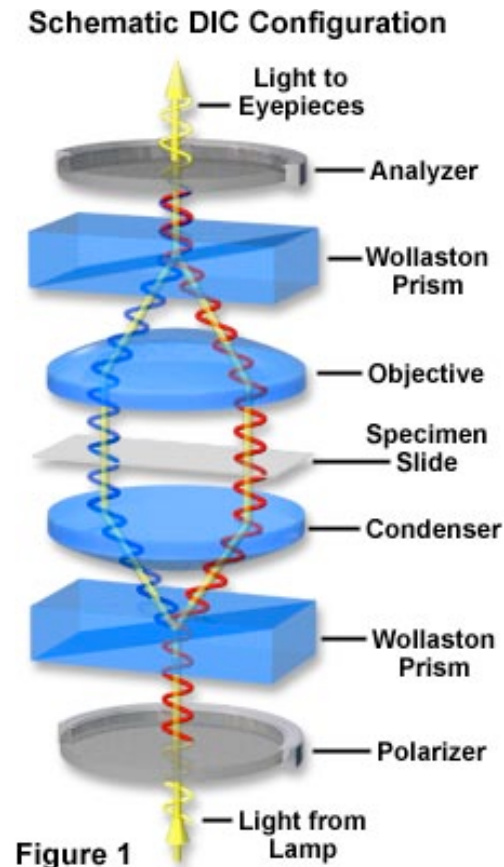
Visual Light Microscopy Contrast Optics: Phase

- 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 (surround or background) by ¼ step, resulting in a ½ wavelength difference with diffracted light. This results in destructive interference, ie. Darkness at edges of refractive sample.

Phase Contrast Light Pathways

Phase Ring — Deflected Light — Objective — Specimen — Condenser — Annular Ring — Light From Source

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.

Schematic DIC Configuration

Light to Eyepieces
Analyzer
Wollaston Prism
Objective
Specimen Slide
Condenser
Wollaston Prism
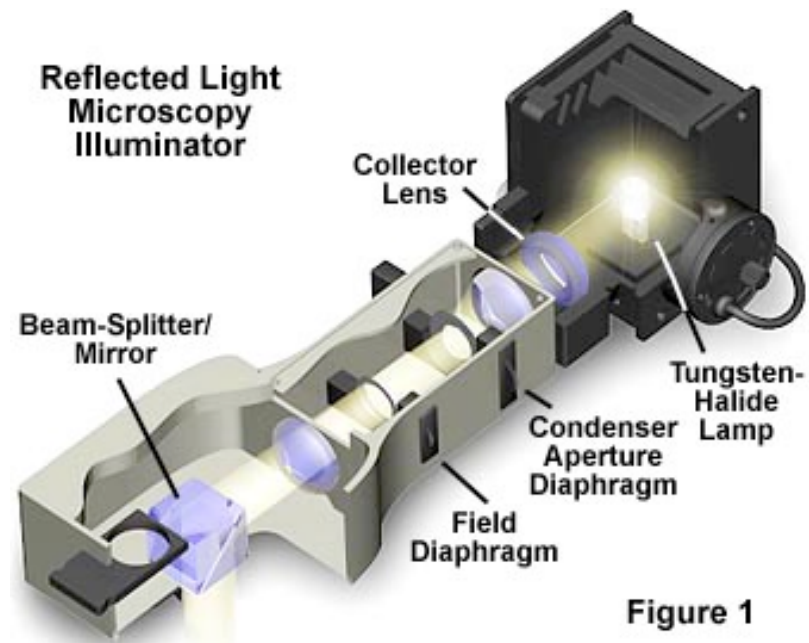Polarizer
Light from Lamp

Figure 1

Comparison of Phase and DIC

- Halos can obscure cell information in phase.
  - Large changes in refractive index give phase halos
- Application should fit your needs:
  - Phase:
    - Lower resolution
    - cheaper
  - DIC:
    - Higher resolution possible

Transparent Specimens in Phase Contrast and DIC

(a)   (c)   (e)

(b)   (d)   Figure 1   (f)
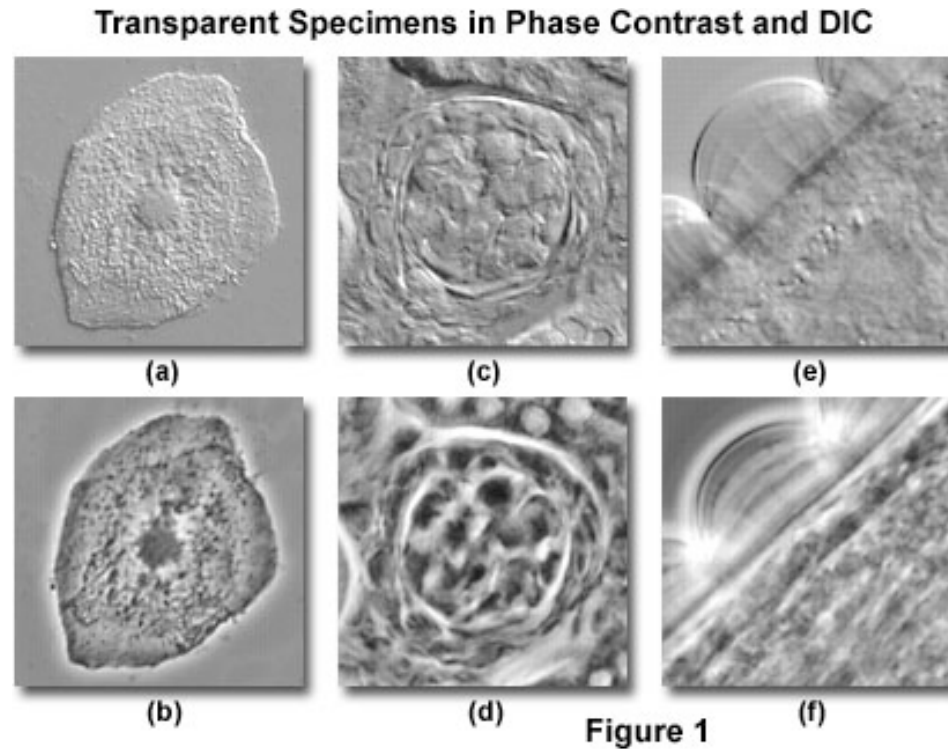
Kohler Illumination: Reflected Light used in epifluorescence microscopy

- 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.

Reflected Light Microscopy Illuminator

Collector Lens

Beam-Splitter/ Mirror

Tungsten-Halide Lamp

Condenser Aperture Diaphragm

Field Diaphragm

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
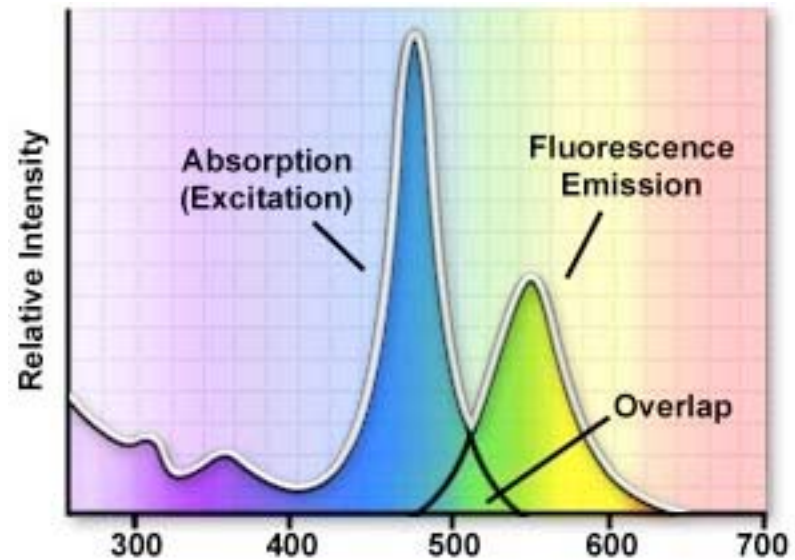   - Use oxygen scavengers in mounting medium (1% n-propylgallate and others)

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

Barrier Filter

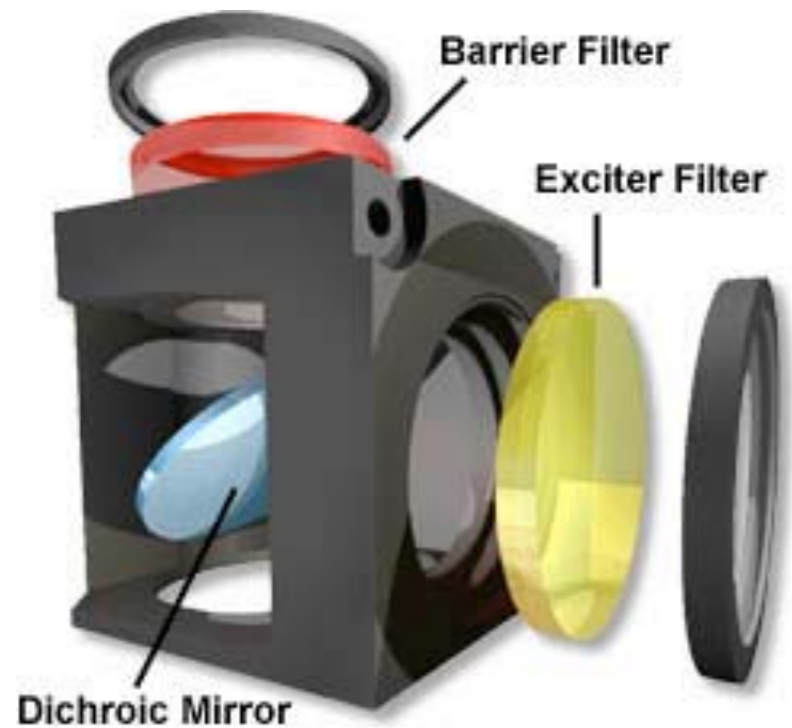Exciter Filter

Dichroic Mirror

Figure 1

Fluorescent microscopy issues

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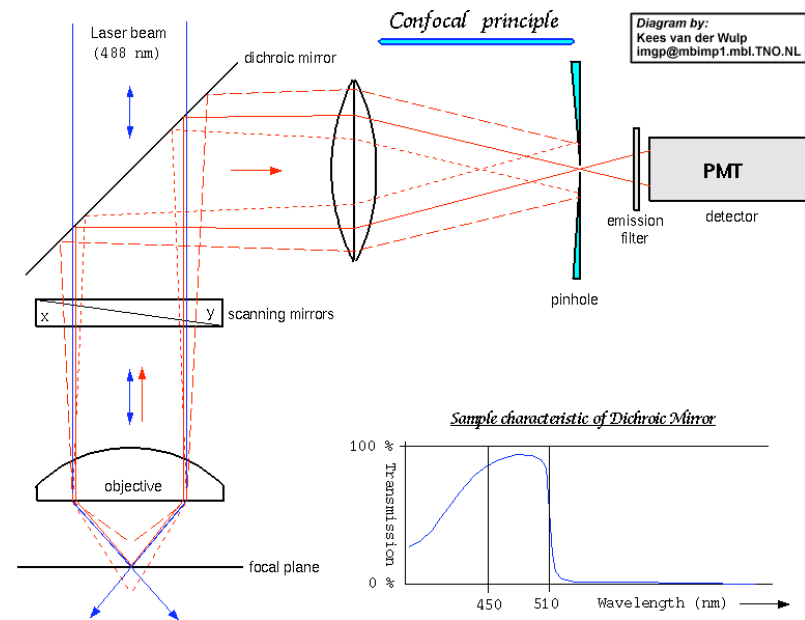 - Fast acquisition
  - Sensitivity

GFP Variants:

Company	Fluorescent proteins available				
	Blue, Cyan	Green	Yellow	Red	Photoactivatable
Amaxa (www.amaxa.com)		pmaxFP-Green[a]	pmaxFP-Yellow[a]	pmaxFP-Red[a]	
BD Biosciences Clontech (www.clontech.com)	AmCyan1	AcGFP1 ZsGreen1	ZsYellow1	DsRed2 DsRed-Express DsRed-Monomer Timer AsRed2 HcRed1	
Evrogen (www.evrogen.com)	PS-CFP2	TurboGFP	phiYFP	JRed	KFP-Red PS-CFP2
Invitrogen (www.invitrogen.com)	BFP CFP	EmGFP	YFP		
Lux Biotechnology (www.luxbiotech.com)		RmGFP PtGFP RrGFP			
MBL International (www.mblintl.com)	Midoriishi-Cyan	Azami Green		Kusabira-Orange	Dronpa Green Kaede KikGR
NanoLight Technology (www.nanolight.com)		RmGFP PtGFP RrGFP			
Promega (www.promega.com)		Monster Green			
Stratagene (www.stratagene.com)		hrGFP			

[a]pmaxFP-Green, pmaxFP-Yellow, and pmaxFP-Red are other names of TurboGFP, phiYFP, and JRed proteins, respectively.

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.

Confocal principle

Diagram by:
Kees van der Wulp
imgp@mbimp1.mbl.TNO.NL

Laser beam (488 nm)

dichroic mirror

PMT

emission filter   detector

pinhole

x   y   scanning mirrors

objective

focal plane

Sample characteristic of Dichroic Mirror

Transmission

100 %

0 %

450   510   Wavelength (nm)

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
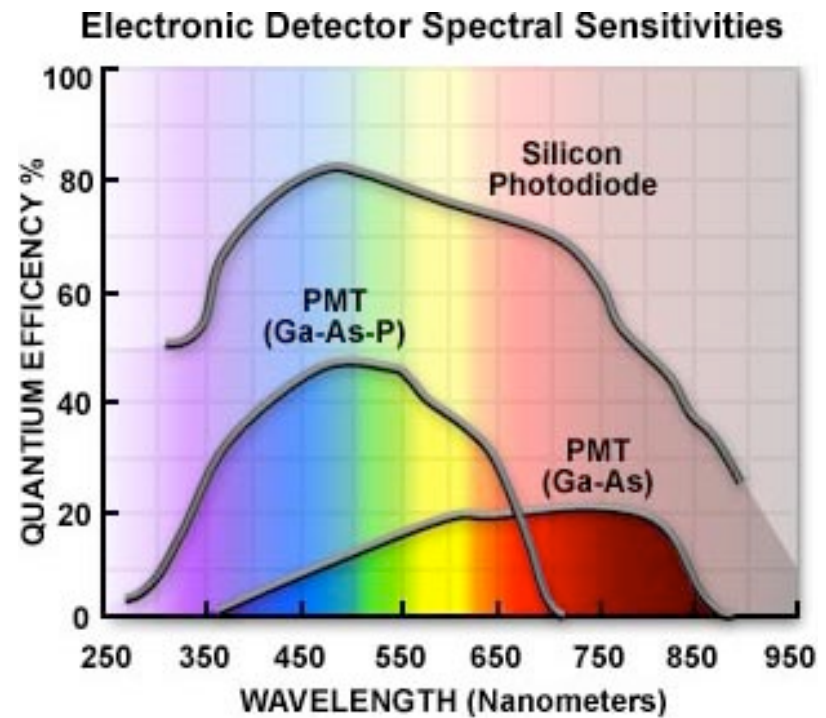 - Only detect about 1/3 of the incident light.

Electronic Detector Spectral Sensitivities

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
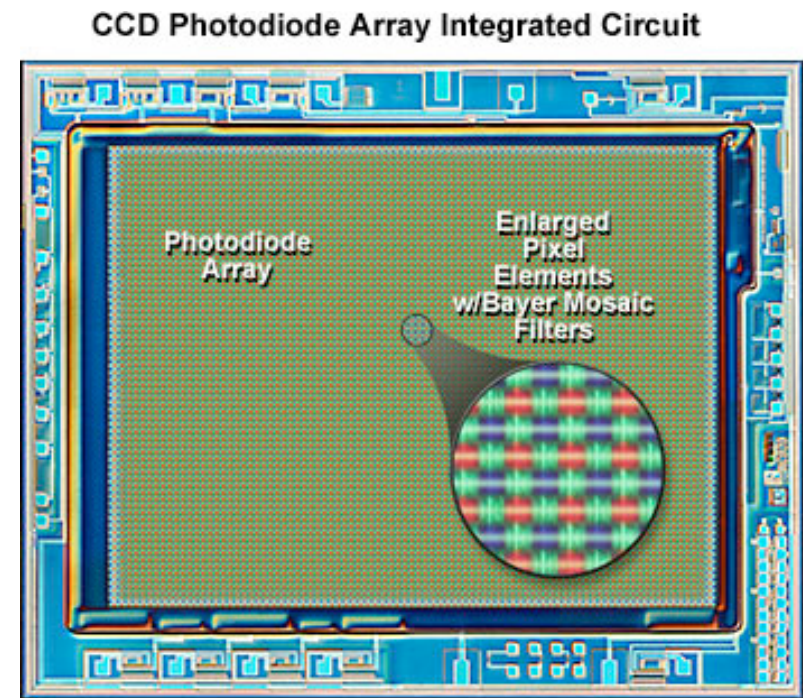   - Acquisition time
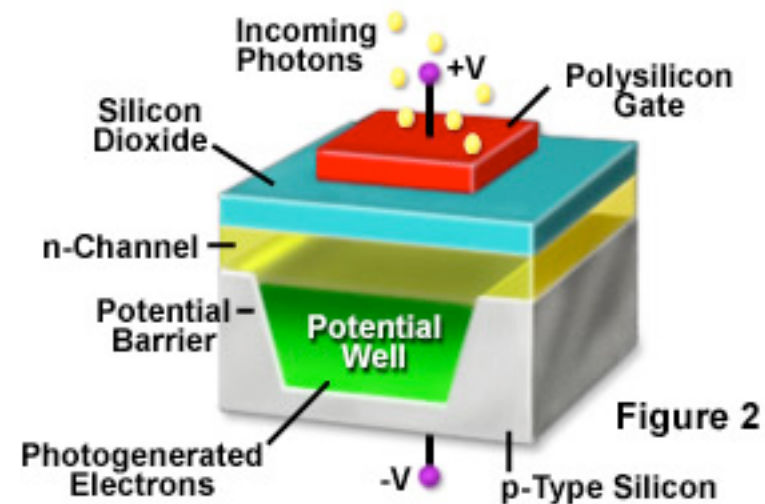   - Noise (read and dark)

CCD Photodiode Array Integrated Circuit

Photodiode Array

Enlarged Pixel Elements w/Bayer Mosaic Filters

Figure 2

Fluorescence Microscopy:
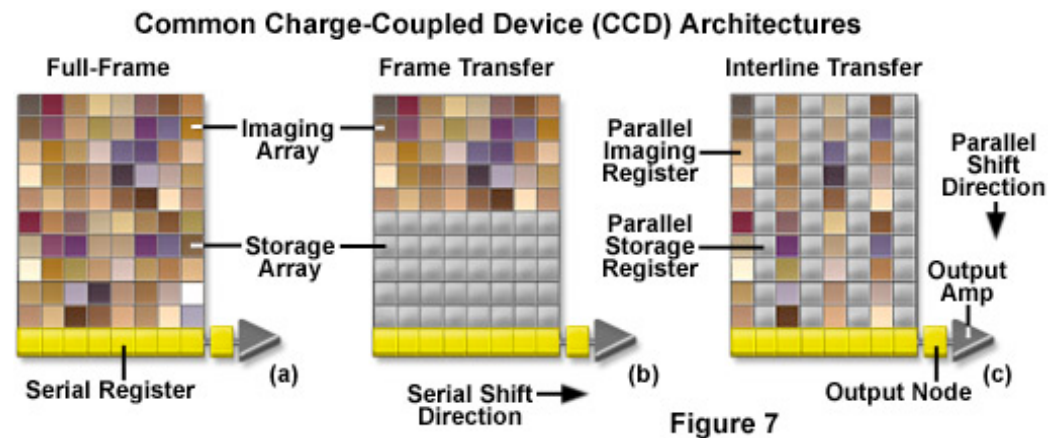# CCD Detectors

- Each Pixel: 2 to 4 wells/pixel:
  - Electronically gated wells for photos to be converted to electrons
  - 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^oC dependent)

Metal Oxide Semiconductor (MOS) Capacitor

Incoming Photons

Silicon Dioxide

n-Channel

Potential Barrier

Photogenerated Electrons

+V

Polysilicon Gate

Potential Well

-V

p-Type Silicon

Figure 2

Fluorescence Microscopy:
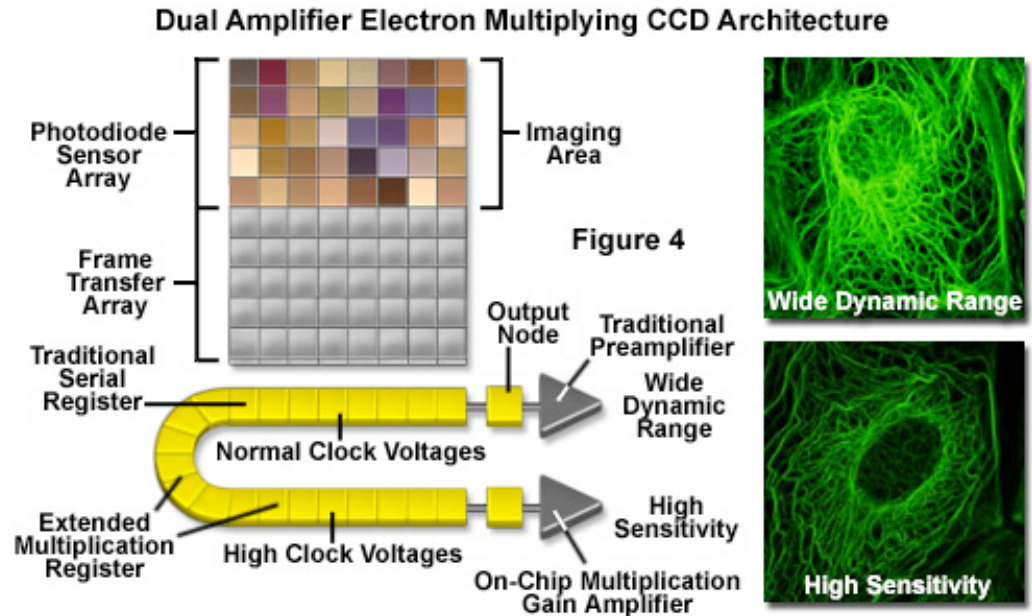# CCD Detectors

- Speed
  - Transfer of electrons to the edge of the chip accomplished before the next intergration
    - Full frame (high res, slow)
    - Frame transfer (faster but lower res)
    - Interline transfer (Fastest and even less resolution)
- CCD issues:
  - Read noise vs. signal
  - Read noise increases as readout increases.
  - Trade off.

Common Charge-Coupled Device (CCD) Architectures

Full-Frame — Imaging Array — Storage Array — Serial Register (a)

Frame Transfer — Serial Shift Direction (b)

Interline Transfer — Parallel Imaging Register — Parallel Storage Register — Parallel Shift Direction — Output Amp — Output Node (c)

Figure 7

Fluorescence Microscopy: CCD Detectors

- CCD format: to reduce noise and increase QE - on chip amplification
  - ICCD: external amplification
    - Incoming photons amplified (lenses)
    - Detected by standard CCD
    - Psec to nsec gating
    - Lower spatial resolution
    - High background
    - Susceptible to damage.
  - EMCCD: (electron multiplying) on chip amplification
    - Extended serial register
    - Amplification takes place after photons detected
    - Electrons accelerated from pixel to pixel using high voltage clock voltages
    - Impact ionization.
    - Less worry about photon noise amplification
    - Concern about dark noise - cool to low temp
  - Can be integrated with back thinned CCD for higher QE

Dual Amplifier Electron Multiplying CCD Architecture

Photodiode Sensor Array — Imaging Area

Frame Transfer Array

Figure 4

Traditional Serial Register

Output Node — Traditional Preamplifier — Wide Dynamic Range

Normal Clock Voltages

Extended Multiplication Register — High Clock Voltages — On-Chip Multiplication Gain Amplifier — High Sensitivity

Wide Dynamic Range

High Sensitivity

Fluorescence Microscopy:
CCD Detectors

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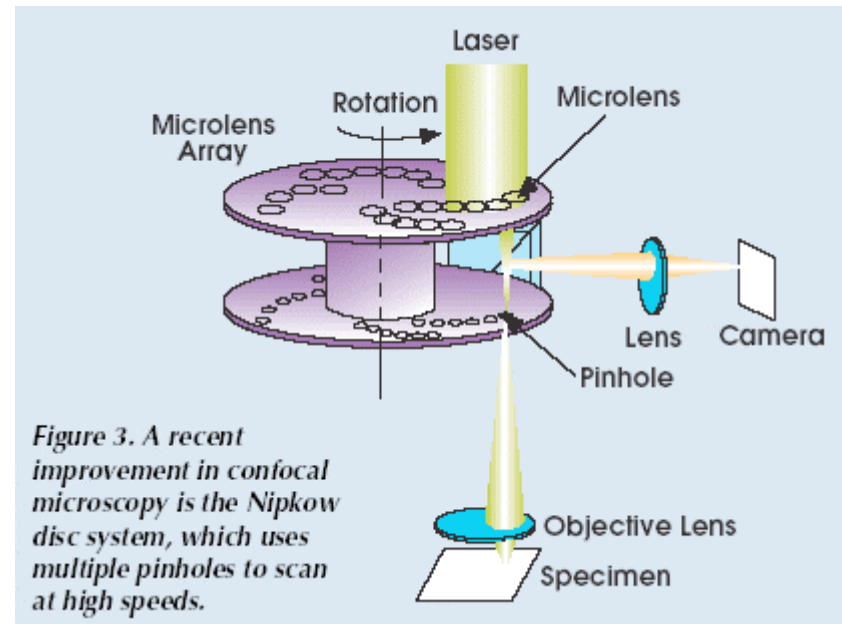- Price vs. function
- Test drive models

EMCCD and ICCD Quantum Efficiencies
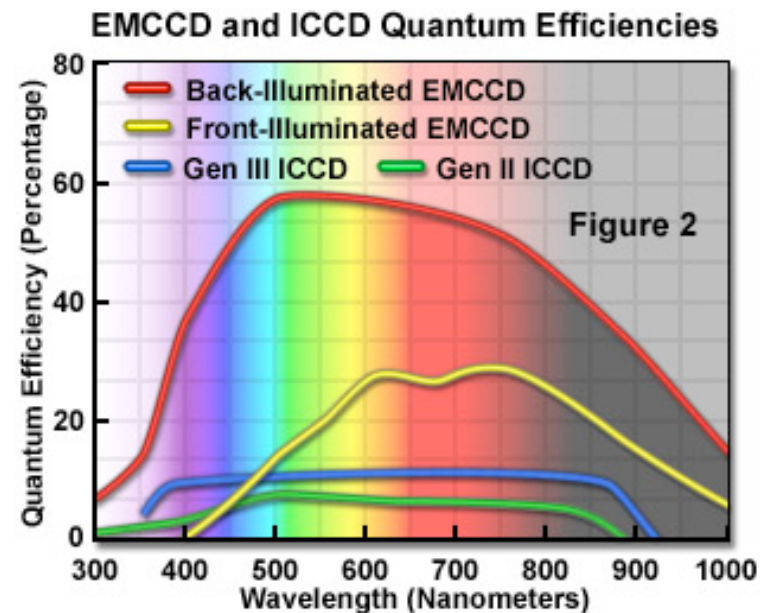
For complete discussion of live cell imaging issues:

http://www.microscopyu.com/articles/livecellimaging/imagingsystems.html

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
- Lower light exposure
  - 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)
  - Filters to subtract uv.
- **Fast acquisition EMCCD**
  - 10X increased linear range compared to confocal
- **Deconvolution algorithm**
  - Removes out of focus light
  - High resolution observed (<0.2μm)
- **Motorized stage**
  - Collect optical sections (0.2 μm).
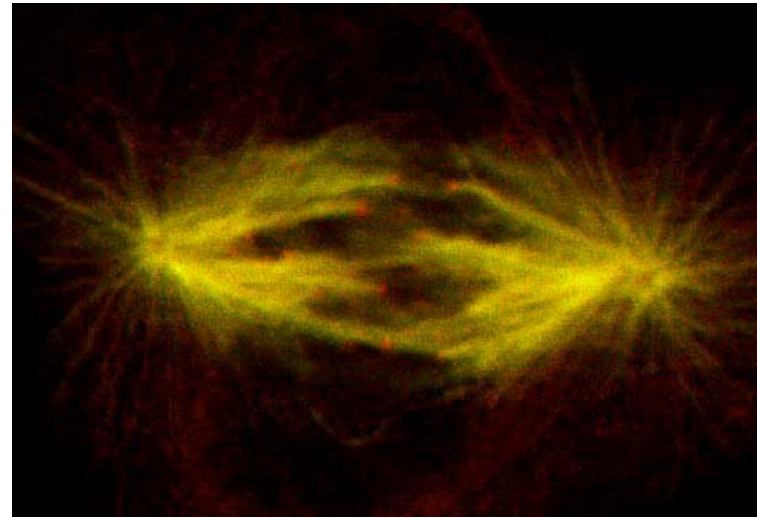
Confocal projection
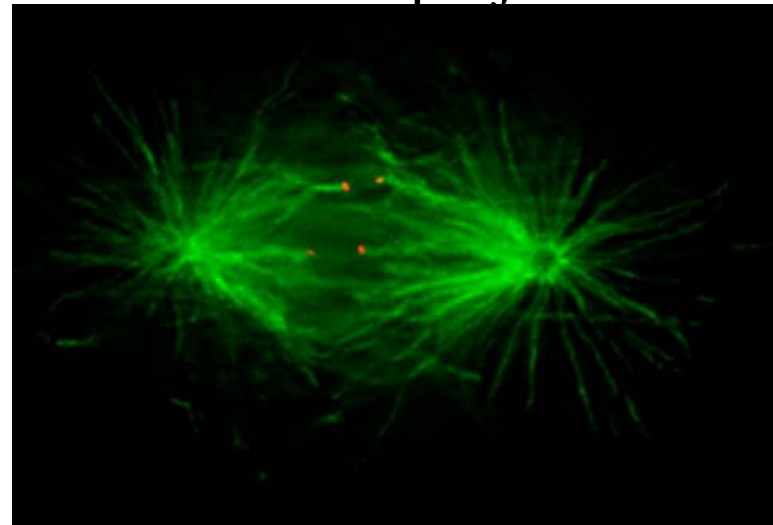
Deconvolved projection

Total Internal Reflection Fluorescence Microscopy

- Laser light focused by mirror into prism
- Angle of light adjusted to generate the evanescent (electromagnetic field) wave at glass-sample interface
  - Decays exponentially
- Wide field can also be used
- Fluorescence emitted is collected by EMCCD
- Excellent for live-cell imaging
  - Reduces photo damage
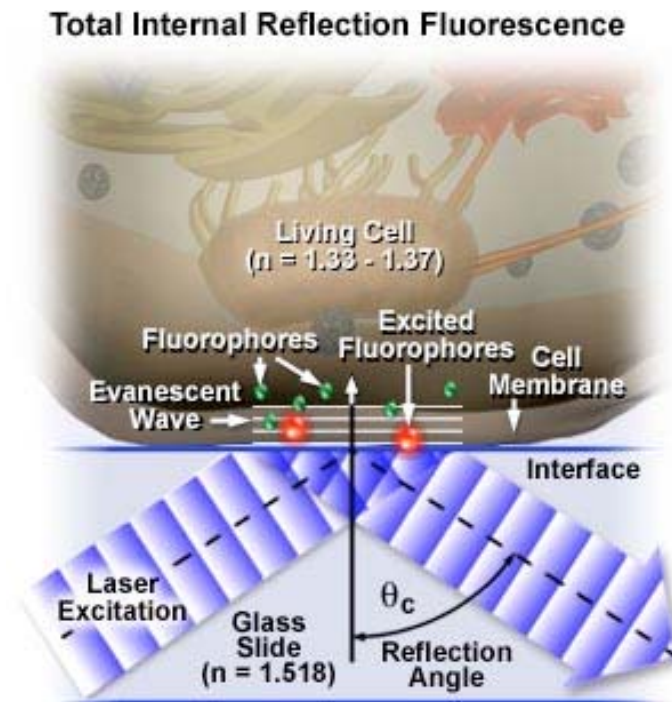 - Eliminates out-of-focus excitation of fluorophores

Total Internal Reflection Fluorescence

Living Cell (n = 1.33 - 1.37)

Fluorophores Excited Fluorophores Cell Membrane

Evanescent Wave →

Interface

Laser Excitation

Glass Slide (n = 1.518)

θ_c

Reflection Angle

Figure 1

Total Internal Reflection Fluorescence Microscopy

- Laser light focused by mirror into prism
- Angle of light adjusted to generate the evanescent (electromagnetic field) wave at glass-sample interface
  - Decays exponentially
- Wide field can also be used
- Fluorescence emitted is collected by EMCCD
- Excellent for live-cell imaging
  - Reduces photo damage
  - Eliminates out-of-focus excitation of fluorophores

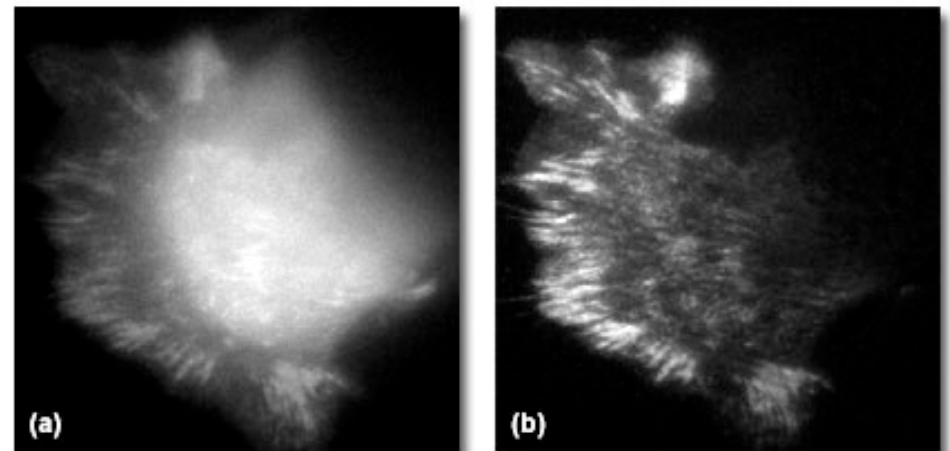Cell Focal Adhesions in Widefield and TIR Fluorescence

(a) (b)

Figure 5

Fluorescence Microscopy:
Two Photon Confocal

- Chromophore is excited by two lower-energy photons (infrared).
  - Requires twice as many photons of twice the wavelength
  - 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.

One-photon CLSM

Two-photon Microscopy

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
  - Deconvolution
  - TIRF
  - Two photon