LOW-LIGHT-LEVEL IMAGING

1.The Problem

2. Low-light-level Cameras

3. Solutions

How Much Light? I.

- Illumination system:
- 75 W Xenon Arc (~1mW/nm in visible)
- 490/10 nm exciter filter (60 % T)
- 505 nm dichromatic mirror (85% Reflect)
- 100X/1.32 objective (95 % T)
- 10 mW*0.6*0.85*0.95=4.8 mW
- Field 40 microns diameter, area = 12.6×10^{-6} cm^2
- Flux = $4.8 \times 10^{-3} W / 12.6 \times 10^{-6} cm^2 = 380$ W/cm^2

How Much Light? II.

- 380 W/cm^2 is about 2500 times the flux of sunlight on the brightest day.
- Fluxes in a confocal may be as high as 8 x 10^5W/cm^2 (1mW in a 0.2 micron diameter spot, area = 12.6 x 10^{-10} cm$^{2)}$.
- In a 2-photon confocal, the light flux is 10^6 higher than in a single photon confocal.

How Much Light? III.

- Detection system:
- Objective lens NA (30% collection)
- Dichromatic (80% T)
- Barrier Filter (85% T)
- Detector QE (10-80%)
- Overall: 2%-16%
- Fluorescein emission is 36,000 photons. Detection ranges from 720 to 5760 photons

Low-Light Fluorescence Microscopy Imaging Paradox

- Photodamage is reduced by lowering the incident light flux.
- As the light flux diminishes, the detected signal decreases with resultant degradation of image quality and signal/noise.

Image Intensifiers II.

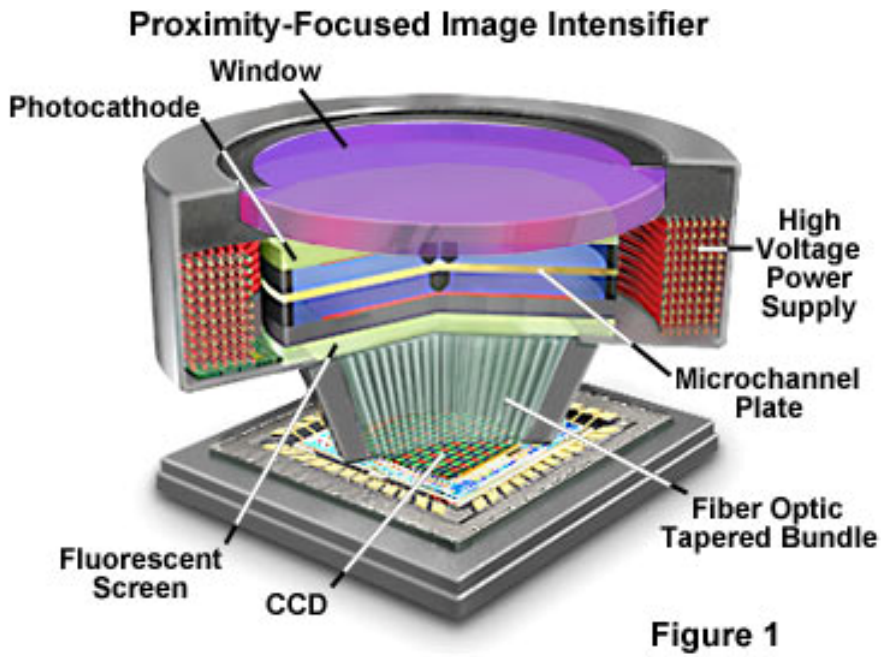
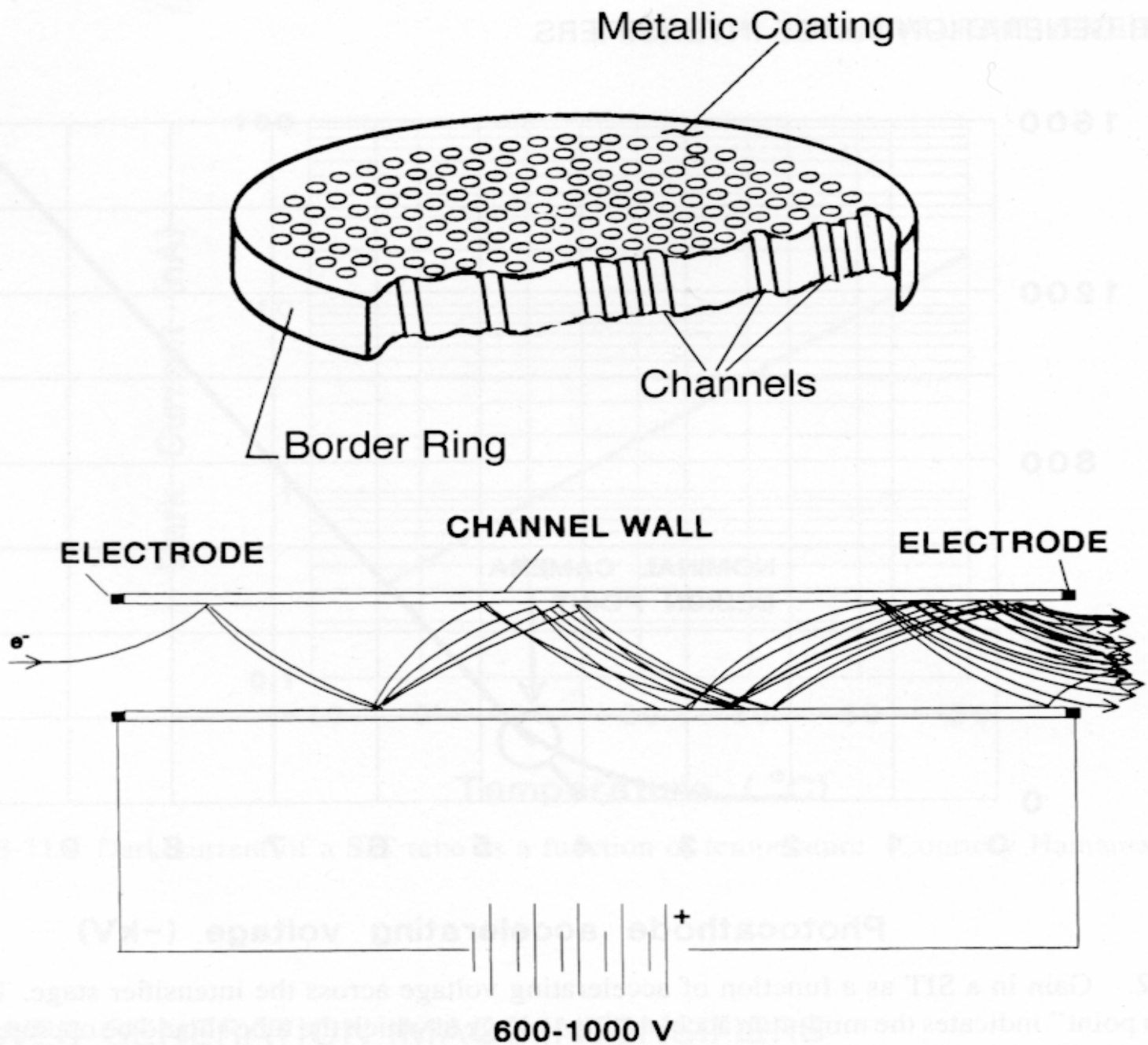


Figure 1


Metallic Coating
Channels
Border Ring
ELECTRODE
CHANNEL WALL
ELECTRODE
e⁻
+
600-1000 V

Responsivity

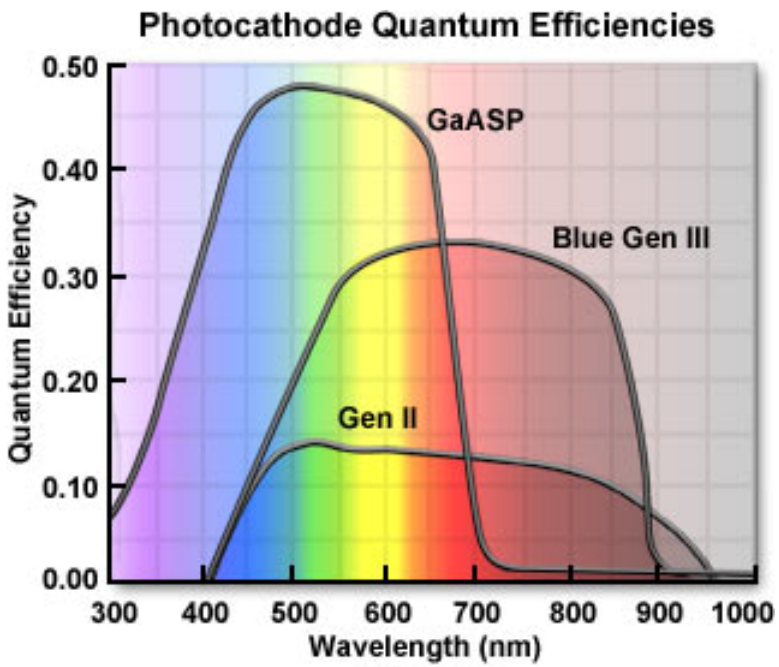
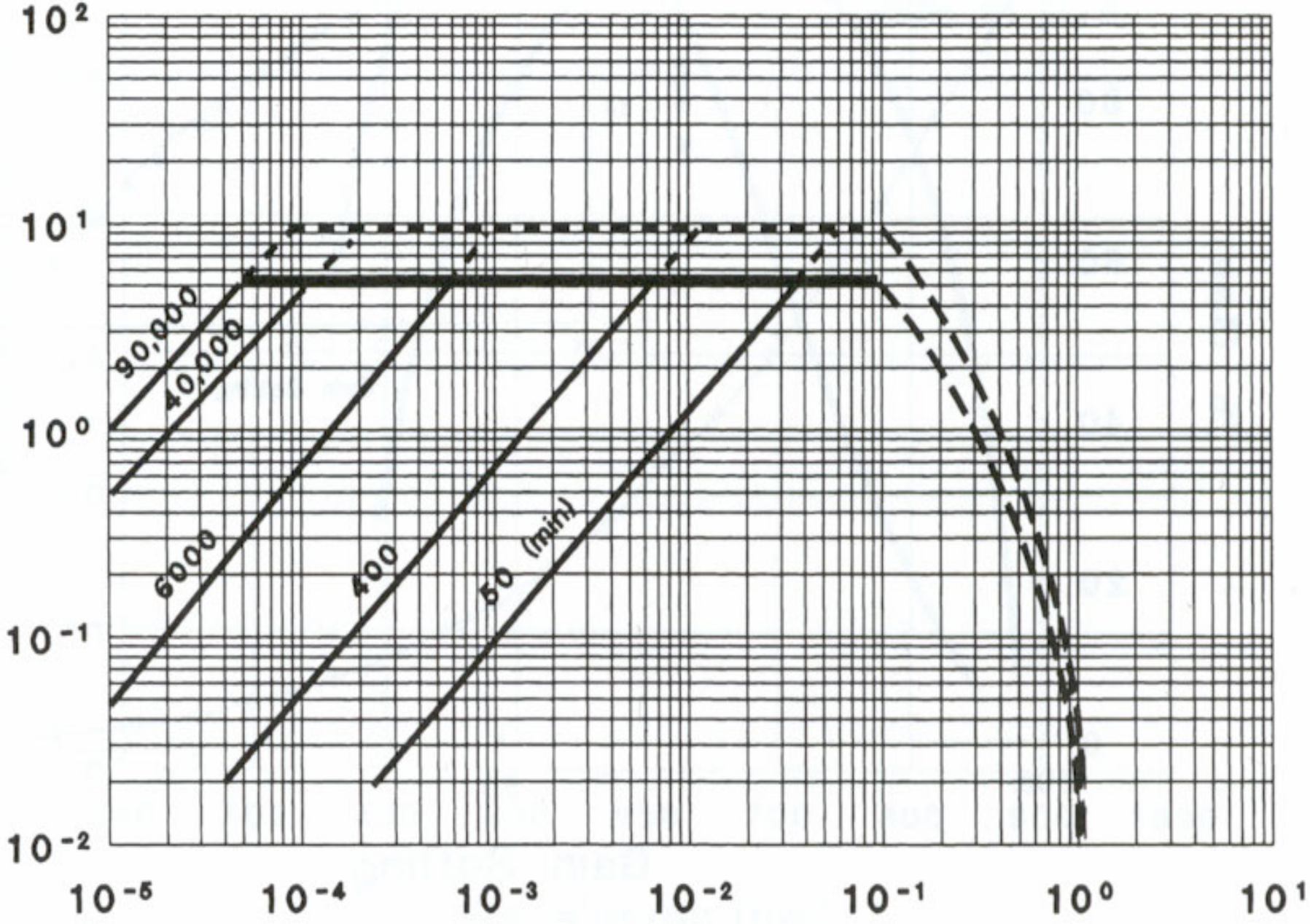
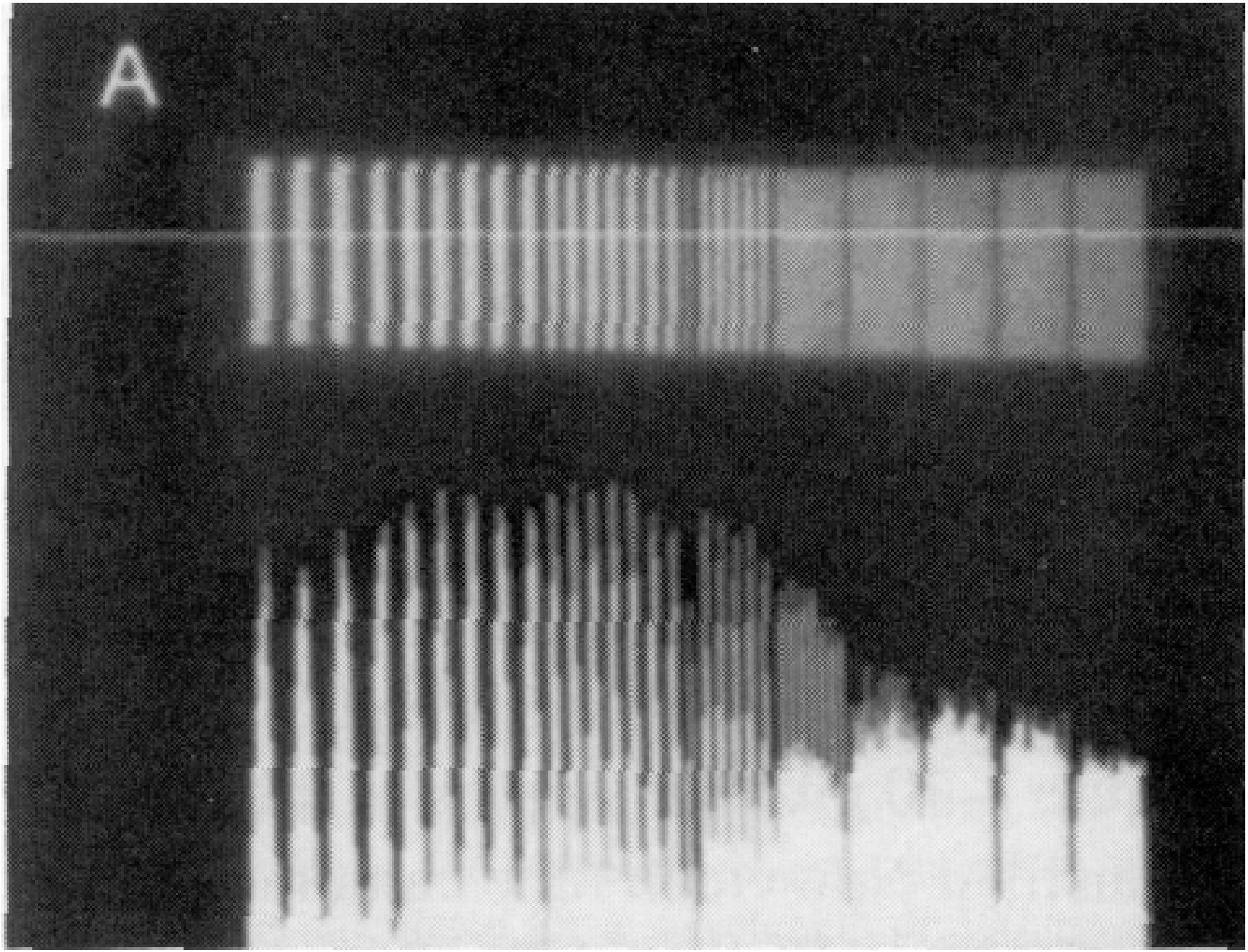
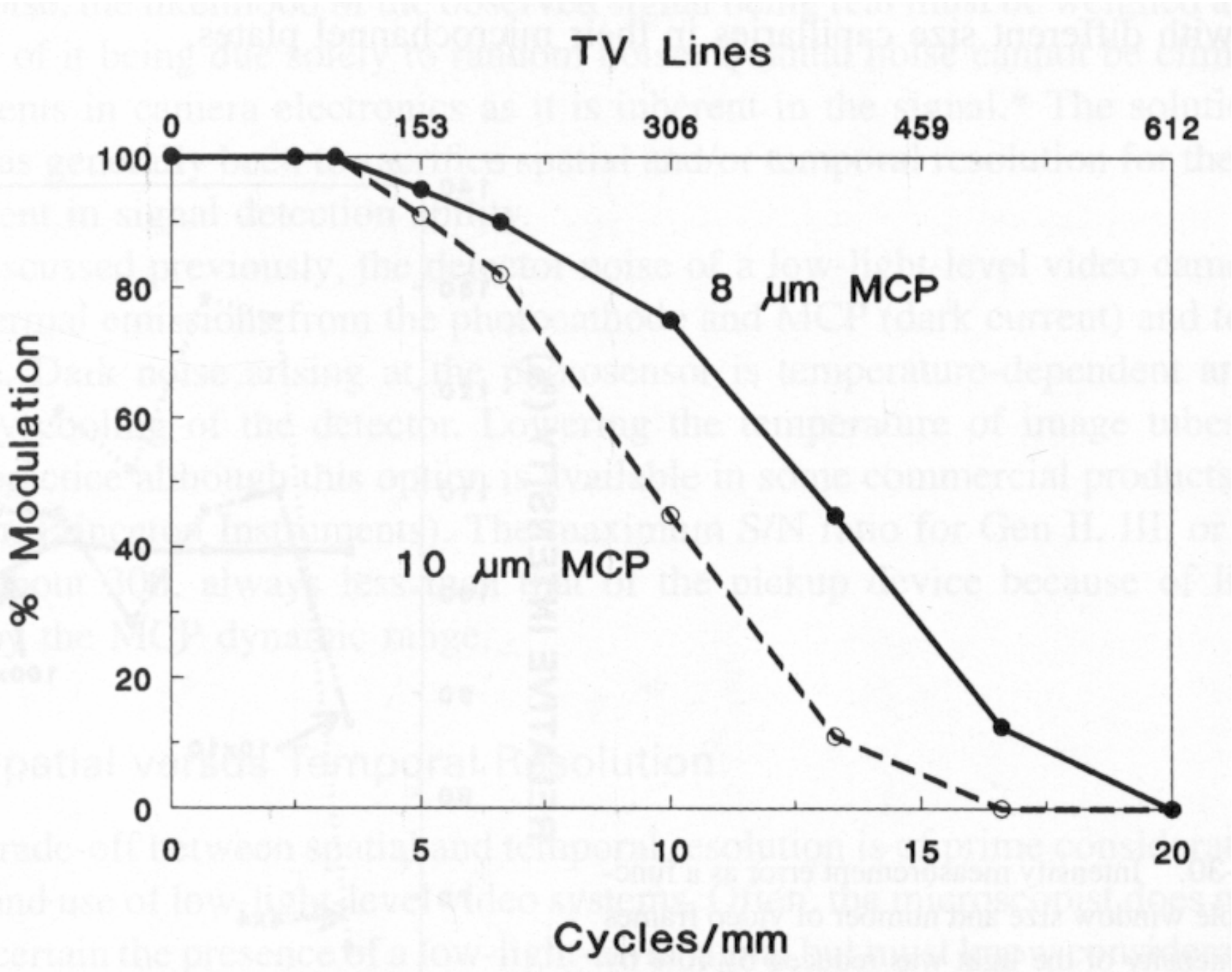


Figure 2

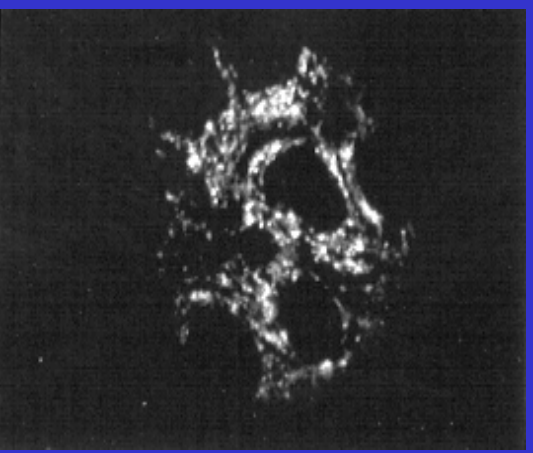

10²
10¹
10⁰
10⁻¹
10⁻²
90,000
40,000
6000
400
50 (min)
10⁻⁵
10⁻⁴
10⁻³
10⁻²
10⁻¹
10⁰
10¹
photocathode illuminance (lx)


A


TV Lines
0
153
306
459
612
100
80
60
40
20
0
% Modulation
8 μm MCP
10 μm MCP
0
5
10
15
20
Cycles/mm

THE INTENSIFIED PROGRESSIVE SCAN CCD CAMERA

- Couple, with a tapered fiber optic, a blue-green sensitive Gen III image intensifier to a 1K x 1K or higher resolution CCD sensor.
- Strengths: 40-50% QE

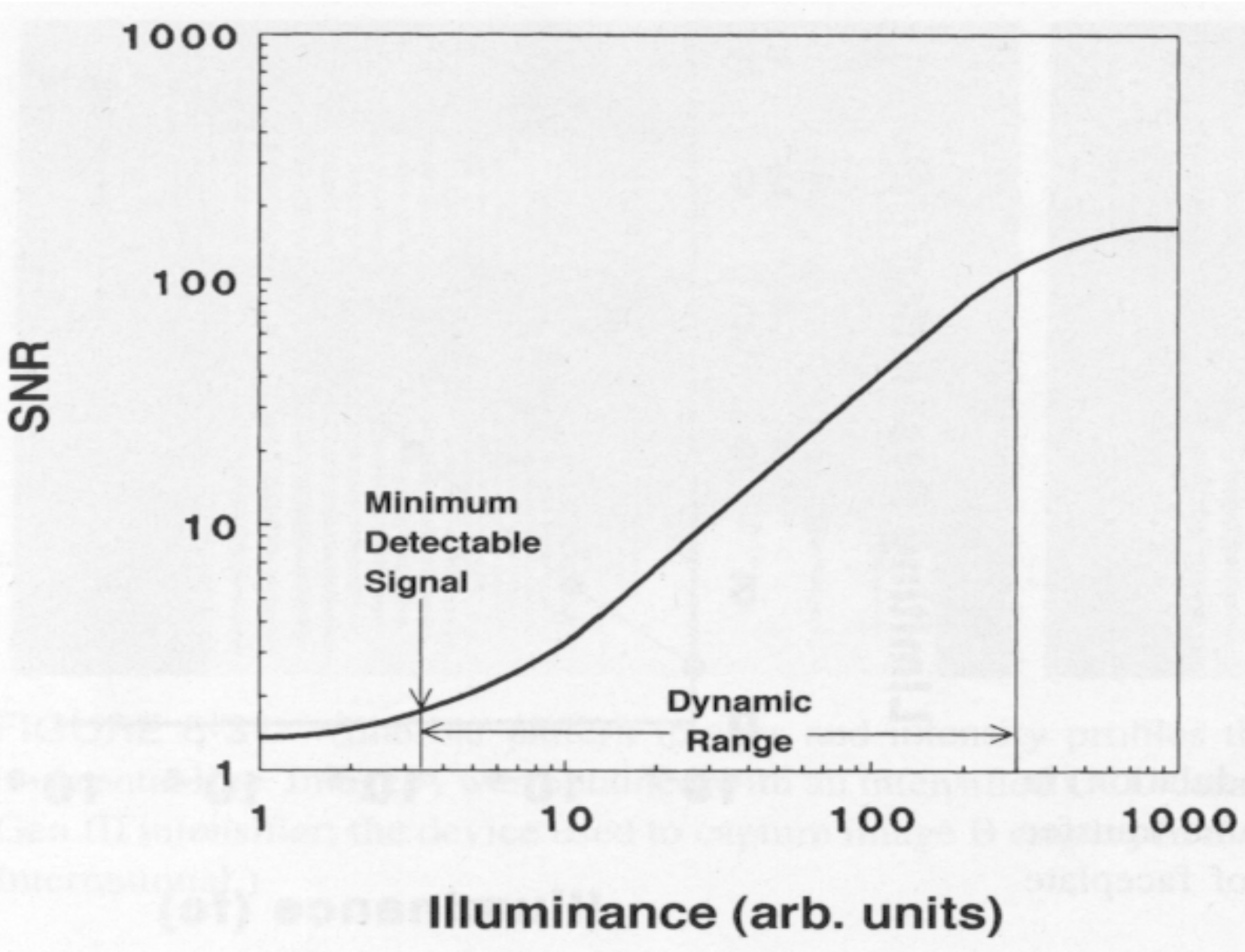


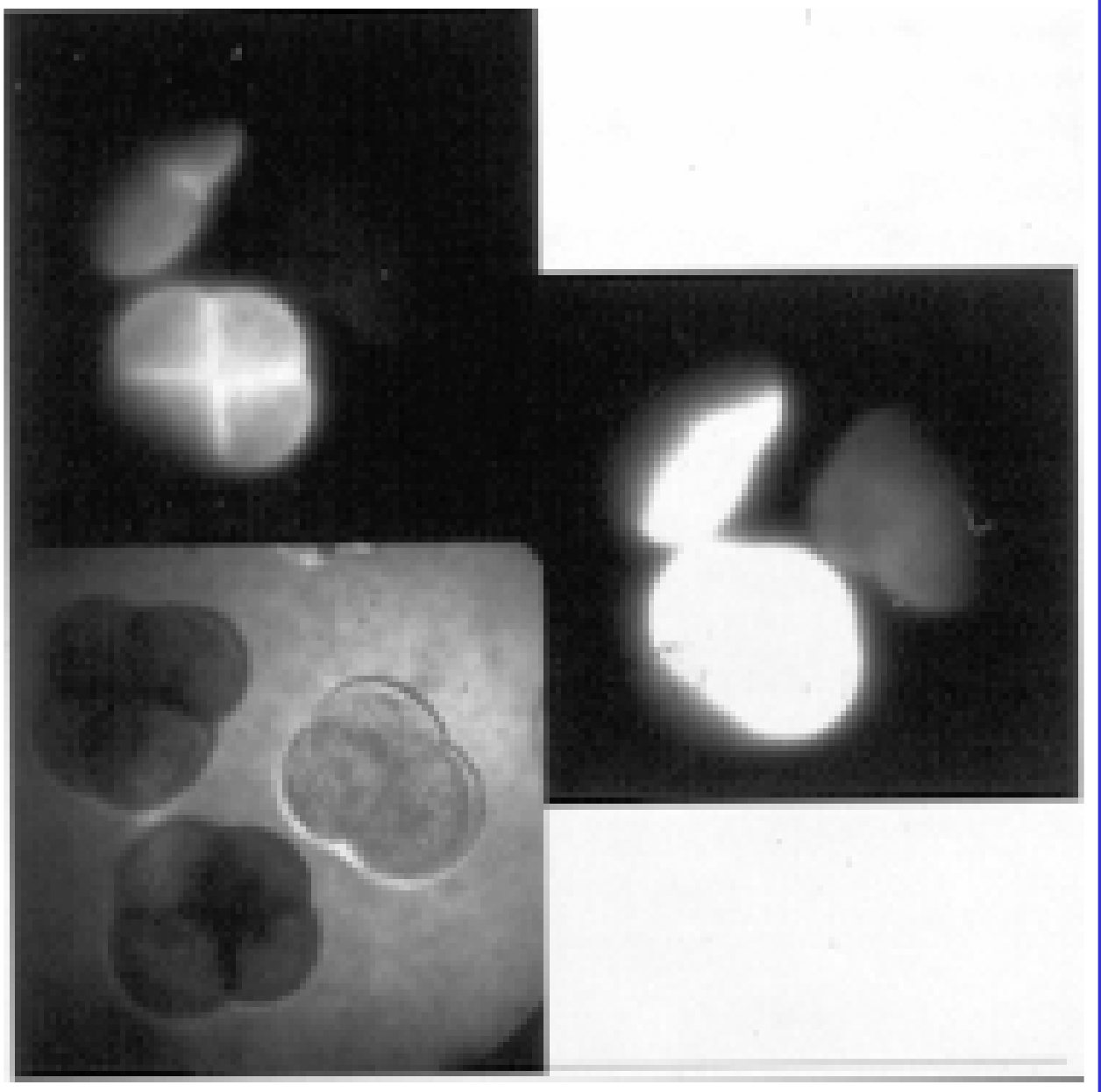
700 TV line resolution (H & V)

No fixed pattern noise (chicken wire)

80,000 Gain, low EBI

Video-rate or higher output at 10-12 bits


1000
100
10
1
SNR
Minimum
Detectable
Signal
Dynamic
Range
1
10
100
1000
Illuminance (arb. units)



LIMITATIONS OF THE PROGRESSIVE SCAN INTENSIFIED CCD CAMERA

- Dynamic range limited-10 bit resolution is possible, 12 bit is questionable
- Burning and sticking from overexposure
- Few manufacturers
- Limited range of image formats and read-out formats

Electron-bombarded CCD

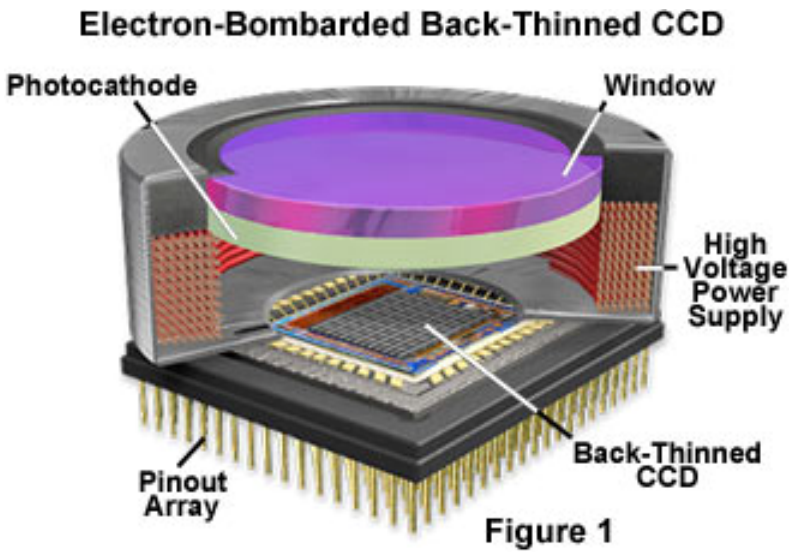
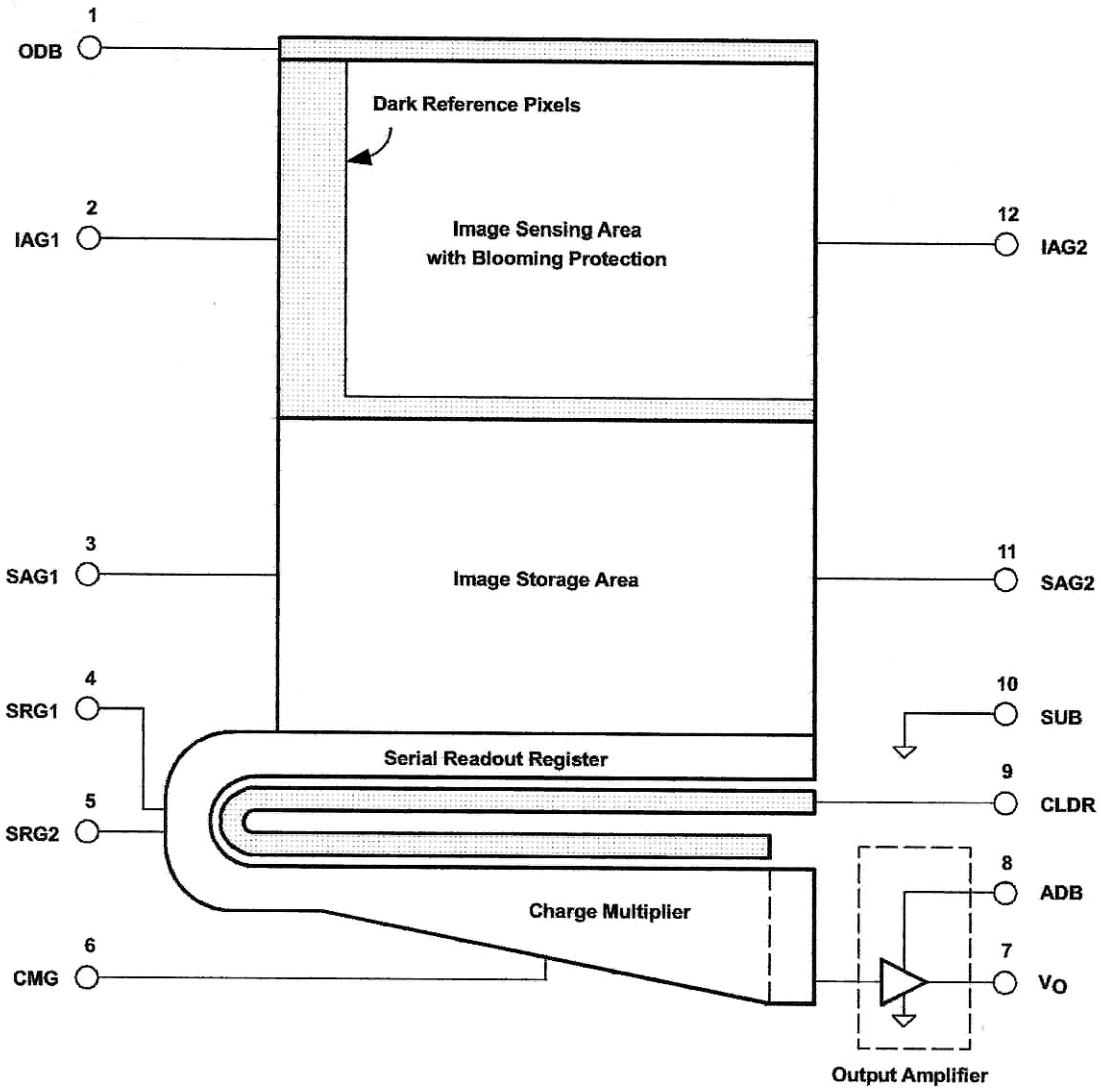
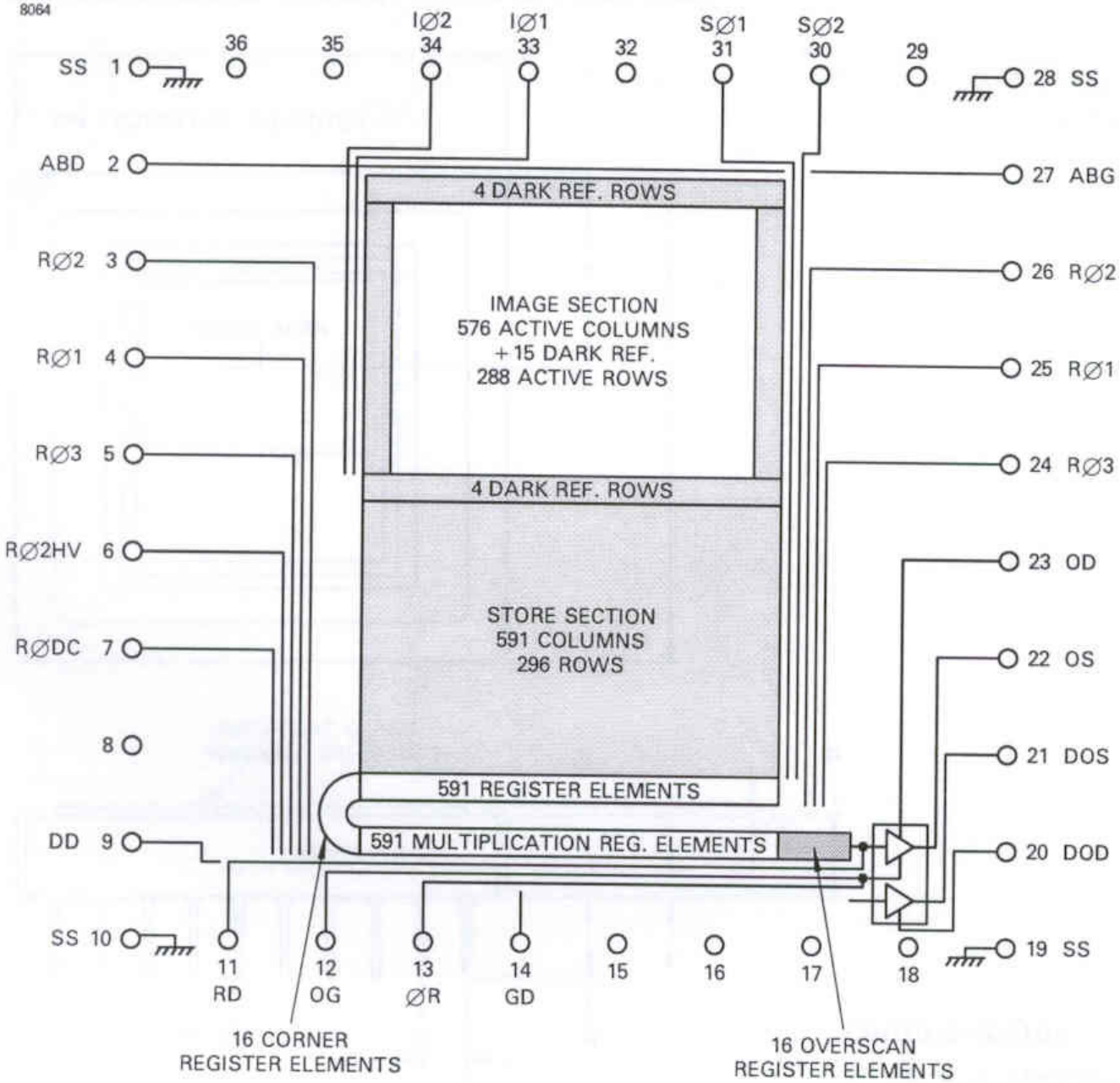


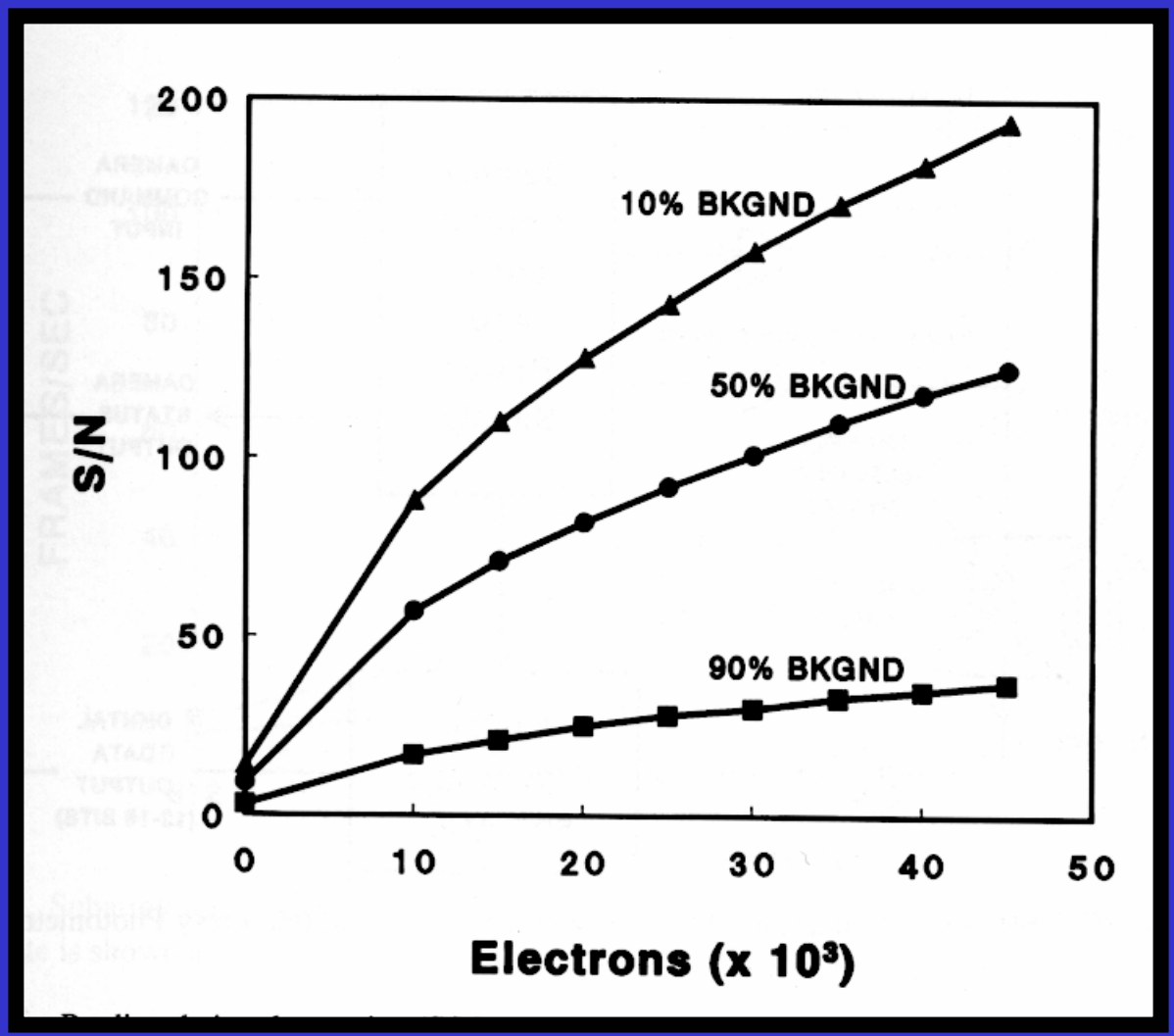
Figure 1

functional block diagram

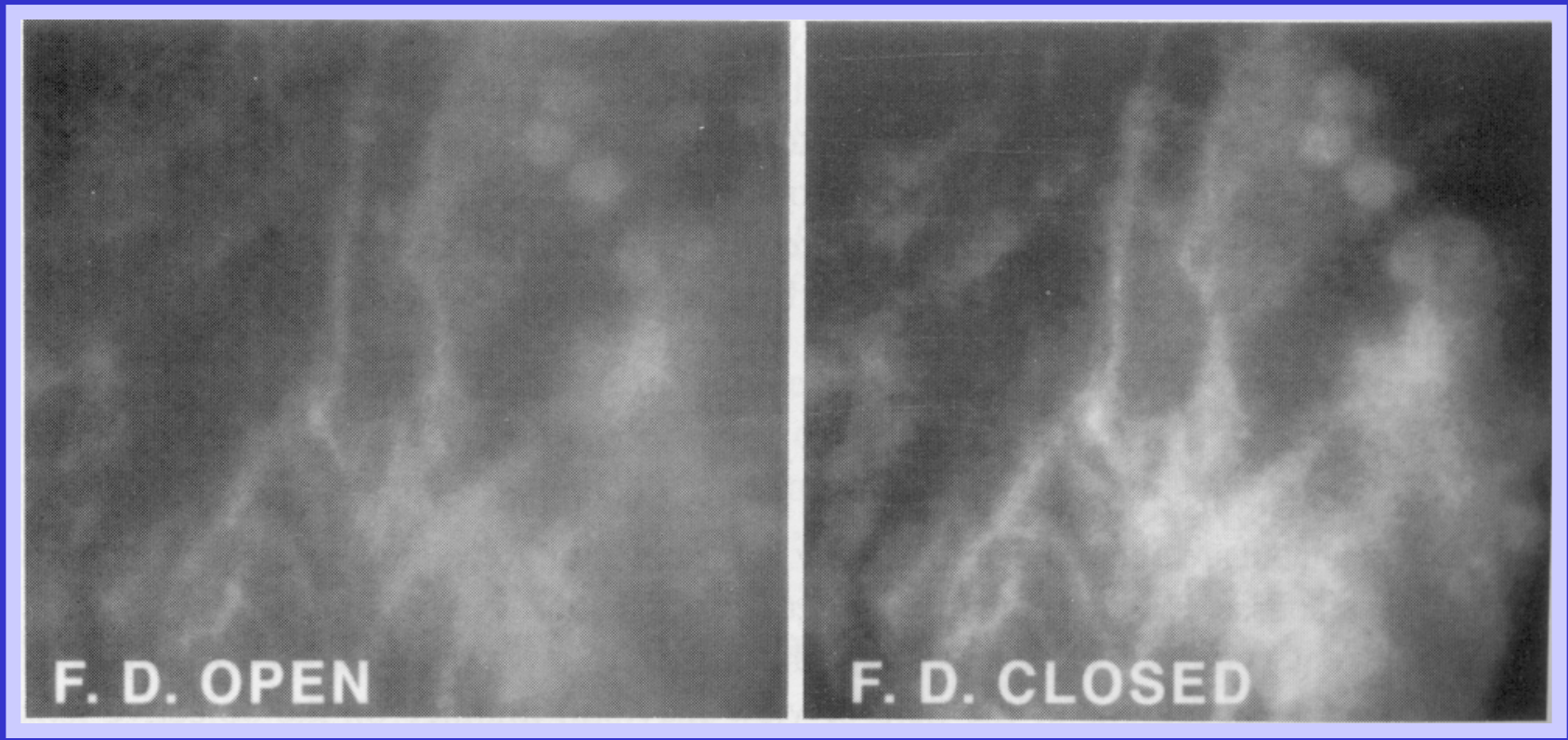


Marconi CCD65

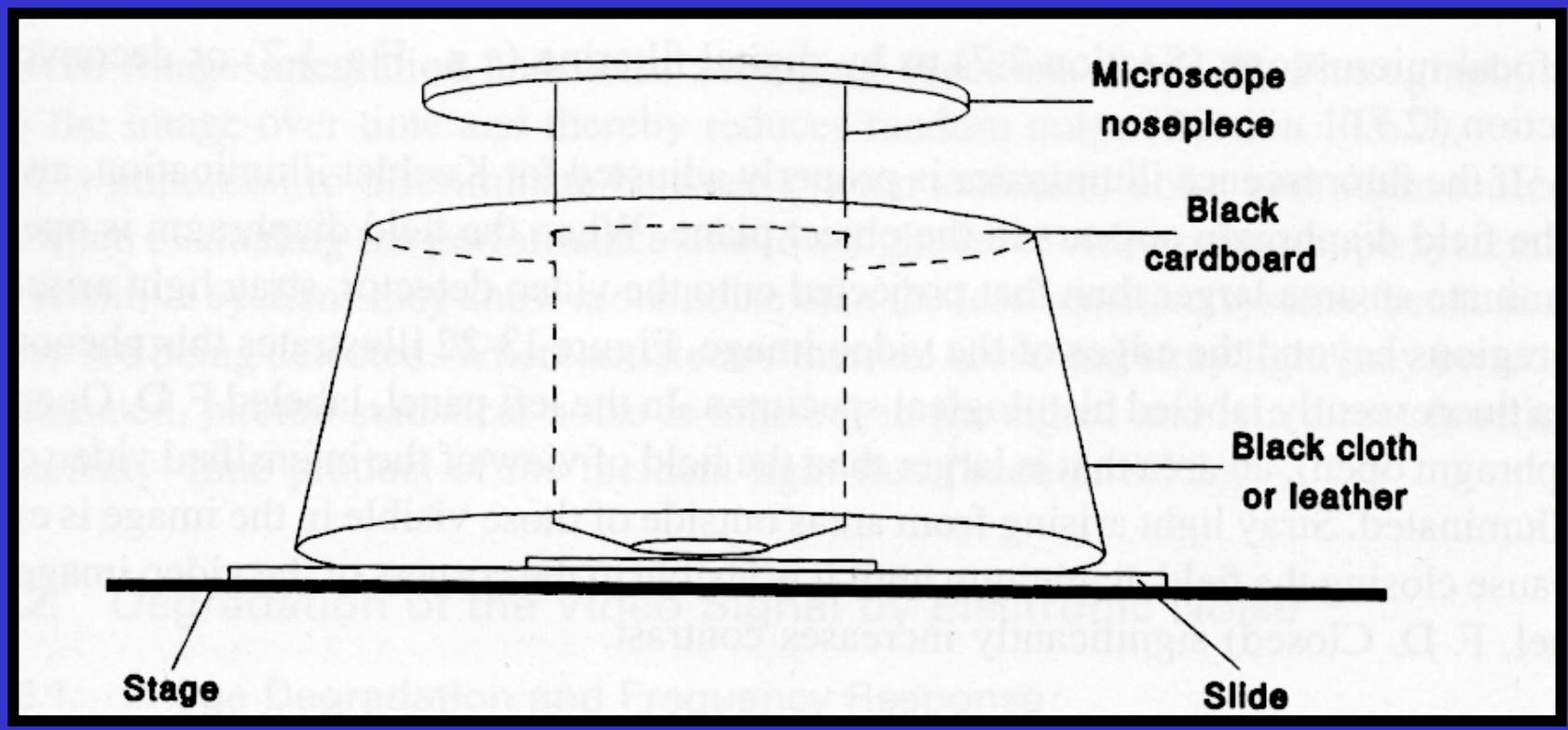



200
150
100
50
0
S/N
10% BKGND
50% BKGND
90% BKGND
0
10
20
30
40
50
Electrons (x 10³)

Evils of Stray Light I



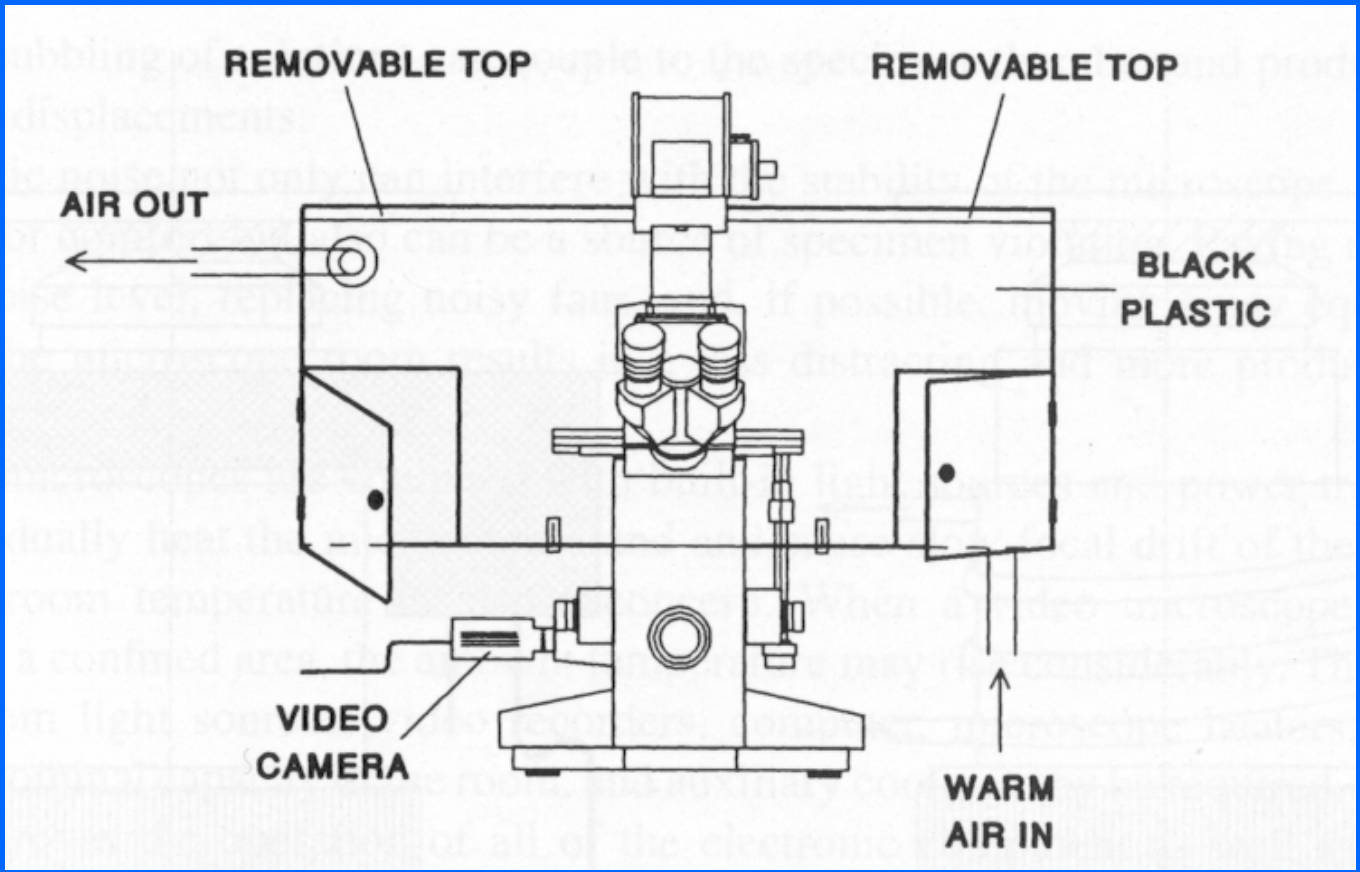
Evils of Stray Light II



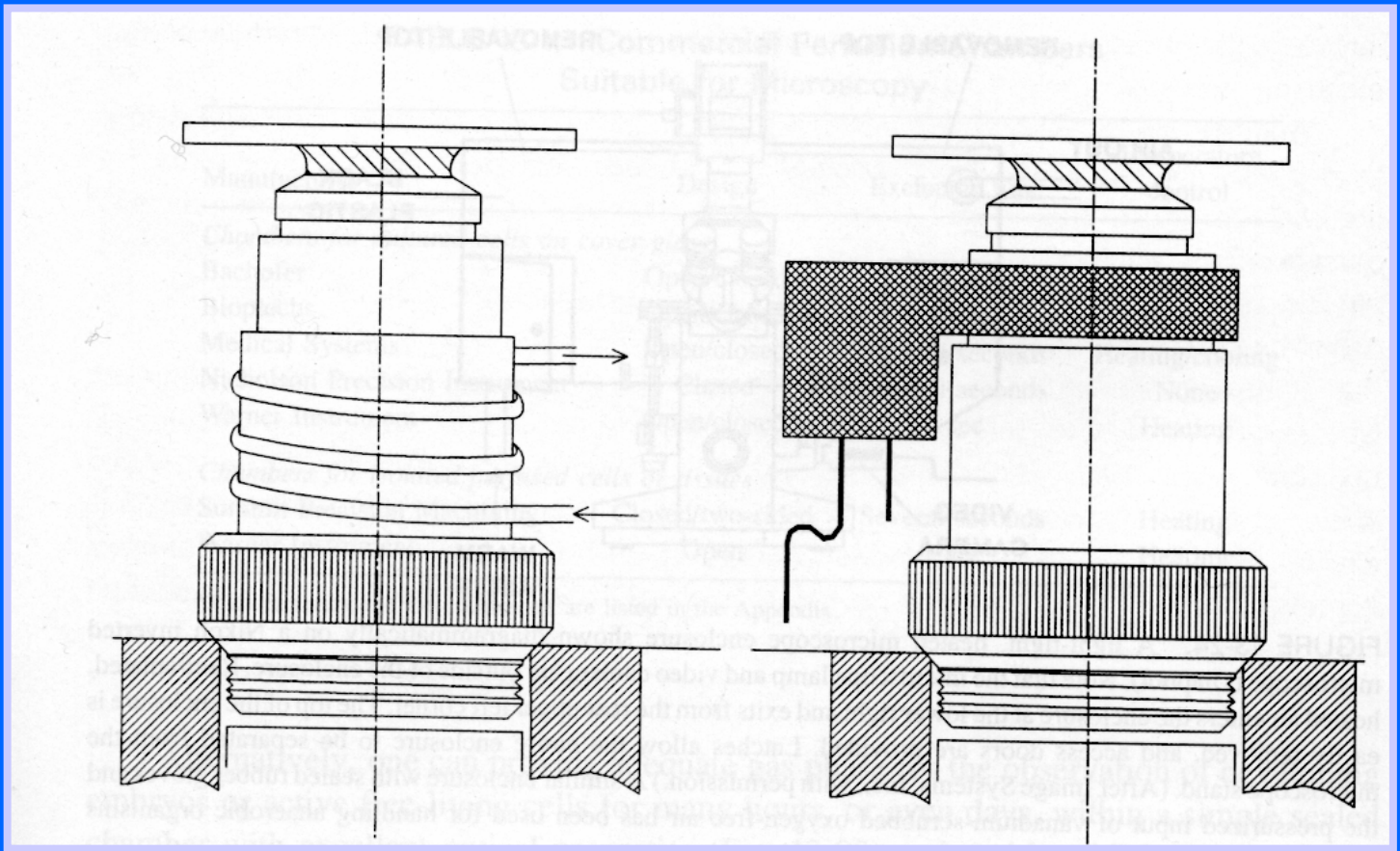
Problems in Imaging Live Cells

- **Temperature control**—chamber, objective lens—prevention of focus drift
- **Perfusion**—changing composition without specimen movement or focus shift
- **Reduction of stray light**—necessitated by the need for dim illumination of specimen
- **Speed**—detectors, illumination switching

Temperature Controlled Environmental Enclosure and Stray Light Shield



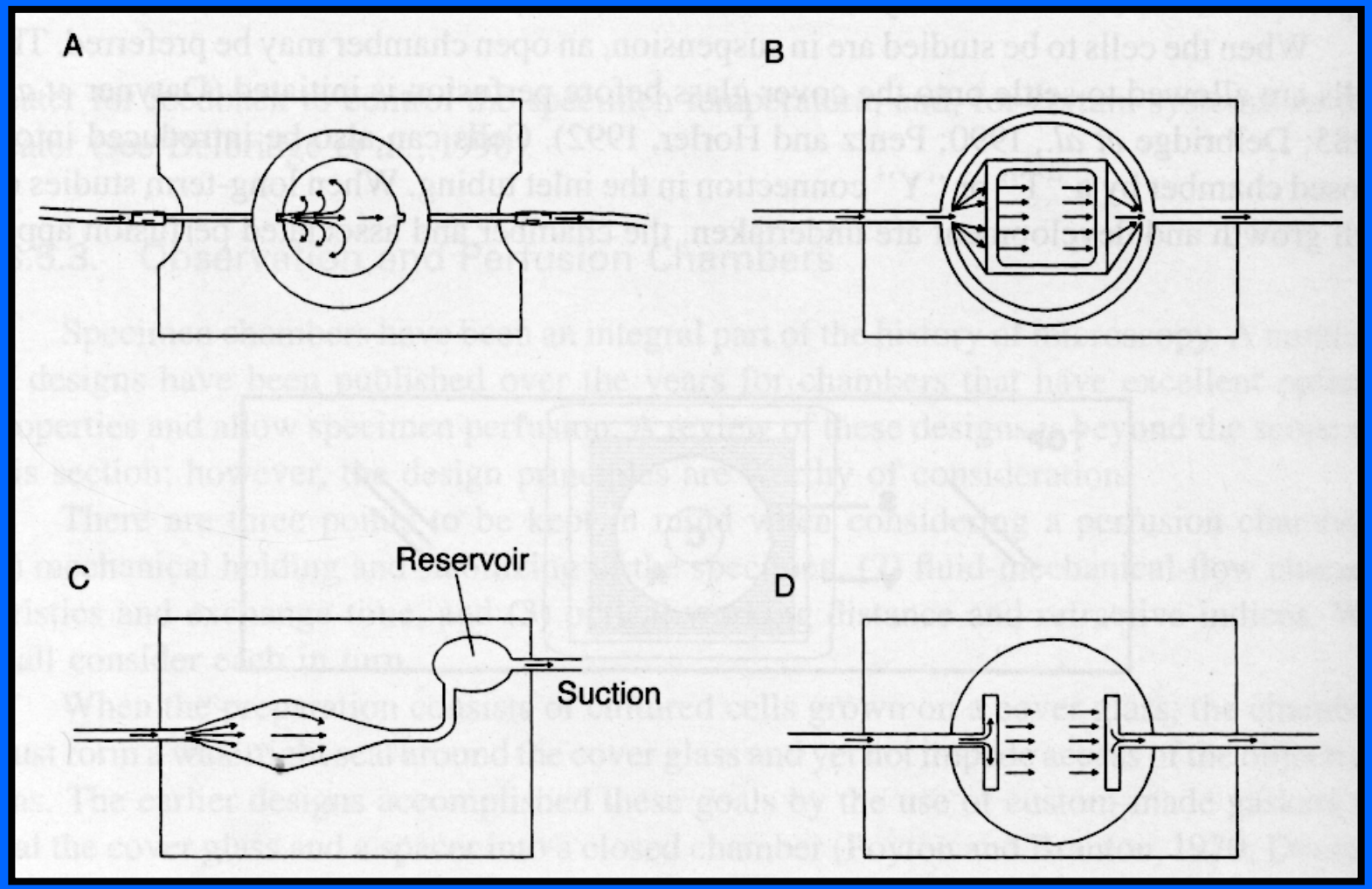
Alternative to enclosure: Objective Lens Heater



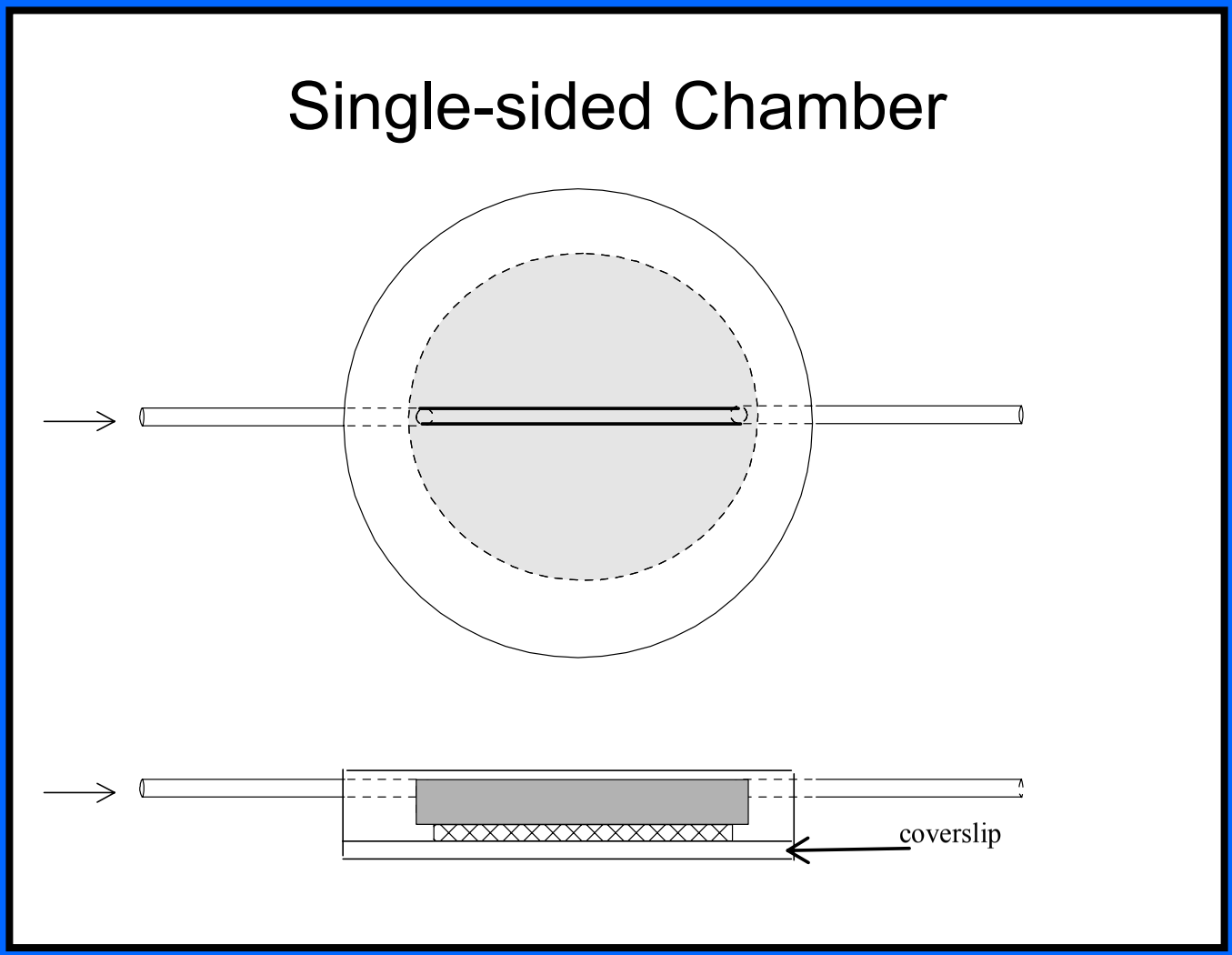
Prevention of Focus Drift

- Keep specimen chamber heater **on** constantly but limit current so that set point is never achieved.
- Heat enclosure to a few degrees **below set point** to reduce temperature gradients between specimen and microscope.
- Warm perfusates to **slightly higher** than set point to allow for cooling and to prevent degassing.

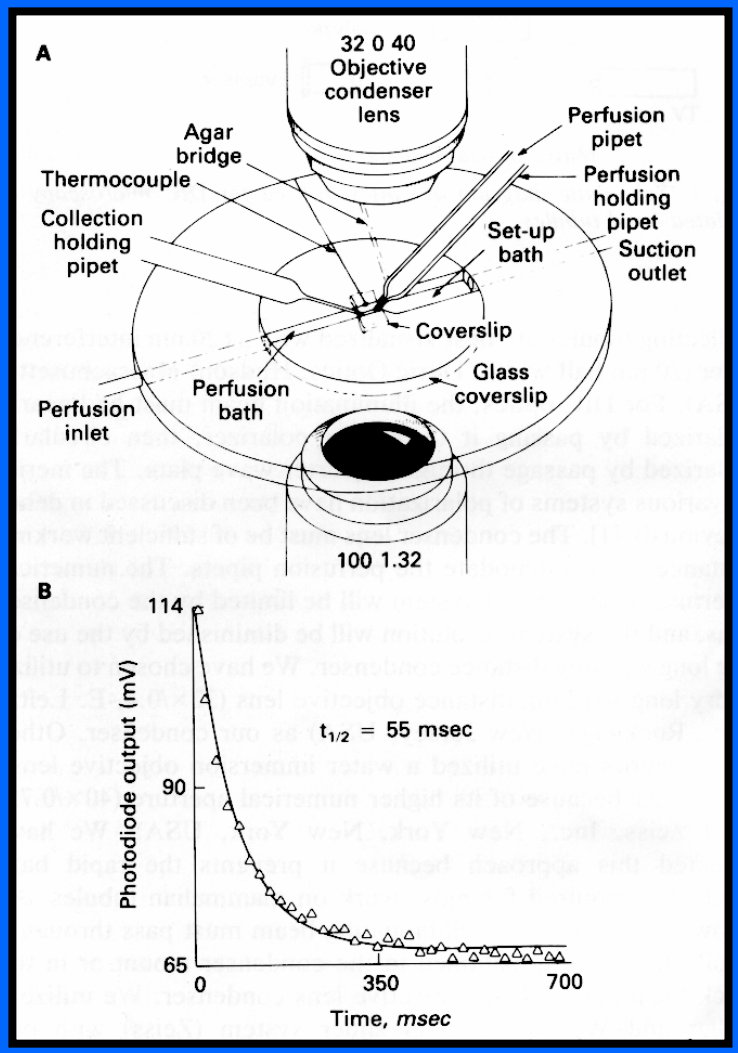
Perfusion Systems—Flow Profiles

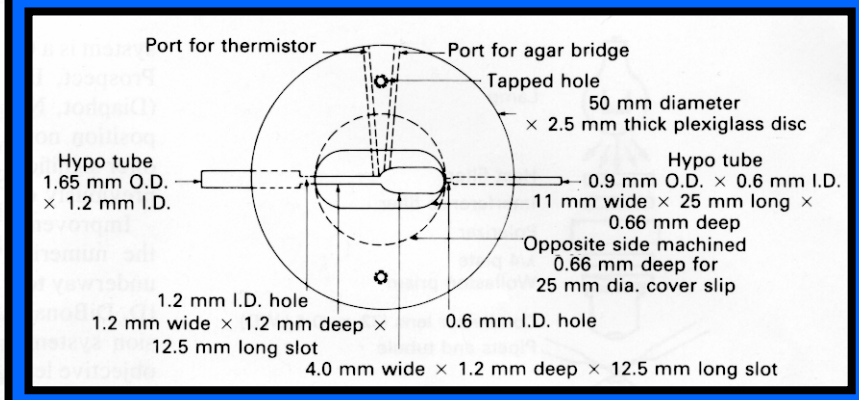


Perfusion Systems—Cultured Cells



Perfusion Systems—Renal Tubules





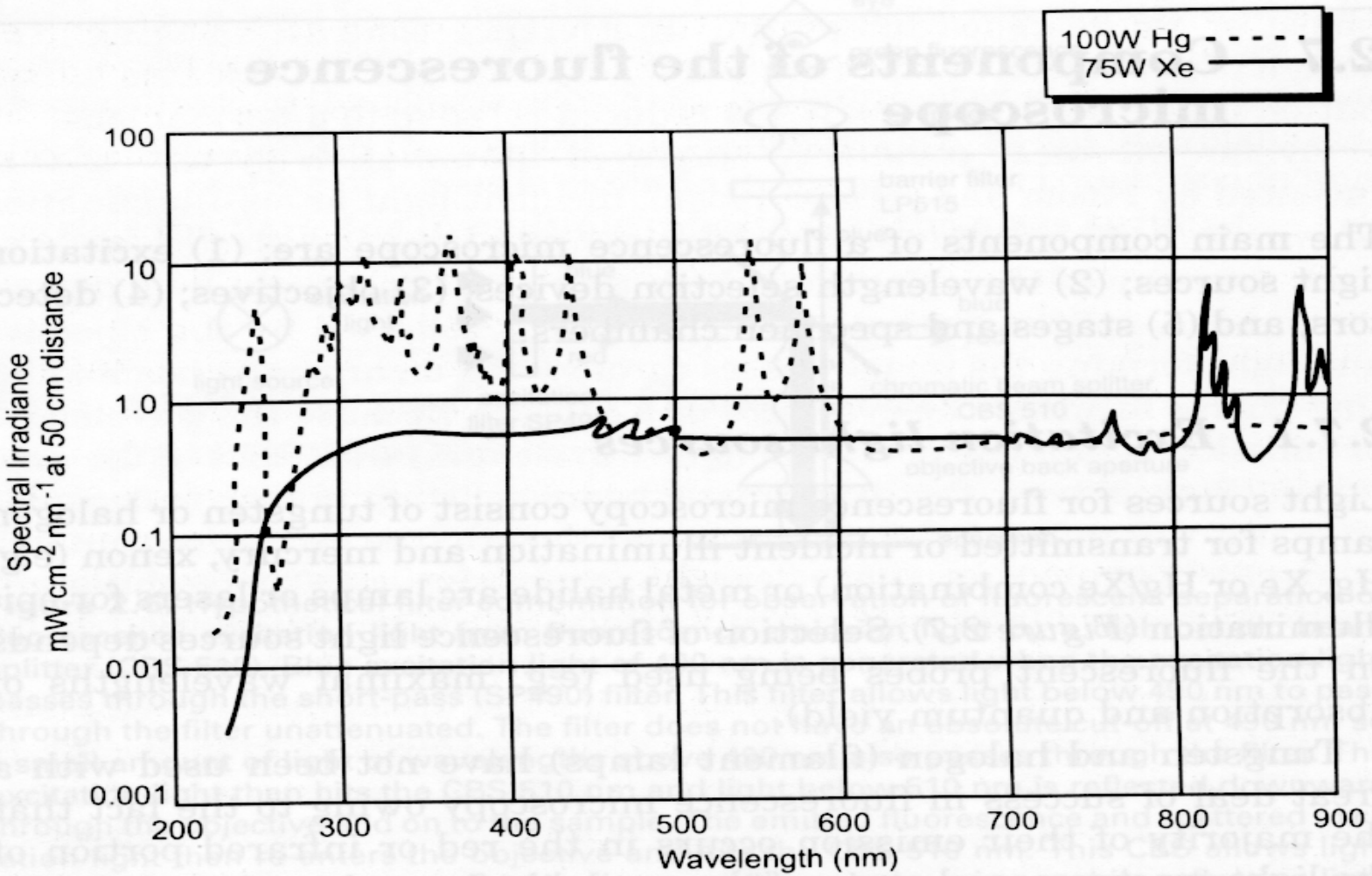
Perfusion Systems—Problems

- Perfusate temperature control
- Perfusate gassing—CO_2 equilibration, solution degassing and bubble formation
- Mechanical disturbances—gravity vs. pump, flow regulation, pressure balance
- Mixing solutions or adding reagents

Summary Live Cell Imaging

- Live cell imaging requires attention to control of temperature and perfusion systems.
- Intervening solution layers can be kept very thin while maintaining adequate control of the rate and composition of the perfusate.
- Illumination intensity must be low, detector sensitivity high, speed matters.

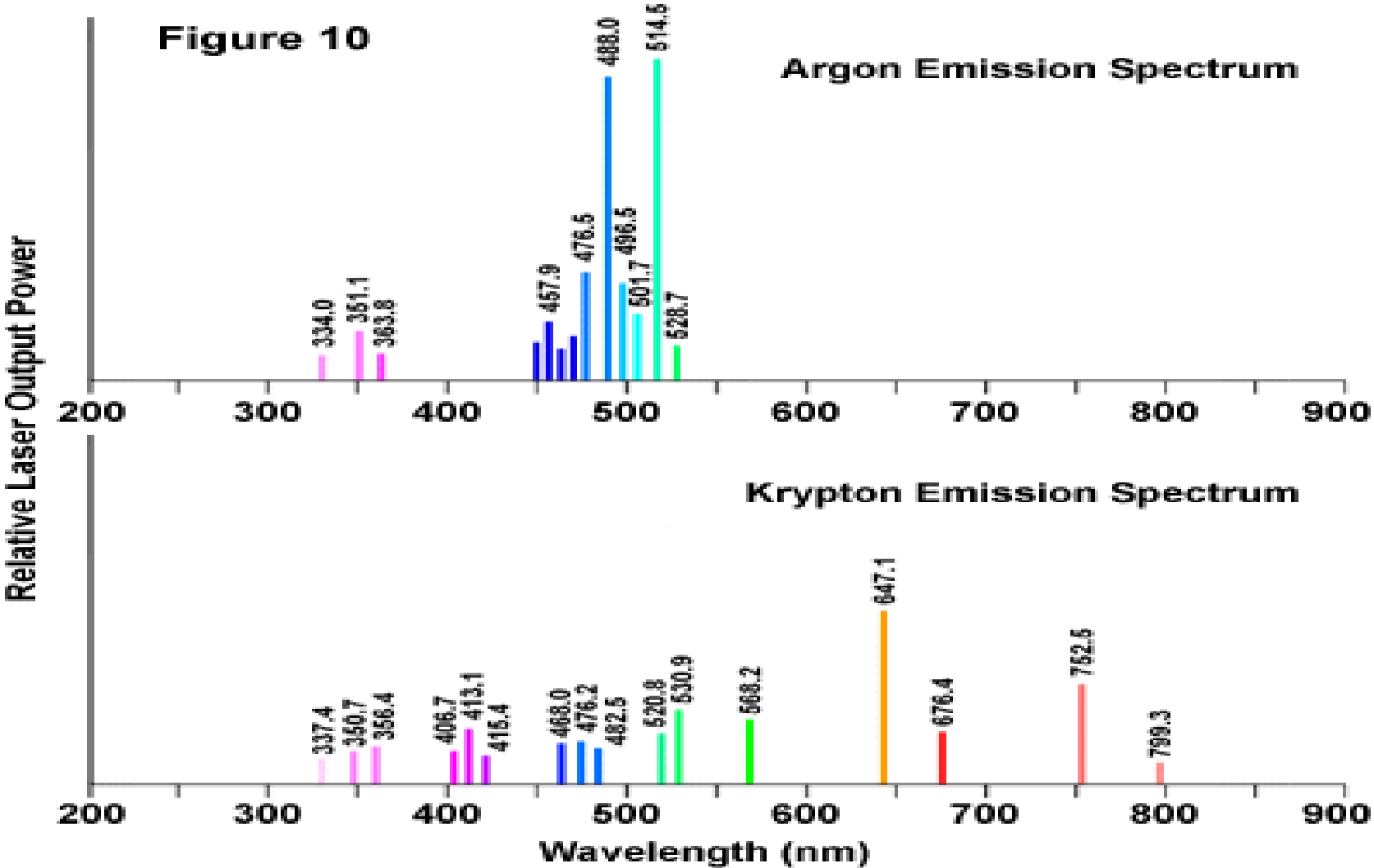
Arc Lamps


100W Hg
75W Xe
Spectral Irradiance
mW cm-2 nm-1 at 50 cm distance
100
10
1.0
0.1
0.01
0.001
200
300
400
500
600
700
800
900
Wavelength (nm)

Energy Output

HBO 100	2200 lumens	1700 cd/mm^2	0.25 x 0.25 mm
XBO 75	850 lumens	800 cd/mm^2	0.25 x 0.50 mm
Tungsten/ Halogen	2800 lumens	45 cd/mm^2	4.2 x 2.3 mm

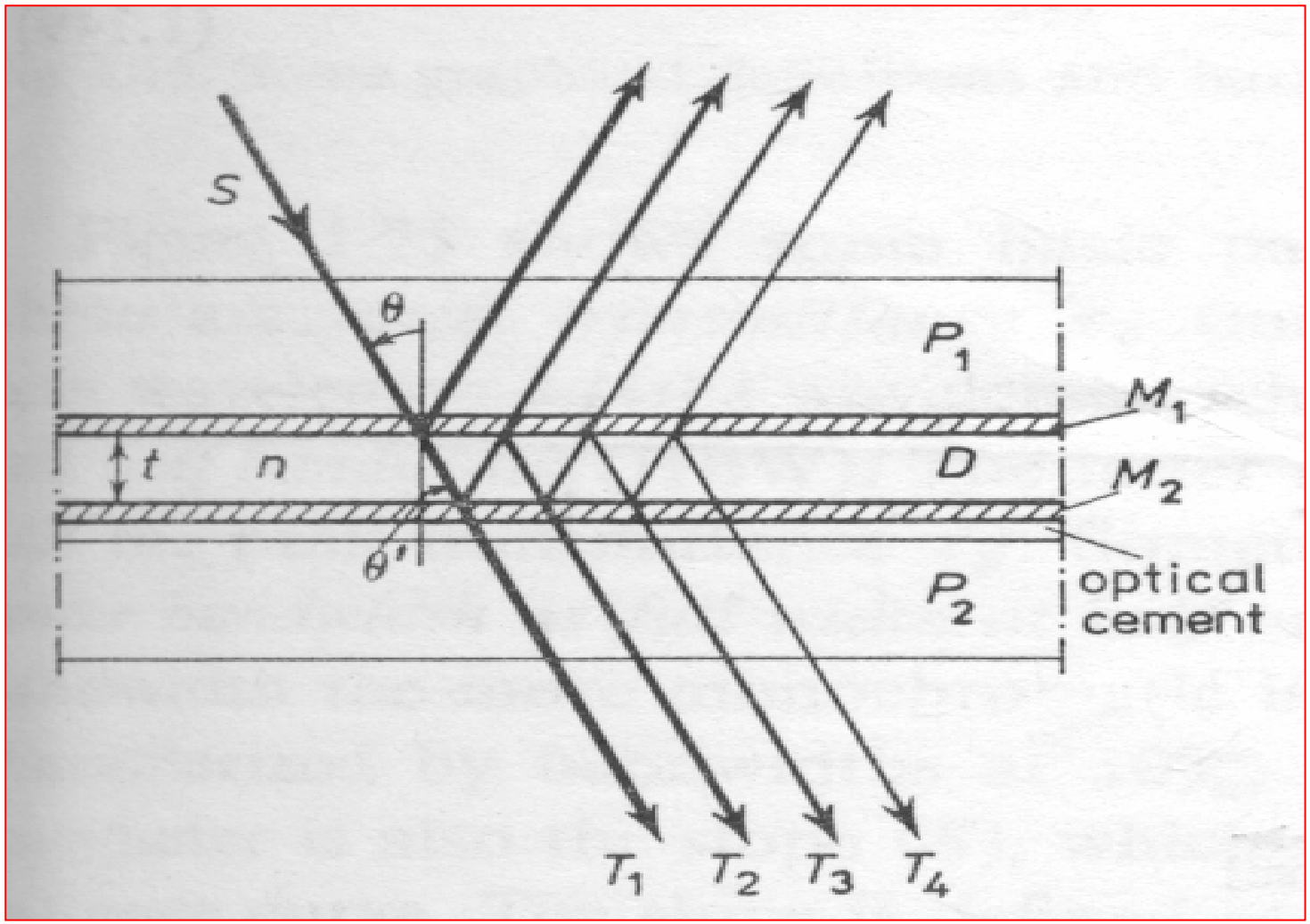
Argon and Krypton Laser Lines



WAVELENGTH SELECTION

- Interference Filters and Filter Wheels
- Electro-optical methods: AOTF and LCTF
- Fiber Optic Coupling to Source
- Double-View Microscopy

Interference Filter Design



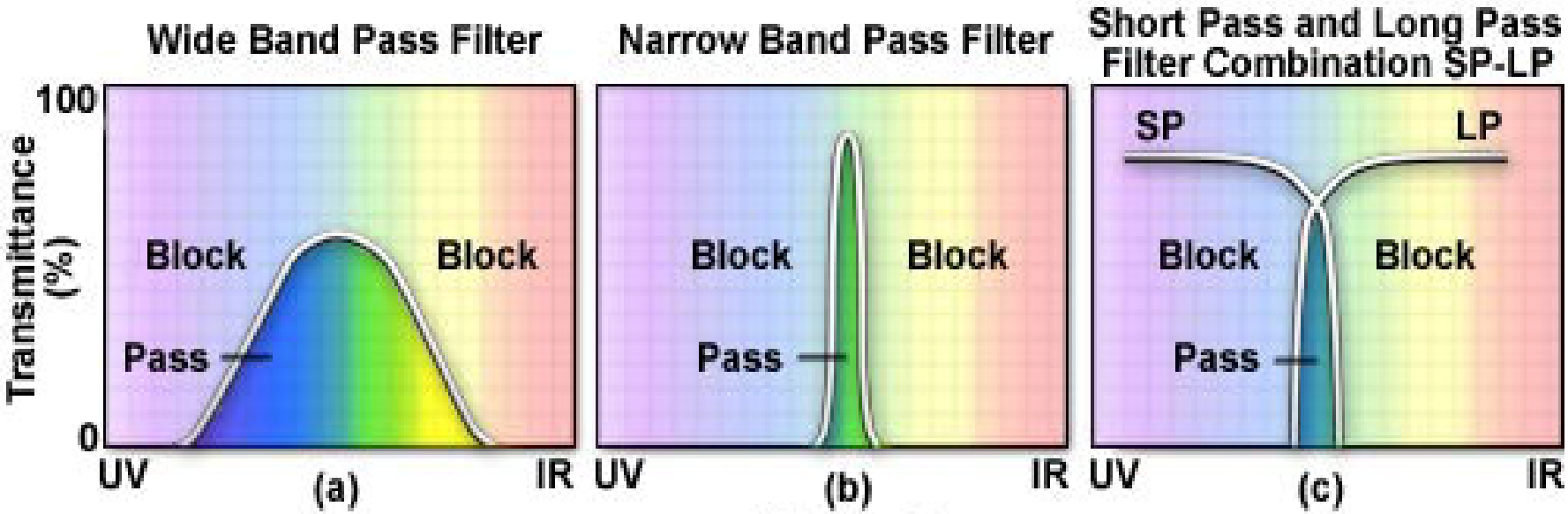
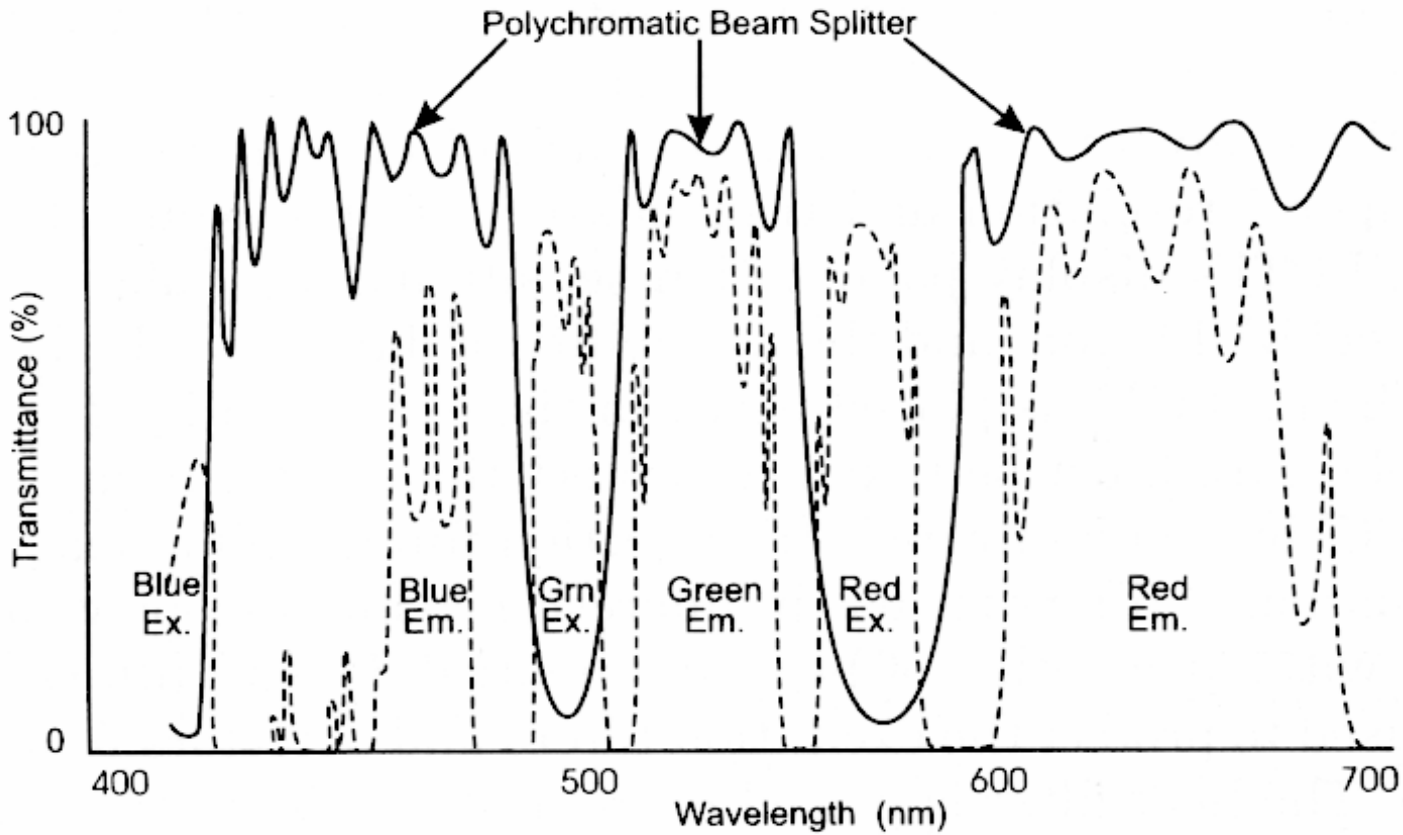
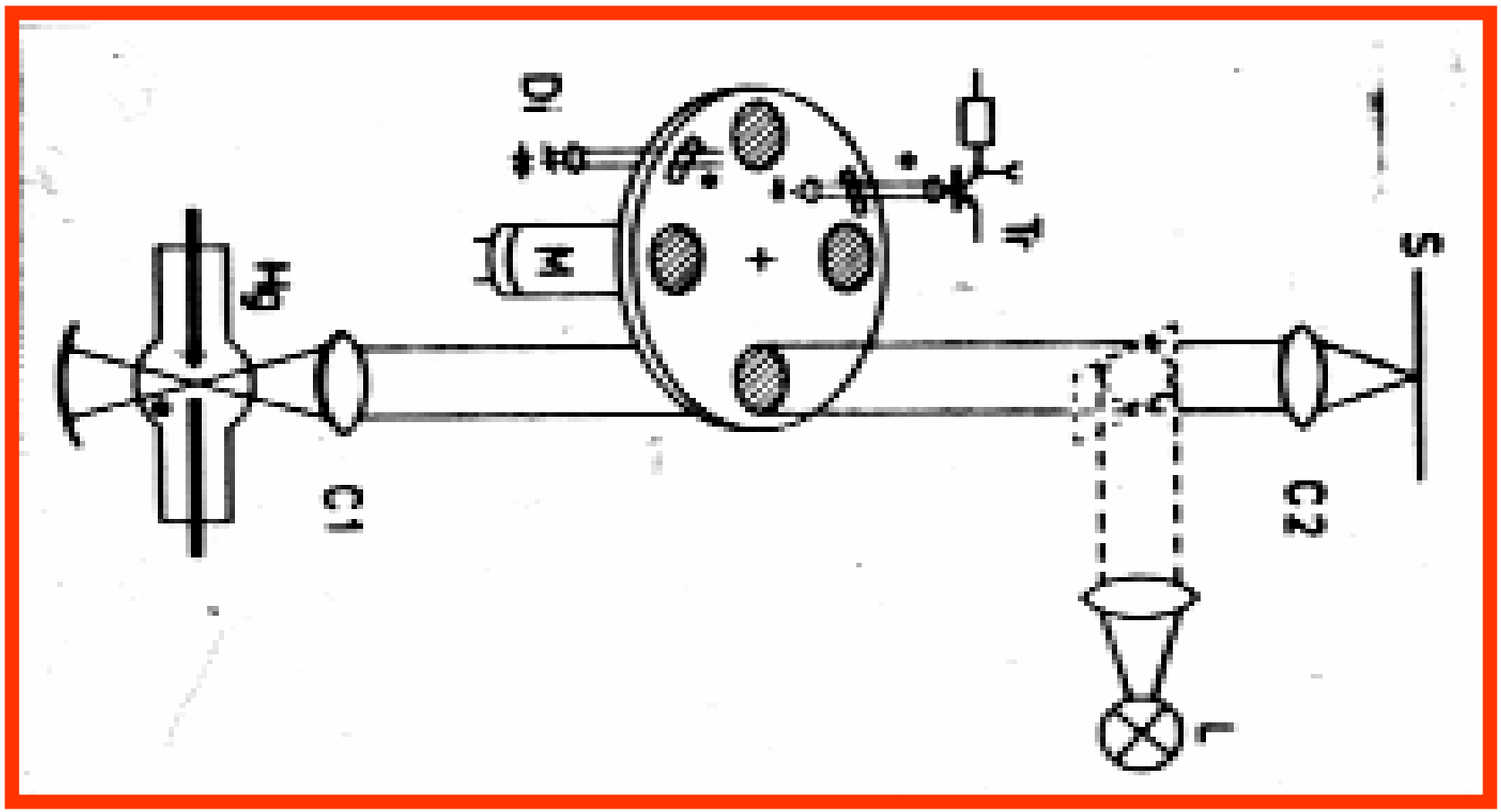

Wide Band Pass Filter
Narrow Band Pass Filter
Short Pass and Long Pass Filter Combination SP-LP
Transmittance (%)
100
0
Block
Pass
Block
UV
(a)
IR
Block
Pass
Block
UV
(b)
IR
SP
LP
Block
Pass
Block
UV
(c)
IR

Figure 3

Multi-Band Filter Set



Wavelength Selection by Filter Wheel



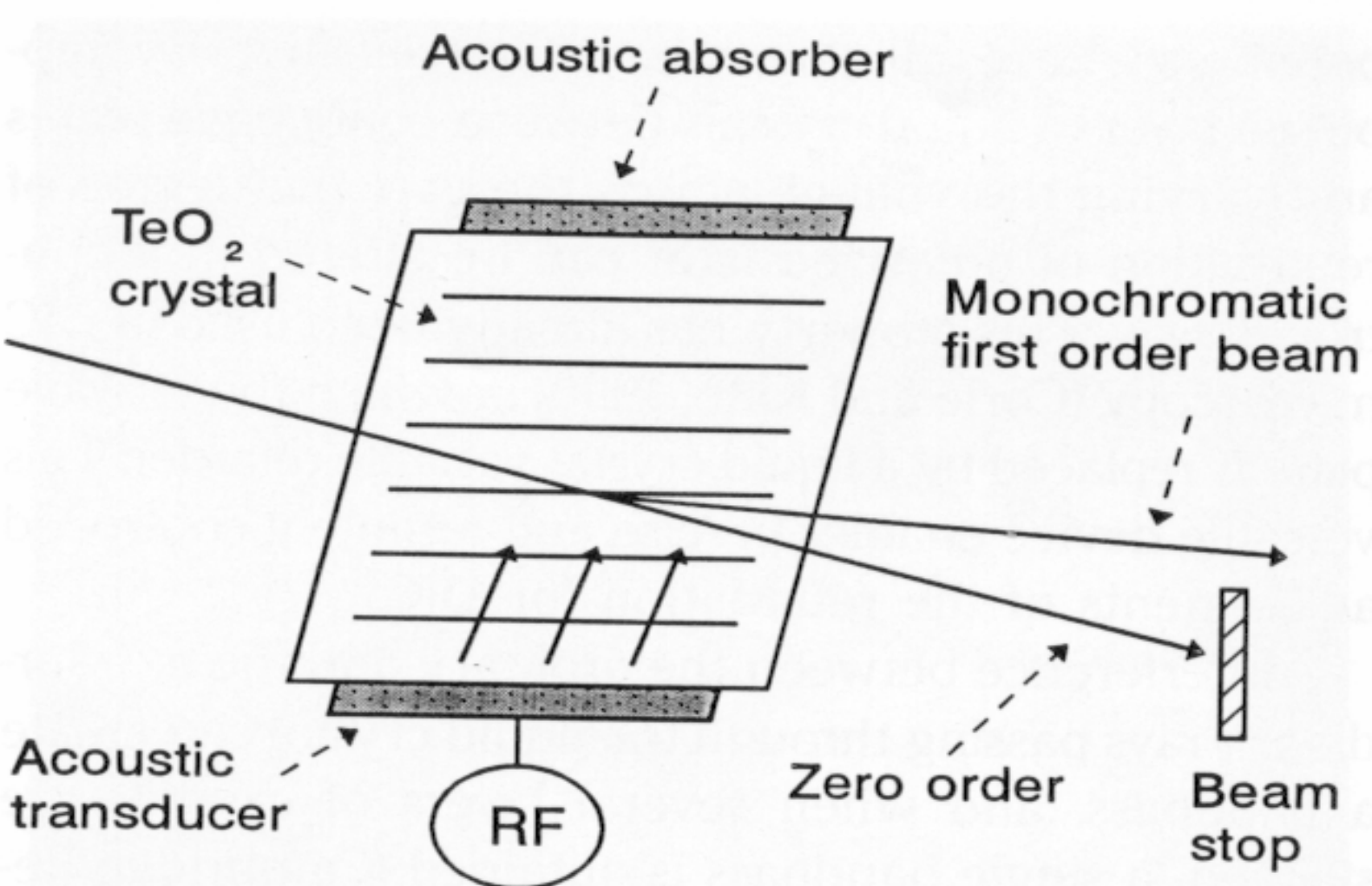
Electro-optic Wavelength Selection

AOTF

LCTF

PRISM

AOTF Principle



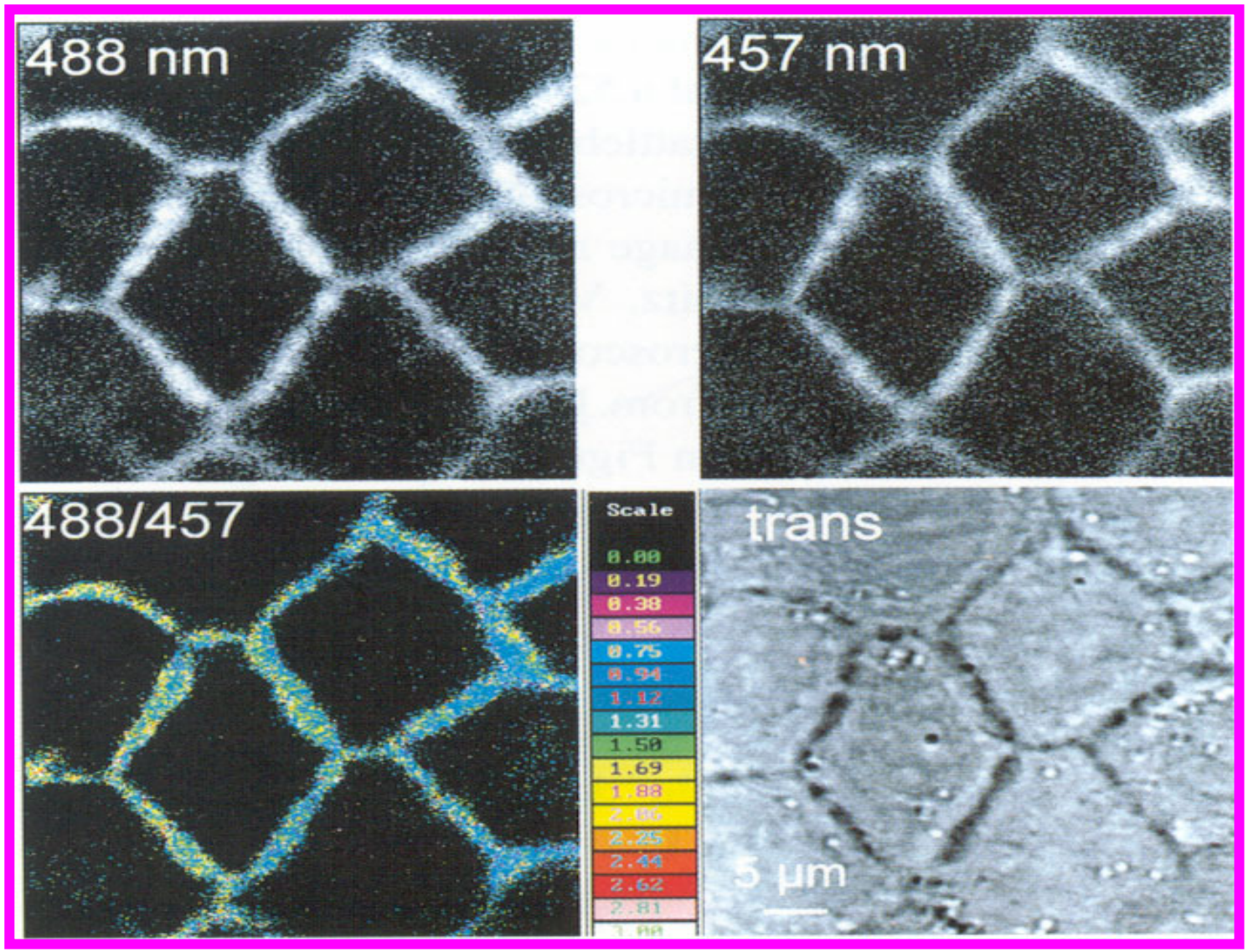
Acousto-optical tunable filter (AOTF) for excitation wavelength selection.

1. Requires a well collimated, polarized light beam of limited dimensions at the Bragg angle.

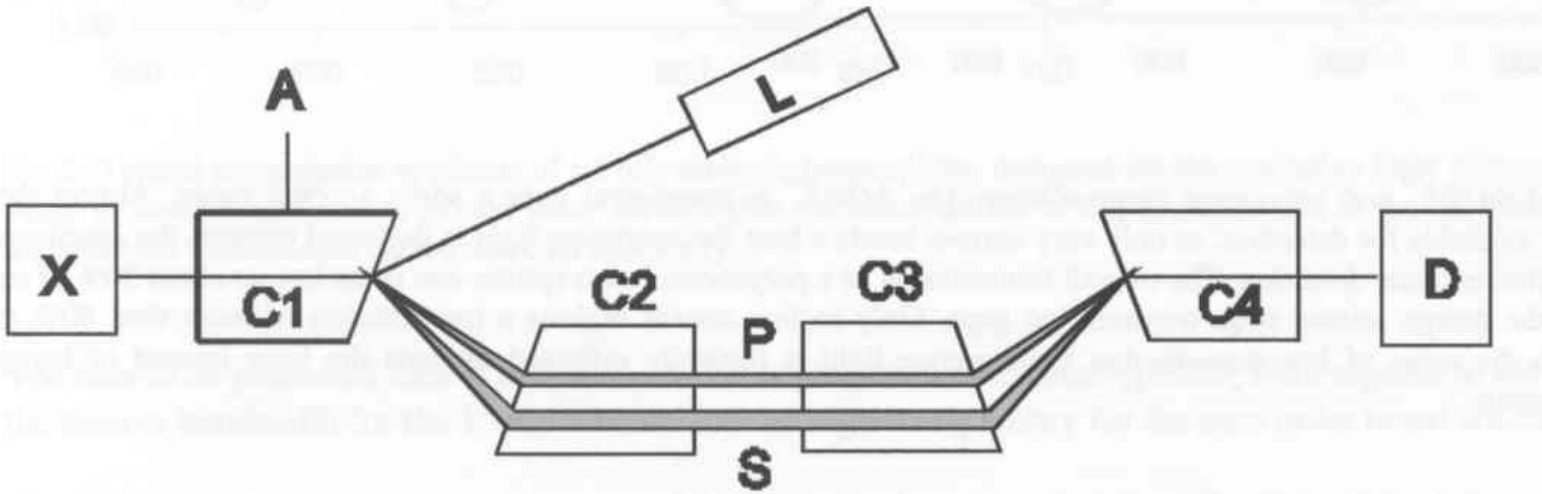
2. Diffraction efficiency of the first order beam can be as high as 85%. High speed (MHz) wavelength and power switching limited by propagation speed of acoustic wave.

3. Best with laser sources although devices for non-coherent sources have been constructed. Rugged.

Excitation Ratio Imaging with an AOTF

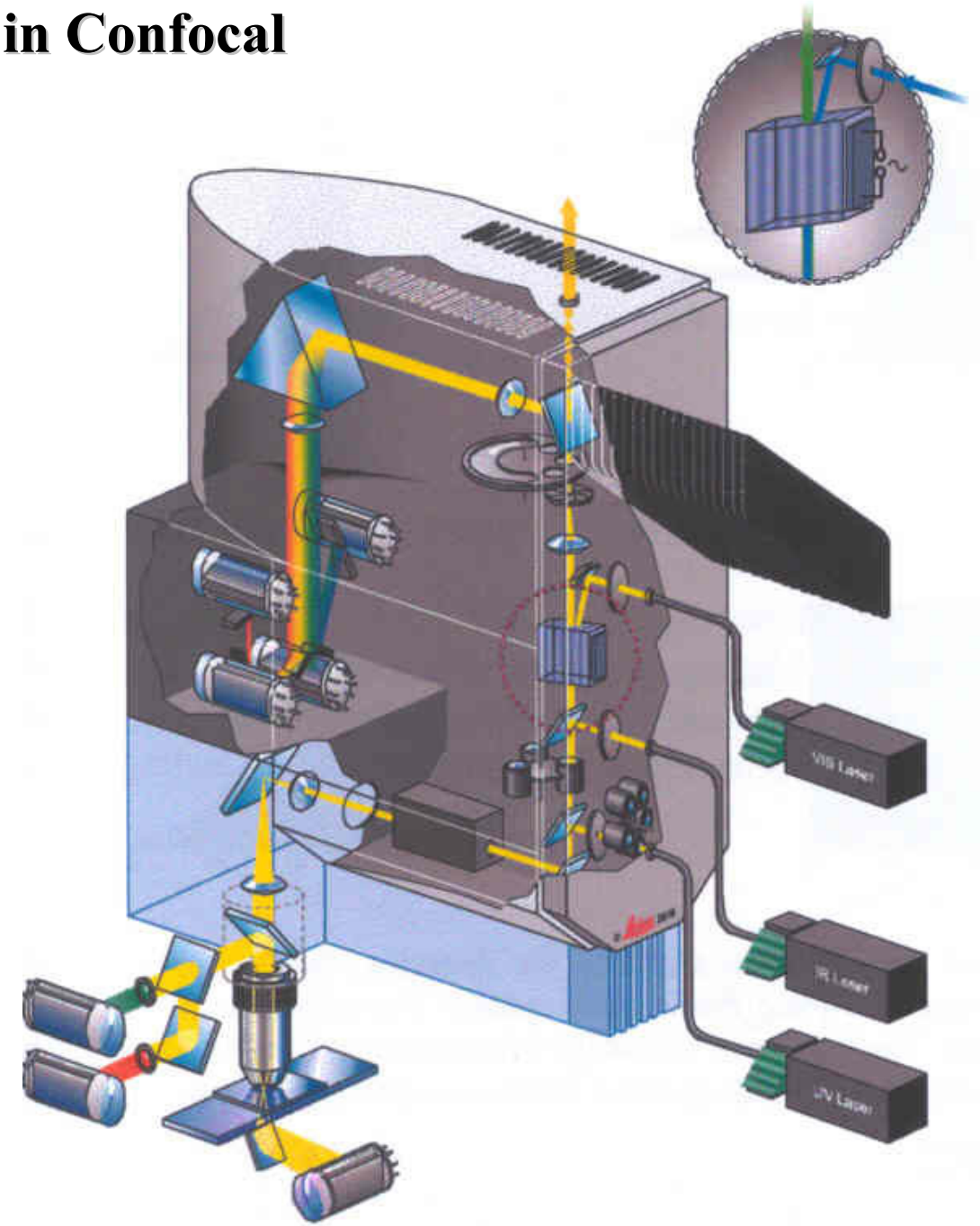


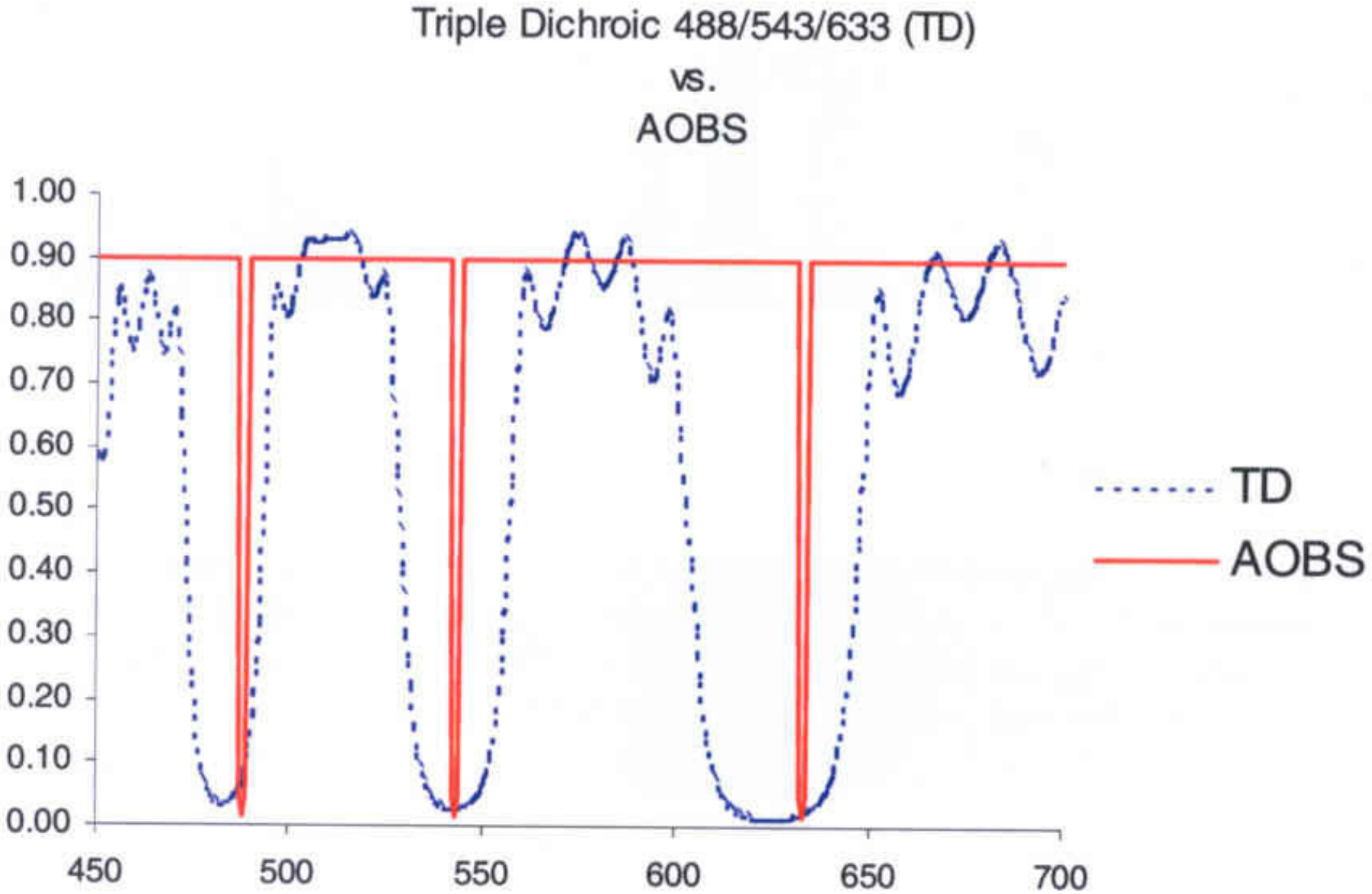
Leica's Acousto-optic Beam Splitter

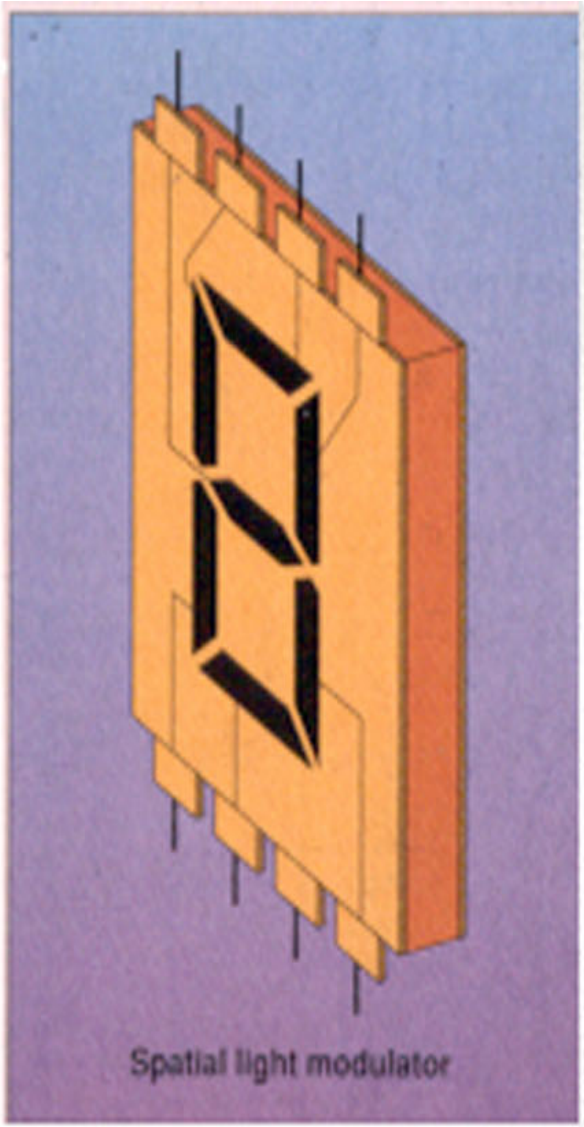


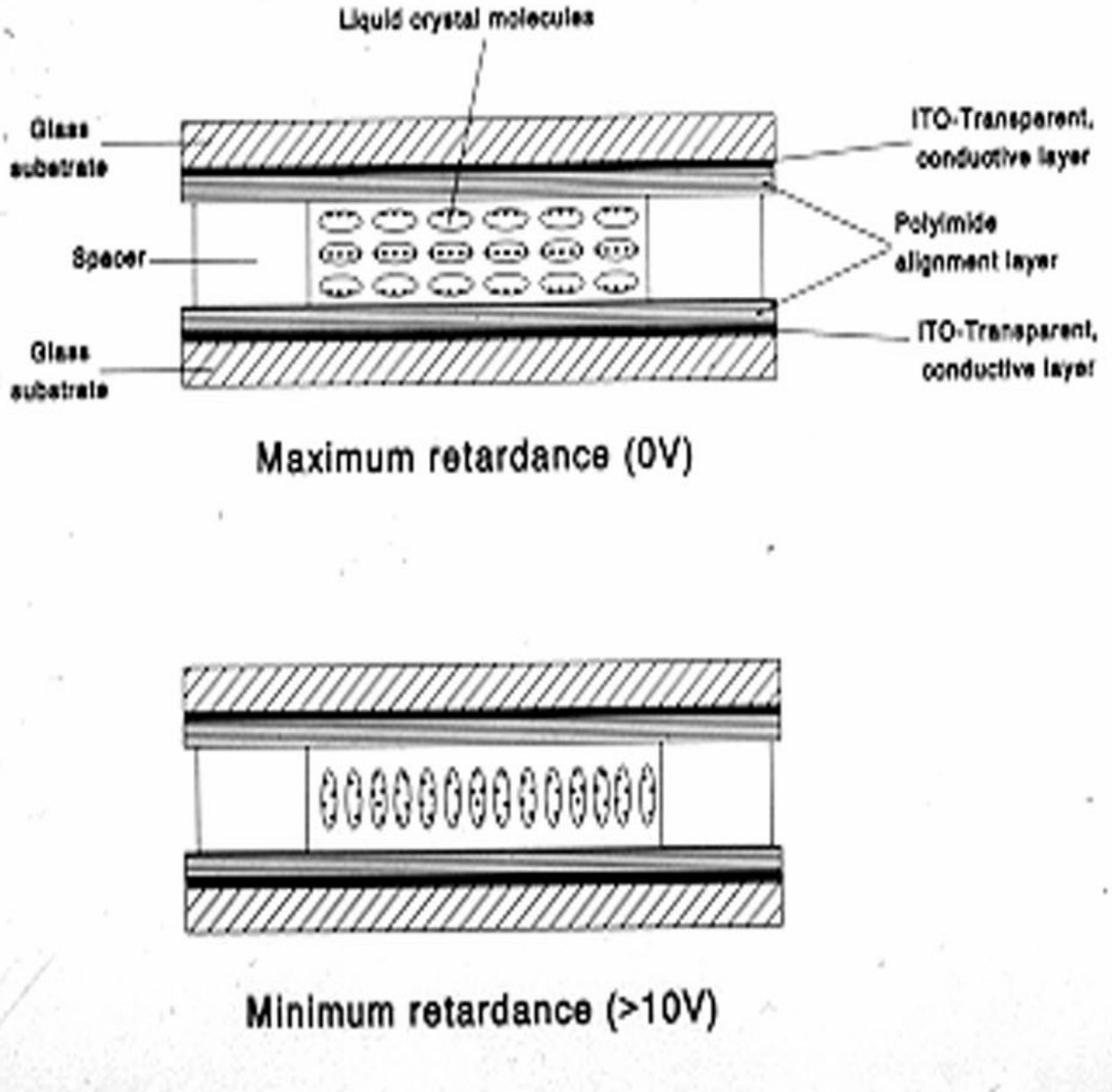
X = specimen, C1=AOBS, C2,C3,C4 = correction prisms, D =detector,
L=laser, A=acoutic input, P,S=polarization directions

Leica AOBS in Confocal

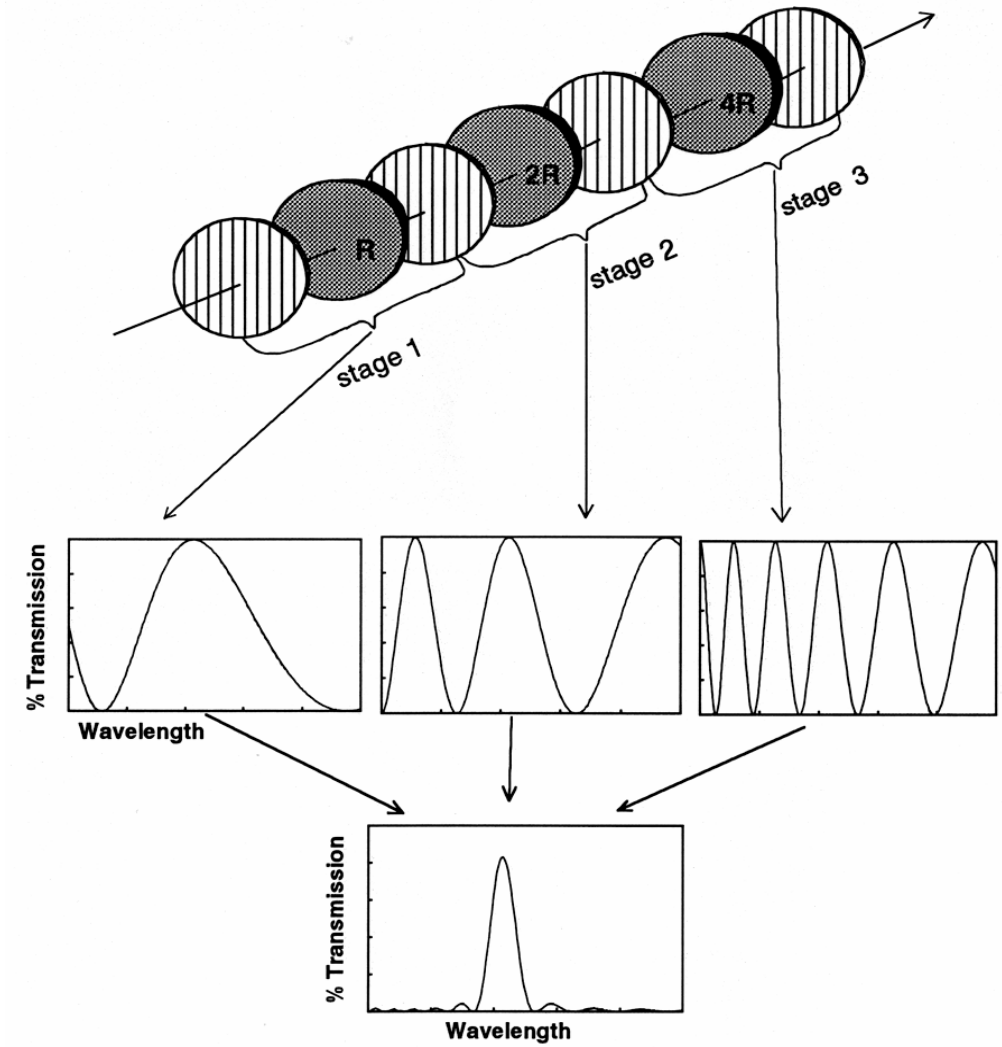



Triple Dichroic 488/543/633 (TD)
vs.
AOBS
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
450
500
550
600
650
700
TD
AOBS

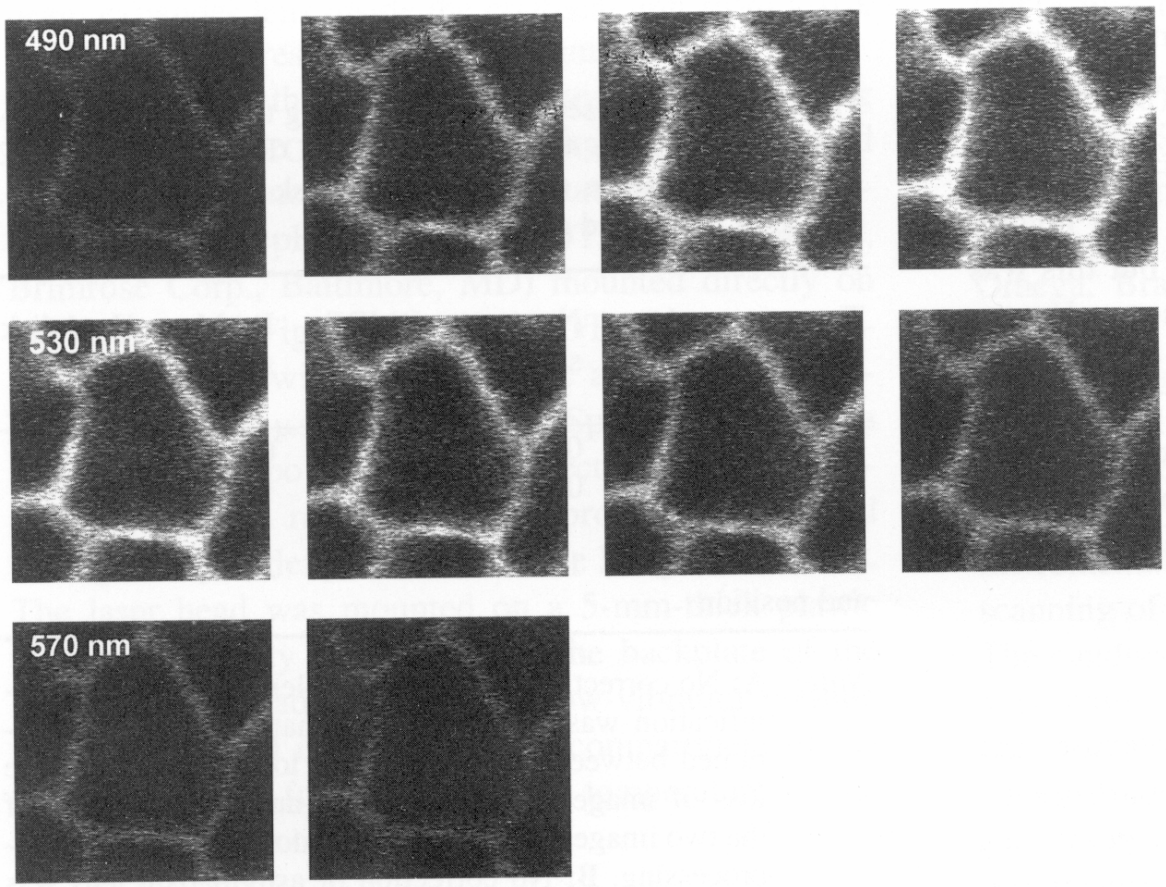

Spatial light modulator

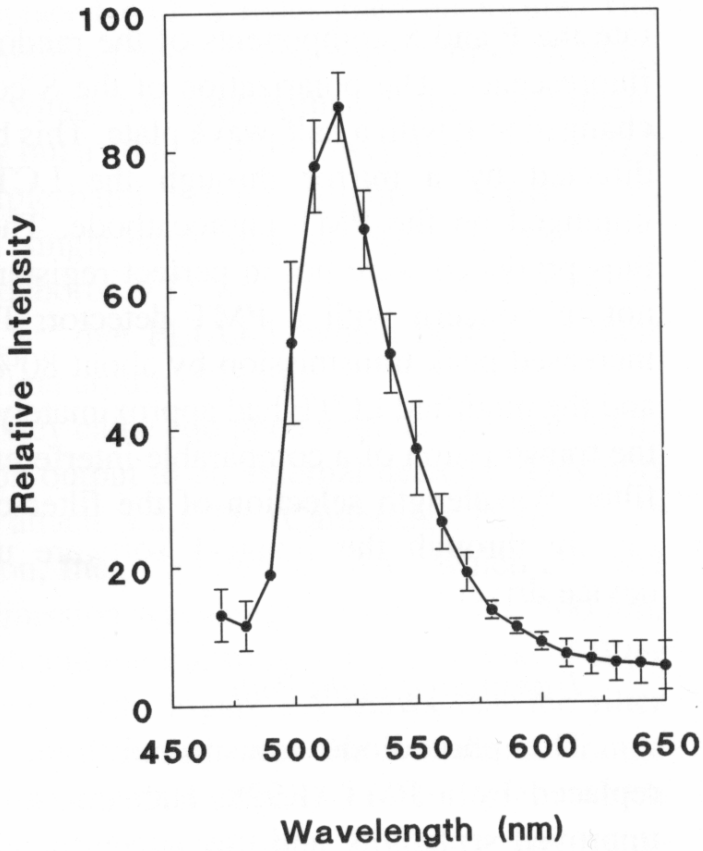

Liquid crystal molecules
Glass substrate
ITO-Transparent, conductive layer
Polyimide alignment layer
Spacer
Glass substrate
ITO-Transparent, conductive layer
Maximum retardance (0V)
Minimum retardance (>10V)

LCTF Design

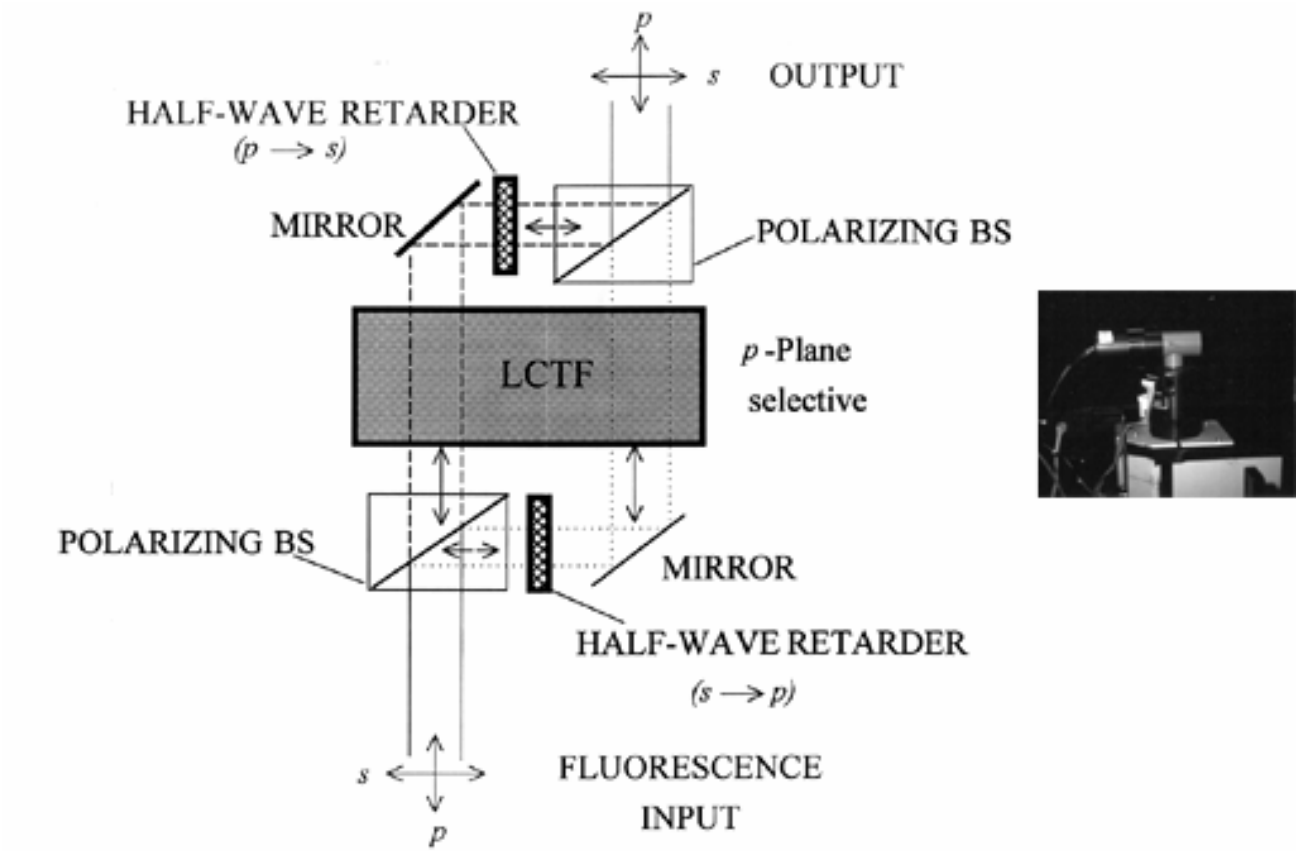

R
2R
4R
stage 1
stage 2
stage 3
% Transmission
Wavelength
% Transmission
Wavelength

Spectral Scanning with a LCTF





Recovering the Lost Signal



Liquid crystal tunable filter (LCTF) for emission wavelength selection.

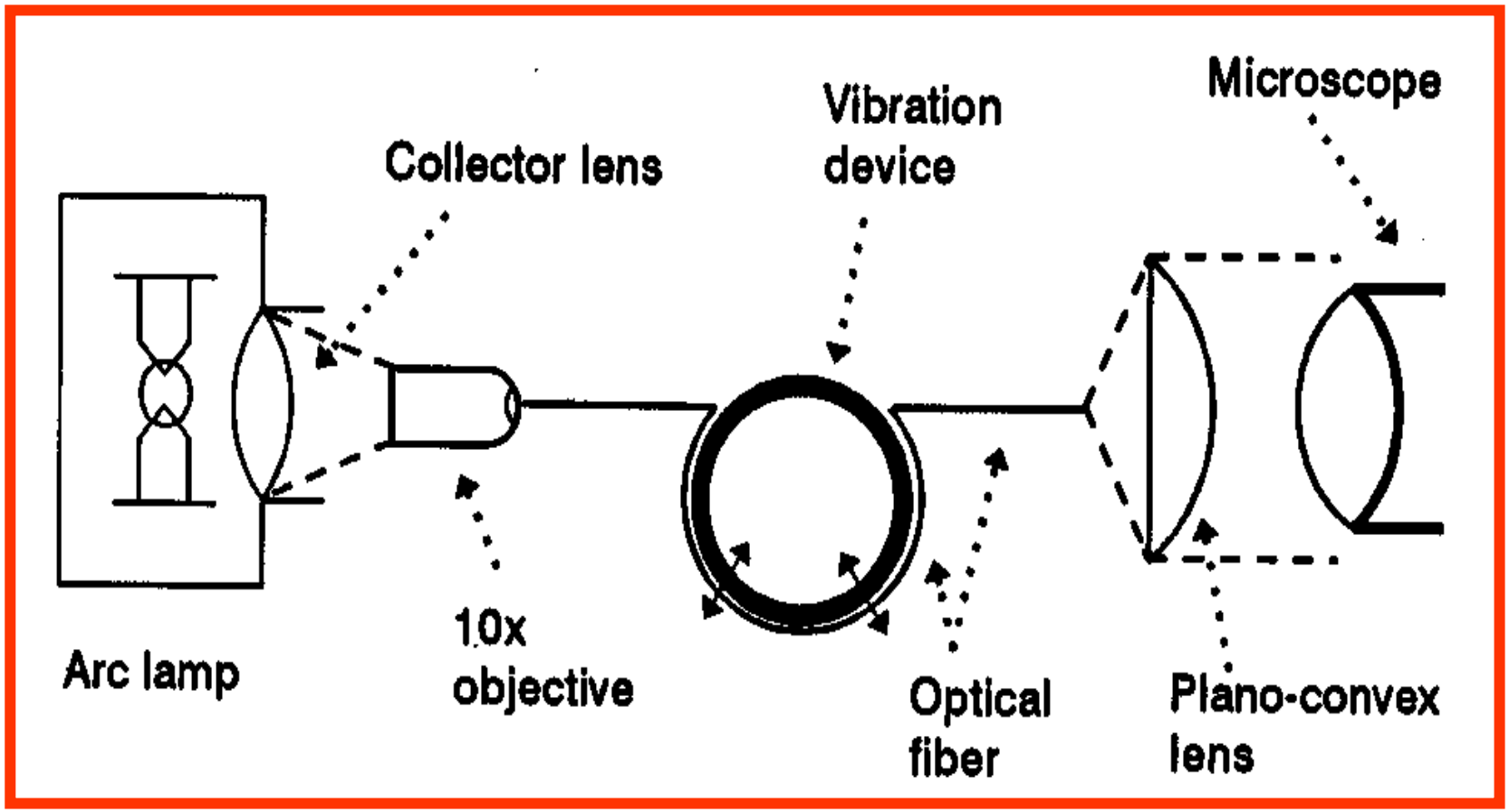
1. Wide acceptance angle, high spatial resolution, large clear aperture are strengths.

2. Limitations include slow switching speed (~25 ms), limited spectral range (400-750 nm) although near IR devices are available (700-1100 nm), requirement for polarized light, variable bandwidth, low transmission (10-30% of unpolarized light), low damage threshold.

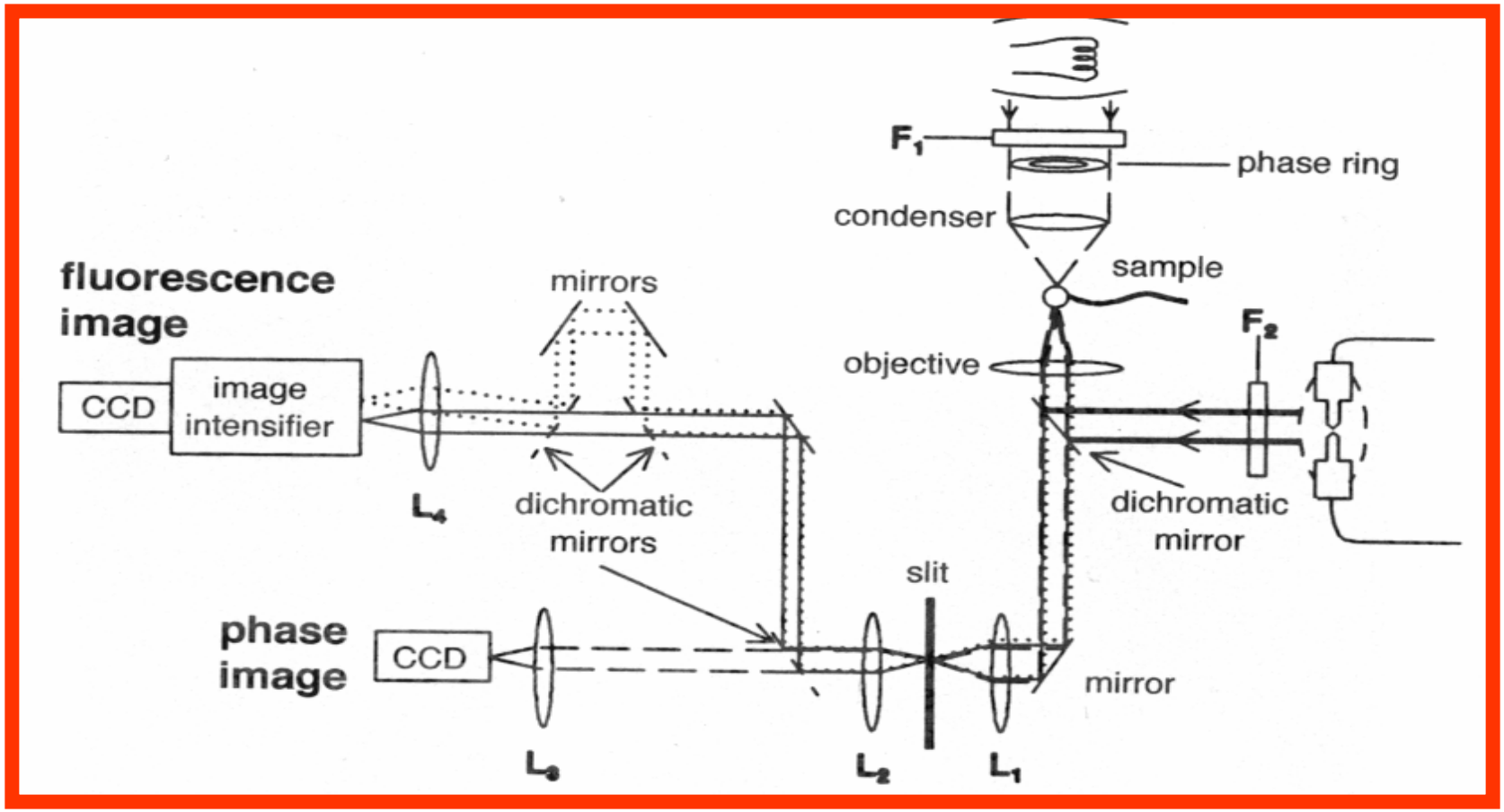
Fiber Optic Coupling in Microscopy

- Two types of fibers: single- and multi-mode
- Single-mode fibers are small (3-8 um ID) and propagate only one mode from the laser and produce a perfect Gaussian beam.
- Multi-mode fibers are large (100-1200 um ID) and propagate many modes. They produce a top-hat profile output.

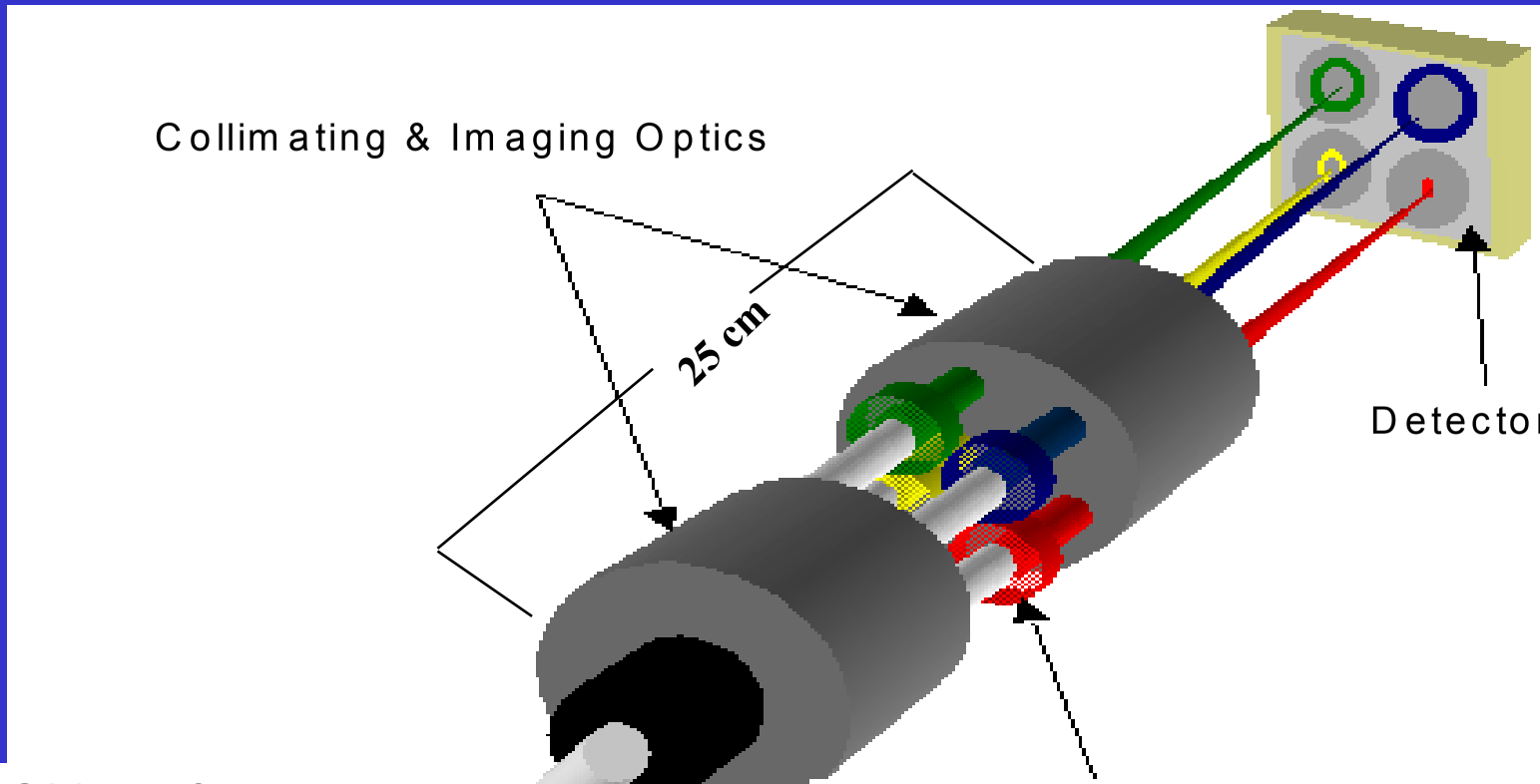
Fiber Optic Coupling of Light Source with a Multi-mode Fiber



Double-View Microscopy (Kinoshita) Optical Insights

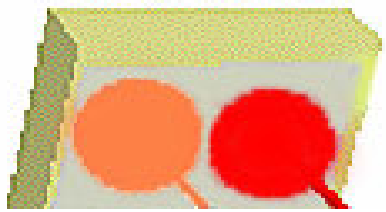


Multi-Channel Imaging Spectrometer: MCIS
Optical Insights



SNARF-1 Analysis

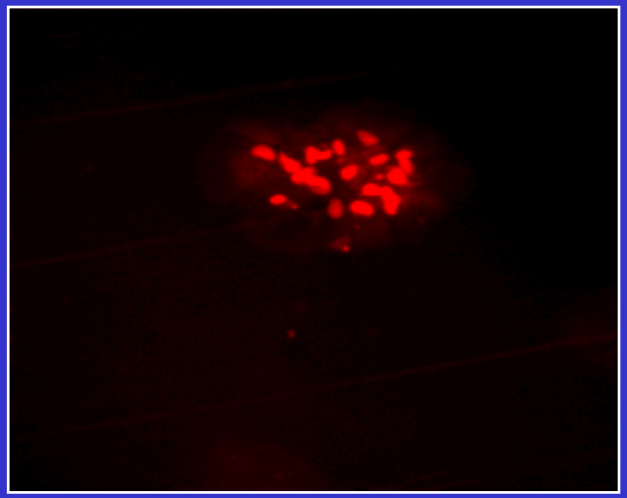
570 nm 640 nm

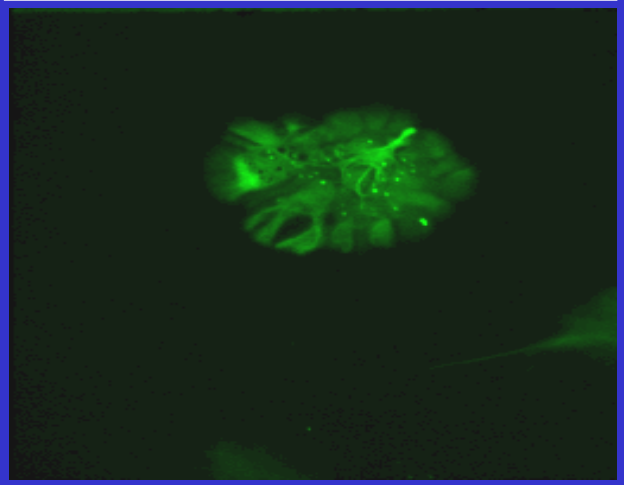


R.M. Lynch et al., U. AZ.

Astroglia and Neurons: GFAP-Alexa and Propidium Iodide

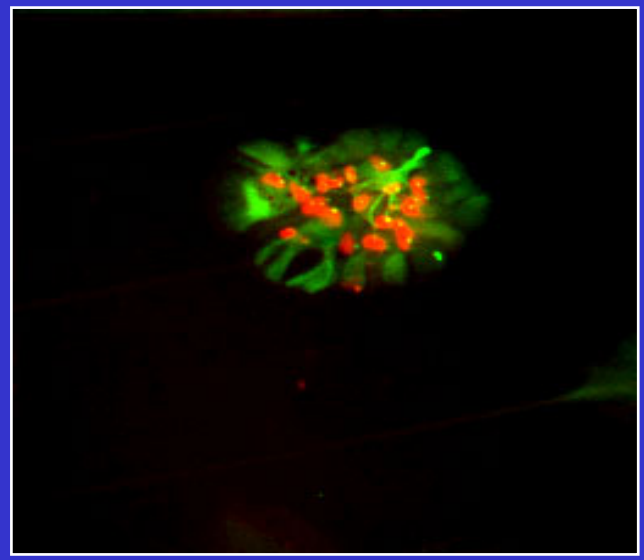
PI : > 600 nm





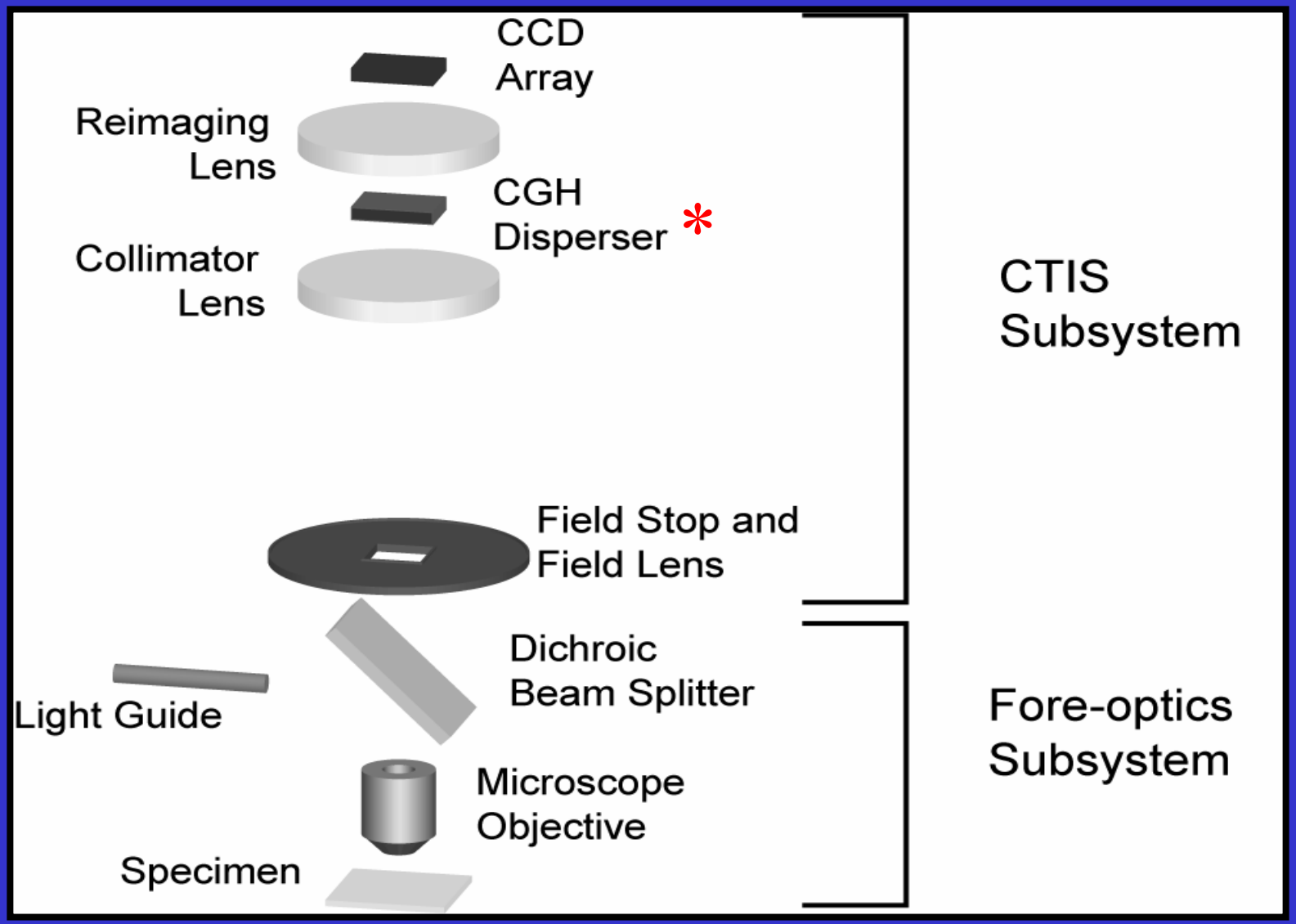
Alexa 530

Overlay

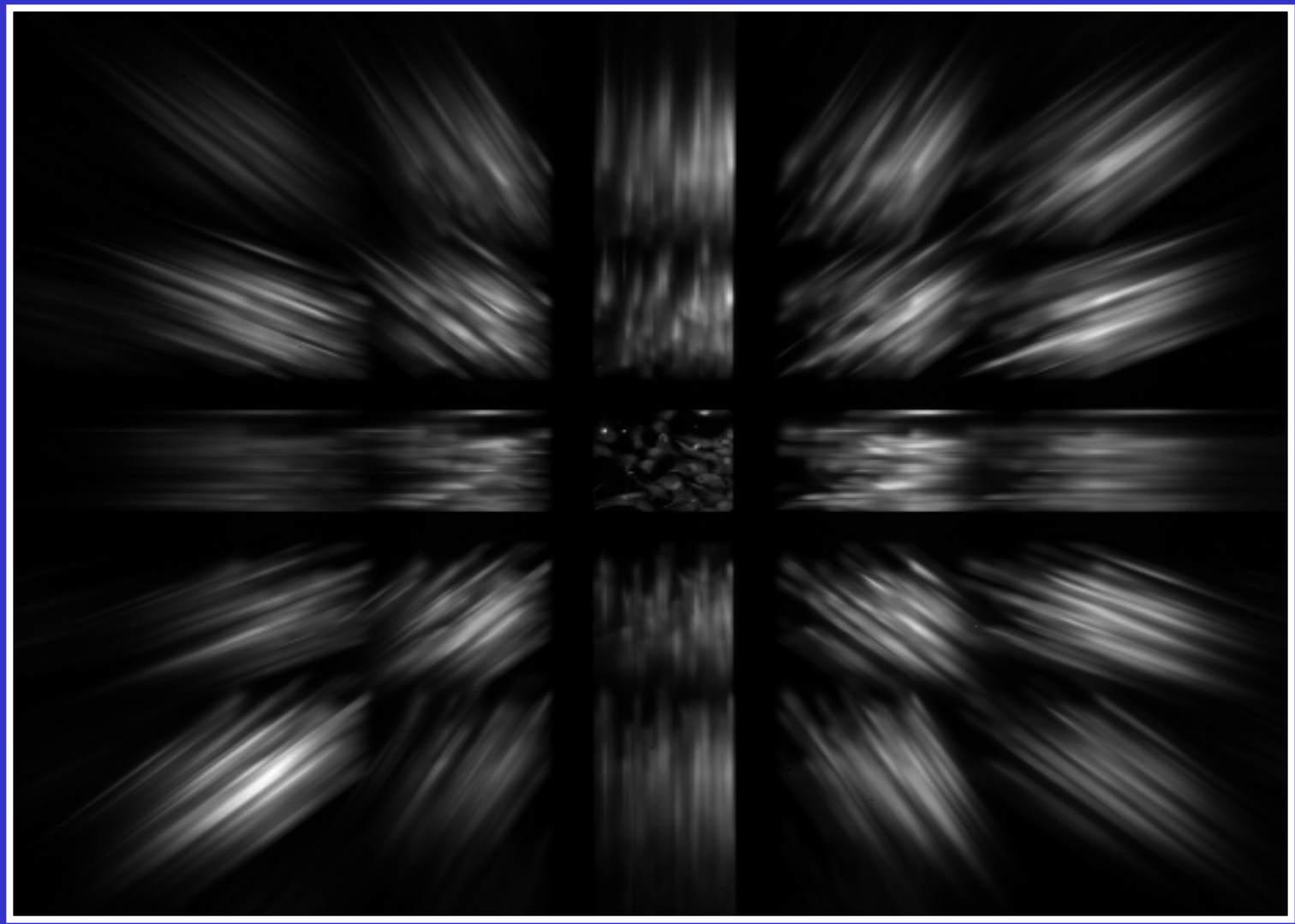


488 nm Excitation

Computed Tomography Imaging Spectrometer

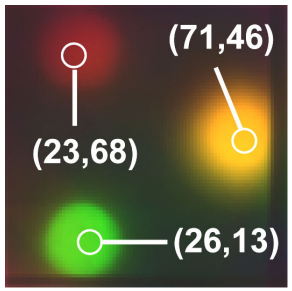


RAW DIFFRACTION IMAGE FROM CTIS MICROSCOPE

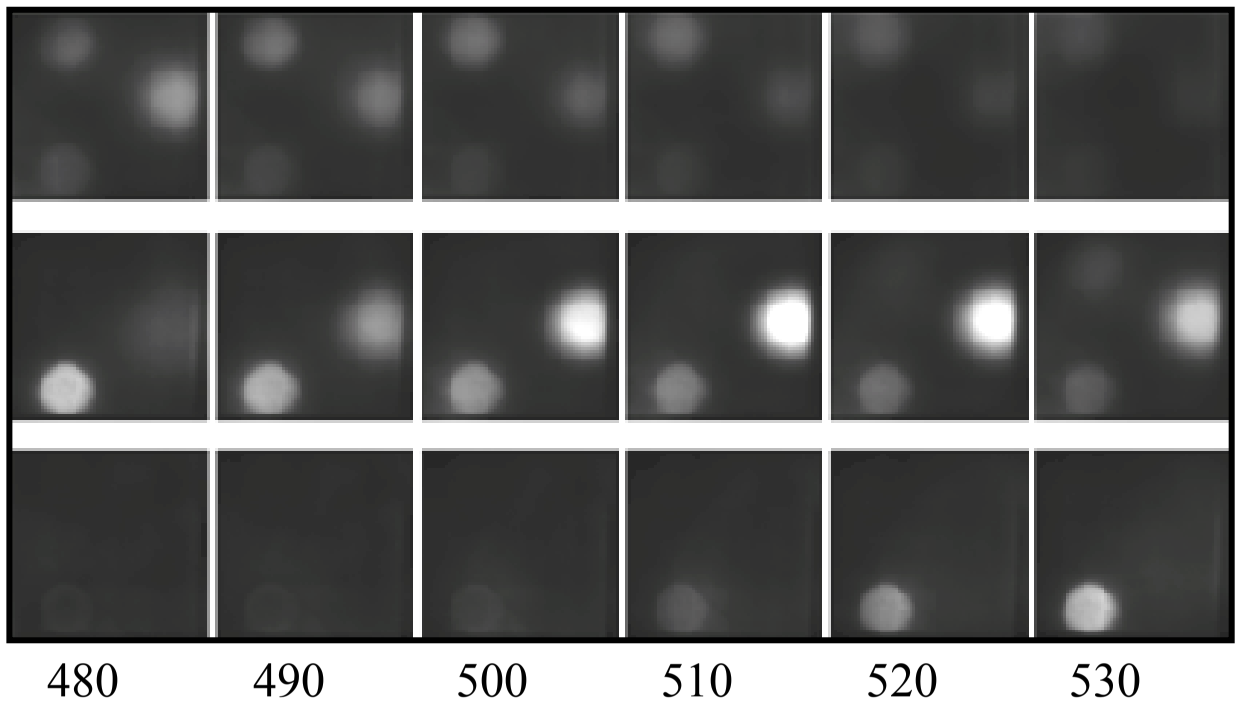


Reconstructed Spectral Images

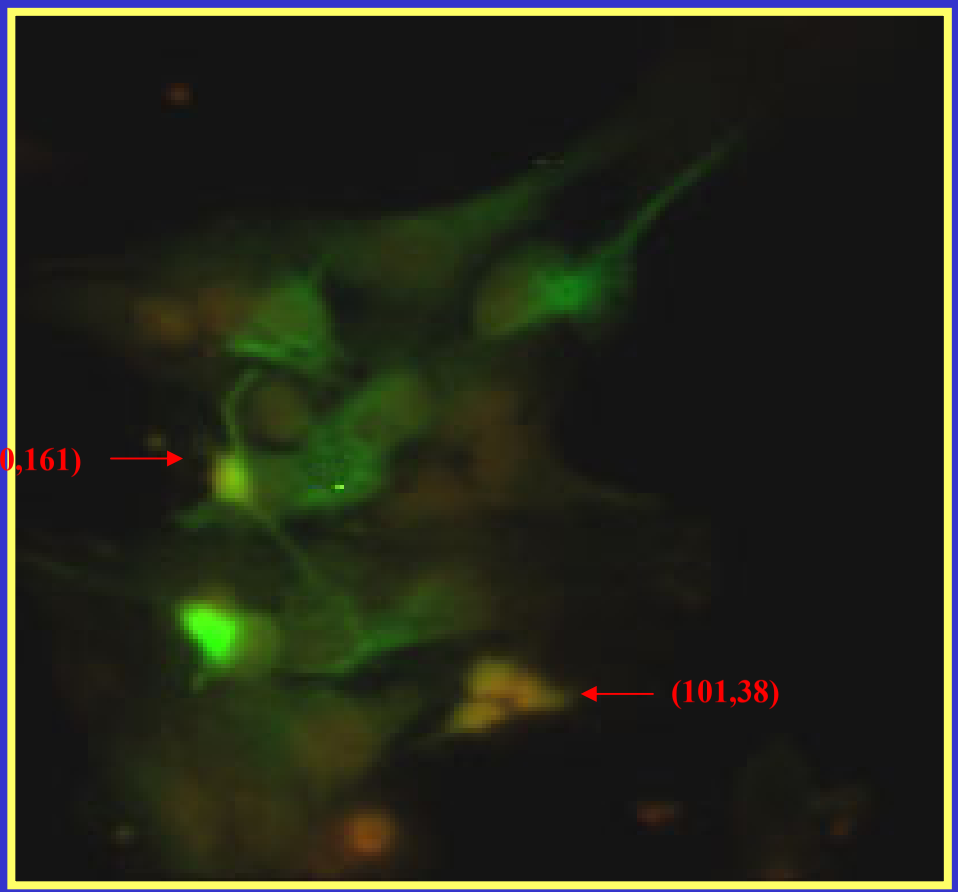
20 μm Beads

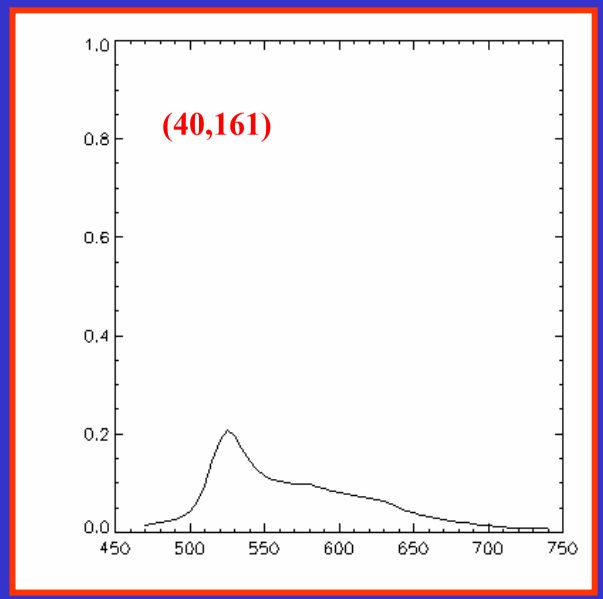


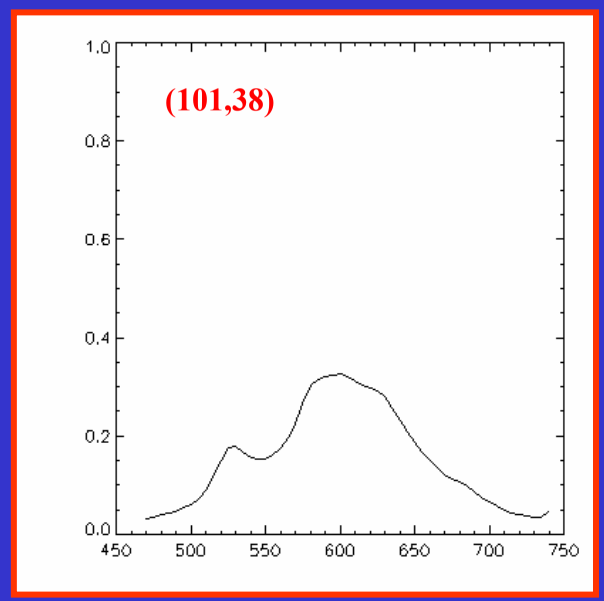
83 ⊛ 83 ⊛ 30 Reconstructed Object cube



GFAP-Alexa and PI Labeling of RIN-3M1 Cells







Limitations:

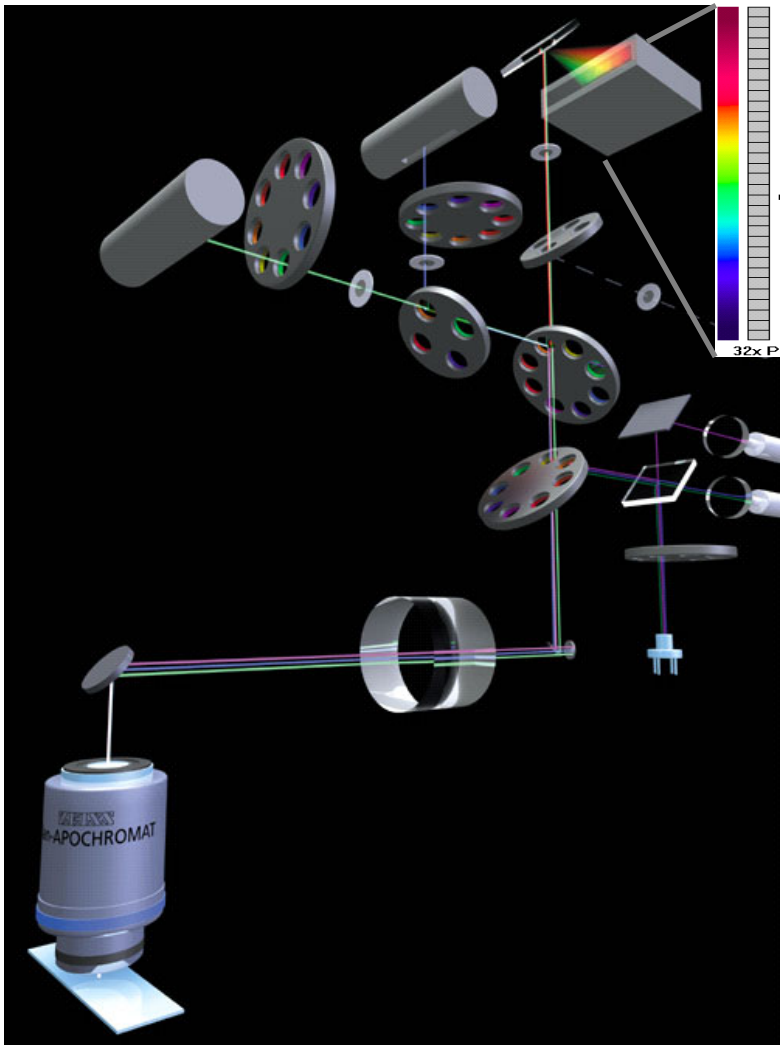
-Limited Range of Excitation Wavelengths

CTIS Limitations:

- Signal to Noise Ratio Limits the Temporal Resolution
-Spatial Resolution is Limited by the Chip Size

- Not Effective on Low Contrast Images.
Implementation of Structured Illumination.

Look inside the solution – LSM 510 META

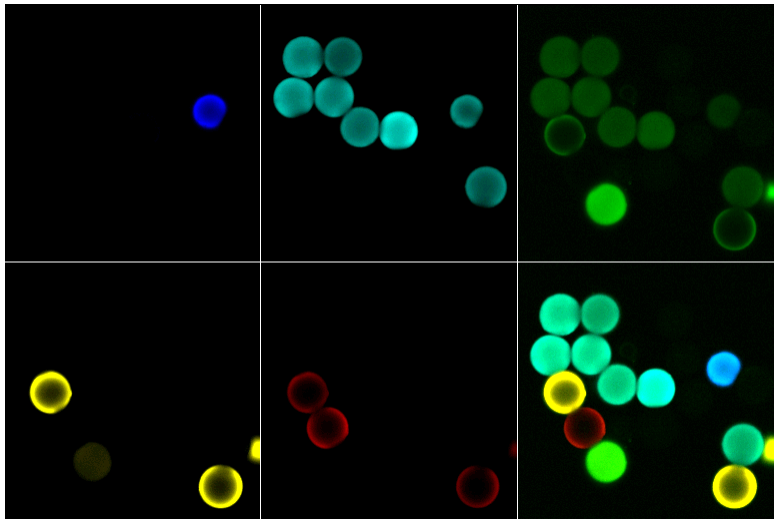


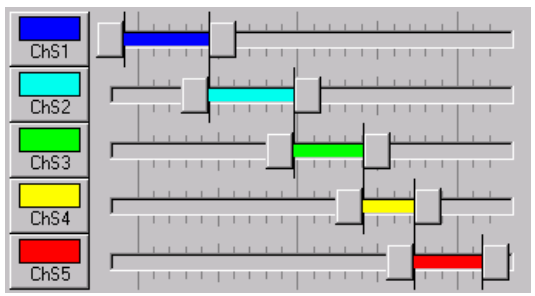
- Multiple pinhole concept
- Adjustable pinholes (x,y, Ø)
- Efficient beam path

- META detector
  - PMT array with 32 elements
  - Reflection grating for even, temperature-insensitive dispersion
  - Capture full emission spectra

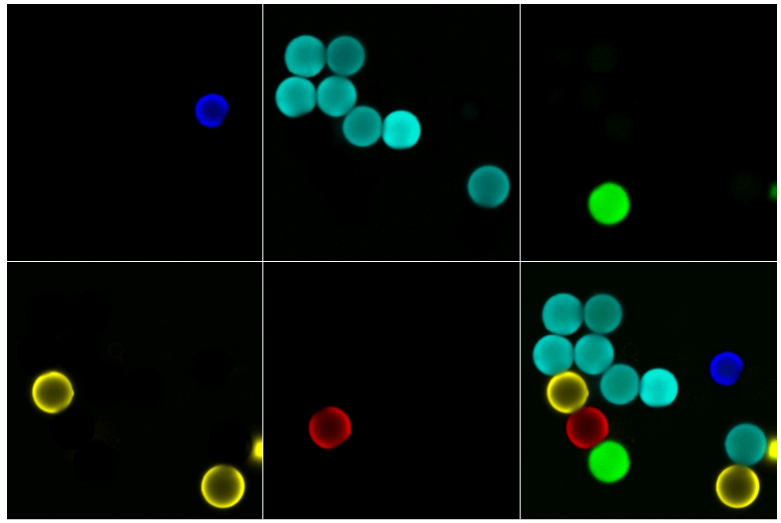
Emission Fingerprinting

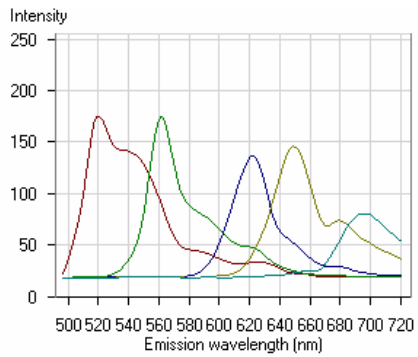
Insufficient separation
using (variable) band pass detection





Crosstalk-free separation
using Emission Fingerprinting



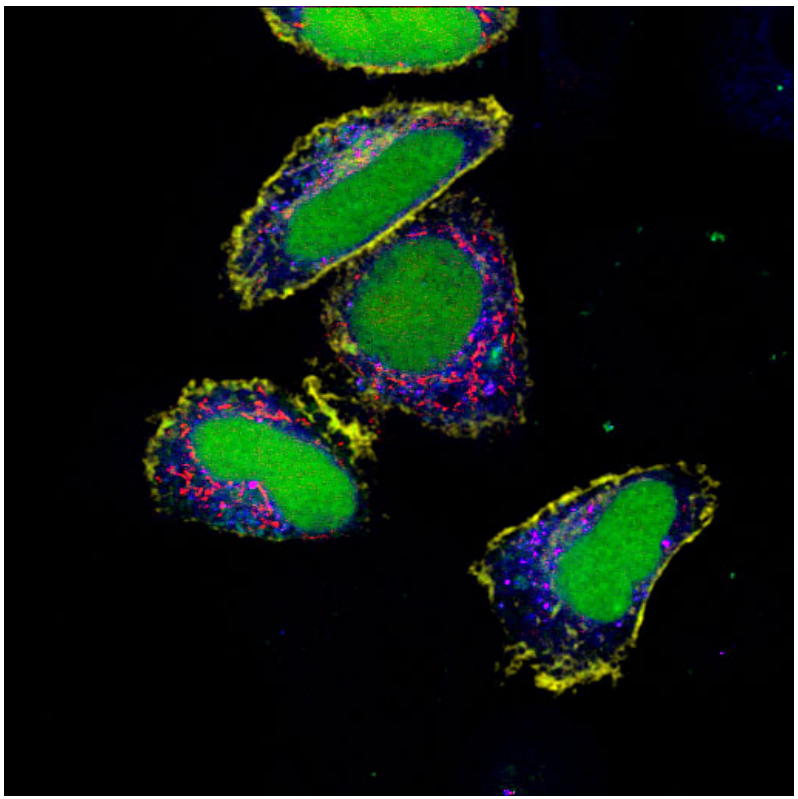


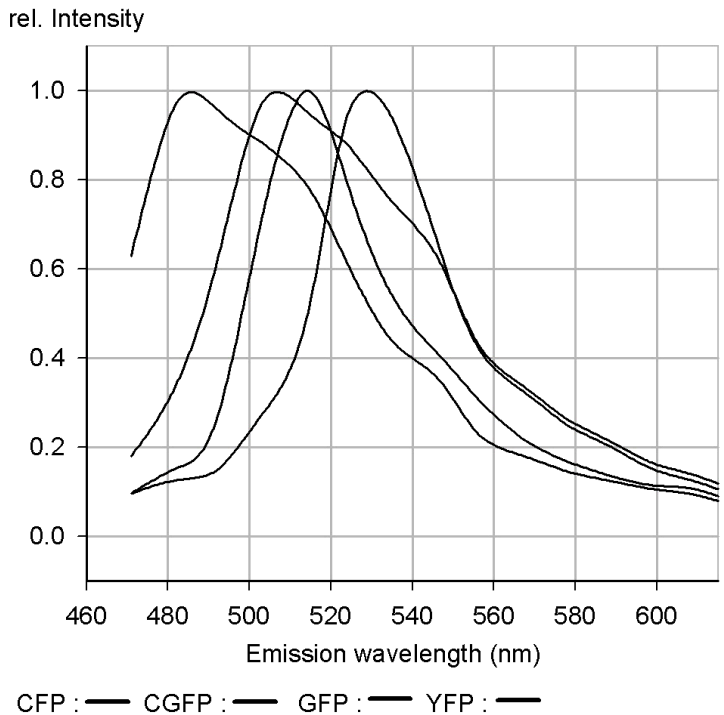
Multi-(5)-color beads

Emission Fingerprinting
4 FPs separated

CFP, CGFP, GFP and YFP

Cultured cells expressing 4 FPs in ER, nuclei, plasma membranes and mitochondria, repectively



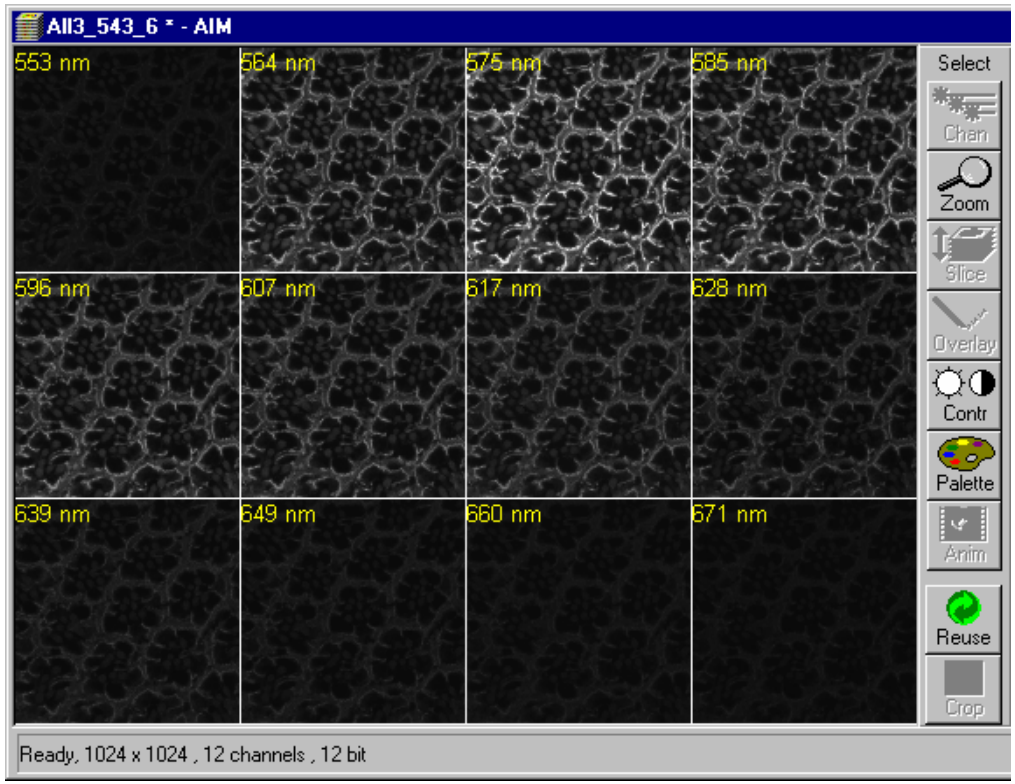


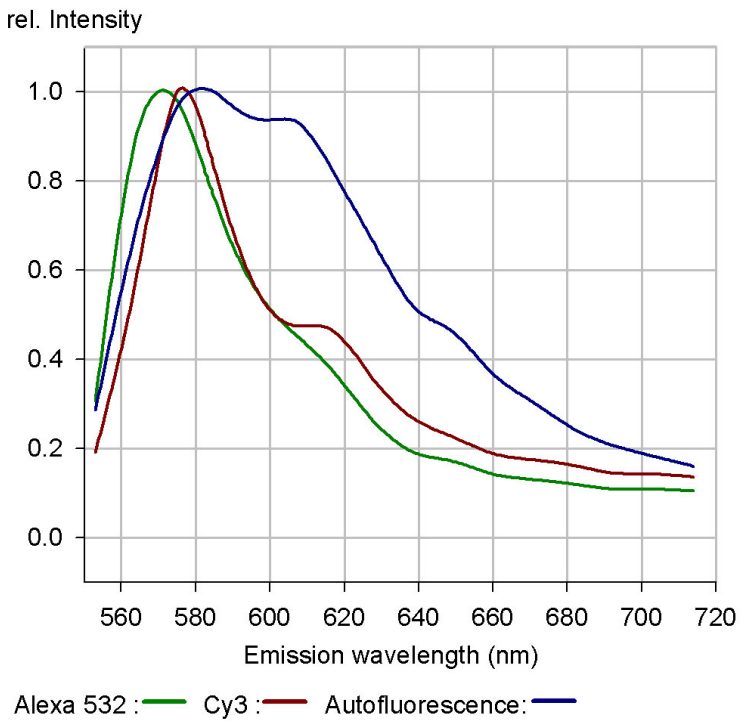
Sample: Drs. Miyawaki, Hirano, RIKEN, Wako, Japan

the auto-fluorescence issue

Alexa 532, Cy3 and autofluorescence

Section through fly (*Drosophila melanogaster*) retina labeled with Alexa 532-phalloidin and Cy3 (Na^+/K^+-ATPase immunostain)



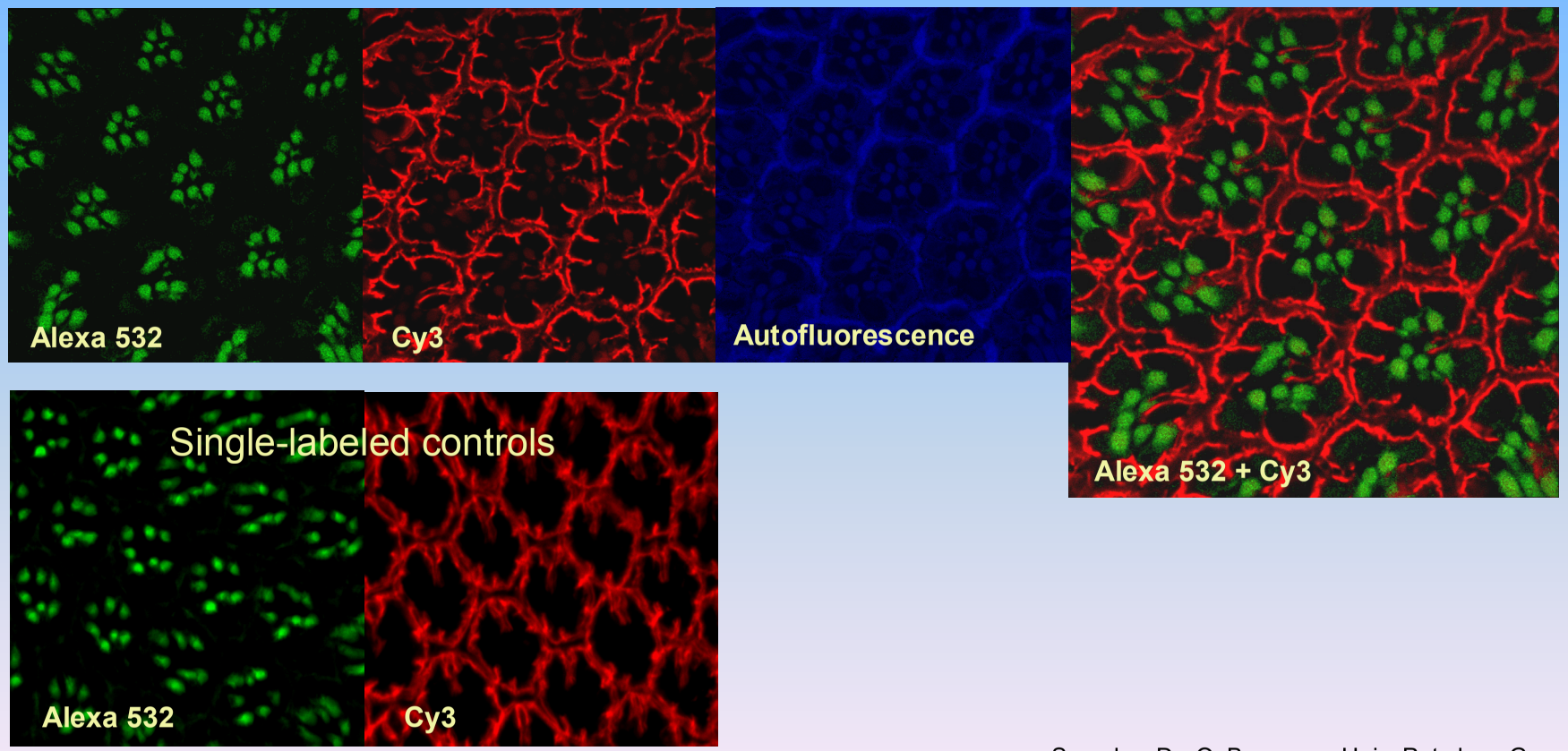


Sample: Dr. O. Baumann, Univ. Potsdam, Germany

False Color Images

Alexa 532, Cy3, and autofluorescence

Section through fly (*Drosophila melanogaster*) retina labeled with Alexa 532-phalloidin and Cy3 (Na^+/K^+-ATPase immunostain)

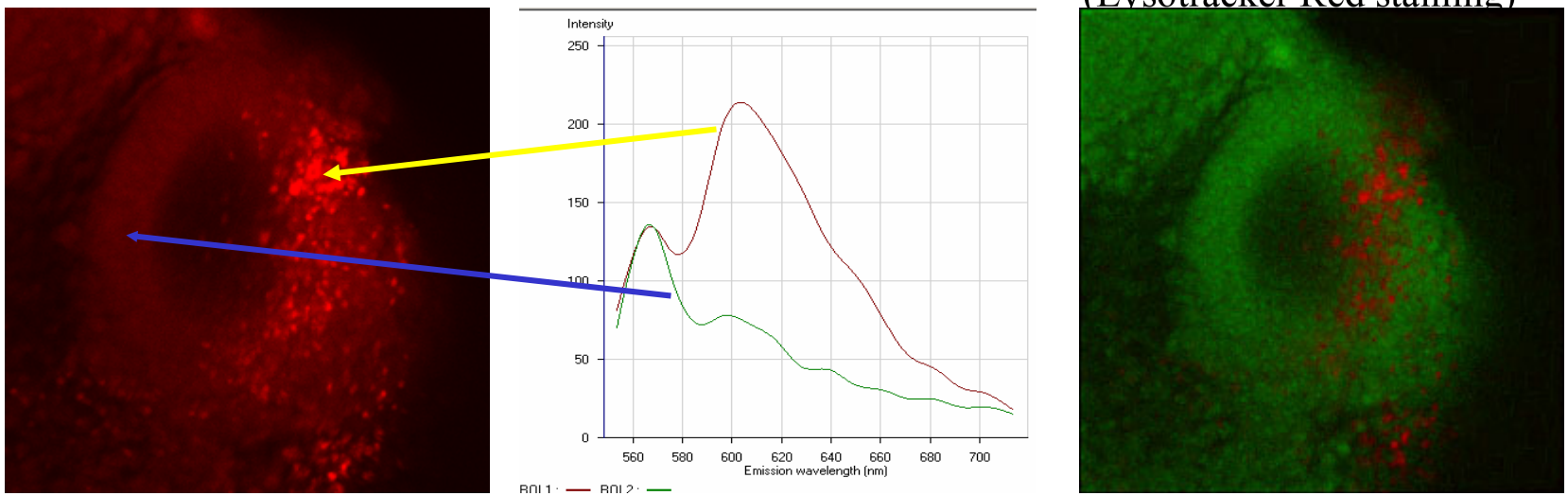


Samples: Dr. O. Baumann, Univ. Potsdam, Germany

Investigation of spectral changes

Identification/visualization of apoptotic cells in mouse embryo

(Lysotracker Red staining)



“bandpass” image	spectral analysis	unmixing
(just shows bright spots)	(reveals emission change	(clearly separates cell types)

using META and Emission Fingerprinting

Sample: Bob Zucker, EPA