LOW-LIGHT-LEVEL IMAGING

1. The Problem
2. Low-light-level Cameras
3. Solutions
How Much Light? I.

Illumination system:
- 75 W Xenon Arc (~1 mW/nm in visible)
- 490/10 nm exciter filter (60% T)
- 505 nm dichromatic mirror (85% Reflect)

\[ 10 \text{ mW} \times 0.6 \times 0.85 \times 0.95 = 4.8 \text{ mW} \]

Field 40 microns diameter, area = 12.6 x 10^{-6} cm^2

\[ \text{Flux} = 4.8 \times 10^{-3} \text{W} / 12.6 \times 10^{-6} \text{cm}^2 = 380 \text{ W/cm}^2 \]
How Much Light? II.

• 380 W/cm² is about 2500 times the flux of sunlight on the brightest day.

• Fluxes in a confocal may be as high as 8 x 10⁵ W/cm² (1mW in a 0.2 micron diameter spot, area = 12.6 x 10⁻¹⁰ cm²).

• In a 2-photon confocal, the light flux is 10⁶ 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.
Responsivity
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
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
Evils of Stray Light II

Diagram:
- Microscope nosepiece
- Black cardboard
- Black cloth or leather
- Stage
- Slide
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

A

B

C

D

Reservoir

Suction
Perfusion Systems—Cultured Cells

Single-sided Chamber

coverslip
Perfusion Systems—Renal Tubules

![Diagram of Perfusion Systems](Image)

- **A**
  - 320 Objective condenser lens
  - Agar bridge
  - Thermocouple
  - Collection holding pipet
  - Set-up bath
  - Coverslip
  - Perfusion pipet
  - Perfusion holding pipet
  - Suction outlet
  - Perfusion bath
  - Glass coverslip

- **B**
  - Photon diode output (mV)
  - $t_{1/2} = 55$ msec

![Graph of Photon Diode Output](Image)

- Port for thermistor
- Port for agar bridge
- Tapped hole
- 50 mm diameter x 2.5 mm thick plexiglass disc
- Hypo tube 1.65 mm O.D. x 1.2 mm I.D.
- 11 mm wide x 25 mm long x 0.66 mm deep
- Opposite side machined
- 0.66 mm deep for 25 mm dia. cover slip
- 1.2 mm I.D. hole
- 1.2 mm wide x 1.2 mm deep x 12.5 mm long slot
- 4.0 mm wide x 1.2 mm deep x 12.5 mm long slot
Perfusion Systems—Problems

- Perfusate temperature control
- Perfusate gassing—CO₂ 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

![Graph showing spectral irradiance of different arc lamps](image-url)
<table>
<thead>
<tr>
<th>Energy Output</th>
<th>HBO 100</th>
<th>XBO 75</th>
<th>Tungsten/Halogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>2200 lumens</td>
<td>0.25 x 0.25 mm</td>
<td>0.25 x 0.50 mm</td>
<td>4.2 x 2.3 mm</td>
</tr>
<tr>
<td>1700 cd/mm²</td>
<td>800 cd/mm²</td>
<td>45 cd/mm²</td>
<td></td>
</tr>
<tr>
<td>850 lumens</td>
<td>2800 lumens</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Argon and Krypton Laser Lines

Figure 10

Argon Emission Spectrum

Krypton Emission Spectrum
WAVELENGTH SELECTION

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

![Graph showing transmittance of different colors across wavelengths.](image)
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=acoustic input, P,S=polarization directions
LCTF Design
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

Collimating & Imaging Optics

25 cm

Interchangeable 25 mm Filters

Object of Interest

Detector Array

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

Overlay

488 nm Excitation

Alexa 530
Computed Tomography Imaging Spectrometer

CTIS Subsystem

Fore-optics Subsystem

Reimaging Lens
Collimator Lens
CCD Array
CGH Disperser *
Field Stop and Field Lens
Dichroic Beam Splitter
Microscope Objective
Specimen
Light Guide
RAW DIFFRACTION IMAGE FROM CTIS MICROSCOPE
Reconstructed Spectral Images

20 µm Beads

<table>
<thead>
<tr>
<th></th>
<th>600</th>
<th>610</th>
<th>620</th>
<th>630</th>
<th>640</th>
<th>650</th>
</tr>
</thead>
<tbody>
<tr>
<td>480</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>490</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>500</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
<tr>
<td>510</td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td>520</td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
<td><img src="image28.png" alt="Image" /></td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
</tr>
<tr>
<td>530</td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
<td><img src="image33.png" alt="Image" /></td>
<td><img src="image34.png" alt="Image" /></td>
<td><img src="image35.png" alt="Image" /></td>
<td><img src="image36.png" alt="Image" /></td>
</tr>
</tbody>
</table>

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, respectively

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

using META and Emission Fingerprinting

Sample: Bob Zucker, EPA