Lecture:

Instrumentation in Fluorescence Microscopy

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Microscope: Instrument in Microscopy

Viewing objects that are too small to be seen

Resolution:
- Optical: 0.2 µm
- TEM: 0.05 nm
- SEM: <1 nm
- AFM: <1 nm
- STM: 0.1/0.01 nm
- SPM: 20/2 nm

http://en.wikipedia.org/wiki/Microscope
Fluorescence Microscopy in Life Sciences

Figure 1

http://www.microscopyu.com/articles/fluorescence/fluorescenceintro.html
http://en.wikipedia.org/wiki/Fluorescence_microscope
Types of Confocal Microscope

• Confocal scanning microscopes
  – Single beam:
    Stage scanning or Laser scanning
  – Advantages/disadvantages:
    • Good image quality and
    • High resolution
    • Slow frame rate (< 3fps)

http://www.olympusconfocal.com/theory/confocalscanningsystems.html

• Spinning-disk confocal microscopes
  – Multi-beam
  – Advantages/disadvantages:
    • Video rate imaging
    • Low resolution

http://www.smt.zeiss.com/
# Confocal Microscopes in LFD

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<th>One-photon laser scanning</th>
<th>One/two-photon</th>
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<td>Fluoview FV1000</td>
<td>LSM 510</td>
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<tr>
<td>Model (Inverted)</td>
<td>IX81</td>
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<td>Axiovert 200M</td>
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<tr>
<td>Microscope (Inverted)</td>
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<td>Laser</td>
<td>Argon Ion HeNe</td>
<td>Argon Ion HeNe</td>
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<td>Extra</td>
<td>FLIM-Box Laser launcher (472/449/405nm)</td>
<td>FLIMBox (471nm)</td>
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<td><strong>Advantages:</strong></td>
<td>High sensitivity.</td>
<td>Fastest Z scan</td>
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<td></td>
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<td>High sensitive</td>
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Detailed instrument components: [http://www.lfd.uci.edu/service/resources/microscopes/](http://www.lfd.uci.edu/service/resources/microscopes/)
2-photon Excitation Fluorescence Microscopy

2-photon excitation

- 2 photons required for excitation
- No out-of-focus excitation
- No pinhole required
- Scattered light is detected

1-photon excitation

- Single-photon excitation
- Fluorescence emission

Excitation

http://www.nature.com/nrg/journal/v4/n8/box/nrg1126_BX4.html
http://research.stowers-institute.org/wiw/external/Technology/Microscopy/
Detector Configurations for TPEF Microscopy

http://www.microscopyu.com/articles/fluorescence/multiphoton/multiphotonintro.html
Non-Descan configuration of TPEF Microscopy

Characteristics:
1. Good collection efficiency.
2. Large area detectors are needed.
Descan configuration of TPEF Microscopy

Characteristics:
1. The signal beam is stationary.
2. Loss of signal.
Effect of a Confocal Pinhole in TPEF Microscope

Use pinhole if your TPEF signal is strong
# Two-photon microscopes in LFD

<table>
<thead>
<tr>
<th>Two-Photon Scanning</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M5</th>
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<tbody>
<tr>
<td>Microscope (Inverted)</td>
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<tr>
<td>Targets:</td>
<td>membrane study</td>
<td>everything</td>
<td>everything</td>
<td>tracking</td>
<td>Larger area detector for deep tissue</td>
</tr>
</tbody>
</table>

Detailed instrument components: [http://www.lfd.uci.edu/service/resources/microscopes/](http://www.lfd.uci.edu/service/resources/microscopes/)
Instrumentations in TPEF microscope

Laser
- Ti:Sapphs

Scanner
- Mirror laser scanner
- Pizo stage scanner

Detector
- PMT/APD
- CCD

Others
- Optics
- Electronics
- ...
Multiphoton Transition Necessitates High Excitation Intensity at the Focus

Photon pairs absorbed per laser pulse

\[ n_a \approx \frac{d}{\tau} \left( \frac{p \pi A^2}{f h c \lambda} \right)^2 \]

- \( p \): Average power
- \( \tau \): Pulse duration
- \( f \): Laser repetition frequency
- \( A \): Numerical aperture
- \( \lambda \): Laser wavelength
- \( d \): Two photon absorption cross-section
  \( (10^{-50} \text{ cm}^4 \text{ sec photon}^{-1} \text{ molecule}^{-1}) \)

Peak power:

\[ P_{\text{peak}} = P / (\tau f) \]

http://www.microscopyu.com/articles/fluorescence/multiphoton/multiphotonintro.html
Light Sources: Titanium Sapphire Lasers

Pulse duration of ~100 fs with 80 MHz repetition rate
Wavelength range 680-1080nm
Average power is about 700mW-3.7W @790nm, ~310 kW peak-power

Enough power to saturate absorption in a diffraction limited spot

Coherent Chameleon-Ultra II

Spectra-Physics Mai Tai HP

Chameleon Vision II

Mai Tai DeepSee
Tuning Curves of Ti:Sapphs Lasers

Tuning curves of the Mai Tai HP (black) and the Chameleon-Ultra II (grey)
Data from Oct 20, 2008

Mai Tai HP tuning range: 690 – 1040 nm
Chameleon-Ultra II tuning range: 680 – 1080 nm

By Dr. Oliver Holub
Dispersion Reduces the Efficiency of Excitation

- Short the better?
  - In principle, 150 fs pulses -> 10 fs -> a factor of 15 improvement in signal.
  - In practice, a net decrease of 30% in signal

- Reason: Chromatic dispersion by the high NA objective lens

Pulse Broadening by Dispersion

Dispersion: 0, 5000, 10000, 15000 and 20000 fs$^2$.

Mai Tai HP: 100fs
Chameleon UltraII: 140 fs

By Dr. Oliver Holub
Optimal Laser Input Pulse Width

- Which will result in minimal pulse broadening for a given dispersion value.

\[ \text{Dispersion [fs}^2\text{]} \]

\[ \sim 167 \text{ fs for a dispersion of 10000 fs}^2 \]

By Dr. Oliver Holub
Pulse Broadening w/o Dispersion Compensation

- Dispersion at 700 nm:
  - \(100 \text{ fs} \rightarrow 391 \text{ fs}\) (Mai Tai HP)
  - \(140 \text{ fs} \rightarrow 304 \text{ fs}\) (Chameleon-Ultra II).

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By Dr. Oliver Holub
Two-photon Excitation Efficiency w/o Dispersion Compensation

Average squared intensity 
\[(\text{number of photons})^2 \text{ cm}^{-4} \text{ s}^{-2}\]

Chameleon-Ultra II w/o dispersion compensation

Mai Tai HP w/o dispersion compensation

Wavelength [nm]

By Dr. Oliver Holub
Two-photon Excitation Efficiency with Dispersion Compensation

- Dispersion Compensation

Mai Tai DeepSee
- 690 nm: -22,500 fs$^2$ to -41,700 fs$^2$
- 800 nm: -8,900 fs$^2$ to -24,500 fs$^2$
- 1040 nm: 0 fs$^2$ to -9,600 fs$^2$

Chameleon Vision II
- 680 nm: 0 to -47,000 fs$^2$
- 800 nm: 0 to -22,000 fs$^2$
- 1020 nm: 0 to -10,000 fs$^2$
- 1080 nm: 0 to -9,000 fs$^2$

Average squared intensity
[ (number of photons)$^2$ cm$^{-4}$ s$^{-2}$]
Summary:
Two-photon Excitation Efficiency

• Chameleon-Ultra II has the advantage of higher average power and a larger tuning range over the Mai Tai HP.
• Dispersion compensation increases the two-photon excitation efficiency by 2~5 times.
• At high dispersion, a Mai Tai DeepSee with 80 fs pulses slightly outperforming the Vision II.
Scanning Unit: Mirror Scanner

- Laser scanning is most widely used.
  - Fast
  - Sample is stationary

- Methods:
  - The galvanometric scanner
  - The polygonal scanner
  - The acousto-optical deflector

http://www.celanphy.science.ru.nl/Bruce_web/scanning_microscopy.htm
Configuration of Light Path

- **Telecentric planes:**
  - SP and FAP

- **Scanning lens:**
  - A “θ-ε” lens.
    The displacement of its focal point from the axis is proportional to the incident angle

- **Requirement:**
  - The pivot points of x-scan and y-scan are at the eyepoint of the scan lens and conjugate with the BFP.

Configuration of Light Path

- Single scanning mirror:

- Two scanning mirrors:

http://www.olympusconfocal.com/theory/confocalscanningsystems.html
Examples

Bio-Rad

Zeiss, Olympus

Leica in one system

http://www.olympusconfocal.com/theory/confocalscanningsystems.html
**Example of Mirror scanners**

**Cambridge technology mirror scanner:**
Moving coil closed loop galvanometer based optical scanner with capacitive position detector, 6033 servo controller

**Model 6350**
- Apertures: 5 / 10 mm
- Angular Excursion: 40°
- Small angle step response time: 1.5 ms
- Position detector linearity: min. 99.9 % over 40°

**Model 6220**
- Apertures: 5 / 8 / 9 / 10 mm
- Angular Excursion: 40°
- Small angle step response time: 0.2 ms
- Position detector linearity: min. 99.9 % over 20°; 99.5% typical over 40°.

http://www.camtech.com/products/6450/6350.html
Scanning Unit: Stage Scanner

• Sample scanning
  – Piezo stage scanners.
  – Sample movement, beam stationary.

• Specifications:
  – Nanometer resolution
  – May cause the change of samples.
  – High imaging speed is difficult to achieve.

http://www.celanphy.science.ru.nl/Bruce_web/scanning_microscopy.htm
Problem in Fast Scanning of Piezo Stage

Distorts the driving function upon voltage reversal due to the capacitance of the stage.

Typical: driver function
stage movement

Modified: linear stage movement
“trained” driver function

*Globals for images* now allows the insertion of a specific driving file, which completely linearizes the piezo stage response. Linear response has been achieved for fast scanning speeds down to **8 us per pixel**.
Examples: Actual Driver Files for Three Different Scanning Speeds

Using a “trained” driver function effectively increases the useful range for linear scanning!
Piezo Stages Scanner in LFD

**XY-stages:**

Physik Instrumente (PI) xy piezo nanopositioning stage
P-730.20 with PI piezo servo controller (E-500.00 + E-505.00 + E-509.C2A)

- Travel @ 0 to 10 V: 50 µm
- Resonant frequency $f_0$: 694 Hz
- Rise time: 1.66 ms
  
  \[T_{\text{min}} = 1/(3f_0) = 0.48 \text{ ms}\]

- Scanning speed @ 1V (=5µm): 330 Hz (= 3 ms) per line
- Resolution: 0.1 nm
- Full range repeatability: 1 nm

Mad City Labs (MCL) piezoelectric xyz-nanopositioner
Nano-PDQ MCLS 01338 with Nano-Drive 85 controller

- Travel @ 0 to 10 V: 50 µm
- Resonant frequency $f_0(x,y)$: 2.5 kHz
- Rise time: not given
  
  \[T_{\text{min}} = 1/(3f_0) = 0.13 \text{ ms}\]

- Scanning speed @10V(=50µm): 400 Hz (= 2.5 ms) per line
- Resolution: 0.1 nm
Which Scanning Method?

- **Mirror scanners:**
  - Large area can be scanned (500 µm typ, while the area of stage scanners is restricted to 50-75µm).
  - Immobile sample (no vibrations or movements distorting the processes under investigation).

- **Piezo stage scanners:**
  - No change in the optics, which could lead to detection artifacts or changes in the point spread function.
  - Nanometer resolution.

- **Other methods:**
  - Acoustic optical deflector
  - Resonant galvanometer
  - etc.
Detectors for Fluorescence Signal

• PMT/APD
  – PMT: Photomultiplier tubes
  – APD: Avalanche photodiodes

• CCD/EMCCD
  – CCD: charge-coupled device
  – EMCCD: electron-multiplying CCD
Photomultipliers

**Elements:**
- Vacuum tube with glass window.
- Photocathode: a negatively charged electrode for electron release at photon abs.
- Dynodes: Electrodes for electron multiplication (up to 18)
- Anode: collection electrode
- appr. 100 V between two dynodes, about 1000 V between cathode and anode
- Current from anode to ground is proportional to cathode photoelectron flux over large range

Photomultiplier Gain

- **Current amplification (gain) estimation:** $\text{Gain} = E^n$
  - $E$ secondary emission ratio for the dynodes
  - $n$ number of dynode stages
  - Photoelectron on first dynode $\rightarrow$ 5-10 additional electrons.
    - 10 stage photomultiplier with a secondary emission ratio of five, the current amplification would be about 10 million

- **ns current pulses up to 100 $\mu$A from a single photon (detectable without amplification)**

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/photomultipliers.html
PMT Spectral Response

• **Spectral response (R):**
  - \( R(\lambda) = QE(\lambda) \times e \times \lambda \div (hc) \)
  - \( QE \) quantum efficiency of photocathode
  - \( \lambda \) wavelength of light
  - \( e \) electron charge
  - \( h \) Planck’s constant
  - \( c \) speed of light

• **Photocathode composition determines:**
  - spectral response
  - quantum efficiency
  - overall uniformity of the photomultiplier sensitivity
  - dark current

• **Materials:**
  - most materials good response at 200-400 nm

Advantages & Disadvantages

• Advantages:
  – Very fast response time (ns), bandwidth 1-1.5 GHz.
  – Extremely high sensitivity: Large gain (one million) without sacrificing bandwidth.
  – Low dark current: very high S/N.
  – Excellent performance @ UV/blue.
  – Large dynamic range.

• Disadvantages:
  – Photocathode quantum efficiency: 30-40 %.
  – No spatial discrimination at detector.
  – Gain and dark current performance of individual photomultipliers from the same production run can vary widely (factor of 2 to 5).
  – Can be damaged by high illumination levels.
  – Require stabilized high voltage power supply.
Side-on or Head-on PMT

**Side-On:**
- High performance, low cost
- An opaque and relatively thick reflection-mode photocathode surrounded by a circular cage-like dynode element chain
- Incident photoelectrons are ejected from the front face and angled toward the first dynode element

**Head-On:**
- Photocathode must be of precise thickness and is semi-transparent
- If the photocathode is too thick, more photons will be absorbed but fewer photoelectrons will be emitted from the rear surface. Alternatively, if the photocathode is too thin, photons may pass straight through without being absorbed.

Contd.: PMT Sensitivity over Surface Area

**Head-On:**
- Larger and more uniform photosensitive regions
- Exhibit a far more uniform response across the entire photosensitive area.

**Side-On**
- Faster rise times, a higher level of responsivity (do not suffer the optical losses associated with the semi-transparent materials)
- Upper half of the photocathode is typically 20 to 30% more sensitive than the lower half

Stability of the electron multiplication relies on the electrons following well-defined trajectories

PMT Noise

• If large noise is present in the photon generated signal, the PMT will simply amplify that signal.

• Noise sources:
  – Photon shot noise ($N_S$): stochastic fluctuations in photon arrival times
  – Detector dark noise ($N_D$)
  – Amplifier noise: signal processing electronics

• Dark current (Dark noise): Noise in the absence of any illumination.
  – Primarily to thermal emission of electrons from the photocathode and first few dynodes.
  – Smaller contribution from leakage current between the dynodes and ground.
  – Cosmic rays and radioactive decay of materials inside the tube or external high-energy radiation from nearby sources.
  – System electronic noise also contributes to dark current and is often included in the specifications for the dark current value.
  – Cooled to reduce the levels of dark current and to prevent fluctuations in room temperature from altering device gain or dark current. $\Rightarrow$ Improved S/N
Hamamatsu H7422P-40:

- High sensitivity @ 300-720 nm
- QE 40% @ 580 nm
- Thermoelect. cooled at const. temp. \([\Delta T=35^\circ\text{C}]\) → red. thermal noise
- GaAsP photocathode (effect. area: diam. 5 mm); Head-on
- 8 dynodes
- High gain for photon counting \((2\times10^6)\)
- Rise time: 1 ns
- electron transit time spread: 5.4 ns
Avalanche Photodiode (APD)

APDs: the semiconductor (silicon-based) analog to PMTs.

It contains a pn junction consisting of a positively doped \textbf{p} region and a negatively doped \textbf{n} region sandwiching an area of neutral charge termed the depletion region. These diodes provide gain by the generation of electron-hole pairs from an energetic electron that creates an "avalanche" of electrons in the substrate.

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/avalanche.html
Contd. : Avalanche Photodiode (APD)

• When a reverse bias (voltage) applied and the crystal junction between the p and n layers is illuminated, a current will flow in proportion to the number of photons incident upon the junction.
  - Modest gain (500-1000)
  - Depletion layer is thin
  - Strong electrical field across junction
  - Very high reverse-bias voltages (up to 2500 V) increases energy of the created electrons multiple collisions avalanche of electrons (electron multiplication)
  - substantial dark current (increasing with bias)

• Advantages:
  - High quantum efficiency (90 %)
  - Broad spectral range
  - Uniform detection surface
  - High dynamic range
  - Require low currents
  - Immune to magnetic fields

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/avalanche.html
http://micro.magnet.fsu.edu/primer/digitalimaging/digitalimagingdetectors.html
Avalanche Photodiodes in the LFD

- **PerkinElmer Photon counting module SPCM-AQR-13-FC (and model SPCM-AQR-15):**
  - broad wavelength range extending (400-1060 nm)
  - circular active area of 180 um
  - quantum efficiency > 65% (@ 650 nm)
  - thermoelectrically cooled and temperature controlled
  - 10 million counts/s
  - 50 ns dead time between pulses
  - TTL output pulse (width 35 ns)
  - FC model with fiber-optic receptacle
  - gating function
  - dark counts (AQR-13: 250 counts/s; AQR-15: 50 counts/s)
  - Afterpulsing
  - high light level will damage the module
The HPM-100-40 Hybrid Detector

- A large part of the gain within a single step -> a narrow amplitude distribution.
- Low transit time spread (120ps).
- Count efficiency.
- Extremely low afterpulsing.

- Wavelength Range: 300 nm to 730 nm
- Quantum efficiency at 500 nm: 45%
- Dark Count rate, T = 22°C: 560 s⁻¹
- Cathode Diameter: 3 mm
- Transit Time Spread: 120 ps, FWHM
- Single Electron Response Width: 850 ps FWHM
- Max. Count Rate (Continuous) > 10 MHz
Comparison of Selected Photodetectors

High sensitivity, slow
→ Low light level imaging

APD
SPCM-AQR
Perkin-Elmer
QE 65%, 35 ns

QE low, gain high (10^7),
→ analog detection

R928
Hamamatsu
QE 25.4%, 10 ns

Low sensitivity, fast rise
time (0.78 ns)
→ Photon counting for FLIM

R7400U-04
Hamamatsu
QE 18.6%, 2 ns

H7422P-40
Hamamatsu
QE 40%, 2 ns

QE high, good timing
→ Photon counting

HPM-100-40
QE 45%, 850 ps
# Electron Transit Time Spread of Selected Photodetectors

Fluctuation in electron transit time between individual pulses
FWHM of frequency distribution of electron transit times

<table>
<thead>
<tr>
<th>Photodetector</th>
<th>Transit Time Spread</th>
<th>Pulse Width</th>
<th>Dead Time</th>
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<tbody>
<tr>
<td>R928 Hamamatsu</td>
<td>10 ns</td>
<td></td>
<td>1.2 ns</td>
</tr>
<tr>
<td>HPM-100-40</td>
<td>850ps</td>
<td></td>
<td>120 ps</td>
</tr>
<tr>
<td>R7400U-04 Hamamatsu</td>
<td>2 ns</td>
<td></td>
<td>0.28 ns</td>
</tr>
<tr>
<td>H7422P-40 Hamamatsu</td>
<td>2 ns</td>
<td></td>
<td>5.4 ns</td>
</tr>
<tr>
<td>APD SPCM-AQR Perkin-Elmer</td>
<td>35 ns</td>
<td></td>
<td>0.35 ns</td>
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</table>
Rise Time of Selected Photodetectors

Anode output-pulse rise-time: time to rise from 10 to 90 % of the peak amplitude when the entire photocathode is illuminated by a delta function light pulse.

- **R928** Hamamatsu: 2.2 ns
- **R7400U-04** Hamamatsu: 0.78 ns
- **H7422P-40** Hamamatsu: 1 ns
- APD SPCM-AQR Perkin-Elmer: APD discrimin. electronics
MODE OF PMT OPERATION:

ANALOG OR PHOTON COUNTING?
The Level of Incident Illumination

**Digital mode:**
- At bandwidths below 100 MHz (10 ns), the signal can be detected as a series of pulses on the anode and processed digitally.
- Signal eventually needs pre-amplification and discriminator electronics.
- Advantages at low light intensities.
- At low light intensities the low level noise of the signal reduces image contrast and increases background intensity (c). Using of discriminator increases image contrast.

**Analog mode:**
- At increasing light intensities, the interval between the photons arriving at the PMT becomes so short that they overlap to produce a continuous waveform.
- Easy to sample with a conventional analog-to-digital converter.
- Broad dynamical range (adjustable with dynode voltage).

PMT catalog, Hamamatsu
Analog or Photon counting?

Analog sampling with fast A/D conversion
Data processing

3 photons
Discriminator photon counting

Discriminator level

Pixel
Time
Analog Single Photon Pulse Height Distribution

The amplitude of the single-photon pulses of PMTs varies from pulse to pulse. Photon counting might discard a large number of photons depending on the setting of the discriminator threshold.

Two cases have to be distinguished:
1) The PMT design effectively separates amplitude levels of electronic noise and photon counts. SPC with correct threshold level improves S/N and filters out the noise distribution.
2) If dark noise cannot be separated from photon counts, rapid analog sampling should be advantageous, because all photon counts are recorded.

\[
\sigma_{\text{spc}}^2 = I \\
\sigma_{\text{analog}}^2 = \sigma_0^2 + S \cdot I
\]

For constant intensities:

\( I \) photon counts
\( S \) analog collection factor (typically around 100)
\( \sigma_0^2 \) read noise

Therefore the analog noise will always be greater than the photon counting noise if \( S > 1 \) and the read noise > 0.

Pulse amplitude distribution of H7422P-40 for different gain. W.Becker, Adv. TCSPC techniques, Springer 2005, Fig.6.14, p.228
Charge Coupled Device (CCD)

- A dense matrix of photodiodes incorporating charge storage regions.
  - A pixel (a silicon diode photosensor) is coupled to a charge storage region. The stored charge is sequentially transferred through the parallel registers to a linear serial register and then to an output node adjacent to the read-out amplifier (Only one amplifier at the corner of the entire array)

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/ccdanatomy.html
http://micro.magnet.fsu.edu/primer/digitalimaging/digitalimagingdetectors.html
Back-thinned CCD

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/ccdanatomy.html
http://micro.magnet.fsu.edu/primer/digitalimaging/digitalimagingdetectors.html
CCD noise, Signal-to-noise Ratio

Main CCD noise sources:
- Photon shot noise $[\text{Sq}rt(S)]$
- Dark noise: thermal electron fluctuation $[\text{Sq}rt(\text{thermal electrons})]$  
- Read-out noise: converting stored charges into voltage, output amplification (on-chip pre-amplifier) and A/D conversion
  - pixel non-uniformity in photo response and dark current

Signal depends on:
$P$ incident photon flux; $QE$ quantum efficiency; $t$ integration time;
$D$ dark current; $NR$ read-out noise; $B$ background light

$$\text{SNR} = \frac{P \cdot QE \cdot t}{\sqrt{(P+B) \cdot QE \cdot t + D \cdot t + NR^2}}$$

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/ccdsnr.html
Electron Multiplying CCD (EMCCD)

CCD disadvantages:
- Very low signal levels typically fall beneath the read noise floor of the sensor

EMCCD:
- Rapid frame-rate capture at extremely low light levels
- Inclusion of a specialized extended serial register on the CCD chip that produces multiplication gain through the process of impact ionization in silicon
- Quantum efficiency >90%
- Read noise < 1 electrons rms
- At 30 frames/s
- A/D conversion with 14 bit

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/emccds.html
CCD Noise Reduction and Linearity

Cooling reduces the dark noise:
One-half reduction every 5-9 °C

Nonlinearity of few tenths of one percent over a signal range of 4 or 5 orders of magnitude

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/ccdsnr.html
http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/ccdlinearity.html
OTHERS:

OPTICS
New Titanium Single-substrate/ion-beam Sputtered Optical Filters

A new line of fluorescence filters and beamsplitters (offered e.g. by Semrock and Chroma) provides:

- Highest possible transmission of >95%
- Steepest possible edges
- Precise wavelength accuracy
- Extreme blocking for optimal S/N
- Beamsplitter: steep transition and flat transmission and reflection bands

http://www.semrock.com/Catalog/SpectralComparison.htm
Contd.

> 95% flat transmission for fluorescence

8 OD blocking of laser excitation

Steep transition @ 680 nm

Chroma et680sp-2p8 custom titanium/sputtered 2-photon blocking emitter[OD 8+ @720-1064 nm]
Problem: Every optical surface reflects 4% of the incident light.
Solution: For multi-element optical system (as microscopes) use antireflection coating.

100
differentiated into:

- Broadband coated (0.5 % per surface)
- Uncoated (4% per surface)

17 surfaces = 8.5 lenses (or optical elements)

Reflection = 4% per surface
100*(1-Reflection/100)^(SurfaceNumber)
OTHERS:

ELECTRONICS
Discriminators

Main types:

- **Leading edge discriminator**
- **Constant fraction discriminator**

Given input pulses of different amplitude, the leading edge discriminator produces an output pulse at the time when the input pulse crosses a given threshold voltage. This, however, causes a problem in situations where the timing is important.

“Time walk”

![Figure 1: Leading Edge Discriminator and “Time Walk”](http://galileo.phys.virginia.edu/research/groups/hep/aag/memos/memoCFD.doc)
Constant-fraction Discriminators (CFD)

CFD uses a constant fraction $f$ of the input pulse to precisely determine the timing of the output pulse relative to the input signal:

Input pulse

Top: Delayed and inverted pulse

Bottom: attenuated pulse (by fraction $f$)

Sum

Compute zero crossing → Output pulse

Figure 2: Input Pulse to CFD

Figure 3: Attenuated Pulse

Figure 4: Delayed and Inverted Pulse

Figure 5: Sum of Attenuated and Delayed and Inverted Pulses

galileo.phys.virginia.edu/research/groups/hep/aag/memos/memoCFD.doc
“Failure of the constant fraction discriminator”, Kirstin Luery, 2003
Zero crossing for a linear ramp:

delay = \( t_d \), \ fraction = f, \ initial \ amplitude = A, 
\ input \ pulse \ V_i = -At, 
\ attenuated \ pulse \ V_a = -fAt, 
\ delayed \ and \ inverted \ pulse \ V_d = A(t - t_d). 

To find the zero crossing, set \( 0 = V_a + V_d \) and solve for:

\[ 0 = -fAt + A(t - t_d), \]

\[ t_{cross} = \frac{t_d}{(1 - f)}. \] \( \rightarrow \) Amplitude independent
Standard discriminator at the LFD:

**CFD: Phillips Scientific Model 6915**
Constant-fraction timing discriminator

- Time walk plus input slewing of ±75 ps for inputs from threshold to 100 times threshold
- threshold adj. -25mV to -1 V
- delay adjusted by delay cable
- Signal output: TTL (among others) of width 5ns – 250 ns and rise- and falltimes of 1.2 ns
- Pulse pair resolution better than 10 ns (@ min output width and 1ns delay cable)
- Continuous repetition rate: > 100 MHz (@ min output width and 1ns delay cable)

\[ f = \frac{1}{3} \]
Others

Data acquisition, e.g. via FLIM-box

Acquisition software:
SimFCS (part of *Globals for Images*)

Flim-Box:
Rapid data processing electronic hardware for time-resolved fluorescence microscopy
FLIM, FCS, PCH, RICS
Others

Standard pre-amplifier at the LFD:

Becker & Hickl GHz wide band amplifier ACA-4-35-N:
- 35 dB gain
- bandwidth 5kHz – 2.2 GHz
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