Instrumentation in Fluorescence Microscopy

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Microscopy in Biomedical Research

- Microscopy is the technical field of using microscopes to view samples and objects that cannot be seen with the unaided eye.
Imaging Techniques: Optical Microscopy

• **Widefield microscopy**
  - Bright Field
  - Fluorescent
  - More...

• **Laser Scanning microscopy**
  - Confocal
  - Multiphoton

http://micro.magnet.fsu.edu/primer/techniques/fluorescence/anatomy/fluoromicroanatomy.html
Basic Principles

Principle

Typical infinity color-corrected optical system

$$\frac{\sin \theta_1}{\sin \theta_2} = \frac{v_1}{v_2} = \frac{n_2}{n_1}$$

http://en.wikipedia.org/wiki/Optical_microscope
http://zeiss-campus.magnet.fsu.edu/
http://www.olympusmicro.com/
Typical Microscope

[Image: Reflecting Light Microscope Tube Length]

[Image: 60x Plan Apochromat Objective]

http://www.olympusmicro.com/primer/anatomy/specifications.html
Fluorescence Microscopy in Life Sciences

Fluorescence Microscopes offer:

Spatial resolution: ~0.2 µm
Different probes for multi-color imaging

J. Lichtman etc, Nature Methods, 2005, 2:910-919
http://rsb.info.nih.gov/ij/images/
http://zeiss-campus.magnet.fsu.edu
From Widefield to Confocal Microscope

Widefield fluorescence microscopy

Confocal fluorescence microscopy

http://www.olympusconfocal.com/theory/confocalintro.html
Confocal Fluorescence Microscope

http://www.olympusconfocal.com/theory/confocalintro.html
Types of Confocal Microscopes

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

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

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

http://www.smt.zeiss.com/
2-photon Excitation Fluorescence Microscopy

3-Photon excitation occurs in the same way

http://www.nature.com/nrg/journal/v4/n8/box/nrg1126_BX4.html
http://research.stowers-institute.org/wiw/external/Technology/Microscopy/
Charactersitics:
1. The signal beam is stationary.
2. Loss of signal.
## Confocal Microscopes in LFD

<table>
<thead>
<tr>
<th>One-photon laser scanning</th>
<th>One/two-photon</th>
<th>Model</th>
<th>Microscope</th>
<th>Laser</th>
<th>Extra</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Olympus</strong></td>
<td><strong>Zeiss</strong></td>
<td><strong>Zeiss</strong></td>
<td><strong>Olympus</strong></td>
<td><strong>Olympus</strong></td>
<td><strong>FV1000 (Keck)</strong></td>
<td><strong>FLIMBox Tracking system</strong></td>
</tr>
<tr>
<td>Fluoview FV1000 (LFD)</td>
<td>LSM 510</td>
<td>LSM 710</td>
<td>Fluoview FV1000 (Keck)</td>
<td>Argon Ion, 405/559/635nm, Ti:Sapphs with Deepsea</td>
<td>Argon, HeNe, Diode laser, Ti:Sapphs with Deepsea</td>
<td>High sensitivity, 3D tracking</td>
</tr>
<tr>
<td>IX81</td>
<td>Axiovert 200M</td>
<td>Axio Observer.Z1</td>
<td>IX81</td>
<td>Argon Ion, HeNe, Ti:Sapphs</td>
<td>Argon, HeNe, Ti:Sapphs with Deepsea</td>
<td>Fastest Z scan, 3D Piezo scanning</td>
</tr>
<tr>
<td>Microscope IX81</td>
<td>Diode lasers</td>
<td></td>
<td>Extra FLIMBox Tracking system</td>
<td></td>
<td></td>
<td>High sensitive, Spectral detectors</td>
</tr>
<tr>
<td>Laser</td>
<td>White laser</td>
<td></td>
<td>Extra FLIMBox Tracking system</td>
<td></td>
<td></td>
<td>High Sensitivity, 3D tracking</td>
</tr>
<tr>
<td>Argon Ion</td>
<td>HeNe Ti:Sapphs</td>
<td></td>
<td>Extra FLIMBox Tracking system</td>
<td></td>
<td></td>
<td>For animal study, 3D tracking</td>
</tr>
<tr>
<td>HeNe Diode laser</td>
<td></td>
<td></td>
<td>Extra FLIMBox Tracking system</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Detailed instrument components: [http://www.lfd.uci.edu/service/resources/microscopes/](http://www.lfd.uci.edu/service/resources/microscopes/)
Non-Descan configuration of TPEF Microscope

Characteristics:
1. Good collection efficiency.
2. Large area detectors are needed.
# 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>
<th>Olympus upright</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscope</td>
<td>Zeiss Axiovert 35</td>
<td>Zeiss Axiovert 135TV</td>
<td>Zeiss AxiovertS100TV</td>
<td>Olympus IX70</td>
<td>Olympus</td>
</tr>
<tr>
<td>Laser</td>
<td>Mira900 MaiTai HP</td>
<td>MaiTai HP</td>
<td>MaiTai HP</td>
<td>Chameleon-Ultra II</td>
<td>MaiTai HP with deepsea</td>
</tr>
<tr>
<td>Scanner</td>
<td>Mirror scanner 6350 (Cam. Tech.)</td>
<td>Mirror scanner 6220 (Cam. Tech.)</td>
<td>Mirror scanner 6350 (Cam. Tech.)</td>
<td>Mirror scanner 6350 (Cam. Tech.)</td>
<td>Mirror scanner 6220 (Cam. Tech.)</td>
</tr>
<tr>
<td>Software &amp; Data acquisition</td>
<td>SimFCS FLIMBox ISS-FCS</td>
<td>SimFCS FLIMBox ISS-FCS</td>
<td>SimFCS FLIMBox BH-SPC830</td>
<td>SimFCS ISS-FCS</td>
<td>SimFCS FLIMBox ISS-FCS</td>
</tr>
<tr>
<td>Targets:</td>
<td>Non-descan mode, FLIM and more.</td>
<td>Non-descan mode, FLIM &amp; on tracking, 3D tracking and more.</td>
<td>Non-descan mode, FLIM, for everything</td>
<td>Non-descan mode</td>
<td>Non-descan mode, 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/)
Components in a Laser Scanning Microscope

Laser:
- Ti:Sapphs
- Other lasers

Scanner:
- Mirror laser scanner
- Pizo stage scanner

Detector:
- PMT/APD
- CCD/ICCD/EMCCD for one photon

Others:
- Optics
- Electronics
- ...
Wavelengths of commercially available lasers

http://en.wikipedia.org/wiki/Laser
Multiphoton Transition Necessitates High Excitation Intensity at the Focus

Photon pairs absorbed per laser pulse

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

- \( p \): 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:

\[ P_{\text{peak}} = \frac{P}{\tau f} \]

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

Major TPEF Laser sources at LFD:
1x Chameleon-Ultra II
6x MaiTai (Two equipped with Deepsee)
1x Tsunami
Tuning Curves of Ti:Sapphs Lasers

Chameleons-Ultra II tuning range: 680 – 1080 nm
Mai Tai HP tuning range: 690 – 1040 nm
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 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
Example of Mirror scanners

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

Model 6350

- Angular Excursion: 40°
- Small angle step response time: 1.5 ms
- Position detector linearity: min. 99.9 % over 40°

Model 6220

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/
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
Piezo Stages Scanner in LFD

XY-stages:

**PI xy piezo nanopositioning stage**
P-730.20 with PI piezo servo controller
- 0 to 10 V: 50 µm
- Resolution: 0.1 nm

**MCL piezoelectric xyz-nanopositioner**
Nano-PDQ MCLS 01338 with Nano-Drive 85 controller
- Travel @ 0 to 10 V: 50 µm
- Resolution: 0.1 nm

**MCL piezoelectric z-nanopositioner**
Nano-Z50HS with Nano-Drive 85 controller
- Travel @ -10 to 10 V: 50 µm
- Resolution: 0.1 nm
Which Scanning Method?

• **Mirror scanners:**
  – Fast scanning. Large area can be scanned.
  – Immobile sample.
  – XY plane only

• **Piezo stage scanners:**
  – Slow scanning. Small area.
  – No change in the optics.
  – Nanometer resolution.
  – 3D capability

• **LFD 3-D particle tracking**
  – Combination of the xy Mirror scanner and fast Piezo Z- positioner
Detectors for Laser Scanning Microscopes

• Point detectors (single channel)
  – PMT: Photomultiplier tubes
  – APD: Avalanche photodiodes

http://www.prairie-technologies.com/resources/techniques/2photon.html
Photomultipliers

Elements:
- Photocathode: a negatively charged electrode for electron release at photon abs.
- Dynodes: Electrodes for electron multiplication (up to 18)
- Anode: collection electrode
- Very fast response time (ns), bandwidth 1-1.5 GHz.
- Extremely high sensitivity
- Very high S/N.

PMT Gain and Spectral Responses

• **Current amplification (gain) estimation:** \[ \text{Gain} = E^n \]
  - \( E \) secondary emission ratio for the dynodes
  - \( n \) number of dynode stages
  - electron gains of **10 million** can be achieved.

• **Photocathode composition determines:**
  - spectral response
  - quantum efficiency: 30-40 %.
  - overall uniformity of the photomultiplier sensitivity
  - dark current

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

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

It contains: a positively doped “p region”, a negatively doped “n region”, and an area of neutral charge “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, a current will flow in proportion to the number of photons incident upon the junction.
  - Gain: 500-1000
  - Depletion layer is thin
  - Very high reverse-bias voltages, increases energy of the created electrons, multiple collisions avalanche of electrons (electron multiplication)

- Advantages:
  - High quantum efficiency (90 %)
  - Broad spectral range
  - Uniform detection surface
  - Require low currents
  - Immune to magnetic fields
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.
Comparison of Selected Photodetectors

- **Low sensitivity, fast rise time (0.78 ns)** → Photon counting for FLIM
  - R7400U-04 Hamamatsu QE 18.6%, 2 ns

- **High sensitivity, slow** → Low light level imaging
  - APD SPCM-AQR Perkin-Elmer QE 65%, 35 ns

- **QE low, gain high (10⁷)**, → analog detection
  - R928 Hamamatsu QE 25.4%, 10 ns

- **QE high, good timing** → Photon counting
  - H7422P-40 Hamamatsu QE 40%, 2 ns
  - HPM-100-40 QE 45%, 850 ps
MODE OF PMT OPERATION: ANALOG OR PHOTON COUNTING?

**HIGHER LIGHT LEVEL** (Multiple Photoelectron State)

1. Arrival of photons
2. Photoelectron emission
3. Signal output (pulses)
4. Signal output (pulse overlapped)

**LOWER LIGHT LEVEL** (Single Photoelectron State)

5. Arrival of photons
6. Photoelectron emission
7. Signal output (discrete pulses)
The Level of Incident Illumination

**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).

**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.
- 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 (d).
Imaging Detectors (Multi-channel) for Widefield Microscope

- **CCD/ICCD/EMCCD**
  - CCD: charge-coupled device
  - ICCD: Intensified CCD
  - EMCCD: electron-multiplying CCD

Commonly used to acquire wide-field, spinning-disk confocal, and total internal reflection fluorescence (TIRF) microscopy images.
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
Electron Multiplying CCD (EMCCD)
Intensified CCD (ICCD)

Fast gating: 3~5 ns

Required for most time-resolved fluorescence microscopy applications

http://www.lambert-instruments.com
ICCD system for FLIM measurement
Multi-Frequency Widefield FLIM at LFD
**CCD/ICCD/EMCCD**

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

**ICCD:**
- Faster Gating (ns). For FLIM.

**EMCCD:**
- Rapid frame-rate capture at extremely low light levels
- Quantum efficiency >90%
- Read noise < 1 electrons rms

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http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/emccds.html
BEHIND THE TABLE: ELECTRONICS AND OPTICS

http://zeiss-campus.magnet.fsu.edu
http://www.ISS.com
Control of laser Scanning

3-axis card

IOTech IO card

Drivers
Detection Components

Photon-Counting Unit
- IOTech IO card
- FCS card
- FLIM card
- TCSPC card

Detector
- Pre-amplifier
- Discriminator

“Failure of the constant fraction discriminator”, Kirstin Luery, 2003
Acknowledgements

• Dr. Enrico Gratton
• Members in LFD