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.
Fluorescence Microscopy

Fluorescence Microscopes offer:

**Spatial resolution:** \(~0.2 \, \mu m\)

**Different probes for multi-color imaging**

J. Lichtman etc, Nature Methods, 2005, 2:910-919

http://homepages.wmich.edu/~eversole/widefield.htm

http://zeiss-campus.magnet.fsu.edu/articles/basics/fluorescence.html
Super-resolution Microscopy

- **"true" super-resolution techniques**
  - "capture information contained in evanescent waves"
  - Pendry Superlens, near field scanning optical microscopy, the 4Pi Microscope and structured illumination microscopy

- **"functional" super-resolution techniques**
  - "clever experimental techniques and known limitations"
  - Deterministic super-resolution
    - STED, GSD, RESOLFT and
  - Stochastic super-resolution
    - SOFI, and all single-molecule localization methods: SPDM, SPDMphymod, PALM, FPALM, STORM and dSTORM.

Imaging Techniques: Optical Microscopy

• Widefield microscopy

http://micro.magnet.fsu.edu/primer/techniques/fluorescence/anatomy/fluoromicroanatomy.html
http://microscopy.duke.edu/learn/introtomicroscopy/fluorescencescopes.html
Wide-field imaging Systems at LFD

TIRF/FLIM imaging

- EMCCD
- ICCD
- SCMOS FLIM Camera

SPIM: Selective/Single Plane Illumination Microscopy

- SCMOS Camera
Imaging Techniques: Optical Microscopy

• Laser Scanning microscopy
  – Confocal
  – Multiphoton

Available at LFD
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.smt.zeiss.com/
http://www.olympusconfocal.com/theory/confocalscanningsystems.html
2-photon Excitation Fluorescence Microscopy

- **Single-photon excitation**
  - Laser illumination everywhere
  - Excitation everywhere
- **Multi-photon excitation**
  - Laser illumination everywhere
  - Excitation only at highest photon flux

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

3-Photon excitation occurs in the same way
Characteristics:
1. The signal beam is stationary.
2. Loss of signal.
# Confocal Microscopes at LFD

<table>
<thead>
<tr>
<th>One-photon laser scanning</th>
<th>One/two-photon</th>
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<td>LSM 510</td>
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<tr>
<td>Frame</td>
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<td>Fluoview FV1000 (Keck)</td>
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<tr>
<td><strong>Frame</strong></td>
<td><strong>Zeiss</strong></td>
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<td>Argon, 405/559/635nm, Ti:Sapph with Deepsea</td>
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<td>FLIMBox Tracking system</td>
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<td><strong>Advantage</strong></td>
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<td>For animal study, 3D tracking</td>
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<td>High sensitivity, 3D tracking</td>
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<td>High sensitive, Spectral detectors</td>
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<td>Super resolution, Spectral detectors</td>
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</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>
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<th>M5</th>
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<tr>
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<td>MaiTai HP</td>
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<tr>
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<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>
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<tr>
<td>Software &amp; Data acquisition</td>
<td>SimFCS FLIMBox ISS-FCS</td>
<td>SimFCS FLIMBox ISS-FCS</td>
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<td>SimFCS FLIMBox ISS-FCS</td>
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<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, For particle tracking</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 typical Microscope for Biomedical Imaging

Sources:
- Laser
  - Ti:Sapphs
  - Other lasers

Scanner
- Mirror laser scanner
- Pizo stage scanner

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

Others
- Optics
- Electronics
- ...

Electronics...
Wavelengths of Commercially Available Lasers

http://en.wikipedia.org/wiki/Laser
Typical Lasers in Confocal Microscope

- Helium Neon Lasers
- Argon ion laser
- Diode Lasers
- Diode Pumped Solid State

FLIM?

http://mellesgriot.com/products/Lasers
Supercontinuum Lasers

White Lasers at LFD:
5x from Fianium/NKT at 20MHz from 390nm to 2000nm

- Total power: up to 20W
- Internal Repetition Rate: 0.1-200MHz
- External Triggerable: 1-80MHz
- Wavelength: 400nm-2400nm
- Pulse Energy: >1.5uJ
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: Multiphoton Lasers

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

Enough power to saturate absorption in a diffraction limited spot

Coherent Chameleon-Ultra II

Spectra-Physics Mai Tai HP

Spectra-Physics Tsunami

Spectra-Physics Insight DS+ dual

Multiphoton Lasers at LFD: 1x Chameleon-Ultra II, 5x MaiTai, 1x Tsunami, 1x Insight DS+ dual
Tuning Curves of Ti:Sapphs Lasers

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

Typical power required for TPEF imaging:
<5 mW
Lasers with Extended Tuning Range

Ytterbium as the gain medium
Scanning Unit: Mirror Scanner

• Scanning the laser beam by mirrors:
  – Fast
  – Sample is stationary

• Methods:
  – Galvanometric scanner
  – Resonant galvanometer scanner
  – Polygonal scanner
  – Acousto-optical deflector

http://www.microscopyu.com/articles/confocal/resonantscanning.html
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 capacitive 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°.
Examples of scanning unit at LFD

ISS scanning Unit

LFD homebuilt scanning Unit
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.
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
Scanning and Lock-in Particle Tracking

- **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
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
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 (10^7), → 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

- **Timing (pulse width)**
  - 35 ns
  - 15 ns
  - 10 ns

- **Sensitivity/QE**
  - 100 %
  - 10 %
Mode of PMT Operation:
Analog & Photon Counting

Higher Light Level (Multiple Photoelectron State)

1. Arrival of Photons
2. Photoelectron Emission
3. Signal Output (Pulses)
4. Signal Output (Pulse Over Lapped)

Lower Light Level (Single Photoelectron State)

5. Arrival of Photons
6. Photoelectron Emission
7. Signal Output (Discrete Pulses)

Time
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/sCMOS
  - CCD: charge-coupled device
  - ICCD: Intensified CCD
  - EMCCD: electron-multiplying CCD
  - sCMOS: Scientific CMOS

Commonly used in wide-field, spinning-disk confocal, and total internal reflection fluorescence imaging

Electron Multiplying CCD (EMCCD)
EMCCD at LFD

Evolve for TIRF

iXon Ultra 897 For Hyberspectral
Intensified CCD (ICCD)

Intensifier principle:
Photons converted to electrons, accelerated and then multiplied in the MCP. The electron clouds get converted back to photons at anode screen.

Fast gating: 3~5 ns

Required for most time-resolved fluorescence microscopy applications

http://www.lambert-instruments.com
ICCD system for FLIM measurement
Scientific CMOS (sCMOS)

sCMOS (1.5 e\textsuperscript{-} noise)  Interline CCD (5 e\textsuperscript{-} noise)  Back-illuminated EMCCD (<1 e\textsuperscript{-} noise)

http://www.scmos.com/
sCMOS as LFD for SPIM and FLIM

95% QE Backside Illuminated sCMOS

https://www.photometrics.com/products/scmos/prime95B
CCD/ICCD/EMCCD/sCMOS

**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

**sCMOS:**
- Larger view
- Fast imaging speed
- High QE

http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/emccds.html
Other Parts: Electronics & Optics
Control of Laser Scanning at LFD

IOTech IO card

3-axis card

Drivers

IOTech IO card
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
Optical Filters

Interference Filter

Colored glass filters

http://www.olympusmicro.com
http://www.schott.com/advanced_optics
http://www.hoyaoptics.com
Thank You ~