Special Techniques 1

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**Special Techniques**

- Multi-photon microscopy
- Second Harmonic Generation
- FRAP
- FRET
- FLIM
- In-vivo imaging
**Two-photon Microscopy**

- Alternative to confocal and deconvolution microscopy
- Two photons of half the energy combine
- Emission equivalent to being excited with a single photon laser

![Two-Photon Jablonski Energy Diagram](image)
TWO-PHOTON MICROSCOPY

Leica SP5 Upright

Newport Spectra-Physics Mai Tai Laser
690-1040nm

Leica SP5 Inverted
Two-photon Microscopy

- Much higher energy required
  - ~1,000,000x greater
- Pulsed laser
- Femtosecond pulses
  - High power per pulse
  - Low average power
- Wave-form power output (2.7W peak)
TWO-PHOTON MICROSCOPY

- No pinhole required (no out of focus light excited)
  - NDD detectors – higher sensitivity (less light path = less light lost)
- Less photo-bleaching/photo-toxicity (lack of excitation above and below the focal plane)
- Less scattering of light (Red vs Blue)
- Deeper penetration
- Protein uncaging – specialist application

- Image resolution/Thin specimens
TWO-PHOTON MICROSCOPY
## Two-photon Stain Selection

- Spectral profile differs (peak may change)

<table>
<thead>
<tr>
<th>Dye</th>
<th>Single Photon Ex/Em</th>
<th>Two Photon Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>488/507nm</td>
<td>860&lt;960nm</td>
</tr>
<tr>
<td>DsRed</td>
<td>543/580nm</td>
<td>900&lt;1064nm</td>
</tr>
<tr>
<td>DAPI</td>
<td>350/470nm</td>
<td>780&gt;820nm</td>
</tr>
<tr>
<td>FITC</td>
<td>490/525nm</td>
<td>780&gt;820nm</td>
</tr>
<tr>
<td>CY3</td>
<td>550/570nm</td>
<td>780nm</td>
</tr>
<tr>
<td>CY5</td>
<td>649/670nm</td>
<td>780&gt;820nm</td>
</tr>
</tbody>
</table>
THREE-PHOTON EXCITATION

- Only 10x more power needed from 2-photon, not the million-fold increase going from 1-photon to 2-photon excitation.
- Requires 3 photons
  - ~1/3 of normal excitation
- 1020nm → 340nm
  - (510nm)
SECOND HARMONIC GENERATION

- (Frequency Doubling)
- Photon's interact to form single photons (double the wavelength)
- Only non-centrosymmetric structures
SECOND HARMONIC GENERATION

- Gives structural information without staining
- Useful for morphological information in whole tissue
  - Gain structural information of surrounding when tracking cells in-vivo without needing staining (live specimens)
- Can be combined with other microscopy techniques (Anisotropic imaging, Autofluorescence, Lifetime imaging)
SECOND HARMONIC GENERATION

- Wavelength dependency for different structures
- Useful to distinguish between tissue composition without staining (with other techniques)
- Commonly imaged structures:
  - Collagen I/III (840/930nm)
  - Elastin (740nm)
  - NAD(P)H (680nm)
SHG 840nm

Collagen I antibody staining
Pancreatic Islets showing two-photon SHG generation of intracellular NAD(P)H
SECOND HARMONIC DYES

- SHRIMPS (Second Harmonic Radiation Imaging Probes)
- Development of structurally significant molecules
- Specific wavelengths of SHG generation used to excite
- Structural interference/amplification
- Excellent possibilities for extreme long term imaging
Photo-bleaching

- Photochemical destruction of fluorescent molecules
- Differs between fluorophores
- 2P excitation reduces this bleaching by limiting exposure
FLUORESCENCE RECOVERY AFTER PHOTO-BLEACHING

- FRAP
- Bleaching of specific areas
- Diffusion/transport experiments
Forster Resonance Energy Transfer

- **FRET**
- Transfer of energy between fluorophores
- Typically under 15nm
- Quantitative measure of co-localisation
  - Only molecules within strict distances will FRET
  - FRET efficiency determined by distance between molecules

![Resonance Energy Transfer Jablonski Diagram](image)
FRET

Intramolecular Fluorescence Resonance Energy Transfer (FRET)

Protein Labeled with Two Fluorochromes

(a) Protein Conformational Change
(b) Donor Excitation 2 Nanometer Separation Distance

No Acceptor Fluorescence

12 Nanometer Separation Distance

Figure 1

FRET Detection of *in vivo* Protein-Protein Interactions

Blue Fluorescent Protein 380 Nanometer Excitation

Separated Protein Molecules

GFP

No Green Fluorescent Protein Emission at 510 Nanometers

Figure 2

Intermolecular Association

Blue Fluorescent Protein 380 Nanometer Excitation

BFP

GFP

Green Fluorescent Protein Emission at 510 Nanometers
FRET

- Specific fluorophore pairings
  - Based on spectral overlap
    - Emission of Donor must overlap Excitation of Acceptor
    - FRET efficiency determines Forster distance

Common Donor-Acceptor Pairs:

- FITC – Rhodamine (4.9nm)
- CY3 – CY5 (>5.0nm)
- PE – CY5 (7.2nm)
- CY5 – CY5.5 (>8.0nm)
FRET

- Photobleaching effects the FRET transfer
- Ratio-metric analysis
- Acceptor bleached, Donor emission increases

- Two-photon bleaching more precise
  - Only bleaches in plane of focus so able to specify the 3D area of bleaching much more precisely
Fluorescence Lifetime Imaging Microscopy

- FLIM
- Pulsed mode-locked laser (2P – FILM Confocal 3 and 4)
- Measure of excitation decay
FLIM for FRET

- Lifetime of fluorophores effected by energy transfer
- Lifetime also changes with conditions of sample
IN-VIVO IMAGING

- Confocal 3
- Imaging whole tissue or animals
- Problematic due to light scatter, movement...
**IN-VIVO IMAGING**

- High quality lens
  - 25x water immersion lens
  - 0.95 N.A.
- High confocal zoom possible
- Allows low power rapid scanning and high power area imaging
**In-vivo Imaging**

- 63x Objective – No zoom
- 25x Objective – 6x zoom
IN-VIVO IMAGING

- Ensure specimen does not move
- Longer scan time possible
- Reproducibility increased
IN-VIVO IMAGING

- 2P laser excitation – deeper penetration, less scatter, less damage...
- Steady specimen holder – increased scan time possible
- Multiple imaging techniques able to be combined
IN-VIVO IMAGING
Thank You...