Rice/TCU REU on Computational Neuroscience

Fundamentals of Molecular Imaging

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Objectives

- Brief discussion of optical resolution and lasers as excitation sources
- Multiphoton excitation-advantages/disadvantages
- Molecules inside cells
- Applications of MPE to the study of intracellular diffusion and biochemistry
- Photobleaching Recovery
- The concept of single molecule analysis
- Fluorescence Correlation Spectroscopy
- Fluorescence Cross-Correlation Spectroscopy
Resolution = $0.61\lambda / NA$

$\lambda = \text{wavelength of electromagnetic radiation}$
Spatial and Temporal Scales of Microscopy

Temporal scales - picoseconds to months
What Limits Resolution in Microscopy?

Numerical Aperture (NA)

\[ \text{NA} = n \sin(\theta) \], where \( n \) is the index of refraction and \( \theta \) the half angle of the illumination cone.

Long working distances

- NA = 0.12
  - \( \theta \approx 7^\circ \)

Short working distances

- NA = 0.90
  - \( \theta \approx 64^\circ \)

Rayleigh criterion:

Resolution = \( 0.61\lambda / \text{NA} \)

\[ R = 1.22\lambda / (\text{NA}_{\text{obj}} + \text{NA}_{\text{cond}}) \]

Destructive Interference
Characteristics of Light from Lasers

Light Amplification by Stimulated Emission of Radiation
Following absorption, a number of vibrational levels of the excited state are populated. Molecules in these higher vibrational levels then relax to the lowest vibrational level of the excited state (vibrational relaxation). From the lowest vibrational level, there can be an emission of photon of lower energy (fluorescence).
Two (or more) photons can interact simultaneously with a molecule adding their energies to produce an excitation equal to the sum of their individual energies.

i.e. 2 red photons can = 1 blue photon

What is Two Photon Excited Fluorescence?
Two/(Multi)-Photon-Excitation

Idea:
Simultaneous \((10^{-15}s)\) absorption of \(n\) photons of wavelength

Major advantage:
Inherent spatial sectioning by \(I^n\) - dependency of excitation probability.
Excitation only in vicinity of focal spot

Pulsed excitation = (100 fs, 80 MHz)
MPE is inherently localized to the focus of a high NA objective.

The intensity (squared) declines from z (red arrows) as $z^{-4}$.

Calculated intensity of 740 nm light near focus of 1.2 NA objective.
Pulsed laser excitation enhances two-photon absorption

![Graph showing laser intensity over time](image-url)
Two Photon Excitation (2PE)

**Advantages**

For intracellular work:
1. Small focal volume
2. Decreased photobleaching
3. Decreased phototoxicity
4. Increased viability
5. Increased focus depth

For cross-correlation work:
6. Single laser line
7. No pinhole necessary
8. Good S/N ratio

**Disadvantages**

1. Greater average Illumination intensities
2. Loss of resolution
3. High cost of pulse laser
Uses of fluorescent molecules:

1. **Labels** - free dyes that may partition to a specific region of a cell or tissue, or fluorescent molecules that are bound to antibodies, receptor proteins or other biomolecules of interest.

2. **Indicators dyes** - the probes dynamically bind an ion (Ca$^{++}$, H$^+$, Mg$^{++}$) and then change in either fluorescence intensity, emission or excitation spectrum.

3. **Fluorescent proteins** such as GFP, that are produced by the organism after the DNA for GFP, or more commonly a GFP fusion protein, is introduced into the cell.
Problems with Fluorescence - Photobleaching and Blinking

Molecular fluorophores do not emit fluorescence photons indefinitely - they have a limited lifetime that depends on their chemical structure and the chemical environment they’re in.

For example, a single rhodamine molecule will emit $10^5 - 10^6$ fluorescence photons before it becomes irreversibly photobleached. Some intrinsically fluorescent biological molecules such as tryptophan (UV excitation) emit, on average 1 photon before the molecule is irreversibly photodamaged.

Reversible photobleaching (triplet state, other dark states) can also occur, which limits the photon yield per unit time since the molecule spends a percentage its time in an non-excitable state. (Example - eGFP)
Fluorescent Probes

- Molecules: 1 nm
- Single fluorophores: 3 nm
- Quantum dot: 6 nm
- Silica nanoparticle (rhodamine): 50 nm

**EGFP**
Two Photon Laser Scanning Microscopy Coupled to Spectroscopy Techniques
Optical Methods Applied to Study Protein Dynamics

1. Two-Photon Fluorescence Photobleaching Recovery (TPFPR)

2. Two-Photon Fluorescence Correlation Spectroscopy (TPFCS)

3. Two-Photon Fluorescence Dual-Color Cross-Correlation (TPCCS)
<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass</td>
<td>$166 \times 10^{-24}$ kg</td>
<td>Mass of one mole/Avogadro's constant</td>
</tr>
<tr>
<td>Density</td>
<td>$1.38 \times 10^3$ kg/m$^3$</td>
<td>1.38 times the density of water</td>
</tr>
<tr>
<td>Volume</td>
<td>$120$ nm$^3$</td>
<td>Mass/density</td>
</tr>
<tr>
<td>Radius</td>
<td>$3$ nm</td>
<td>Assuming a spherical shape</td>
</tr>
<tr>
<td>Drag Coefficient (in water @ 20°C)</td>
<td>$60$ pN.s/m</td>
<td>From Stoke's Law</td>
</tr>
<tr>
<td>Diffusion Coefficient (in water @ 20°C)</td>
<td>$67$ μm$^2$/s</td>
<td>From the Stoke's-Einstein relationship</td>
</tr>
<tr>
<td>Average Speed</td>
<td>$8.6$ m/s</td>
<td>From the Equipartion principle</td>
</tr>
</tbody>
</table>
Distance/Time Relationship for One-Dimensional Diffusion of Different Sized Objects in Water

<table>
<thead>
<tr>
<th>Object</th>
<th>1 ( \mu \text{m} )</th>
<th>100 ( \mu \text{m} )</th>
<th>10 mm</th>
<th>1 m</th>
</tr>
</thead>
<tbody>
<tr>
<td>K(^+)</td>
<td>0.25 ms</td>
<td>2.5 s</td>
<td>2.5 ( \times 10^1 ) s (7 hours)</td>
<td>2.5 ( \times 10^8 ) s (8 years)</td>
</tr>
<tr>
<td>Protein (3 nm radius)</td>
<td>5 ms</td>
<td>50 s</td>
<td>5 ( \times 10^5 ) s (6 days)</td>
<td>5 ( \times 10^9 ) s 150 years</td>
</tr>
<tr>
<td>Organelle (0.5 ( \mu \text{m} ) radius)</td>
<td>1 s</td>
<td>10(^4) s (3 hr)</td>
<td>10(^8) s (3 years)</td>
<td>10(^{12}) s (30 million years)</td>
</tr>
</tbody>
</table>

\[ \langle x^2 \rangle = nDt \quad n = 2, 4 \text{ or } 6 \text{ for one, two and three dimensional diffusion} \]
Two-Photon Fluorescence Photobleaching Recovery (TPFPR)

- Low intensity monitoring beam
- Short (<ms) bleaching pulse

![Diagram showing the process of Two-Photon Fluorescence Photobleaching Recovery (TPFPR)]

- Focal volume
- Bleaching pulse
- Recovery

Graph showing the change in fluorescence intensity over time:
- \( F/F_0 \) vs. Time (ms)
- \( F_0 \), \( F(0) \), \( F(t) \), \( F(\infty) \)
- Half-time \( t_{1/2} \)
Diffusion Mapping of Alexa-488-Labeled Calmodulin in Neurons Using MPFPR

Alexa-488-CaM in solution

$D(t) = 54 \mu m^2/sec$

$D(t)$ of faster diffusing species

<table>
<thead>
<tr>
<th>Species</th>
<th>Soma</th>
<th>Neurite</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 kD dextran</td>
<td>29.2</td>
<td>29.0</td>
</tr>
<tr>
<td>Alexa-488-CaM</td>
<td>28.5</td>
<td>22.3</td>
</tr>
</tbody>
</table>

Alexa-488 labeled CaM in solution

$D = 2.7 \times 10^{-7} \text{cm}^2 \text{s}^{-1}$

$D_1 = 28.5 \mu m^2/s (88\%)$
# Comparison of Diffusion Coefficients from *in Vitro* and *in Situ* FPR Measurements

<table>
<thead>
<tr>
<th>Protein</th>
<th>Radius (nm)</th>
<th>$D_s$ (in solution)</th>
<th>$D_c$ (in cytoplasm)</th>
<th>$D_c/D_s$</th>
<th>% mobile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calmodulin</td>
<td>2.1</td>
<td>102</td>
<td>&lt;4</td>
<td>0.039</td>
<td>81</td>
</tr>
<tr>
<td>GFP</td>
<td>2.5</td>
<td>87</td>
<td>27</td>
<td>0.31</td>
<td>82</td>
</tr>
<tr>
<td>BSA</td>
<td>3.2</td>
<td>67</td>
<td>6.8</td>
<td>0.1</td>
<td>77</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>3.3</td>
<td>65</td>
<td>&lt;4.5</td>
<td>0.07</td>
<td>50-80</td>
</tr>
<tr>
<td>Enolase</td>
<td>3.8</td>
<td>56</td>
<td>13.5</td>
<td>0.24</td>
<td>100</td>
</tr>
<tr>
<td>IgG</td>
<td>4.7</td>
<td>46</td>
<td>6.7</td>
<td>0.15</td>
<td>54</td>
</tr>
</tbody>
</table>

$D = \frac{kT}{6\pi\eta r}$

D= diffusion coefficients (μm²/s); modified from Luby-Phelps, 2001
Intracellular Diffusion: Far from Simple
Applications of Single Molecule Approach to Biochemistry and Cell Biology

Fluorescence Correlation Spec./Fluorescence Cross Correlation Spec.
How to detect single molecules?

Low concentrations of fluor (<10^{-9} M)

Small volume elements achieved through confocal or multiphoton optics

Primary measurement parameter is *signal fluctuations* induced by:
- Diffusion of molecules through the open measurement volume
- Intramolecular dynamics which affect the fluorescence emission

```
“bursts”

I_{em}

time
```
Experimental Apparatus
Analysis of Fluorescence Fluctuations

Temporal analysis of spontaneous fluorescence fluctuations $\delta F$-

Signal fluctuations are induced by:

- Diffusion of molecules through the open measurement volume
- Intramolecular dynamics which affect the fluorescence emission
Parameters Provided by Fluorescence Correlation Spectroscopy (FCS)

**CONCENTRATION**

- **Few Molecules in Observation Volume**
  - Fluctuations from single molecule
  - Low average fluorescence
  - Large fluorescence fluctuations, large correlation amplitude

- **Many Molecules in Observation Volume**
  - Smaller relative fluctuations from each molecule
  - Higher average fluorescence
  - Effect of a single molecule is reduced, “washed out”
  - Smaller fluctuations, small correlation amplitude

**DIFFUSION COEFFICIENT**

- **Short Residence Time**
  - Increasing \( \tau_d \)
  - Decreasing Diffusion Coefficient

- **Long Residence Time**
  - Effect of a single molecule is reduced, “washed out”
  - Smaller fluctuations, small correlation amplitude

Graphs show the correlation function over time for different concentrations and diffusion coefficients.
Examples for fast internal dynamics: “flickering”

Molecules under study: GFP (Green Fluorescent Protein) and its mutants: many of them show pH-dependent emission

![Graph showing pH-dependent emission and structure diagram](image-url)
FCS measurements of GFP-a pH sensor

GFP “blinks” on a single molecule scale. Fast dynamics are strongly pH dependent

> reversible protonation

GFP can be employed as single molecule pH meter!

![Diagram](image)

\[ \text{R} - \text{O}^\ominus + H^+ \xrightleftharpoons[k_f]{k_b} \text{R} - \text{OH} \]

\[ pK = 5.7 \]

\[ \lambda_{\text{abs,deprot}} = 488\text{nm} \]

\[ \lambda_{\text{abs,prot}} = 400\text{nm} \]
What to determine by diffusion analysis?

Analysis of molecular structure: diffusion properties depend on hydrodynamic radius

Analysis of association/dissociation processes by change in molecular mass

\[ D = \frac{kT}{6\pi \eta R_h} \]

Correlation \( G(\tau) \)

\[ \tau_1, \tau_2 \]

\[ Y(t) \]

\[ k_{ass} = 10^4 \text{ M}^{-1}\text{s}^{-1} \text{ to } 10^6 \text{ M}^{-1}\text{s}^{-1} \]
Assessing molecular mobility in different cellular compartments

Requirement: specific labeling of regions of interest

Precision: 0.3 um in XY
1.0 um in Z

Determination of “molecular speed”
Detection of single molecules in membranes

⇒ Only labeled regions contribute to the measured signal
The viscosity of the membrane has been likened to that of olive oil, some 50-100 times that of water.

\[ D = \frac{kT}{6\pi \eta r} \]
Diffusion in Membranes
**Dual-color cross-correlation analysis FCCS**

Advantage: mobility independent analysis of molecular interactions

\[
D = \frac{kT}{6\pi \eta R_h}
\]

RECALL

\[R_h \sim (MW)^{1/3}\]

Principle: only doubly labeled species contributes to cross-correlation signal

Red channel:  

Blue channel:  

Denominator  

Numerator  

\[G_{ij}(\tau) = \frac{\langle \delta F_i(t) \cdot \delta F_j(t + \tau) \rangle}{\langle F_i(t) \rangle \cdot \langle F_j(t) \rangle}\]
Experimental setup for TPCCS

Inherent overlap of excitation volumes

Simplified alignment of detection volumes (no pinholes required)
Dual-color two-photon cross-correlation (TPCCS)

Concept: Excitation of spectrally separable fluorophores with a single IR line

Requirement: both dyes show similar emission on a single molecule scale
Analysis of DNA-DNA association

Greater specificity for reaction product observation

\[ G_{RB}^{x}(\tau) = \frac{C_{RB} \text{Diff}_{BR}}{V_{eff}(C_{R} + C_{RB})(C_{B} + C_{RB})} \]
Intracellular FCCS applications: The toxin system

System: Bacterial protein toxins entering the cell in a retrograde fashion
Objective: to simultaneously study the endocytic trafficking of Cholera (red label) and Shiga (green label) Toxin

Bacia et al., 2002, Biophy. J.
Comparing Endocytic Pathways for CTX and ST

Distinct autocorrelations, no cross-correlation: different pathways

CTX-Cholera

Comparable autocorrelations, existing cross-correlation: same pathway

ST-Shigella
FCCS reveals where the subunits dissociate

Dissociation of A and B$_5$ subunits required to induce toxicity of A

Cross-correlation finally decays to zero in the Golgi

(1) Cell membrane

(2) Endosomes

(3) Golgi
Assessing Binding Ratios of CaM and CaMKII

\[ \frac{1}{G(0)} = N : \text{absolute particle number} \]

<table>
<thead>
<tr>
<th>Condition</th>
<th>+ EGTA</th>
<th>+ MgATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFP-CaMKII</td>
<td>( N = 0.73 )</td>
<td>( N = 0.73 )</td>
</tr>
<tr>
<td>Alexa 633CaM</td>
<td>( N = 130 )</td>
<td>( N = 128 )</td>
</tr>
<tr>
<td>Cross-Correlation (KK)</td>
<td>( \sim 0 )</td>
<td>( 251.1% )</td>
</tr>
</tbody>
</table>

\[
\frac{KK}{AK_2} = \frac{n \cdot C_{20}}{C_{20} + n(n-1)C_{10}} = \frac{n \cdot 130}{130 + n(n-1)0.73}
\]

For

- \( n = 1 \)  \( \rightarrow \) \( KK = 100\% \)
- \( n = 2 \)  \( \rightarrow \) \( KK = 198\% \)
- \( n = 3 \)  \( \rightarrow \) \( KK = 296.7\% \)

Therefore, experimental data means an average of 2.5 Alexa 633 CaMs are bound to 1 eGFP-CaMKII.
Protein Signaling Analyzed with Cross-Correlation

Kim, S.A. et al, 2004 PNAS
Other Applications of MPE

• Uncaging of fluorescent compounds: inherent spatial localization provides excellent spatial selectivity for uncaging

• In vivo imaging over long time scales (months):
  e.g., watching the development of amyloid plaques develop over months in the brain of a living animal