Rice/TCU REU on Computational Neuroscience

Fundamentals of Molecular Imaging

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Objectives

• Introduction to resolution in light microscopy
• Brief discussion of lasers as excitation sources
• Multiphoton excitation-What is it? advantages/disadvantages
• Fluorescent Probes-uses and characteristics
• Applications of MPE to the study of intracellular biochemistry
• The concept of single molecule analysis
Resolution = \(0.61\lambda / NA\)

\(\lambda = \) wavelength of electromagnetic radiation
Temporal scales - picoseconds to months
What Limits Resolution in Microscopy?
Numerical Aperture (NA)

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

Rayleigh criterion:
Resolution = \( 0.61\frac{\lambda}{NA} \)
\[ R = 1.22\frac{\lambda}{(NA_{obj} + NA_{cond})} \]

Destructive Interference
Characteristics of Light from Lasers

Light Amplification by Stimulated Emission of Radiation
How Lasers Work

[Diagram of a laser illustration with labeled parts: Flash Tube, Mirrored Surface, Atoms, Partially Mirrored Surface, Emitted Light, Excited Atom]
Fluorescence

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

One Photon vs. Two Photon Florescence?

Lifetimes ~ns timescale

Increasing Wavelength

Increasing Energy

1 photon excitation

Fluorescence

2 photon excitation
Two/(Multi)-Photon-Excitation

S0 → S1

hv/2

S1 → S1'

“virtual” state ca. $10^{-15}$ s

Fluorescence $h\nu_f$

hv

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

Single photon excitation (488 nm)
Two photon excitation (900 nm)

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
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
Fluorescent Probes

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.
Fluorescent Probes

- **molecules**: 1 nm
- **EGFP**: 3 nm
- **quantum dot**
- **silica nanoparticle (rhodamine)**: 50 nm

Single fluorophores

Multiple fluorophores
Two Photon Laser Scanning Microscopy Coupled to Spectroscopy Techniques
How do you generate an image?

Scanning mirrors move the laser beam back and forth across the sample.

A detector collects the photons that come out of a single area and map them onto an X-Y Image as pixels.
In vivo imaging - example: transgenic mouse models of Alzheimer's disease.

β amyloid plaque stained with Thio-S, excitation at 760 nm
Two-photon Imaging in Live Animals

Neurons imaged in the brain of a live animal 18 months apart
Arrows-spines eliminated; Arrowheads, spines formed

From Zuo et al., Neuron 2005
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)
Two-Photon Fluorescence Photobleaching Recovery (TPFPR)

- **low intensity monitoring beam**
- **short (<ms) bleaching pulse**

![Diagram showing the process of two-photon fluorescence photobleaching recovery](image)

**Graph showing bleaching and recovery process**

- **F₀**: Initial fluorescence
- **F(t)**: Fluorescence at time t
- **F(∞)**: Fluorescence at equilibrium
- **t₁/₂**: Time for half-recovery
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>
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<tbody>
<tr>
<td>10 kD dextran</td>
<td>29.2</td>
<td>29.0</td>
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<tr>
<td>Alexa-488-CaM</td>
<td>28.5</td>
<td>22.3</td>
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</table>

\[ D_1 = 28.5 \, \mu m^2/s \, (88\%) \]
Comparison of Diffusion Coefficients from \textit{in Vitro} and \textit{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>
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<tbody>
<tr>
<td>Calmodulin</td>
<td>2.1</td>
<td>102</td>
<td>&lt;4</td>
<td>0.039</td>
<td>81</td>
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<tr>
<td>GFP</td>
<td>2.5</td>
<td>87</td>
<td>27</td>
<td>0.31</td>
<td>82</td>
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<tr>
<td>BSA</td>
<td>3.2</td>
<td>67</td>
<td>6.8</td>
<td>0.1</td>
<td>77</td>
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<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>
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<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>

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

$D = \text{diffusion coefficients (\text{\mu m}^2/\text{s})}$; 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
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

\[
G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2}
\]
Parameters Provided by Fluorescence Correlation Spectroscopy (FCS)

**CONCENTRATION**
- Few molecules in observation volume: low average fluorescence, large fluctuations from single molecule.
- Many molecules in observation volume: higher average fluorescence, smaller relative fluctuations from each molecule.

**DIFFUSION COEFFICIENT**
- Short residence time: increased diffusion coefficient.
- Long residence time: decreased diffusion coefficient.

Increasing concentration decreases autocorrelation. Increasing $\tau_d$, the diffusion coefficient decreases.
Examples for fast internal dynamics: “flickering”

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

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

$\lambda_{abs,deprot} = 488\text{nm}$

$\lambda_{abs,prot} = 400\text{ nm}$

GFP can be employed as single molecule pH meter!
What to determine by diffusion analysis?

Analysis of association/dissociation processes by change in molecular mass

Analysis of molecular structure: diffusion properties depend on hydrodynamic radius

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

$k_{\text{ass}} = 10^4 \text{ M}^{-1}\text{s}^{-1}$ 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
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: \textcolor{red}{\textbullet} + \textcolor{red}{\textbullet}

Blue channel: \textcolor{blue}{\textbullet} + \textcolor{blue}{\textbullet}

cross-c.

\[ G_{ij}(\tau) = \frac{\left< \delta F_i(t) \cdot \delta F_j(t + \tau) \right>}{\left< F_i(t) \right> \cdot \left< F_j(t) \right>} \]
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

\[ G_{RB}^x(\tau) = \frac{C_{RB} \text{Diff}_{BR}}{V_{eff} (C_R + C_{RB})(C_B + C_{RB})} \]

Greater specificity for reaction product observation
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

Comparable autocorrelations, existing cross-correlation: same pathway

CTX-Cholera

ST-Shigella
FCCS reveals where the subunits dissociate

Dissociation of A and B₅ subunits required to induce toxicity of A

Cross-correlation finally decays to zero in the Golgi
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) deep in living tissue