Time-resolved fluorescence spectroscopy: An old technique to monitor protein higher order structure changes

Sergey Arzhantsev
Division of Pharmaceutical Analysis, Office of Testing and Research
Center for Drug Evaluation and Research, US Food and Drug Administration
645 South Newstead Ave, Saint Louis, MO 63110

This presentation reflects the views of the author and should not be construed to represent FDA’s views or policy.
Outline

• Introduction
• Experimental Setup and Data Analysis
• Results
  – Case Study 1
  – Case Study 2
  – Case Study 3
• Conclusions
Fluorescence spectroscopy cannot provide quantitative information about HOS

- No numeric values can be determine for α-helix and β-sheet

Fluorescence spectroscopy is sensitive to change in HOS

- Any change in fluorescence is indicative of a microenvironment change around tryptophan residues
Web of Science

Search: tryptophan fluorescence protein
Introduction to Fluorescence

Log-normal function

\[ I(x) = \frac{1}{x\sigma\sqrt{2\pi}} e^{-\frac{(\ln x - \mu)^2}{2\sigma^2}} \]

\(\nu_{\text{peak}} ; \sigma\)

- \(Q\) - quantum yield
- \(\tau\) – fluorescence lifetime
- \(\tau_n\) – natural lifetime

\[ \tau_n = \tau / Q \]

✓ Time-resolved fluorescence provides information not available from steady-state data
Intrinsic Protein Fluorescence

Intrinsic fluorescence can be used to monitor changes in HOS

<table>
<thead>
<tr>
<th></th>
<th>Absorption</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau$ (ns)</td>
<td>$\lambda$ (nm)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3.1</td>
<td>280</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.6</td>
<td>274</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>6.4</td>
<td>257</td>
</tr>
</tbody>
</table>
Tryptophan

Tryptophan Fluorescence is sensitive to

✓ pH
✓ Solvent Polarity
Tyrosine

- Distinguishable from tryptophan fluorescence
- Less sensitive to microenvironment
Resonance Energy Transfer

\[ D^* + A \rightarrow D + A^* \]

\[ k_T(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6 \]

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>( R_0(A) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe</td>
<td>Tyr</td>
<td>11.5-13.5</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyr</td>
<td>9-16</td>
</tr>
<tr>
<td>Tyr</td>
<td>Trp</td>
<td>9-18</td>
</tr>
<tr>
<td>Trp</td>
<td>Trp</td>
<td>4-16</td>
</tr>
</tbody>
</table>

✔ Tryptophan is acceptor for all three amino acids: phe, tyr, trp
Sensitivity

Myoglobin
Average Fluorescent Lifetime:
1.06 ns $\rightarrow$ 2.44 ns

Albumin (Bovine)
Average Fluorescent Lifetime:
4.38 ns $\rightarrow$ 2.3 ns
Spectrofluorometer

Things to remember:
- Quantum Efficiency of PMT
- Grating Efficiency
- Signal collected from center of cell (OD < 0.1)

- Simple design, robust method
Time correlated single photon counting

Laser pulse | Fluorescence photon | Laser pulse | Fluorescence photon | Laser pulse

\[ \Delta t \]

Photon Distribution

\[ \text{Statistics} \]
Deconvolution

Convolution

\[ N(t) = \int_{0}^{t} L(t')I(t - t')dt' \]

Model

\[ I(t) = \sum_{i} A_i e^{-t/\tau_i} \]

Goodness-of-fit parameter

\[ \chi^2 = \sum_{k=1}^{n} \frac{|N(t_k) - N_c(t_k)|^2}{N(t_k)} \]

\[ \chi_R^2 = \frac{\chi^2}{n - p} \]
Data Analysis

The graph shows the intensity of the signal over time in nanoseconds (ns) with three exponential decay models labeled as 1 exp, 2 exp, and 3 exp. The residual graph below the intensity graph indicates the difference between the observed data and the fitted model.

www.fda.gov
Models

Multiexponential model: \( I(t) = \sum_i A_i e^{-t/\tau_i} \) Independent species

Stretched exponential model: \( I(t) = \sum_i A_i e^{-(t/\tau_i)^\beta_i} \) Heterogeneous environment

Distribution model: \( I(t) = \int_{-\infty}^{\infty} \rho(\tau)e^{-t/\tau}d\tau \) Dynamic environment

✓ Model should describe the system
Example of Models

Multiexponential model: \( \chi^2 = 0.9879 \)
\( \tau = 2.95 \text{ ns} \)

Stretched exponential model: \( \chi^2 = 0.9500 \)

Distribution model: \( \chi^2 = 0.9832 \)
\( \tau = 2.96 \text{ ns} \)

✔ Model should describe the system
Fluorescence anisotropy

**Excitation light**

**Detector**

Anisotropy measurement can provide information about size of the protein

\[
I_{\parallel}(t) - I_{\perp}(t) = r_0 e^{-\theta t}
\]

**Initial Anisotropy**

\[
r_0 = \frac{2}{5} \left( \frac{3 \cos^2 \beta - 1}{2} \right)
\]

**Rotational Correlation Time**

\[
\theta = \frac{\eta V}{RT}
\]
Time-resolved Anisotropy

Two methods:
- Direct calculation
- Deconvolution

\[ r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \]

\[ r(t) = \frac{I_{VV}(t) - GI_{VH}(t)}{I_{VV}(t) + 2GI_{VH}(t)} \]

✓ Correction by G factor
# Case Study 1

Model protein in solution under stressed condition

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total #AA</th>
<th>Total # Trp</th>
<th>Location of Tryptophan</th>
<th>Average Fluorescent Lifetime, ns</th>
<th>with SDS</th>
<th>2M GuHCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (bovine)</td>
<td>583</td>
<td>2</td>
<td>W-134 α-helix</td>
<td>W-212 α-helix</td>
<td>4.38</td>
<td>2.3</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>152</td>
<td>2</td>
<td>W-7 α-helix</td>
<td>W-14 α-helix</td>
<td>1.06</td>
<td>2.44</td>
</tr>
</tbody>
</table>
Case Study 2

Monoclonal Antibody

Variable number of TRP
Case Study 2

4 Monoclonal Antibodies

Time-resolved fluorescence can distinguish mAbs

✓ Time-resolved fluorescence can distinguish mAbs
Case Study 3

Monoclonal Antibody under stressed condition

- $\langle \tau \rangle = 1.62 \pm 0.05 \text{ ns}$
- $\tau_{\text{heat}} = 1.64 \text{ ns}$
- $\tau_{\text{GuHCl}} = 2.20 \text{ ns}$

✓ Time-resolved fluorescence is sensitive enough to determine degradation of mAb
Conclusions

- Time-resolved fluorescence data provides information about changes in HOS, which is not available from steady-state data
- “Know before you measure”
Acknowledgements

• David Keire, Ph.D., Director DPA
• Connie Ruzicka, Ph.D., Lab Chief
• Stephanie Capsuto