Ultrasensitive and ultrahigh resolution fluorescence spectroscopy and imaging for fundamental biomolecular studies and towards clinical diagnostics

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Experimental Biomolecular Physics, KTH

- 11 researchers
- 1 professor
- 2 senior researchers
- 1 postdoc
- 6 PhD students
- 1 Masters student
Fluorescence as a readout parameter

- **Sensitivity**

  ![Diagram of fluorescence sensitivity](image)

  \[ \Delta E = h \nu \]

  Boltzmann distribution

  \[ \frac{n_1}{n_2} = \exp\left(-\frac{\Delta E}{kT}\right) \]

- **Specificity**

  ![Diagram of specificity](image)
Use of multiplexed fluorescence readouts in bioscience

DNA arrays

Fluorescence microscopy

Protein chips

Flow cytometry
Single-molecule Multi-parameter Fluorescence detection (smMFD)
Why Single-Molecule Spectroscopy?

**Ensemble**
- Static and dynamic heterogeneity

**Individual Molecules**
- Direct measurement of the distributions
- Direct access to molecular dynamics and mechanisms
Förster Resonance Energy transfer:

Transfer of excitation energy from a donor to an acceptor fluorophore

\[ E = \frac{R_0^6}{R_0^6 + R_{DA}^6} \]

\[ R_0 = \left[ 8.79 \times 10^{-5} J(\lambda) \Phi_{FD} n^4 \kappa^2 \right]^{1/6} [\text{Å}] \]

\[ E(\tau_{FD}, \tau_{FA}, r_D, r_A, F_A, F_D) \]
Fluorescence resonance energy transfer (FRET) is described by the following equations:

\[ E = (1 - \gamma \frac{F_G}{F_R})^{-1} \]

\[ E = 1 - \frac{\tau_{DA}}{\tau_D} \]
Conformation-based identification

\[ E = (1 - \gamma \frac{F_G}{F_R})^{-1} \]

\[ E = 1 - \frac{\tau_{DA}}{\tau_D} \]

Mixture: \( \Delta_{bp} : \) 5; 9; 13; 15 bp

SNARE proteins and membrane fusion
Fluorescence Correlation Spectroscopy

\[ G(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I \rangle^2} = 1 + \frac{\langle \delta(t)\delta(t+\tau) \rangle}{\langle I \rangle^2} \]

Diagram showing the experimental setup with a laser beam, dichroic filter, pinhole, beam splitter, avalanche photo diodes, and correlator. The correlation function is plotted against correlation time (ms) with markers for 1/N and \( \tau_D \). Two graphs illustrate the behavior of the correlation function for different values of N (N<1 vs N>>1).
Categories of molecular dynamics monitored by FCS

Δ Diffusion

Δ fluor. Int.

Cross correlation

\[ I(t) \]

Δ = Diffusion

\[ \Delta \text{fluor. Int.} \]

\[ \Delta \text{correlation} \]

\[ G(t) \]

Free

Bound

\[ k_f \]

\[ k_0 \]

\[ r_f = \frac{1}{k_f + k_0} \]
Categories of molecular dynamics monitored by FCS

Δ Diffusion

Δ fluor. Int.

Cross correlation

\[ G(t) = \frac{1}{k_1 + k_2} \]

\[ \Delta \text{Diffusion} \]

\[ \Delta \text{fluor. Int.} \]

\[ \Delta \text{Cross correlation} \]
FCCS applied to molecular immunology and regulation of NK cell activity

Categories of molecular dynamics monitored by FCS

\( \Delta \text{ Diffusion} \)

\( \Delta \text{ fluor. Int.} \)

\( \Delta \text{ Cross correlation} \)
FCS and ion exchange:

\[
F l^{2-} + H^+ \xrightarrow{k_1} HFl^-
\]

FITC in 1 mM carbonate buffers

- pH 7.5
- pH 7
- pH 6.5
- pH 6
- pH 5.5
- pH 5

\[
BH + Fl^{2-} \xrightarrow{k_1} B^- + HFl^-
\]

Bufffer dependence (phosphate buffer)

- phosphate
- citrate
- HEPES
- NaCl (200)

\[
G(\tau)
\]

Time (ms)

- 0.0001
- 0.001
- 0.01
- 0.1
- 1
- 10
- 100
- 1000

\[
0.01
- 0.1
- 1
- 10
- 100
- 1000
\]

Buffer dependence (phosphate buffer)

- 0.01
- 0.05
- 0.1
- 0.5
- 1
- 2
- 2.5
- 3

\[
G(\tau)
\]

Time (ms)

- 0.01
- 0.1
- 1
- 10
- 100
- 1000

Green Fluorescent protein (BioST)

- pH 8.5
- pH 5.8
- pH 5.4
- pH 4.5

\[
F l^{2-} + H^+ \rightleftharpoons HFl^-
\]

G(\tau)

\[
0.01
- 0.1
- 1
- 10
- 100
- 1000
\]

REF: Widengren and Rigler, J. Fluoresc. 7(1), 211-213, 1997

Monitoring of protonation kinetics on membranes:

What is the mechanism by which certain membrane incorporated proteins can take up protons from bulk water at a rate faster than that limited by proton diffusion in the bulk?

Respiratory chain

\[ \text{CH}_2\text{O} \rightarrow \text{CO}_2 \]

\[ \text{NAD}^+ + \text{H}^+ \rightarrow \text{NADH} + \text{H}^+ \]

\[ \text{Succinate} \rightarrow \text{Fumarate} \]

\[ \frac{1}{2}\text{O}_2 + 2\text{H}^+ \rightarrow \text{H}_2\text{O} \]

\[ 4\text{H}^+ \rightarrow 2\text{H}^+ + \text{H}_2\text{O} \]

\[ \text{ADP} + \text{P}_i \rightarrow \text{ATP} \]
pH jump / proton pulse approach:

- Measurements performed at equilibrium conditions, with no perturbation
- Low fluorophore concentrations, no buffer effects
- Protonation in the immediate environment of the fluorophore

FCS:

- Graph showing correlation time (ms) vs. pH (10^{-6}M) with data points at pH values of 8.2, 8.6, 9.1, and 9.6.
- Equation k_{cal} = 4 x 10^{-6} s^{-1} indicated with corresponding data points and line of best fit.
- Additional information on singlet-triplet, protonation, and diffusion processes shown in the graph.
\[ k_{\text{tot}} = 1/\tau_{\text{tot}} = k_+ [H^+] + k_- \]

**Fluorescein**
- \( k_+ = 6 \times 10^{10} \text{ s}^{-1} \text{M}^{-1} \)
- \( k_- = 3 \times 10^4 \text{ s}^{-1} \)

**DOPGFluorescein**
- \( k_+ = 9 \times 10^{12} \text{ s}^{-1} \text{M}^{-1} \)
- \( k_- = 3 \times 10^4 \text{ s}^{-1} \)

Proton Collecting Antenna

Rapid transfer (↔) between surface groups

Increases the cross-section of proton acceptor

M Brändén, T Sandén, P Brzezinski, and J Widengren
Lateral proton transfer between the membrane and a membrane protein:
- Fluorescein-labelled CytcO
- proton transfer increases ∼400 times upon membrane incorporation

Öjemyr L, Sandén T, Widengren J, Brzezinski P
*Biochemistry*, 48, 2173-2179, 2009

Proton exchange over a broader pH range:
- Protonation exchange kinetics display different modes depending on pH

T Sandén, L Salomonsson, P Brzezinski, J Widengren
*Proc Natl. Acad Sci USA*, 107(9), 4129-4134, 2010
Fluorescence cross-correlation spectroscopy of a pH-sensitive ratiometric dye for molecular proton exchange studies

NK-138 (Atto-tec)

(a) Normalized absorption

(b) Normalized intensity

(c) Normalized intensity vs pH

Diagram showing the setup for fluorescence cross-correlation spectroscopy with a pH-sensitive dye.
Auto-correlation

Cross-correlation (with alt. exc)

Cross-correlation (CW)

Buffer dependence (pH 7.7)

Persson G, Sandén T, Sandberg AS, Widengren J
BLINKING - Transient non-fluorescent states

Triplet state formation  Charge transfer  Isomerization

- Widengren, Rigler and Mets J. Fluoresc. 4(3), 255-258, 1994
**Fluorescence output optimisation**

**Photo-oxidation**

\( \mu \text{M conc of nPG:} \)

![Graph showing interactions between Rh6G and nPG in the ms time range](image)

**Diagram:**

- \( F \) (Fluorescence)
- Photooxidation
- Reduction
- \( e^- \)
- \( R^+ \)
- \( nPG^+ \)
- \( nPG \)
\[ k_{\text{ox1}} = 2.1 \times 10^3 \text{ s}^{-1} \Rightarrow \Phi_D = 0.8 \times 10^{-6} \]

\[ K_{\text{ox2}} = 8 \times 10^8 \text{ s}^{-1} \Rightarrow \Phi_{D2} = 1.6 \times 10^{-4} \]

\[ k_{\text{red}} = 2.3 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \]

Widengren J, Chmyrov A, Eggeling C, Löfdahl PÅ, Seidel CAM
Additional dimensions of fluorescence information!

μs-ms

High environmental sensitivity
Limitations of fluctuation and single-molecule spectroscopy:

- low conc.
- high fluorescence brightness
- high time resolution
- high detection quantum yield
Ar-Ion laser

AOM

Sample

NI PCI-6602

Fluorescence Intensity Decay Analysis
Rhodamine 6G with 5 mM KI

Concentration Comparison
10 nM vs 10 μM Rhodamine 6G with 2 mM KI

Transient state imaging by laser scanning microscopy

\[
R(t_{px}) = \frac{\overline{F}(t_{px, min})}{\overline{F}(t_{px})} = \frac{1}{t_{exc}(t_{px, min})} \int_0^{t_{exc}(t_{px, min})} F(t) \, dt
\]

\[
R(t_{px}) \approx \frac{1}{1 - T}
\]

Images of immobilized liposomes
TRAST monitoring
By TIRF microscopy

A

\[
\begin{align*}
S_1 & \xrightarrow{k_{isc}} T_1 \xrightarrow{k_{ox}} R^+ \\
S_0 & \xrightarrow{k_{exc}} k_0 \\
\text{Photobleaching} &
\end{align*}
\]

B

\[
\begin{align*}
S & \xrightarrow{k_{isc}'} T_1 \xrightarrow{\frac{k_{ox}}{k_T}} \dot{R}^+ \\
\text{d} \frac{d}{dt} \left( \begin{array}{c}
S(\bar{r},t) \\
T_1(\bar{r},t) \\
\dot{R}^+(\bar{r},t)
\end{array} \right) & = \left( \begin{array}{ccc}
-k_{isc}'(\bar{r},t) & k_T & k_{red} \\
0 & -(k_{ox}+k_T) & 0 \\
0 & k_{ox} & -k_{red}
\end{array} \right) \left( \begin{array}{c}
S(\bar{r},t) \\
T_1(\bar{r},t) \\
\dot{R}^+(\bar{r},t)
\end{array} \right)
\end{align*}
\]

Oxygen Quenching

O₂ atmosphere

Normal air atmosphere

Argon atmosphere

Triplet Population

Radical Population

High quenching

High population

Less quenching

Lower population

~No quenching

Not extractable
TRAST wide-field microscopy on cells:

Setup.

- Function generator: pulse widths: 100 ns ... 1 ms duty cycle: 1%
- Cobalt laser 491 nm, 75 mW
- AOM
- Focusing Lens
- Objective 40x 0.75 Ph2
- Dichroic
- Tube Lens
- CCD

PO₂ = 10%
PO₂ = 3%
PO₂ = 0.5%

Monitoring of low-frequency molecular encounters:

"Differential quenching" of the triplet state:

Monitoring of molecular transit times through an excitation volume:

Chmyrov, Sandén, Widengren, Anal Chem 2011
Ultra-high resolution and ultra-sensitive fluorescence methods for objective sub-cellular diagnosis of early disease and disease progression in breast and prostate cancer.

12 partners

3,5 years

Coordinator: Jerker Widengren, KTH Stockholm, Sweden
Stimulated emission depletion (STED)

"An increase in the resolution in fluorescence microscopy can be obtained by restriction of the volume from which fluorescence is detected"


SW Hell, Nat. Biotechol 21(11), 1347-1355, 2003
SW Hell, 316, 1153-1158, Science 2007
STED microscopy @ KTH:

Collab: SW Hell et al, MPIBPC, Göttingen
Fibroblasts: Phosphotyrosine-green, Actin-red

Confocal

STED+

Scale bars 2 µm

Collab: Annica Gad & Pontus Aspenström, KI
FNA HER1-green, IGF1R-red

Collab: FLUODIAMON project (http://www.biomolphysics.kth.se/fluodiamon/)
Fluorescent imaging of thrombocytes

Confocal | STED | STED+
---|---|---
![Confocal Image](image1)
![STED Image](image2)
![STED+ Image](image3)

Green: VEGF
Red: Actin

Scale bar 1 µm

Collab: Gert Auer, KI
Collaborations:
EU FP7 FLUODIAMON project
(http://www.biomolphysics.kth.se/fluodiamon)
- Claus AM Seidel, HHU Düsseldorf
- Peter Brzezinski, Stockholm Univ
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