Quantitative \textit{in vivo} imaging of molecular distances using FLIM-FRET

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EMBO Practical Course about Quantitative FRET, FRAP and FCS
Heidelberg, 25\textsuperscript{th} September 2009
PicoQuant GmbH

- Founded in 1996
- 43 employees + students
- Key background in Electrical Engineering, Lasers, Physics and Chemistry with high qualified staff
- Situated in the Technology Park Berlin – Adlershof
- PicoQuant Photonics North America Inc. was established in April 2008
- Dedicated to optoelectronic research & development
FLIM
- Time-domain analog to multicolor image
- New parameters independent of system settings and fluorophore concentration

Multi-Staining
- Imaging of multiple dyes with similar emission but different lifetimes
- Discrimination of autofluorescence

Local environment sensing
- Viscosity
- Lipophilic/Hydrophilic environment
- pH sensing
- Oxygen, water or ion concentration

FRET
- Distance measurements (nm range)
- Intra- and intermolecular interactions
- In fixed as well as in living cells and organisms
- Time lapse analysis

Quenching and Anisotropy
- Accessibility and conformational studies (protein folding)
- Molecular Rotation

FLCS / FLCCS
- Correction for background, detector artifacts and spectral bleed trough
Fluorescence Photocycle

Energy

A = Photon absorption
F = Fluorescence (emission)
P = Phosphorescence
S = Singlet state
T = Triplet state
IC = Internal conversion
ISC = Intersystem crossing

Excited state

Fluorophore

Electronic ground state

Sample

Light source

Detector

Fluorescence Lifetime = average time that a molecule remains in the excited state prior to returning to the ground state by emitting a photon

How fast is the photocycle?
→ typ. ps [$10^{-12}$ s] to ns [$10^{-9}$ s]

Observables
- Fluorescence intensity
- Color or wavelength
- Polarization
- Fluorescence lifetime

Electronic ground state

Fluorophore

Excited state

Light source

Ground state

Detector
How to Measure the Fluorescence Lifetime?

One needs:
- a defined “start” of the experiment → pulsed excitation; each laser pulse is a new “start”
- a defined “stop” of the experiment → single photon sensitive detector; photon arrival at the detector is the “stop”
- a fast “stopwatch” to measure the time difference between “start” and “stop”
Time-Correlated Single Photon Counting (TCSPC) to Measure the Fluorescence Lifetime

In principle with a stop watch:
1. Start the clock with a laser pulse
2. Stop the clock with the first photon that arrives at the detector
3. Reset the clock and wait for next start signal
A statistical process!

- Repeat this time measurement very often and count “how many photons have arrived after what time”
- Sort the photons within a histogram into time bins according to their arrival times

Fluorescence lifetime histogram:
Fit a exponential decay to get the fluorescence lifetime
Time-Tagged Time-Resolved (TTTR) Single Photon Detection

PicoQuant data acquisition mode:

The photon records \((t, T, CH)\) are collected continuously. The data stream is recorded to disk. It can be processed immediately for display and analysis. ALL temporal information is preserved!
TTTR File: four pieces of information

- **TCSPC time:**
  - Start-stop photon time
  - Time difference between the excitation and the arrival of the first photon at the detector
  - Measured by a “stop watch” (picosecond resolution)

- **Time tag:**
  - Represents the global arrival time of each photon relative to the beginning of the experiment
  - Measured with nanosecond resolution

- **Marker signal:**
  - External synchronization signal from the LSM scanner given at the beginning and the end of each line and start of each frame with the corresponding global time tag
  - Spatial information of each photon to rebuild the FLIM image

- **Channel information:**
  - In case of a multi-channel detector setup
  - Add a channel identifier to each measured TCSPC time to get the information, on which detector the photon was detected
### Time-Tagged Time-Resolved (TTTR) - Data Display and Analysis Possibilities

<table>
<thead>
<tr>
<th>marker event</th>
<th>photon event</th>
<th>TCSPC time</th>
<th>time tag (global arrival time)</th>
<th>detection channel indicator</th>
<th>marker indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>T CH</td>
<td>t</td>
<td>T</td>
<td>CH</td>
<td>M</td>
</tr>
</tbody>
</table>

#### Point

- **fluorescence lifetime**, time-gated analysis, PIE, coincidence correlation, antibunching
- Temporal intensity fluctuations (blinking, bursts), FCS
- Spectral splitting (FRET), cross correlation
- Temporal lifetime fluctuations (lifetime trace), FLCS, PIE-FRET, lifetime FRET

#### Image

- Intensity imaging
- ... + spectral splitting (FRET)
- Time-gated imaging, PIE-FRET, fluorescence lifetime imaging (FLIM), FLIM-FRET
Advantages of time-domain versus frequency-domain FLIM

- Upgrade of confocal LSM
- Very intuitive approach
- Higher sensitivity: counting single photons is much better suited for biological samples with often relative low fluorescence intensities due to e.g. moderate expression levels that are comparable to endogenous concentrations
- Better timing resolution
- Higher accuracy of multi-exponential decay analysis that is essential for FLIM analysis in the heterogeneous cellular environment
- Possibility of single molecule studies (e.g. FCS)
FLIM & FCS Upgrade Kit for Laser Scanning Microscopes

You have the cup, we have the coffee.

Features:
- One or two detectors (SPAD or PMT)
- Multiple excitation options
- Online FLIM and online FCS
FLIM & FCS Upgrade Kit for Laser Scanning Microscopes: Components

**Single photon counting detector unit:**
- 2 Single Photon Avalanche Detectors (SPAD)

**Router**
- "Stop watch" Time-Correlated Single Photon Counting (TCSPC) unit

**Synchronization** (Line and Frame clock)

**Fiber Coupling Unit (FCU II) with pulsed diode laser heads of LDH series**

**Pulsed laser system**

**Excitation**

**Emission**

**LSM**
Single Molecule Sensitivity in a Complete System: MicroTime 200

- Excitation subsystem
- Objective scanning and DIC prism for two focus FCS
- Confocal excitation and detection optics
- Computer controlled laser driver
- Advanced system and analysis software
- Time-correlated single photon counting unit
Fast Fluorescence Lifetime Imaging (Fast FLIM)

- Online display of the image during data acquisition
- Fast FLIM displays the average photon arrival time
- Facilitates data acquisition and pre-selection photon by photon

Daisy pollen, measured with MicroTime 200 confocal microscope

Intensity

laser pulse

photon

0.5 ns

1.5 ns

Average TCSPC time: 1 ns

Fast FLIM

23 µm

Intensity

laser pulse

photon

0.5 ns

1.5 ns

Average TCSPC time: 1 ns

Fast FLIM
Lifetime Histogram: Tail Fit

- Display of the photon arrival times in a histogram
- Tail fit for lifetime analysis

**Fast FLIM**

- Fast
- Good lifetime contrast
- Less lifetime - noise

**Tail Fit**

- More accurate results for
  - very short lifetimes
  - complex dye mixtures

Daisy pollen, measured with MicroTime 200 confocal microscope
More than one fluorophore with different lifetimes present in sample
Tail fit with multi-exponential decay

FLIM

Lifetime histogram: bi-exponential decay

Short component: 0.8 ns
Long component: 2.4 ns

Daisy pollen, measured with MicroTime 200 confocal microscope
Environmental sensing by FLIM

Living hepatocyte (liver cell) containing a canalicular vacuole, stained with NBD (7-nitrobenz-2-oxa-1,3-diazole).

The FLIM image visualizes the different hydrophobicities and their local variations within the cell.

→ Canalicular vacuole is very likely of bilayer type at the rim (membrane) and of micellar type in the center.

Fluorescence intensity

Fluorescence lifetime

Lifetime distribution

$\lambda_{\text{exc}} = 467 \text{ nm}$

100x, 1.3 N.A. oil immersion filter: LP500

300 × 300 pixels

acquisition time: 3 min.

Sample courtesy of Astrid Tannert, Thomas Korte, Humboldt University Berlin
Förster Resonance Energy Transfer (FRET)

- Detection of protein interaction
- Both proteins labeled with donor and acceptor fluorophores, e.g. CFP and YFP
- (Donor) Fluorescence Lifetime measurement

\[
E = 1 - \frac{\text{Short lifetime}}{\text{Long lifetime}}
\]
Protein Interactions of CENP-A and CENP-B via FLIM-FRET

Human centromere kinetochore complex
- ensures correct chromosome segregation during cell division
- located at the primary constriction of each chromosome
- ~50 kinetochore proteins (CENPs) and underlying DNA (centromere)

Determination of neighbourhood relations of kinetochore proteins by FLIM-FRET in vivo
- Example: CENP-A and CENP-B
- Fluorophores: Cerulean / EYFP
  - Well suited for FRET studies
  - Donor excitation: 405 nm or 440 nm

Sample courtesy of Sandra Orthaus, former member of Leibniz Institute for Age Research, Fritz Lipmann Institute (FLI), Jena
Dye spectra taken from: http://www.tsienlab.ucsd.edu/Documents.htm
Protein Interactions of CENP-A and CENP-B via FLIM-FRET

U2OS cell transfected with CENP-B-Cerulean (donor)

5 µm

similar fluorescence lifetimes in all centromeres

\[ \tau_{av} \sim 2.94 \text{ ns} \]

CENP-A and CENP-B are in direct vicinity at human centromers

U2OS cell transfected with CENP-B-Cerulean (donor) & YFP-CENP-A (acceptor)

3.5 ns

1.8 ns

every centromere shows a specific fluorescence lifetime

\[ \tau_{av} \text{ between } \sim 1.8 \text{ ns and } 2.2 \text{ ns} \]

Sample courtesy of Sandra Orthaus, former member of Leibniz Institute for Age Research, Fritz Lipmann Institute (FLI), Jena

Excitation: 440 nm, 20 MHz

Emission: 480 / 40 bandpass-filter

objective: UPLSAPO 60x O NA1.35

LSM Upgrade Kit
Protein Interactions of CENP-A and CENP-B via FLIM-FRET: Dual Channel detection

Cell 1:
contains only the donor CENP-B-Cerulean
Donor and Acceptor channel:
\( \tau_{av} \approx 3 \text{ ns} \)
(= CENP-B-Cerulean)

Cell 2:
transfected with both YFP-CENP-A and CENP-B-Cerulean
Donor channel:
\( \tau_{av} \approx 1.2 \text{ ns} \)
\( E_{FRET} = 60\% \)
Acceptor channel:
\( \tau = 2.8 \text{ ns} \)
+ rise time of
\( \tau = 0.5 \text{ ns} \)
(all fits including IRF)

Sample courtesy of Sandra Orthaus, former member of Leibniz Institute for Age Research, Fritz Lipmann Institute (FLI), Jena
Protein Interactions of CENP-A and CENP-B via FLIM-FRET

Cell 2:
transfected with both YFP-CENP-A and CENP-B-Cerulean

Donor channel:
\[ \tau_{av} = \sim 1.2 \text{ ns} \]
\[ E_{\text{FRET}} = 60\% \]

Acceptor channel:
\[ \tau = \sim 2.8 \text{ ns} \]
+ rise time of \[ \tau = \sim 0.5 \text{ ns} \]

Sample courtesy of Sandra Orthaus, Fritz Lipmann Institute (FLI), Jena
FLIM-FRET measurements: 2-photon excitation and acceptor photo-bleaching

EGFP-RFP fusion construct expressed in living cells

= POSITIVE CONTROL

<table>
<thead>
<tr>
<th></th>
<th>FRET (D+A)</th>
<th>Bleach (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lifetime 1</td>
<td>2.4 ns</td>
<td>2.4 ns</td>
</tr>
<tr>
<td>Lifetime 1</td>
<td>1.2 ns</td>
<td>1.2 ns</td>
</tr>
<tr>
<td>Amp. 1</td>
<td>51%</td>
<td>85%</td>
</tr>
<tr>
<td>Amp. 2</td>
<td>49%</td>
<td>15%</td>
</tr>
</tbody>
</table>

Leica SP5
Two photon excitation: $\lambda_{\text{exc}} = 850$ nm, 80 MHz
filter: BP (500-540) nm

Lifetime of EGFP alone: 2.4 ns

Sample courtesy of Dirk Daelemans, Thomas Vercruysse, Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium
FLIM-FRET Analysis with Scripting

\[ E_{\text{FRET}} = 1 - \frac{\tau_D(A)}{\tau_D} \]

Sample courtesy of Dirk Daelemans, Thomas Vercruysse, Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium
FLIM-FRET Can Resolve Subpopulations

\[ E = 1 - \frac{\text{Short lifetime}}{\text{Long lifetime}} \]

\[ \tau_{\text{average}} = \frac{\sum_i A_i \tau_i}{\sum_i A_i} \]
Interactions of fluorescent proteins in inside living cells (12V HC Red cells) labeled with EGFP and RFP attached to each other

→ After acceptor bleaching the quenching of the donor is strongly reduced

**Fluorescence lifetime before bleach**

Mean lifetime: 2.2 ns

**Fluorescence lifetime after bleach**

Mean lifetime: 2.9 ns

**Lifetime distribution**

<table>
<thead>
<tr>
<th>Frequency [10^5 counts]</th>
<th>Lifetime [ns]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before bleach</td>
<td>0.5 1.5 2.5 3.5</td>
</tr>
<tr>
<td>After bleach</td>
<td>0.5 1.5 2.5 3.5</td>
</tr>
</tbody>
</table>

Sample courtesy of Philippe Bastiaens, Max Planck Institute for Molecular Physiology, Dortmund, Germany
FLIM-FRET - Separating Quenched from Unquenched Donor Species

\[ E_{\text{FRET}} = 1 - \frac{\tau_{D(A)}}{\tau_D} \]

**Sample courtesy of Philippe Bastiaens, Max Planck Institute for Molecular Physiology, Dortmund, Germany**
Dual colour Pulsed Interleaved Excitation (PIE) to identify FRET artifacts
(effectively only possible at the single molecule level)

Time-gating:
selection of excitation

Spectral separation

Channel 1
Blue
0 50 ns 100 ns

Channel 2
Red
0 50 ns 100 ns

FRET sample
D A

470 nm @ 10 MHz
635 nm @ 10 MHz
Time-Gated Analysis: PIE-FRET

Intact pair → FRET

Intact pair → no FRET

Non fluorescing acceptor
PIE-FRET in RNA Folding Studies

- Folding and unfolding monitored by FRET
- Mg\(^{2+}\) driven
- Important RNA folding motif
- Excitation: 532nm

*in collaboration with J. Fiore and David Nesbitt (JILA, Univ. of Colorado, Boulder)*
PIE-FRET: Analysis of Sub-Populations

Photon Stoichiometry

$$S_{\text{Eff}} = \frac{n_{A-Fret} + n_D}{n_{A-Fret} + n_D + n_{A-Direct}}$$

FRET Efficiency

$$E_{\text{FRET}} = \frac{n_{A-Fret}}{n_{A-Fret} + n_D}$$

Sample courtesy of Julie Fiore and David Nesbitt, University of Colorado, Boulder
Summary

Fluorescence Lifetime Imaging

Förster Resonance Energy Transfer

Fluorescence Correlation Spectroscopy

LSM Upgrade kit / MicroTime 200 enable for...

… and much more …
Acknowledgement

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Astrid Tannert and Thomas Korte
Humboldt University Berlin, Germany

Philippe Bastiaens
Max Planck Institute for Molecular Physiology, Dortmund, Germany

Dirk Daelemans and Thomas Vercruysse
Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium

Julie Fiore and David Nesbitt
University of Colorado, Boulder, USA
PicoQuant Events

2\textsuperscript{nd} European Short Course on “Time-Resolved Microscopy and Correlation Spectroscopy”

16 – 18 February 2010, Berlin-Adlershof, Germany

- Topics: Introduction to Microscopy, Hardware for Time-Resolved Microscopy, FCS, FLIM, FRET, Steady-State Microscopy Techniques
- Course instructors: Jörg Enderlein, Paul French, Johan Hofkens, Fred Wouters
- Hands-On experimentation and lab demonstration by: Leica, Nikon, Olympus and PicoQuant
- www.picoquant.com/_mic-course.htm

7\textsuperscript{th} European Short Course on "Principles & Applications of Time-Resolved Fluorescence Spectroscopy"

9 – 12 November 2009, Berlin-Adlershof, Germany

Topics: Steady state and time-resolved fluorescence spectroscopy and instrumentation, time- and frequency domain measurements, anisotropy, solvent effects, quenching and Förster energy transfer, data analysis, ...
Course instructors: Joseph R. Lakowicz, Karol Gryczynski, Rainer Erdmann, Matthias Patting, Michael Wahl
Hands-On experimentation and lab demonstration by market leading companies
www.picoquant.com/_trfcourse.htm
Thank you for your attention!

Always targeting our customers needs ...
Interactions of protein partners in their natural environment inside living cells can be studied with time-resolved FRET microscopy

→ **Characterization of intra-nuclear dimer formation** for the transcription factor C/EBP α in living pituitary GHFT1-5 cells of mice

Members of the C/EBP family of transcription factors are critical determinants of cell differentiation

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**Förster Resonance Energy Transfer (FRET)**

**Lifetime distribution**

Olympus FV1000

$\lambda_{\text{exc}} = 440 \text{ nm}$, 40 MHz

Apo 60x, 1.4 N.A. oil immersion filter: LP460

512 × 512 pixels

Lifetime of CFP alone: 2.7 ns

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Sample courtesy of Ammasi Periasamy, University of Virginia, USA
Why Fluorescence Lifetime Imaging (FLIM)?

Fluorescence Lifetime Imaging (FLIM) gives you new parameters
- Independent of system settings, fluorophore concentration
- Discrimination between fluorophores with similar excitation spectra (e.g. EGFP and EYFP) and from autofluorescence
- Measurements of environmental parameters
  - hydrophobicity
  - pH value
  - Oxygen, water or ion - concentrations

Förster Resonance Energy Transfer (FRET)
- Distance measurements in the nanometer range
- Can be measured down to the single molecule level
  - Intra- and intermolecular interaction studies
  - Protein folding
  - Moving of molecular motors

Fluorescence Correlation Spectroscopy (FCS)
- Mobility, dynamics and concentration
  - Fluorescence Lifetime Correlation Spectroscopy (FLCS)
  - Time-gated FCS
Advanced Excitation Schemes

Pulsed Interleaved Excitation (PIE)

- coding spectral information in time

\[ 50 \text{ ns} \]

- coding spatial information in time

Laser heads with pulsed and cw Excitation

- Antibunching
- Total correlation from ps to seconds
EGFP-RFP fusion construct expressed in living cells (12V HC Red cells)

Fluorescence lifetime image (FLIM)

Sample courtesy of Philippe Bastiaens, Max Planck Institute for Molecular Physiology, Dortmund, Germany
Conclusion

LSM Upgrade kit / MicroTime 200 enable for:

- Time-Correlated Single Photon Counting with up to two/four detectors (PMT or SPAD) and five laser wavelengths simultaneously
- Spatial, spectral and timing information for every photon ➔ Universal data pre-selection photon by photon

- Fluorescence Lifetime Imaging (FLIM) with online visualization for increased information:
  ➔ Distance measurements, molecular interactions (FRET)
  ➔ Environmental parameters

- Fluorescence Correlation Spectroscopy (FCS) with online visualization for measurements of:
  ➔ Diffusion coefficients
  ➔ Concentration of molecules
  ➔ FLCS measurements
    - more realistic concentrations at high dilutions
    - afterpulsing removal