Sub-exposure time resolution in wide-field time-correlated single photon counting (TCSPC) imaging

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July 3, 2014
Outline

- Background – fluorescence lifetime imaging (FLIM) microscopy
- Wide-field time-correlated single photon counting (TCSPC) with ultrafast cameras
- Photon arrival time from phosphor decay

**Poster 12:** Electron-bombarded CCD (EBCCD) as a parallel-processing photoelectronic time-to-amplitude converter (TAC)
Fluorescence microscopy

- Rejection of reflected light $\rightarrow$ imaging inside cells and tissues
- Tagging of specific components of cell, sub-cellular resolution, single molecule sensitivity
- Visible wavelength $\rightarrow$ less damage, live cell dynamics & function

Fluorescence is multi-dimensional
- Intensity
- Position
- Wavelength
- Polarisation
- Lifetime

Nucleus – actin – mitochondria

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Fluorescence lifetime $\tau$
= average time in excited state

Independent of excitation intensity and fluorophore concentration

Mapping of local environment:
- Ion concentration (Na, Cl, Ca...)
- Oxygen concentration
- Acidity (pH)
- Refractive index
- Viscosity ...

Applications: Cell biology, forensic science, art conservation, remote sensing, temperature sensing...
Scanning TCSPC
- Image build one pixel at a time
- Max one photon per pulse
- High rep-rate lasers
- Nano/picosecond time resolution

Wide-field gated detection

Wide-field TCSPC with ultrafast camera
FLIM implementations (time-domain)

- Scanning TCSPC
- Wide-field gated detection
  - Fast (all pixels parallel)
  - Loss of photons
  - No single photon sensitivity
  - Photobleaching and intensity fluctuations affect measurement
- Wide-field TCSPC with ultrafast camera
Scanning TCSPC
Wide-field gated detection
Wide-field TCSPC with ultrafast camera
  - All pixels parallel
  - No loss of photons
  - Unlimited dynamic range
  - Insensitive to photobleaching and intensity fluctuations
  - Time resolution determined by frame rate (microseconds)
Long lifetime probes

Transition metals

- Ru, Ir, Pt, Pd, Rh, Re, ...
- $\mu$s lifetimes
- Long Stokes shift, bright
- Chemically stable, water soluble
- Visible excitation wavelength

- Lanthanide compounds (Eu, Tb, ...): ms lifetimes
- Nanodiamonds, Quantum dots, ...

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Wide-field TCSPC lifetime microscopy
Microscope setup

- Nikon TE inverted microscope
- Light sources:
  - Picosecond pulsed diode laser
  - Pulsed LED
  - Mercury lamp with chopper
- Camera: Photron SA5 / SA1.1
  - 1 MHz frame rate: 16x64 pixels
  - 54 kHz frame rate: 320x264 pixels
- Camera and light source synchronised
Single photon sensitivity
Photon creates a photoelectron, multiplication of electrons, conversion back to photons
Our intensifier: 3 MCPs, P20 phosphor: long decay ($\mu$s), green
Data processing

\[ I = \exp(-t/\tau) \]

Frame 1  Frame 10  Frame 20

Sum

Raw data

Threshold & centroiding

Histogram & analysis
Euro note

- Star in €20 note
- Lifetimes (biexponential):
  - $568 \pm 4 \, \mu s$
  - $43 \pm 1 \, \mu s$

**Acquisition:** 10000 frames/s, 50 frames/pulse, $\sim 135000$ frames, $\sim 60$ sec;
Exc: UV LED (365 nm) @ 50 Hz; Em: 515nm LP filter; 4× air objective
Europium in living HeLa cells

- 40 nm Europium beads in living HeLa cells.
- 320x256 pixels, 20 kHz frame rate, 92.3 photons/pulse.
- Data collection time: 3.5 seconds.
- Lifetime $\sim 570 \mu$s.

![Graph showing lifetime data](image)

Eu intensity  Eu (red) + transmission (grey)  Lifetime image
Ru(dpp) in water and glycerol

Lifetimes (monoexponential):
- Water: 1.35 µs
- Glycerol: 5.05 µs

Different lifetimes due to different oxygen diffusion rates

**Acquisition:** 1,000,000 frames/s, 50 frames/pulse, ~800,000 frames, <1 s;
Exc: Diode laser (467 nm) @ 100 kHz; Em: 515nm LP filter; 10× air objective
Summary (1st part)

- Image intensifier + ultrafast camera allows wide-field TCSPC imaging.
- Collection of up to 100’s of photons / pulse.
- Time-resolution limited by camera frame rate to $\sim \mu s$.

- How to improve time resolution?
Sub-exposure time resolution from phosphor decay

- Image intensifier phosphor screen has an afterglow.
- Decay time depends on the type of phosphor (ns to ms).
- P20: multi-exp, $\sim 250\mu$s
- Intensity ratio of first and second frame yields photon arrival time.

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Intensity ratio to time conversion

- Convert intensity ratio frame2/frame1 to photon arrival time.
- Measured by reflection and varying the time delay between the frame start time and the laser trigger pulse.
- 54 kHz frame rate (18.5 µs frame exposure time).
Ruthenium in water and glycerol mixtures

- 4 solutions: 100%, 50%, 20% and 0% glycerol mixed with water
- 54 kHz frame rate, 2.6 s
- Decay for each pixel of image
- Ru(dpp) lifetime contrast due to oxygen diffusion

![Intensity image](image1.png)
![Lifetime image](image2.png)
![Lifetime histogram](image3.png)

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Wide-field TCSPC lifetime microscopy
Ruthenium in living HeLa cells

- Ru(dpp) in living HeLa cells
- 54 kHz frame rate, 1.3 s
- Average lifetime from 2-exp fit: 2.7 µs (+ fast component 0.1 µs)
  → Partial protection from quenching by molecular oxygen

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Wide-field TCSPC lifetime microscopy
Iridium beads

- Beads mix: Ir(ppy)$_3$, Ir(BMes) phos, Ir(fppy)$_3$ phos, Pd(OEP), green fluorescence
- 54 kHz frame rate (18.5 $\mu$s frame exposure time)

Colour photo | Intensity image | Lifetime image
--- | --- | ---
200 $\mu$m

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Wide-field TCSPC lifetime microscopy
Image intensifier + ultrafast camera allows wide-field photon counting imaging.

Time-resolution limited by camera frame rate to microseconds.

Sub-exposure time resolution from image intensifier phosphor decay.

Demonstrated with 18.5 $\mu$s exp time and sub-$\mu$s lifetimes (P20 phosphor).

Faster phosphor and frame rate for nanosecond time resolution?
Acknowledgements

- **Klaus Suhling**
- Nicolas Sergent
- Zdeněk Petrášek (Max Planck Institute of Biochemistry, Germany)
- Andrew Beeby (Durham University) – Ir beads
- Medical Research Council
- EPSRC loan pool (Adrian Walker) – camera loan