SPAD arrays: from single-molecule detection to wide-field phasor fluorescence lifetime imaging

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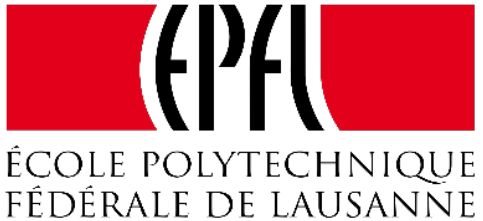


A brief history of past work

- 1996-2008: SPCM-AQR 14 (EG&G, Perkin-Elmer, Excelitas)

- 2008: single-pixel HPD: BiOS, *SPIE Proc.* 6862 (2008) 68620F
- 2010: 8x1 single-color FCS & burst detection: BiOS, *SPIE Proc.* **7571** (2010) 75710G
  OptoE, *SPIE Proc.* **7608** (2010) 76082D
  Biomed. Opt. Expr. **1** (2010) 1408
- 2011: 8x8 (of 32x32 SPADA) single-color FCS: BiOS, *SPIE Proc.* **7905** (2011) 790503
  8x1 single-color FCS DSS, *SPIE Proc.* **8033** (2011) 803316
- 2012: 8x1 smFRET (4 spot): BiOS, *SPIE Proc.* **8228** (2012) 82280B
- 2013: 8x1 smFRET (8 spot): BiOS, *SPIE Proc.* **8590** (2013) 85900E
  1 RE-SPAD (1 spot): BiOS, *SPIE Proc.* **8590** (2013) 85900D
- 2014: SwissSPAD: Photonics Europe, *SPIE Proc.* **9141** (2014) 914109
  Opt. Expr. **22** (2014) 17573 + IISW 2013
- 2015: 4x12 single-color setup: DSS, *SPIE Proc.* **9492** (2015) 949204 (unpublished)
- 2016: sm file format: BiOS, *SPIE Proc.* **9714** (2016) 971405
- 2017: 16x1 single-color TCSPC: BiOS, *SPIE Proc.* **10071** (2017) 100710Q
  more 8x1 smFRET + kinetics: *PLoS ONE* **12** (2017) e0175766
  SwissSPAD 2: IISW 2017
- 2018: 4x12 smFRET (48 spots): *J. Chem. Phys.* **148** (2018) 123304 (+BiOS, unpublished)
  4x12 CCF/crosstalk (48 spots): *NIMA*, DOI: 10.1016/j.nima.2017.11.070
  smFRET review + perspectives: *Science* **359** (2018) eaan1133
  SwissSPAD 2: BiOS, unpublished

Overview

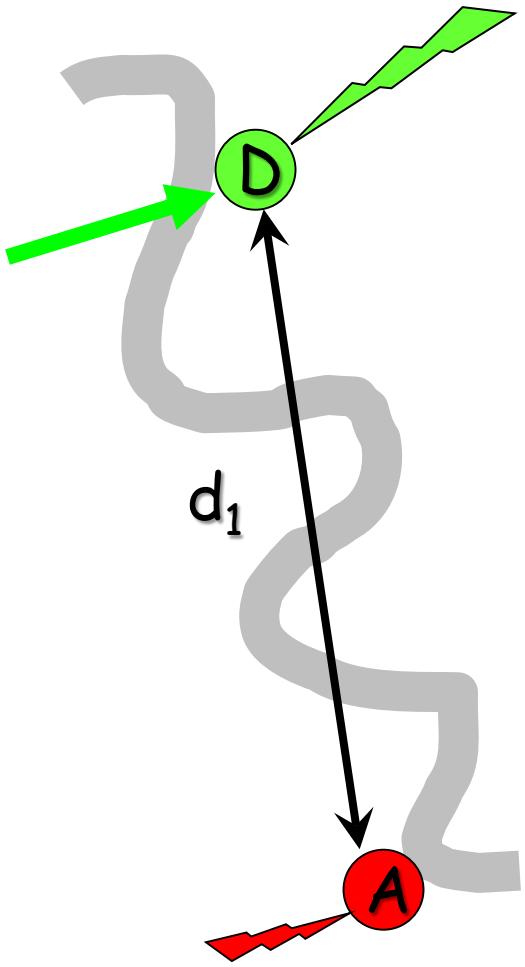
1. Analysis of freely diffusing single molecules in solution
    1. Introduction
    2. Ideal detector requirements
    3. Current status
    4. Perspectives

2. Wide field imaging of single-molecules
    1. Introduction
    2. Ideal detector requirements
    3. Current Status
    4. Perspectives

3. *in vivo* NIR fluorescence lifetime imaging
    1. Introduction
    2. Ideal detector requirements
    3. Current Status
    4. Perspectives

1. Analysis of freely diffusing single molecules in solution

Förster Resonant Energy Transfer (FRET)



Unfolded molecule
(State 1)

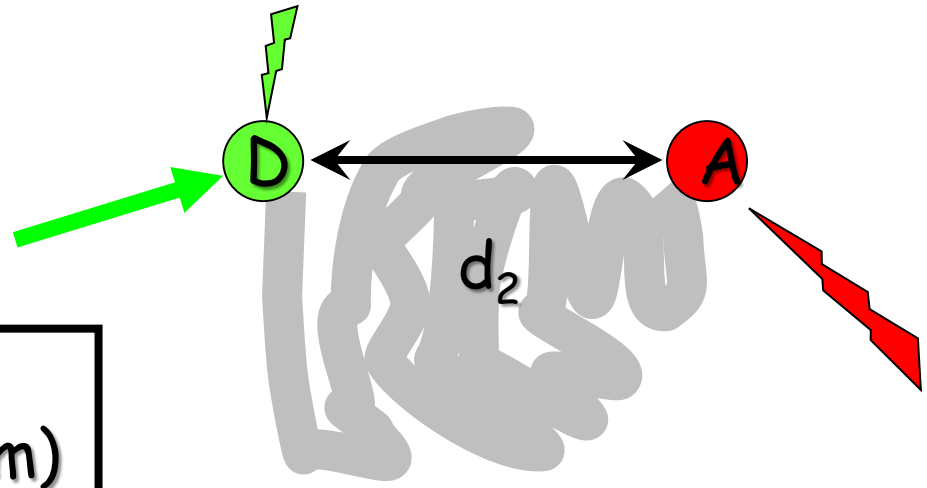
$r = d/R_0$

R_0: Förster Radius (5-8 nm)

$E = I_A/(I_A + I_D) = 1/(1 + r^6)$

$= 1 - \tau_{DA}/\tau_D$

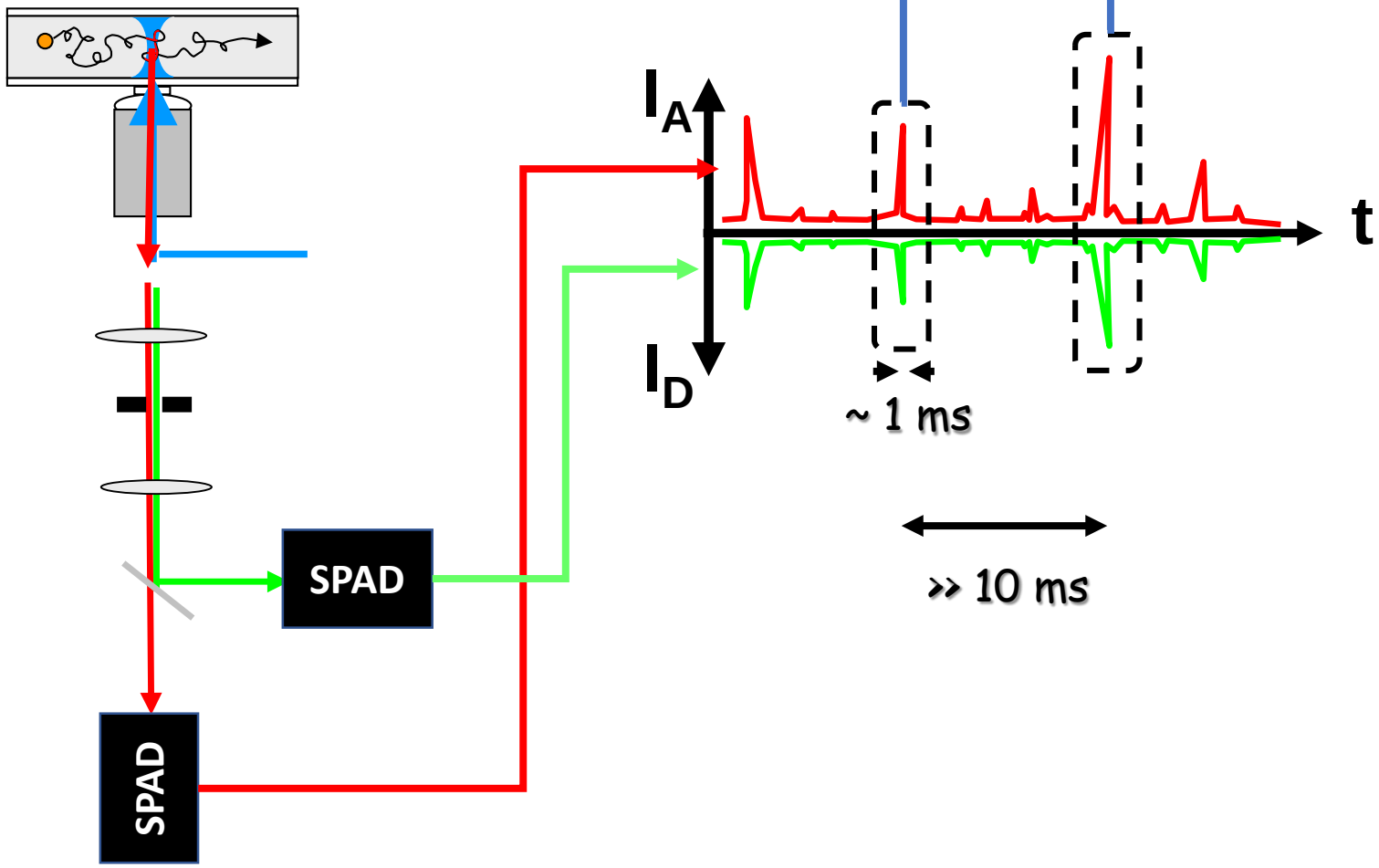
E: Transfer efficiency

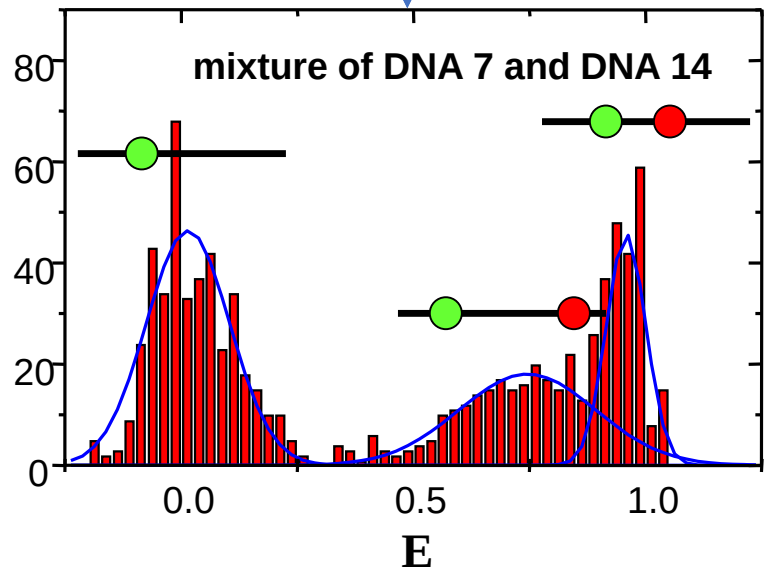


Folded molecule
(State 2)

Single-molecule FRET Measurements: Principles

$$E \sim \frac{I_A}{I_D + I_A}$$



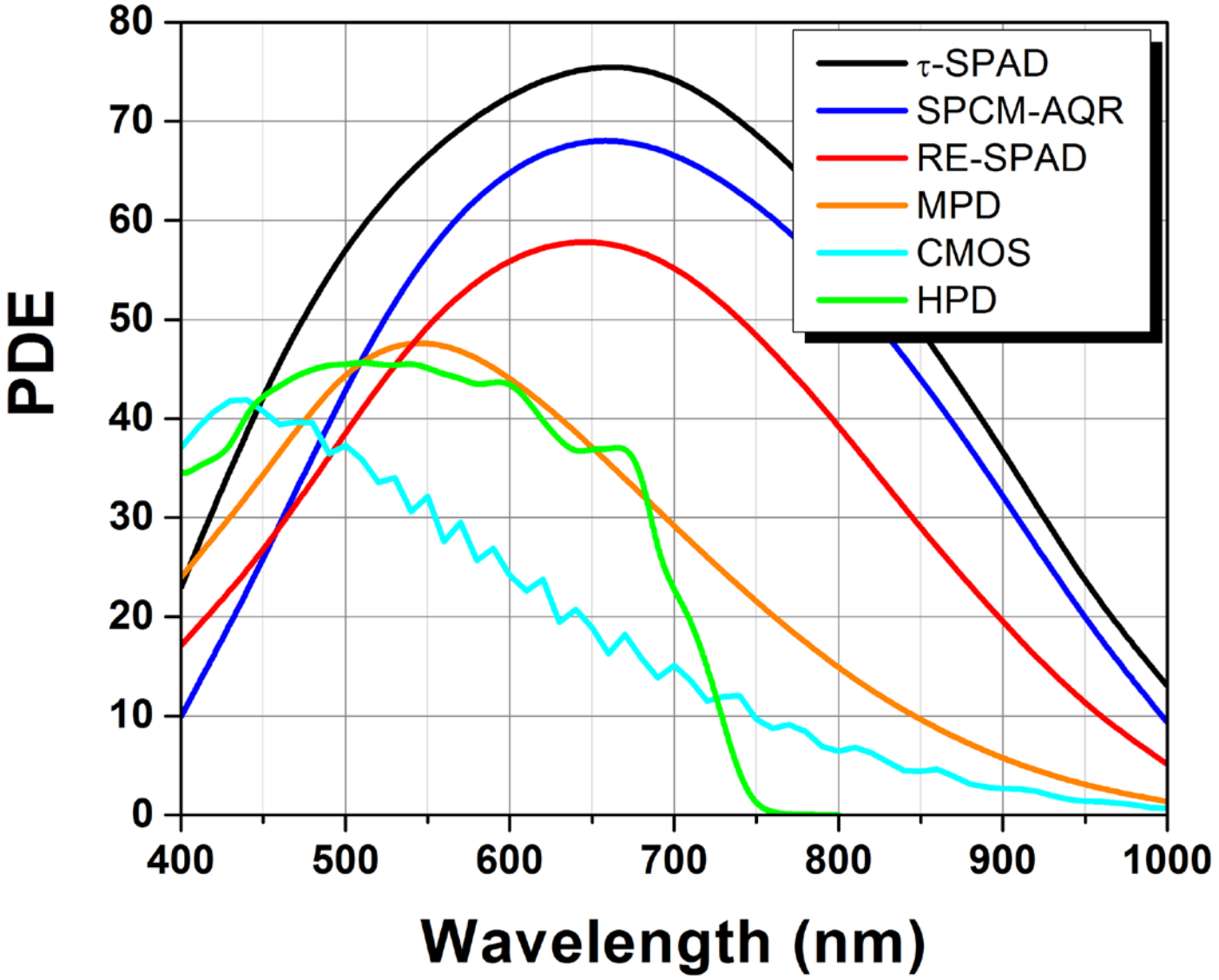


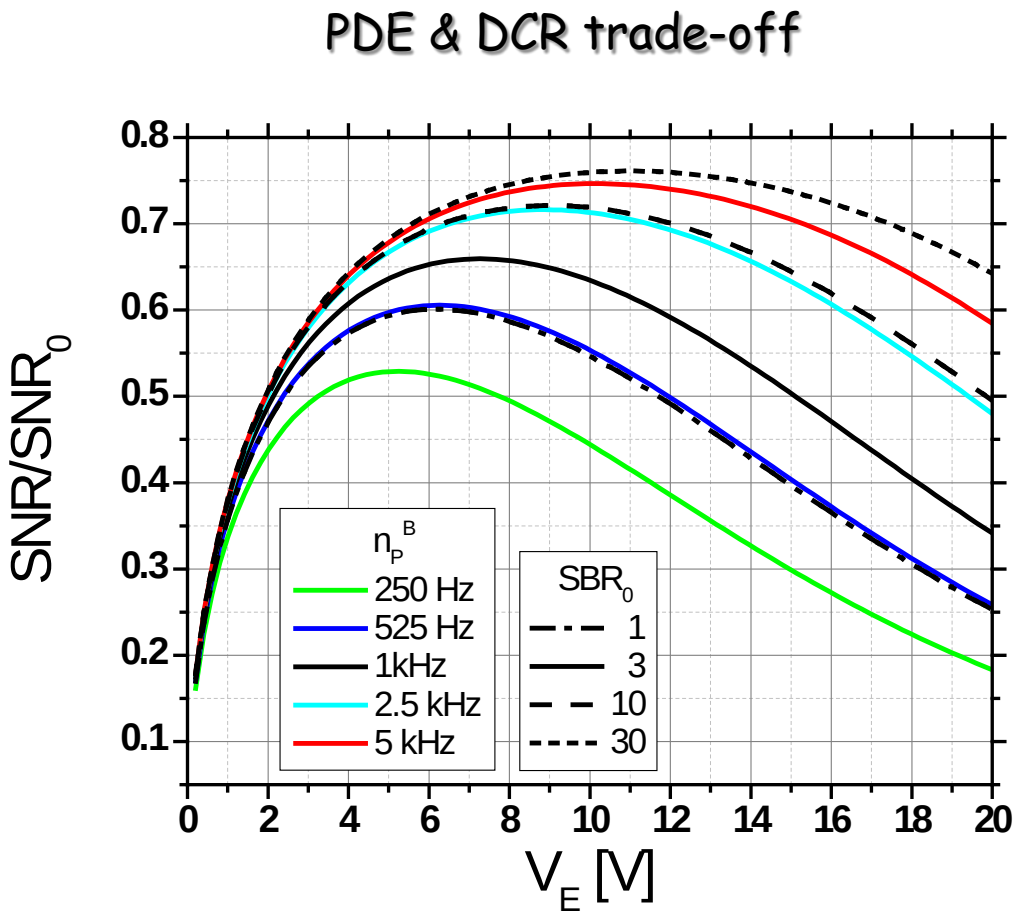
PNAS **96** (1999) 3670

Detector Requirements

- Capture brief bursts of photons (0.1-10 ms, 10-500 ph)
  - Count rate < few MHz
  - Dead-time < 100 ns
- Time-correlated single-photon counting (TCSPC) capabilities
  - ~ 100 ps resolution (basic single burst analysis)
  - Laser repetition rate: 20-80 MHz
- Sensitivity:
  - Range: 500 nm-750 nm (Lasers: 488, 514, 532, 633, 690)
  - DCR < 1 kHz
- Afterpulsing, crosstalk:
  - As low as possible to allow for simple ACF/CCF analysis

PDE



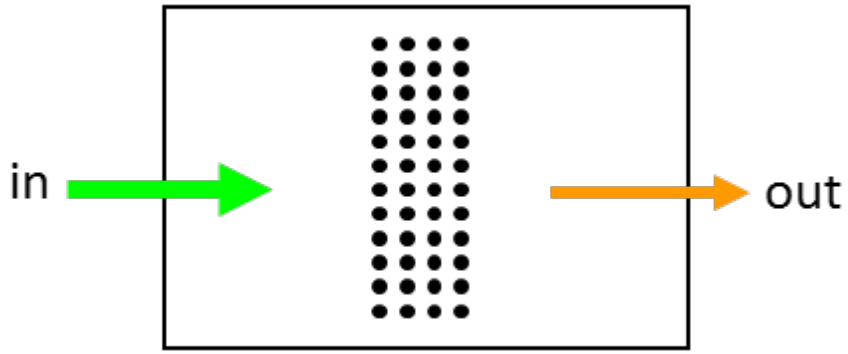


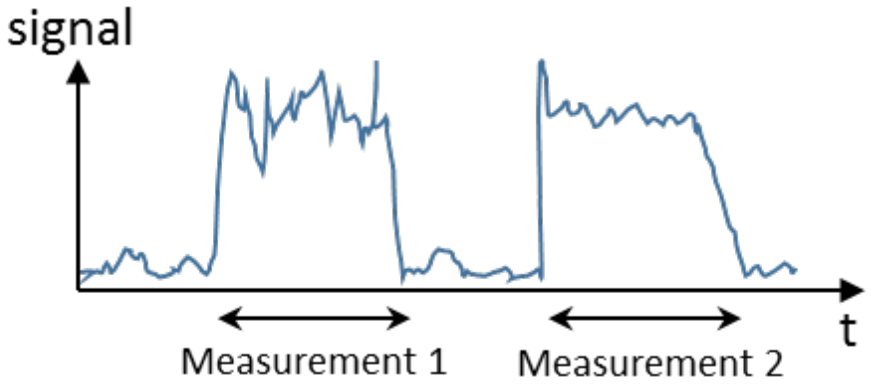
JSTQE **20** (2014) 3804420

Single-spot measurements are slow, calling for parallelization

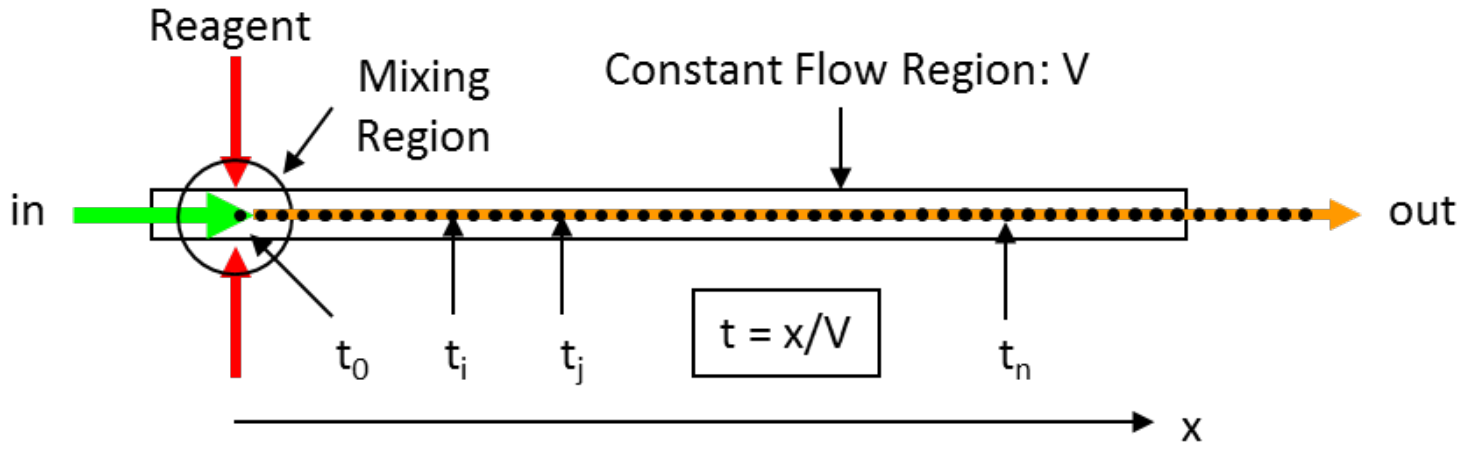
Arrays: Measurement Geometries

1. "Formulator" principle (Mix & Monitor aka M&M)



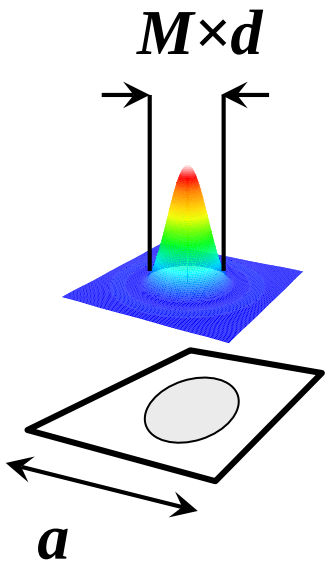


2. "Fast mixer" principle (Position = Time)

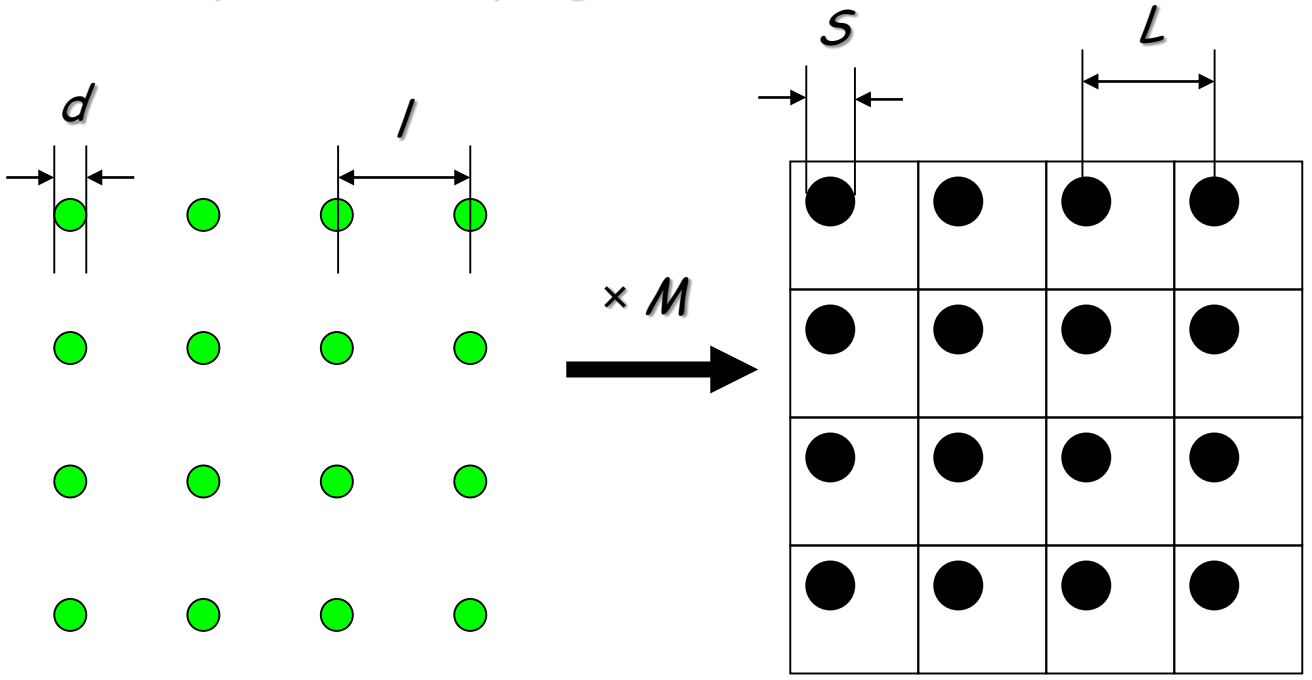


Detector geometry for high-throughput parallel smFRET

1. Confocal detection



2. Independent sampling volumes



Excitation
(sample space)

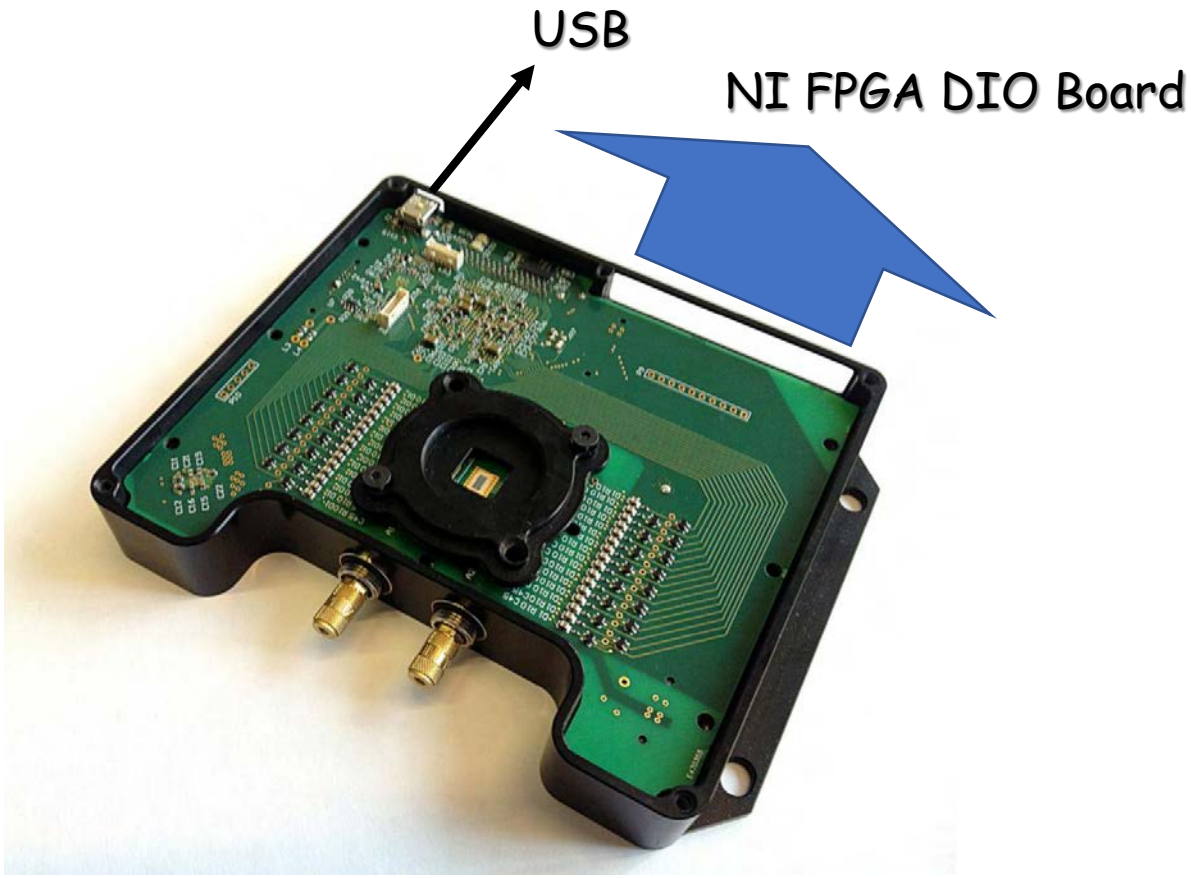
Detection
(image space)

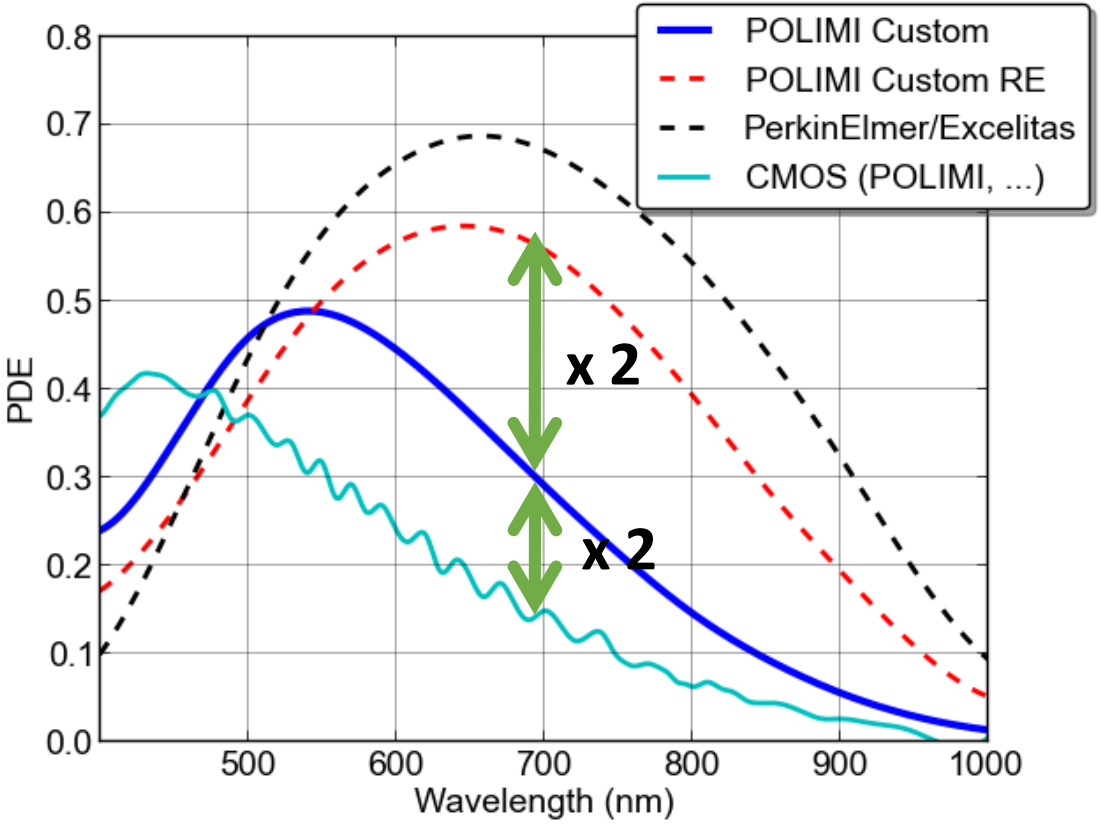
$d \sim \lambda/NA \sim 0.5\ \mu m$
M: 40 - 100
a: 20 - 50 µm

$l \sim 5\ \mu m$

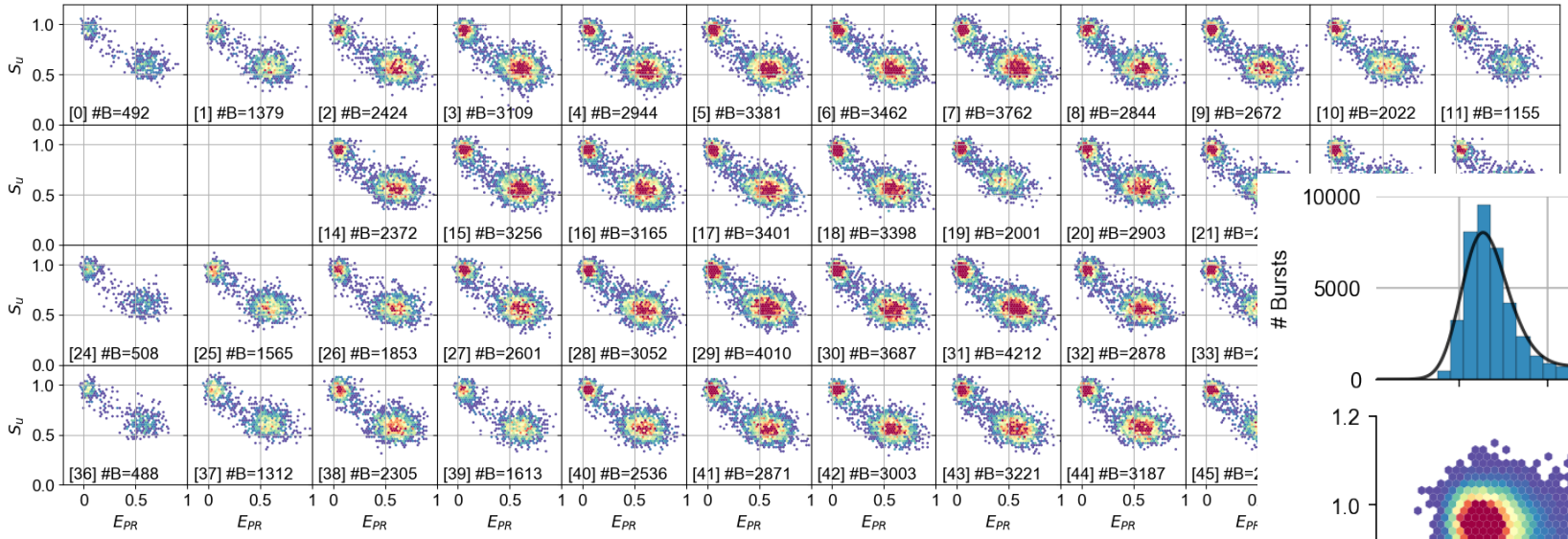
Phil Trans R Soc B **368** (2013) 20120035

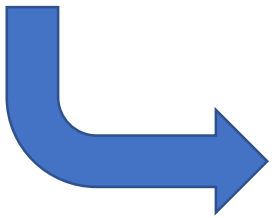
4x12 SPAD array in custom technology

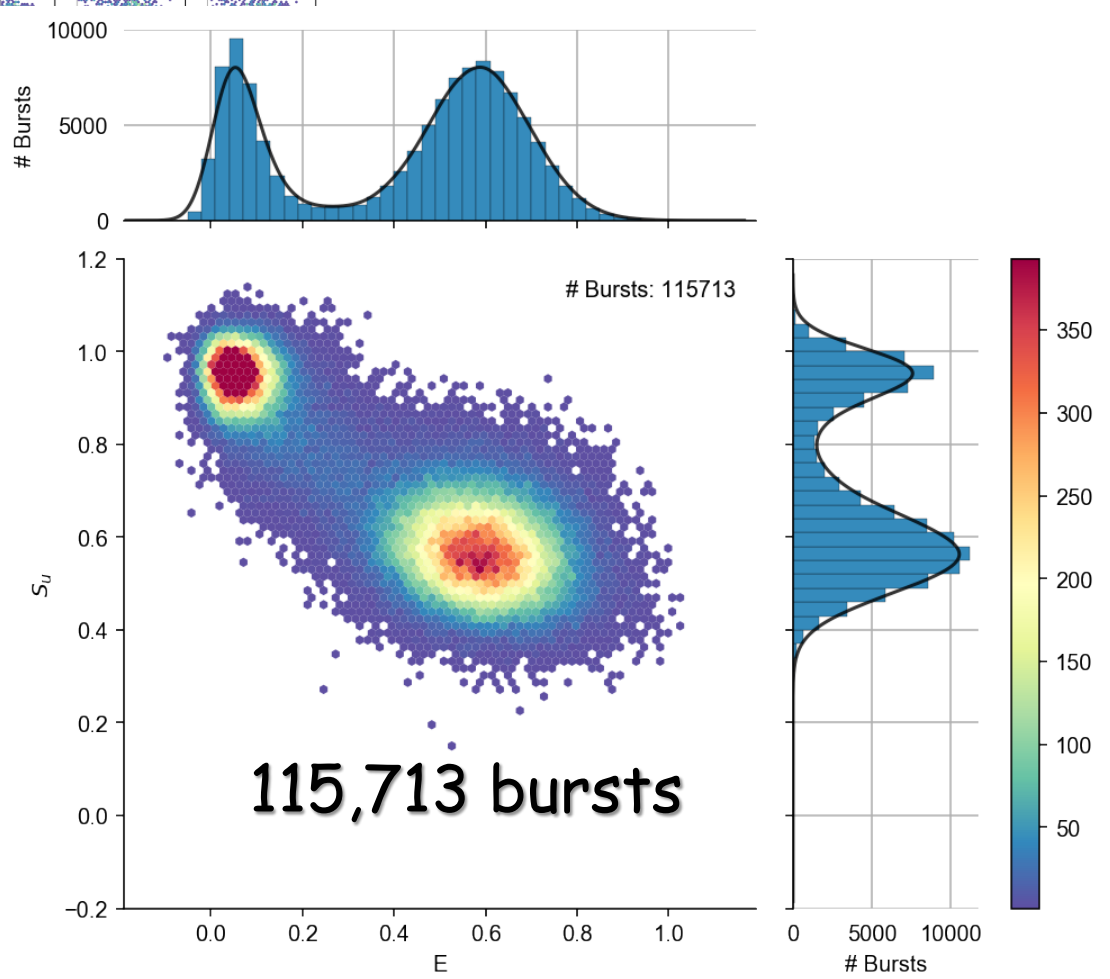




4x12 dsDNA measurements





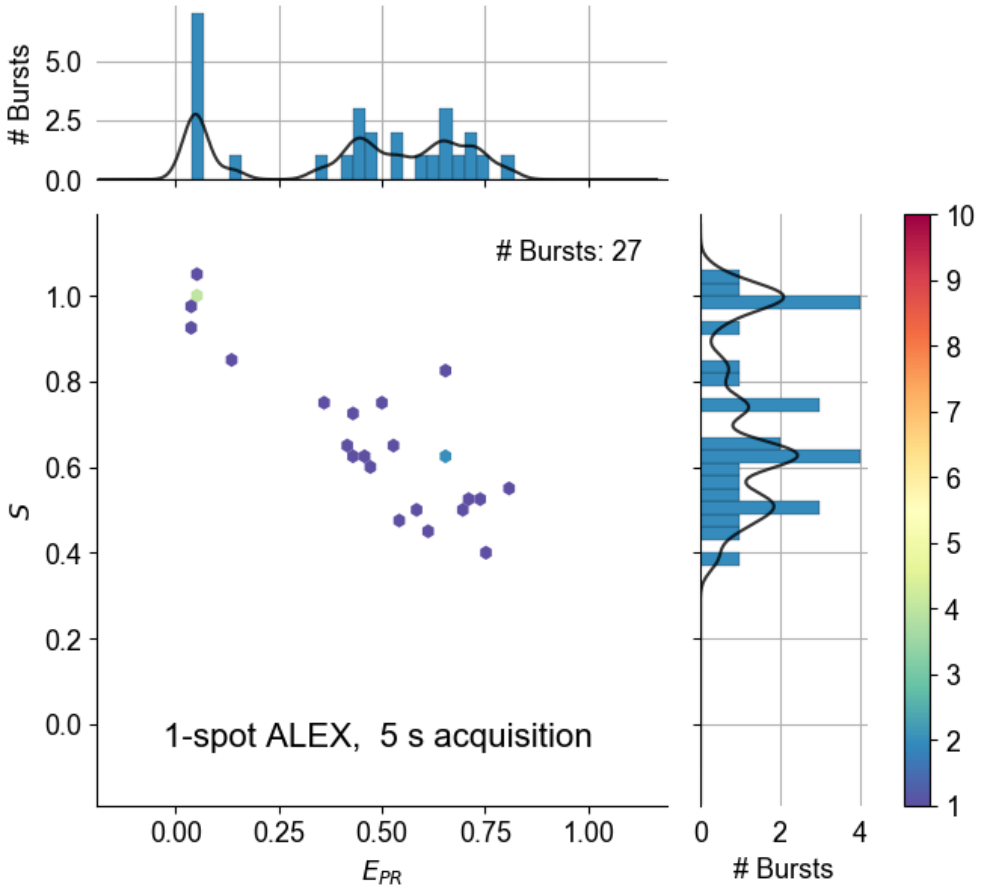


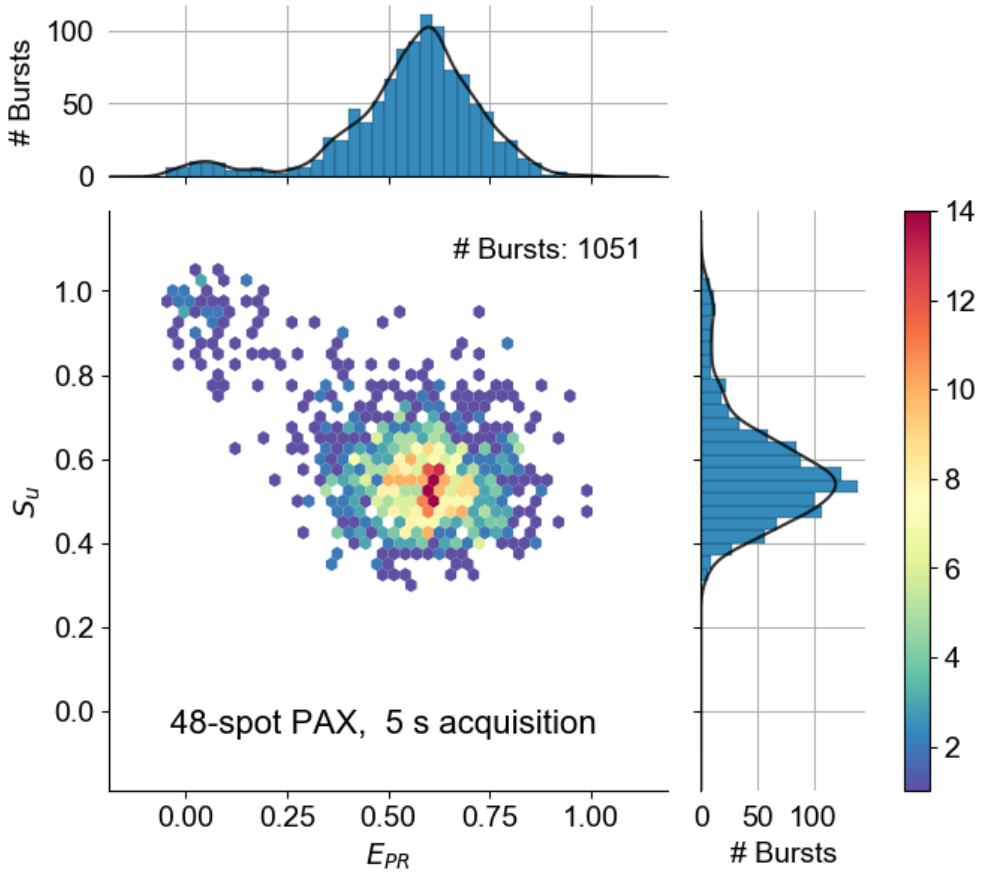
Sample:

- 40 base-pair dsDNA
- 12-bp D-A separation
- D: ATTO550
- A: ATTO647N

- 10 min measurement

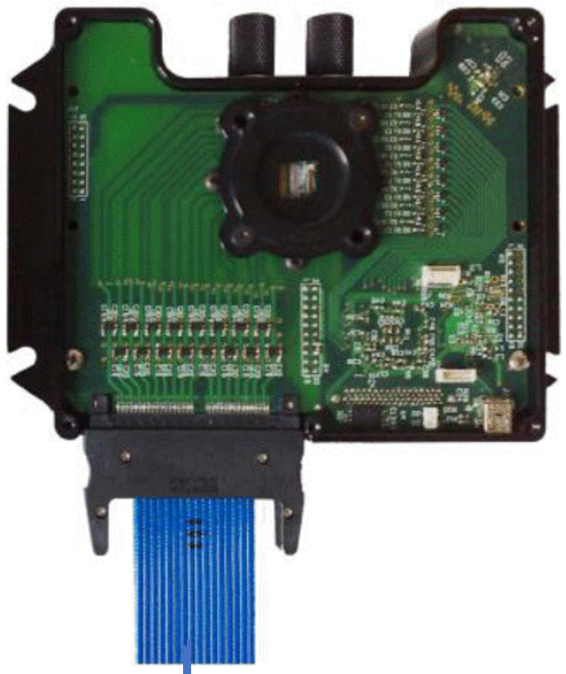
4x12 spots: towards 1 s dynamic resolution



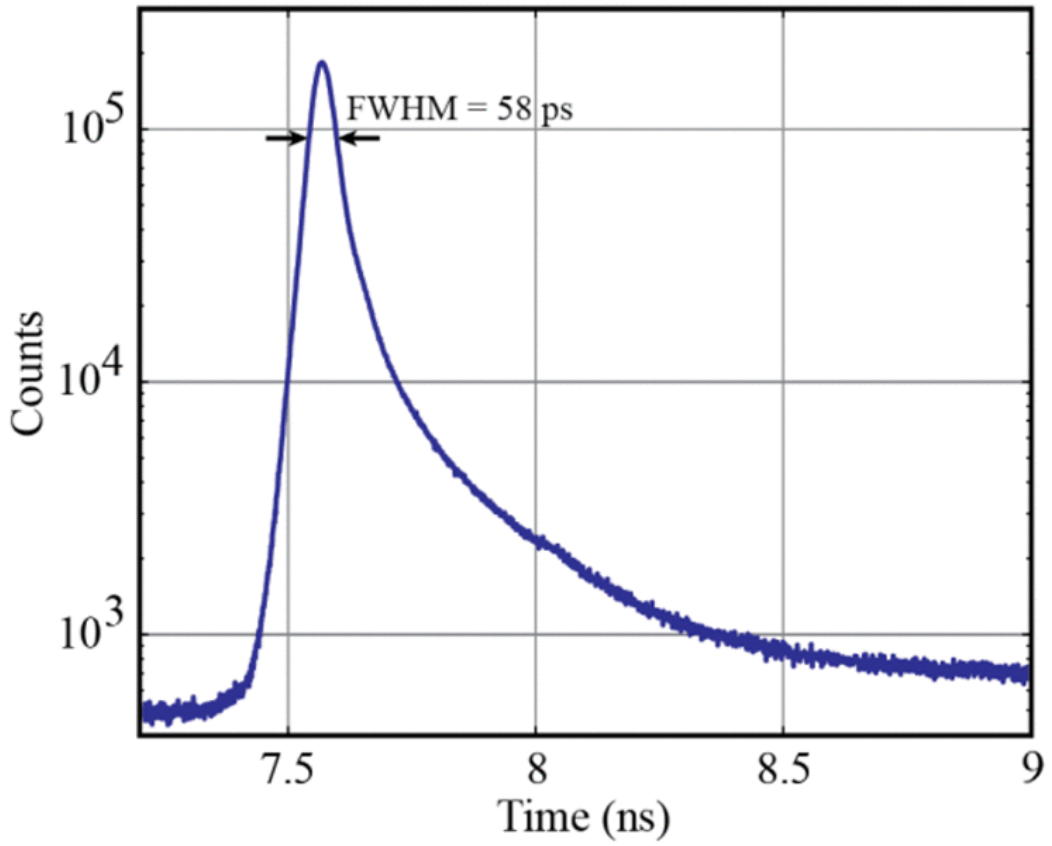


Linear TCSPC capable arrays

32 SPAD array
(custom RE process)



16 ch TCSPC
Hardware

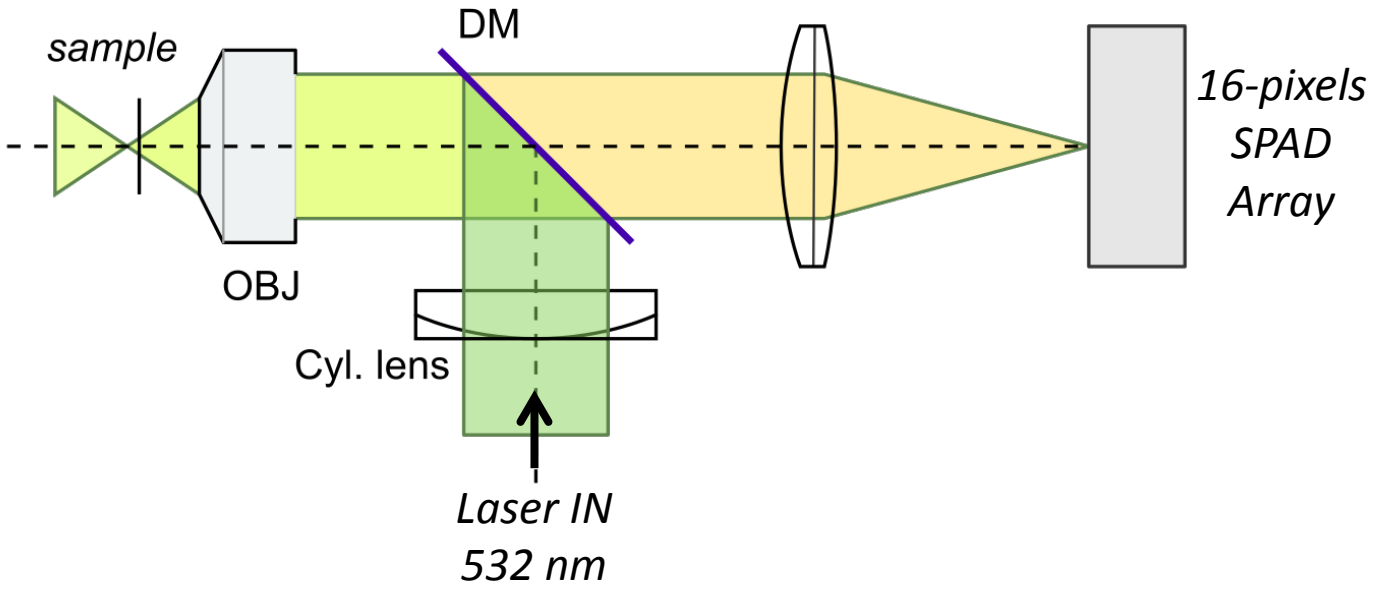


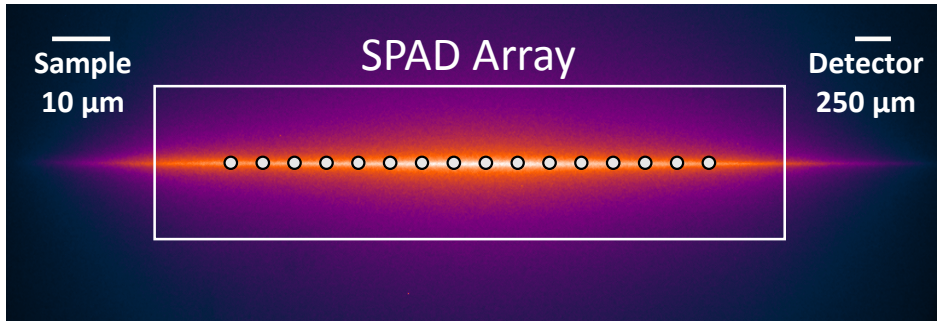
Pixel diameter: 50 μm
Pixel pitch: 250 μm
Geometry: 32 x 1

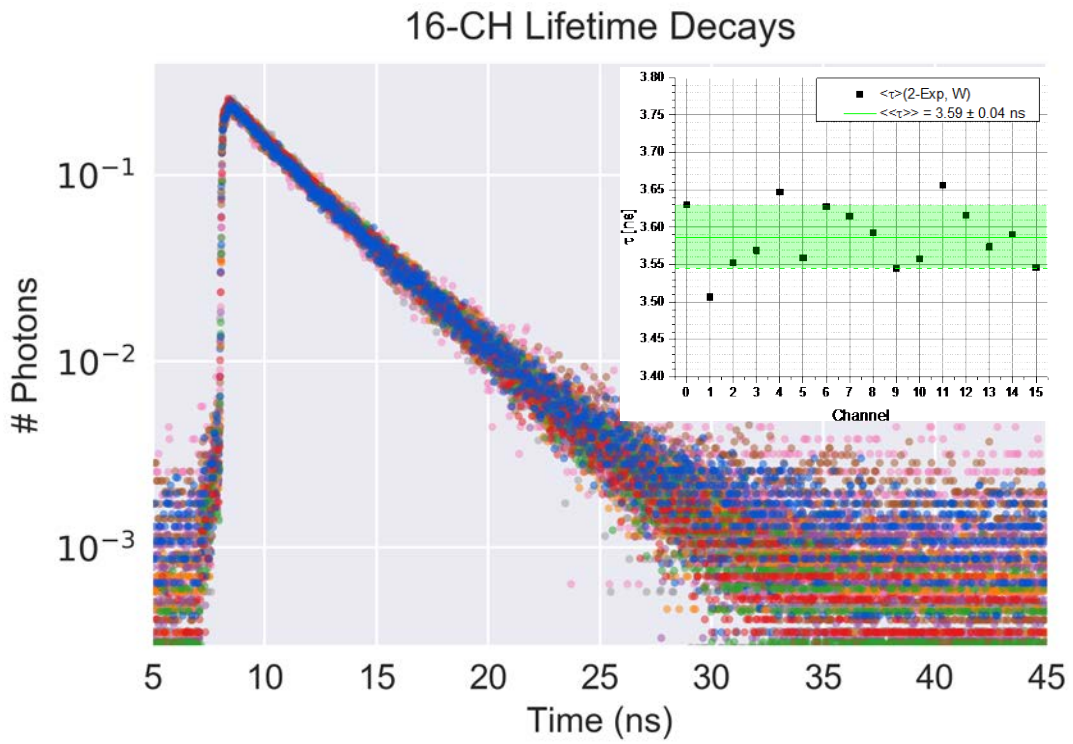
Antonioli *et al.* Rev. Sci. Instrum. **84**, 064705 (2013)
Cuccato *et al.* IEEE Photonics J. **5**(5) (2013)

Linear geometry for mixer experiments: 32x1 TCSPC capable arrays

Time-resolved measurement provide access to information inaccessible in mere counting measurements







80 base pairs dsDNA + ATTO550

Proc. SPIE **10071** (2017) 100710Q

Conclusion & Perspectives (1)

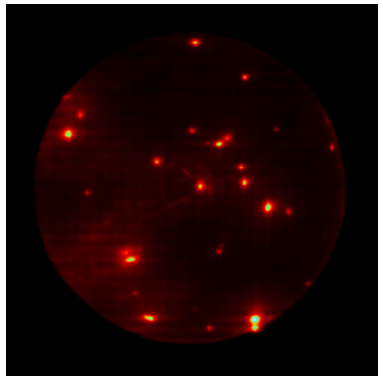
- Red-enhanced version of SPAD arrays will allow faster measurements (and potentially extend the spectral range of usable dyes)
- TCSPC capabilities are useful but not always necessary (and require high power lasers)
- 3D architecture for larger arrays (256x1, 32x32, larger? but for what applications?)
- New illumination & detection schemes needed to afford larger fields of views
- User modifiable FPGA firmware (or auxiliary NI FPGA?) for data preprocessing

3. *in vivo* NIR fluorescence lifetime imaging

Similarities & differences between single-molecule imaging and small animal imaging

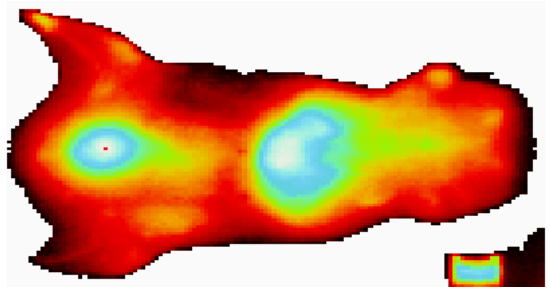
smFRET

- Fluorescence in the visible ($500 < \lambda < 750$ nm)
- Weak and sparse signals
- High fill factor needed
- Low dynamic range, high peak count rate
- Short observation timescales (high resolution macrotime needed)
- Long lifetimes (> 1 ns)
- Narrow IRF (SPAD is often dominating, but rarely an issue)



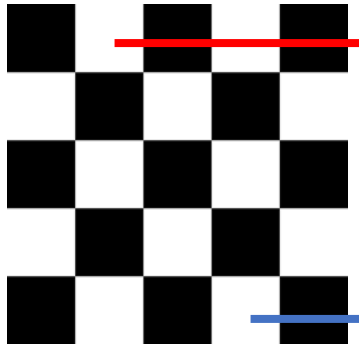
Small animal FLI

- NIR to SWIR
- Spatially extended signal, generally weak
- High fill factor needed
- Low dynamic range but potentially high peak count rate (adjustable by I_{ex})
- No need for high resolution time scale (< 0.1 s is enough to counterbalance breathing artefacts)
- Very short lifetimes (< 1 ns)
- Lots of scattering resulting in broad and spatially dependent IRF



Sparse vs Uniform signal

SPADs in detector: N
"on" SPADs at any time (on average): P
Macrotime stamp resolution: dt
or
Frame readout time: Δt



S,T,L

Readout Bandwidth:
P(S+T+L)/dt

L

Readout Bandwidth:
NL/Δt

Event-driven readout encoding (per pixel):

- S bytes to encode SPAD position
- T bytes to encode macrotime
- L bytes to encode nanotime

Example:

- S+T+L = 8
- 1,000 sm at 10 kHz = 10 Mcps = P/dt

Bandwidth = 80 MB/s

1-bit frame readout encoding:

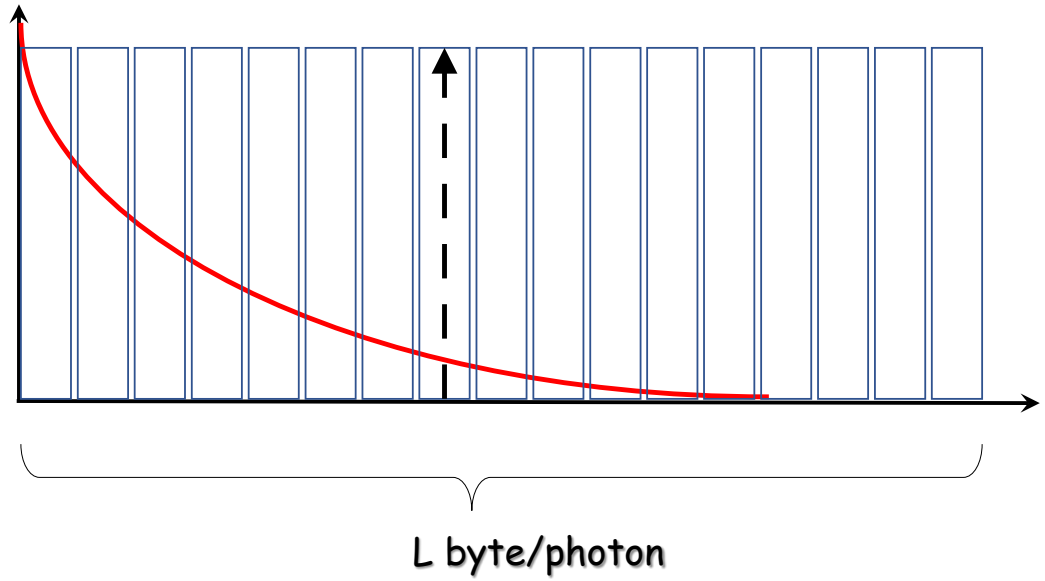
- L bytes to encode nanotime
- Frame rate: 1/Δt

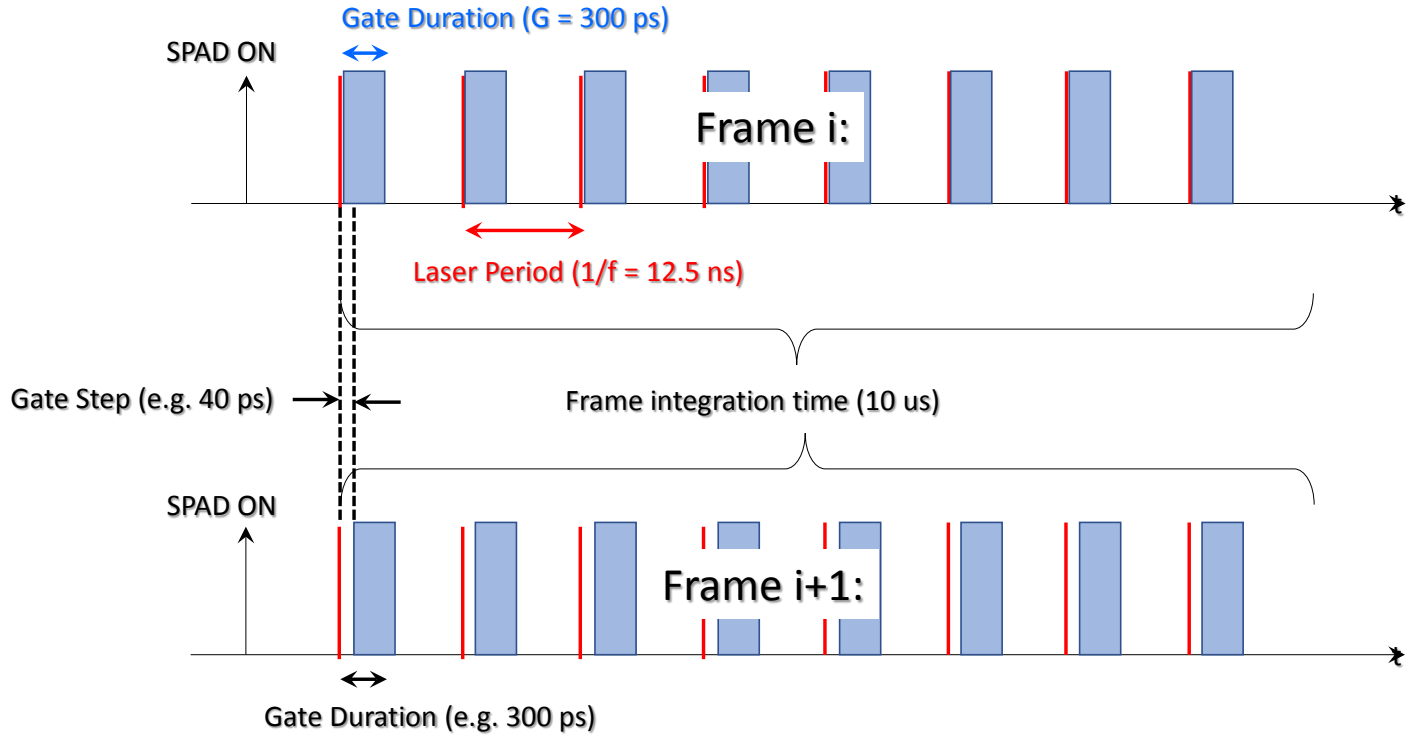
Example:

- L = 1/8 (1 bit), N = 1M
- Δt = 10 us (max count rate/pixel << 100 kHz)
- 1 frame = N/8 bytes = 125KB

Bandwidth = 12.5 GB/s most of which are 0

TCSPC vs Gated

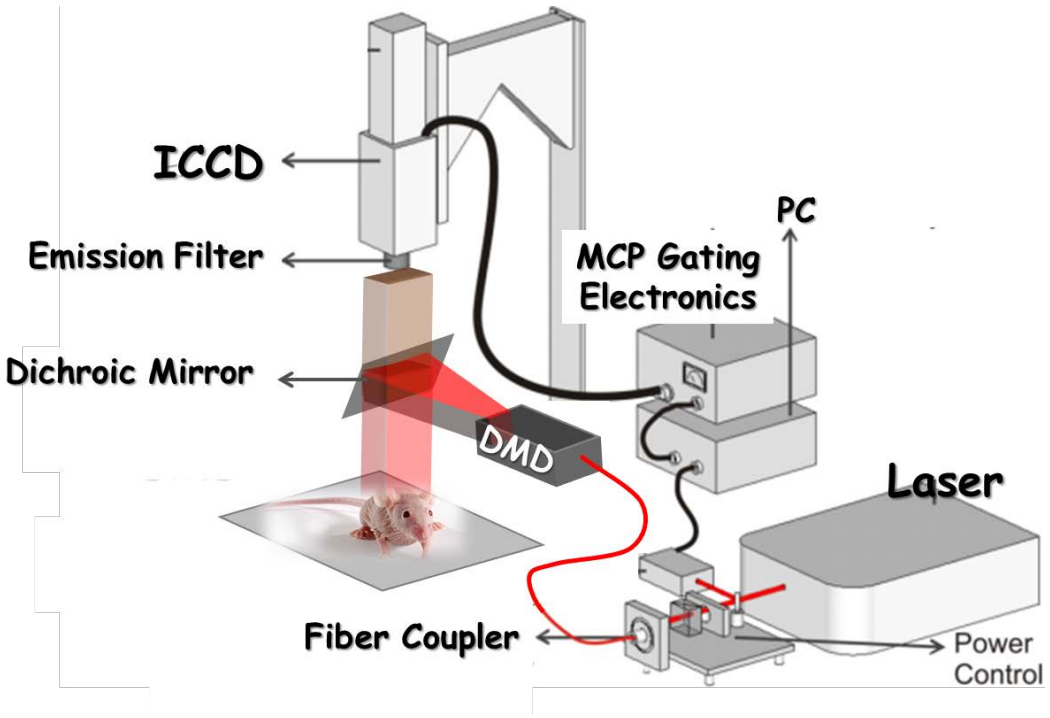




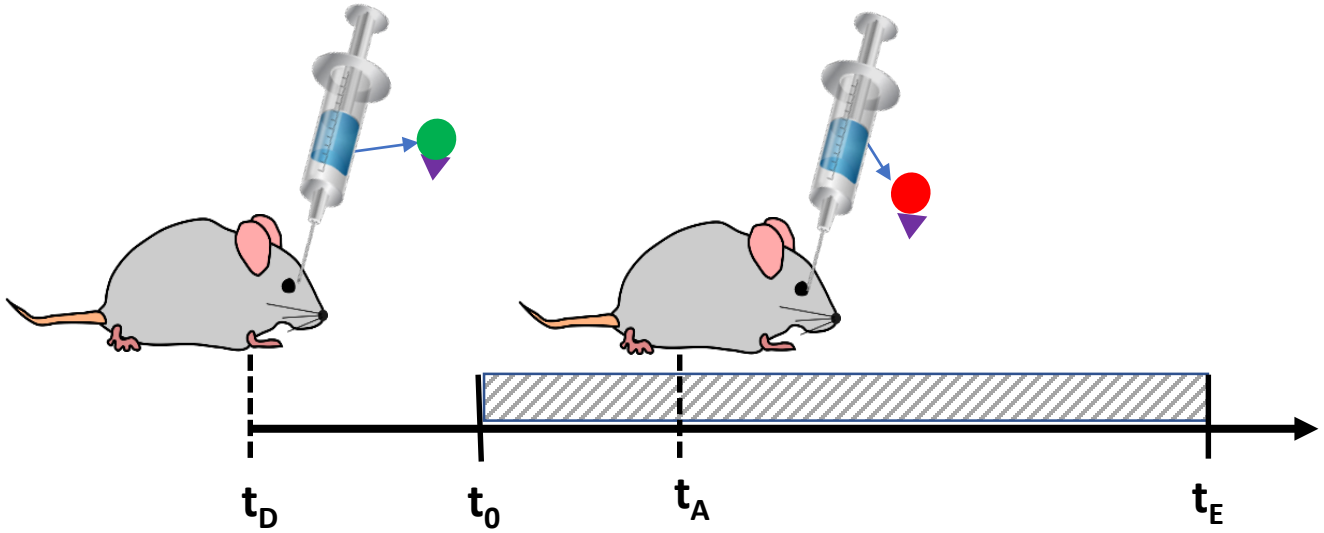
Very demanding technologically and in bandwidth.

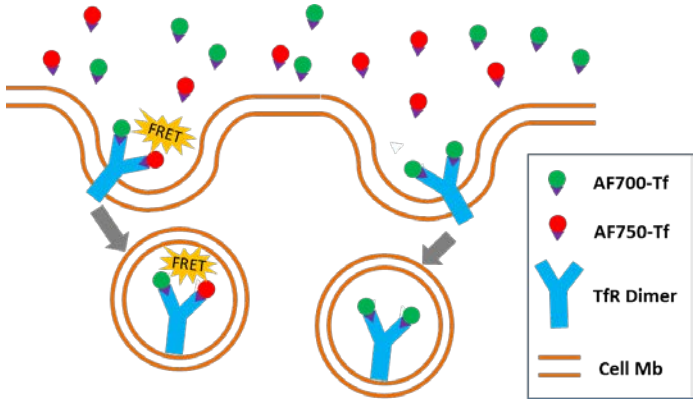
Wasteful acquisition (e.g. detector is on G×f = 2.4% of the acquisition)

in vivo NIR FRET fluorescence lifetime analysis



Gate: 300 ps
Step: 40 ps
Integration: >100 ms



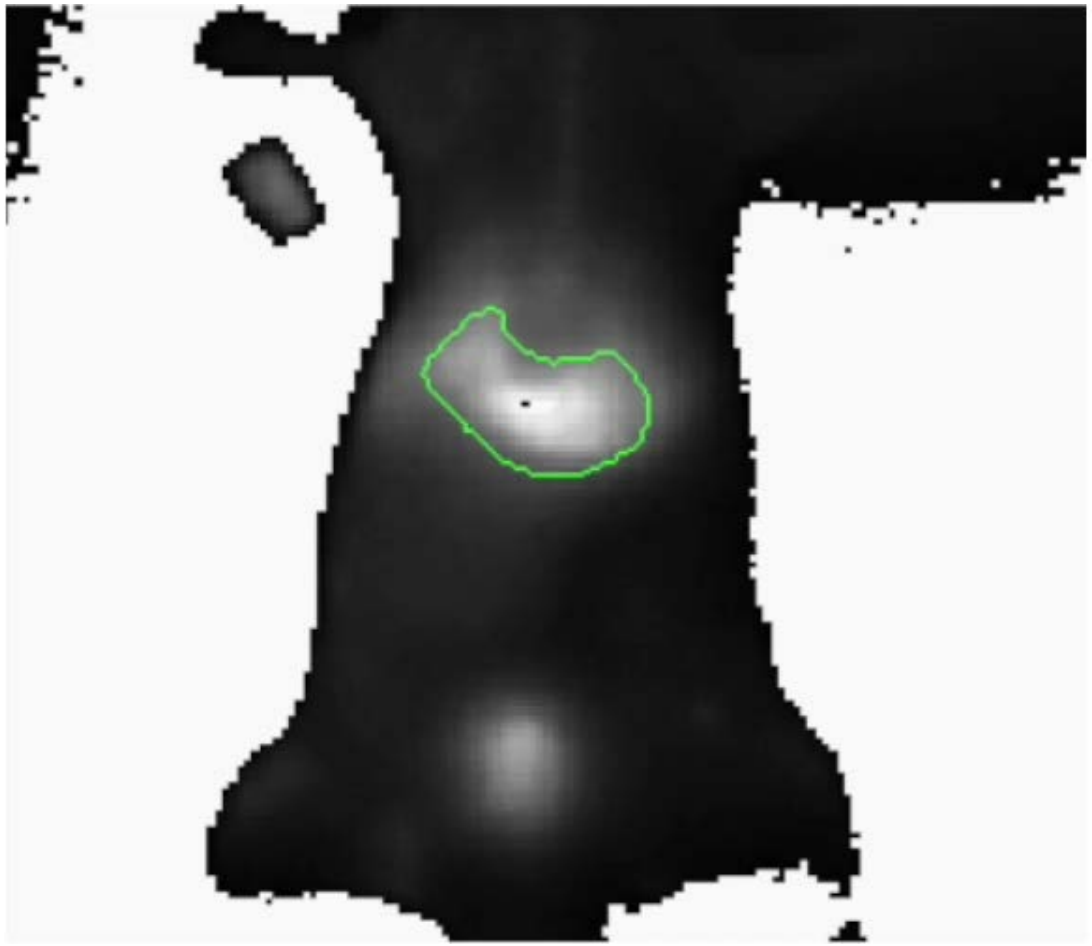


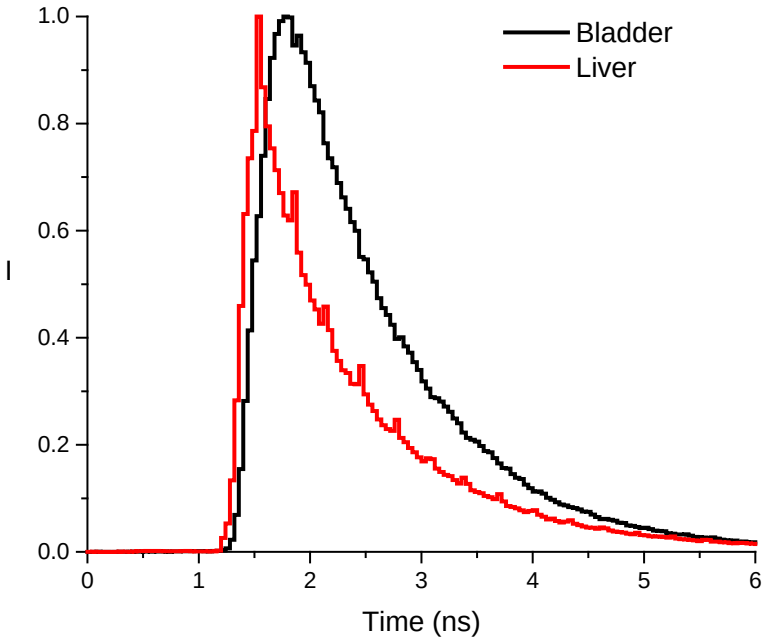
$$\tau_{DA} = \tau_D (1 - E) < \tau_D$$

in collaboration with the Intes & Barroso Labs

Chen et al., BiOS 2018, 10487-17, *in preparation*

FRET analysis by decay fitting





$$\tau_1 = \tau_{DA}$$

$$\tau_2 = \tau_D$$

$$f(t) = N_1 \frac{1}{\tau_1} \exp\left(-\frac{t}{\tau_1}\right) + N_2 \frac{1}{\tau_2} \exp\left(-\frac{t}{\tau_2}\right)$$

$$f_1 = \frac{N_1}{N_1 + N_2}$$

tells about the amount of FRET
i.e. Tf binding to TfR

Brief introduction to phasor analysis

Fourier series of a decay

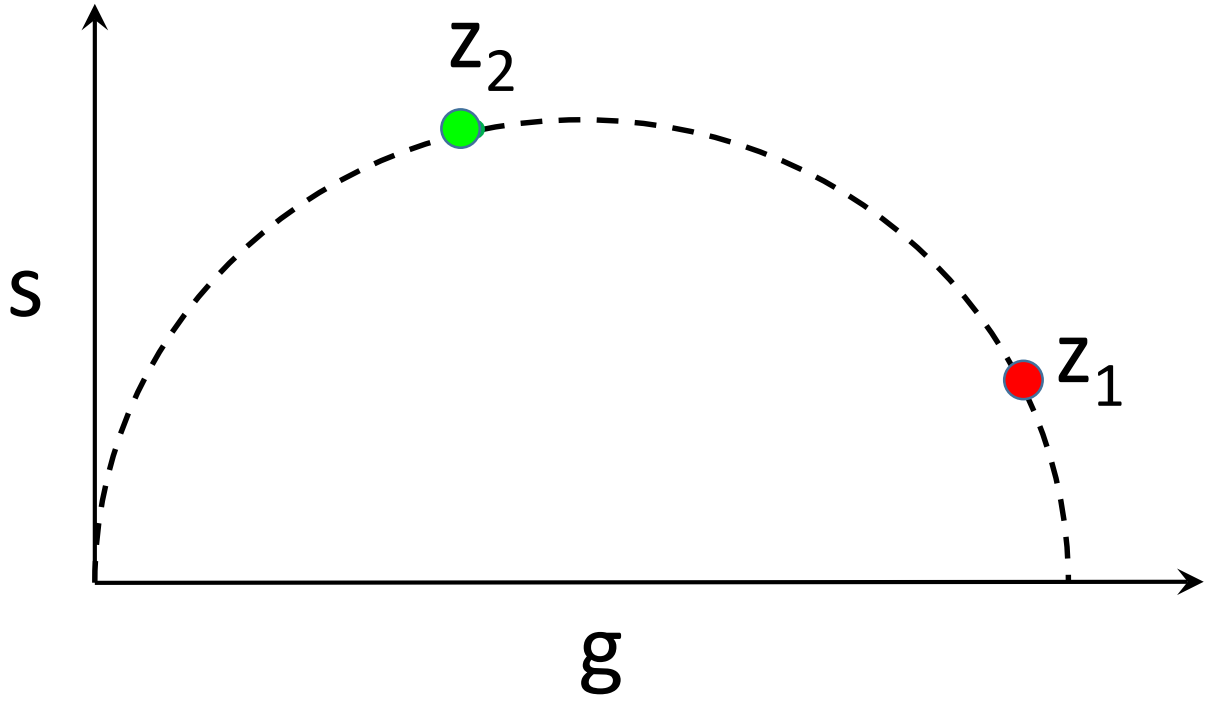
$$f(t)=\frac{1}{\tau}\exp\left(-\frac{t}{\tau}\right) \longrightarrow f(t)=\frac{a_0}{2}+\sum_{n=1}^{\infty} g_n \cos\left(2\pi n\frac{t}{T}\right)+s_n \sin\left(2\pi n\frac{t}{T}\right)$$

Components of the 1st harmonic: g_1, s_1

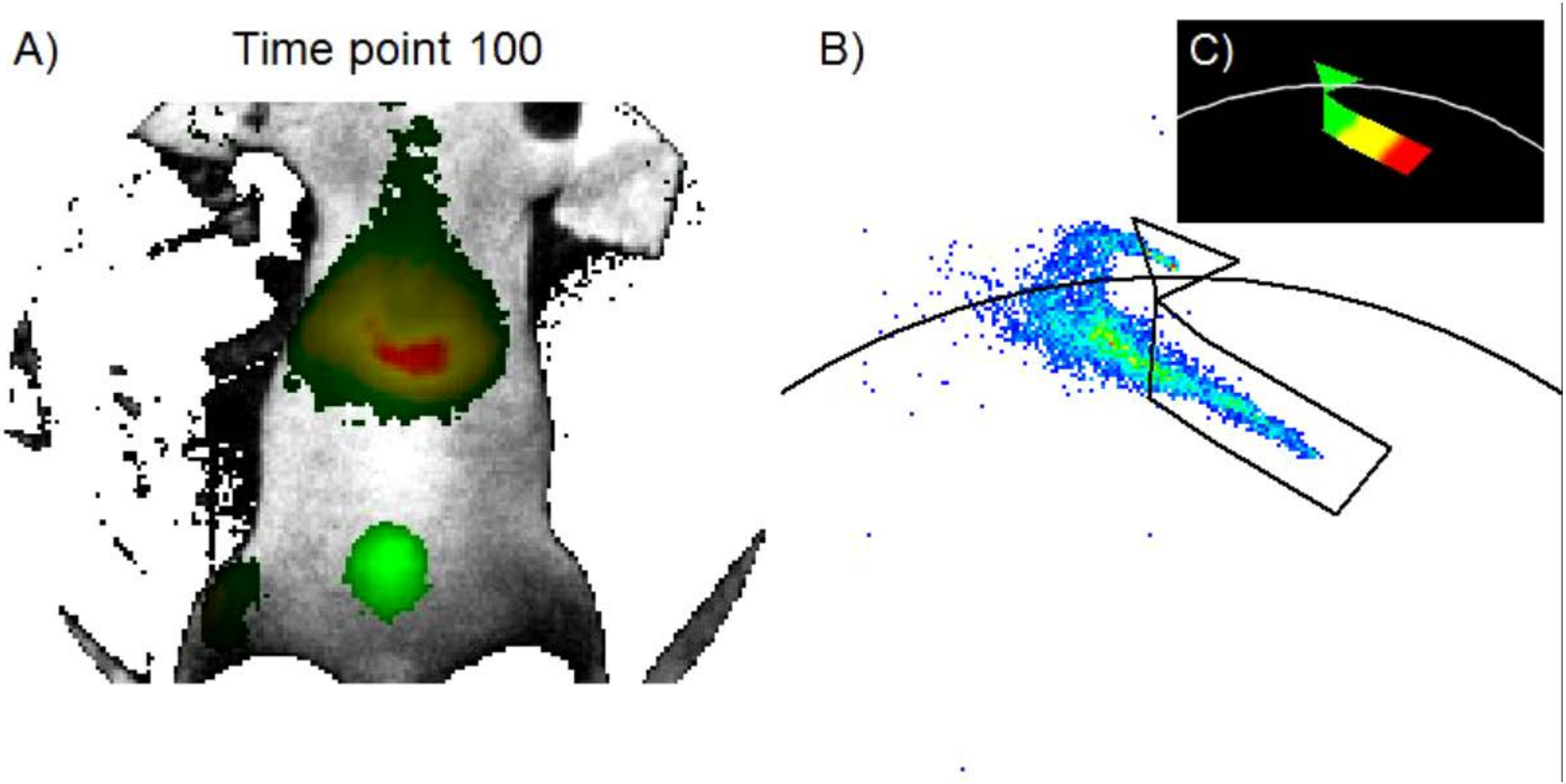
$$g_1=\int_0^T f(t)\cos\left(\frac{2\pi t}{T}\right)dt,\quad s_1=\int_0^T f(t)\sin\left(\frac{2\pi t}{T}\right)dt$$

$$f(t)=N_1\frac{1}{\tau_1}\exp\left(-\frac{t}{\tau_1}\right)+N_2\frac{1}{\tau_2}\exp\left(-\frac{t}{\tau_2}\right)$$

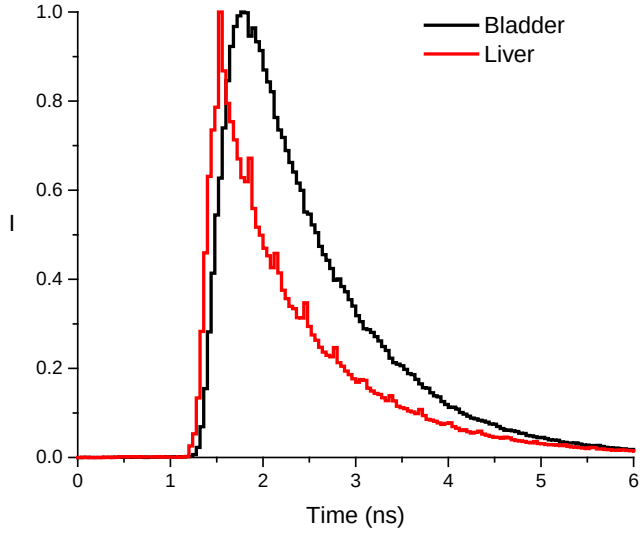
$$f_1=\frac{N_1}{N_1+N_2}=\frac{d_1}{d_1+d_2}$$



FRET analysis with the phasor approach

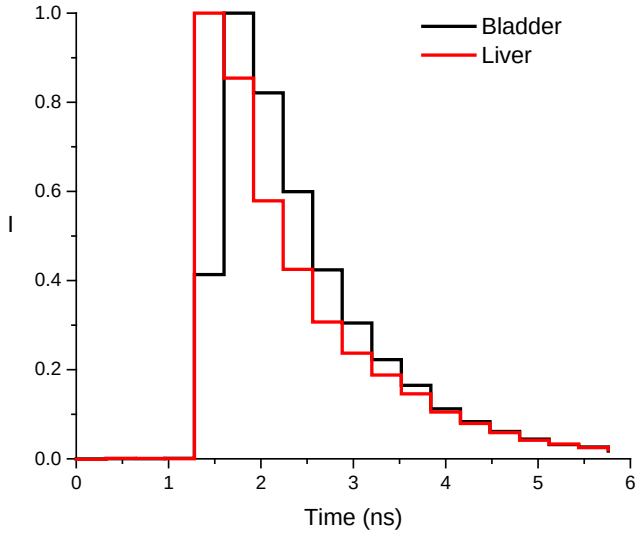


Fewer gates than for decay fitting are needed for quantitative phasor analysis

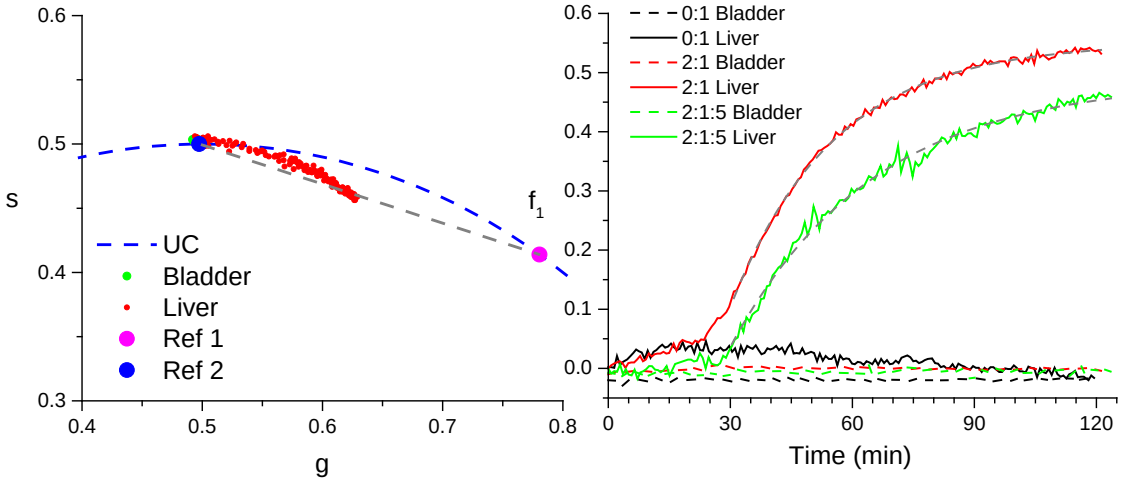


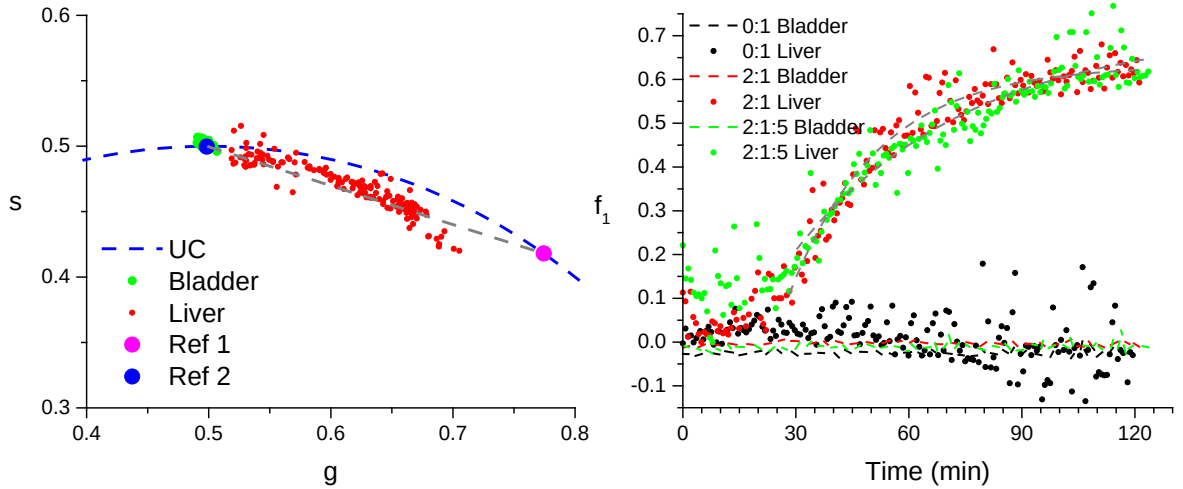
1/8

$\tau_{2:1}$ = 1,528 ± 15 s
$\tau_{2:1:5}$ = 2,109 ± 55 s

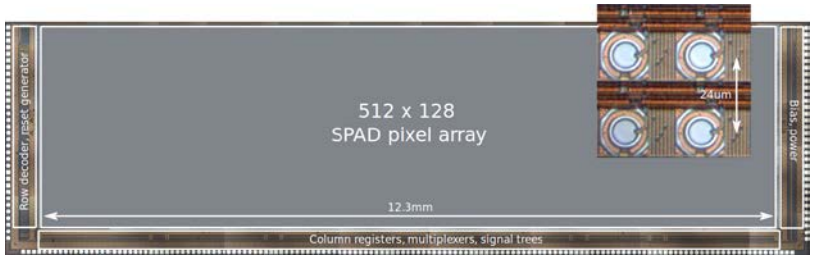


$\tau_{2:1}$ = 1,418 ± 93 s
$\tau_{2:1:5}$ = 2,769 ± 356 s.

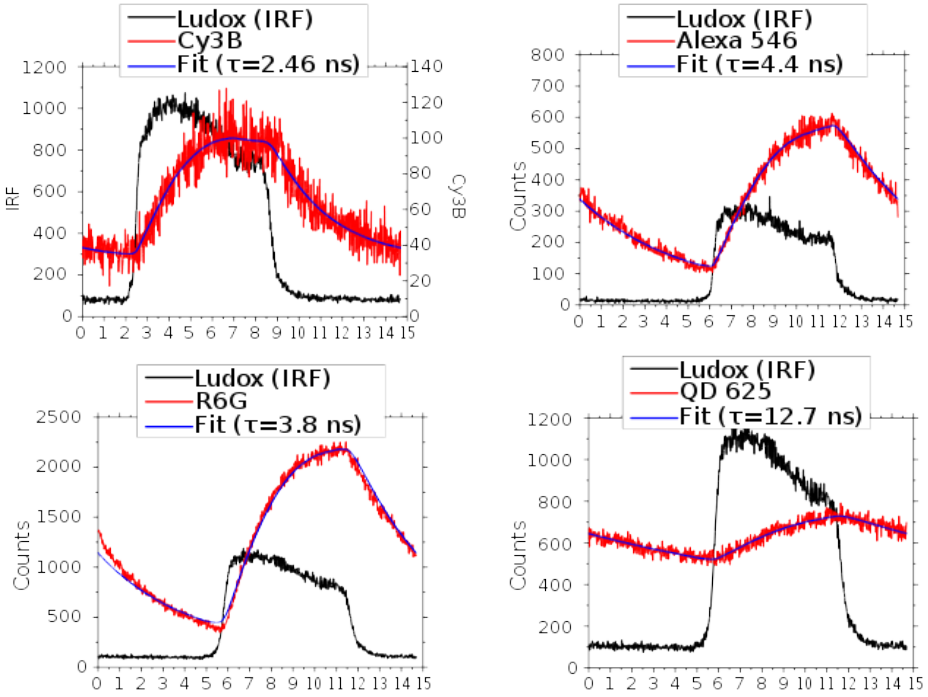




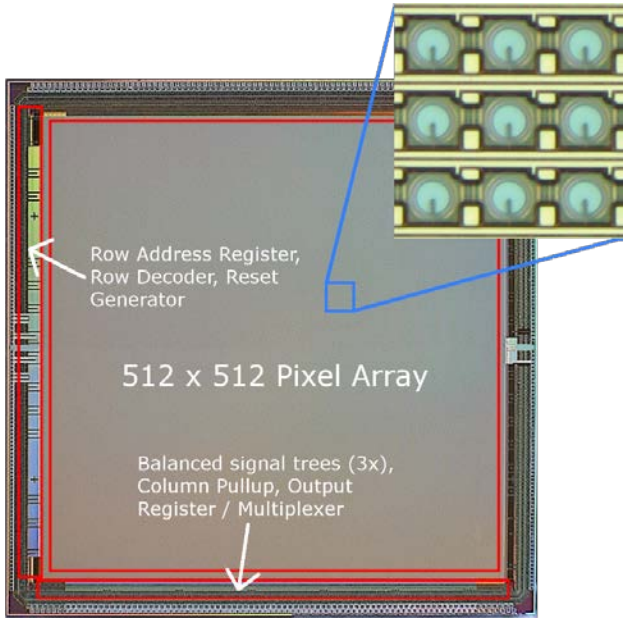
Narrow (or even many) gates are not needed



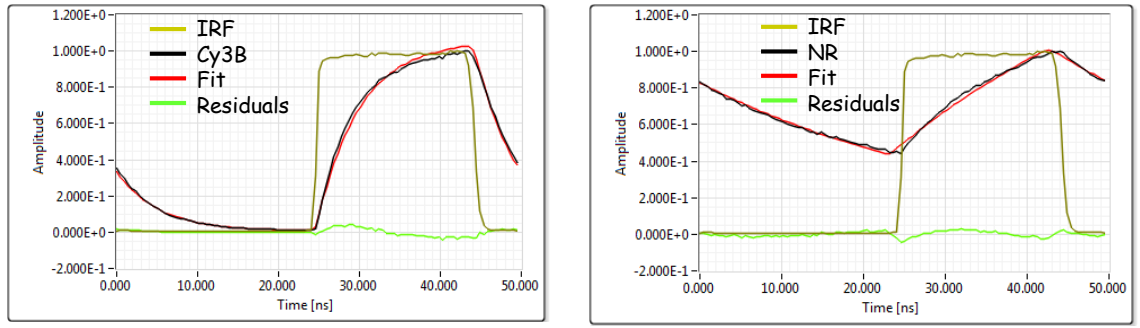
SwissSPAD 1, *Opt. Expr.* **22** (2014)17573



14.7 ns period, 768 gates, 6 ns wide

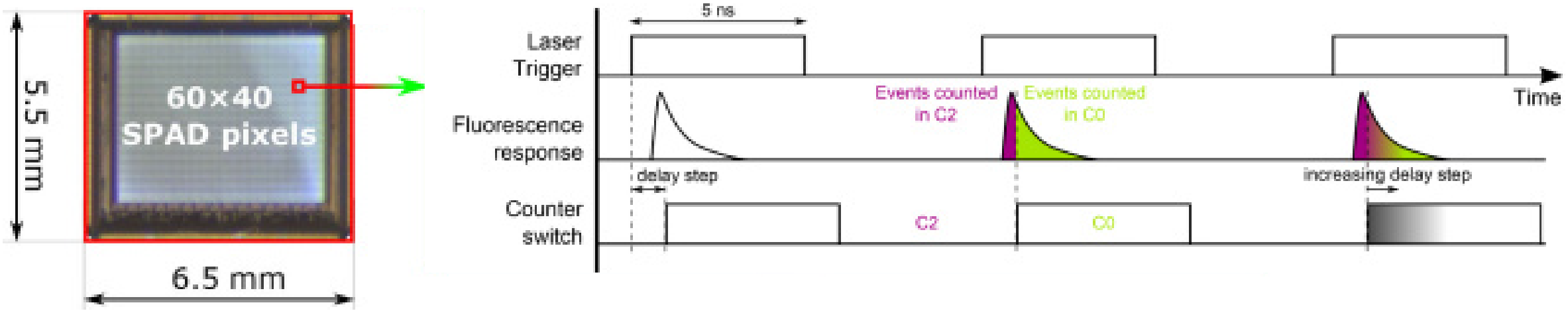


SwissSPAD 2, IISW (2017) 234
SPIE BiOS **10498** (2018) 10498-21



50 ns period, 125 gates, 20 ns wide

Pushing the concept of large gates to the limit: FluoCam



Best of both worlds?

- Technical simplicity
- Photon efficiency

FluoCam, *Biomed . Opt. Expr.* **7** (2016) 1797

Conclusion & Perspectives (3)

- Photon-efficient time-resolved counting schemes are worth developing
- TCSPC resolution does not need to be that of LIDAR applications (ps), because measured observables involve 100s-1000s of photons
- *in vivo* NIR (and SWIR) FLI will benefit from improved PDE
- User-modifiable FPGA firmware (or auxiliary NI FPGA?) for data preprocessing (e.g. phasor computation)

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