Imaging Fluorescence Correlation Spectroscopy: Novel Results on a New Image Sensor (SPAD array) and a Comprehensive New Software Package (QuickFit 3.0)

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50 Years – Research for A Life Without Cancer

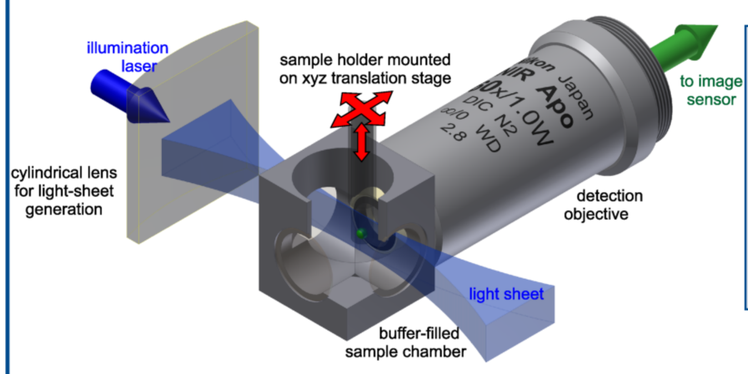
Abstract

Fluorescence (cross-)correlation spectroscopy (FCS/FCCS) is a useful technology to characterize the mobility of molecules inside living cells and the accessibility of cellular compartments. The typically used confocal microscopy based FCS gives us high time-resolution but only for a single-position. To extend this approach to imaging, we typically use a selective plane illumination microscope (SPIM) equipped with high-NA detection optics. With this setup we could demonstrate the feasibility of imaging FCS and FCCS with several fluorescent proteins in all compartments (cytosol, nucleoplasm, membrane) of a living cell [1].

On this microscope we normally use a high-speed, high-sensitivity commercial EMCCD camera (128x128 pixels), which offers high spatial and moderate, but for many samples (e.g. large proteins) sufficient temporal resolution (~500µs temporal resoluation for 128x20 pixels) [1,2]. Here we demonstrate new in vitro and first in vivo results on the use of an alternative, experimental image sensor: an array of single-photon sensitive avalanche diodes (SPAD array, 512x128 pixels) [3]. This novel type of image sensor improves the temporal resolution to 6.4µs for full frames (!), while retaining acceptable photo-sensitivity. It is therefore applicable also to small fluorescent particles, such as single chemical dye molecules. For this sensor, we also developed advanced data evaluation techniques, that can perform the autocorrelation of data from the full sensor in real-time or faster.

We also developed a comprehensive open-source (GPL3) software package QuickFit 3.0, which implements all necessary computational methods to perform confocal and imaging FCS/FCCS. It also contains modules for advanced analysis methods, such as global fits, maximum entropy (MaxEnt) or mean squared displacement (MSD) evaluations of FCS data. QuickFit can be extended on several levels (fit functions/algorithms, raw data types, evaluations, general extensions) by plugins, written in C++. In addition, this software is also used to control our home-built lightsheet microscope. QuickFit 3.0 is available from: http://www.dkfz.de/Macromol/quickfit/.

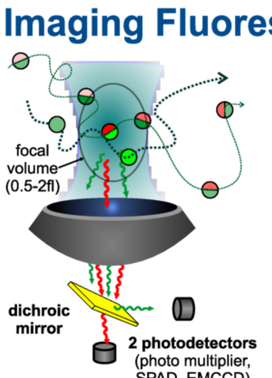
Our Selective Plane Illumination Microscope (SPIM)



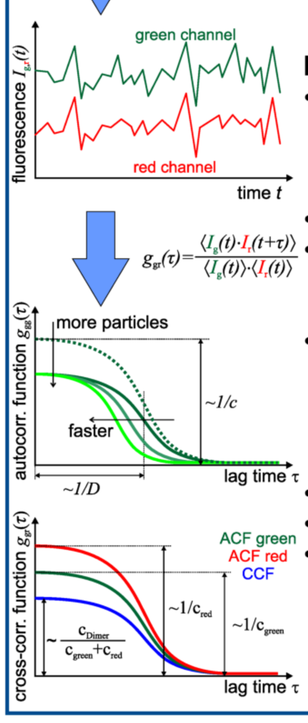
- ✓ good z-sectioning
- ✓ low photodamage
- ✓ easy 3D imaging
- ✓ simultaneous multispot FCS/FCCS
- ✓ high detection efficiency
- ✓ spatial resolution (~0.6µm)
- ✓ temporal resolution (~300µs)
- ✓ good per-cell statistics

- **illumination:** cylindrical lens + NA 0.3 microscope objective (lightsheet width: ~1.5µm$_{FWHM}$)
- **light sources:** 488nm + 561nm lasers, LED
- temperature-controlled sample chamber, filled with low scattering measurement buffer
- **observation:** 60x/NA1.0 water-dipping objective (lateral resolution: ~0.6µm$_{FWHM}$)
- **image detectors:**
  - high-speed EMCCD camera (Andor iXon X3 860, 128×128 pix, ~2-4 kfps)
  - SPAD array detector (CHSPAD, µLenses, 512×128 pix, 100-1000 kfps)
- **image splitter** (DualView) for simultaneous two color detection
- fully automated and computer-controlled setup (uses QuickFit 3.0 as control-software!)

Imaging Fluorescence Correlation Spectroscopy

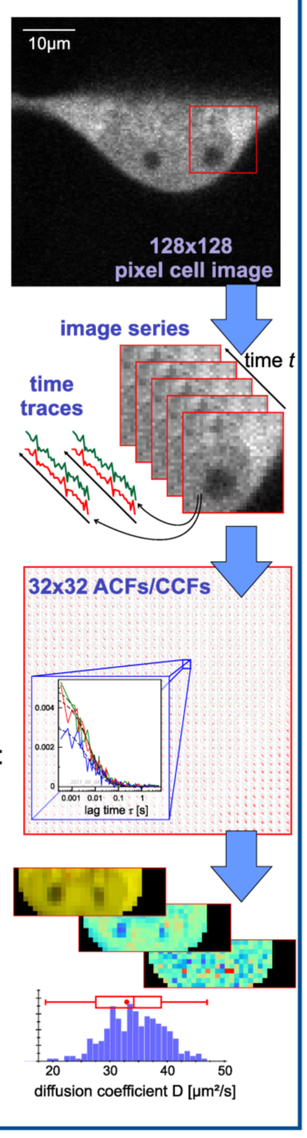


FCS/FCCS:
- measures particle number and mobility (diffusion coefficient, anomality coefficient, flow speeds, ...)
- fluorescence intensity fluctuations from a small volume are analyzed by:
  - autocorrelation analysis: faster particles lead to faster fluctuations and faster decay of the correlation curve
  - 2-color cross-correlation measures molecular interaction
  - 2-pixel cross-correlation measures directed motion (flow), absolute diffusion coefficients

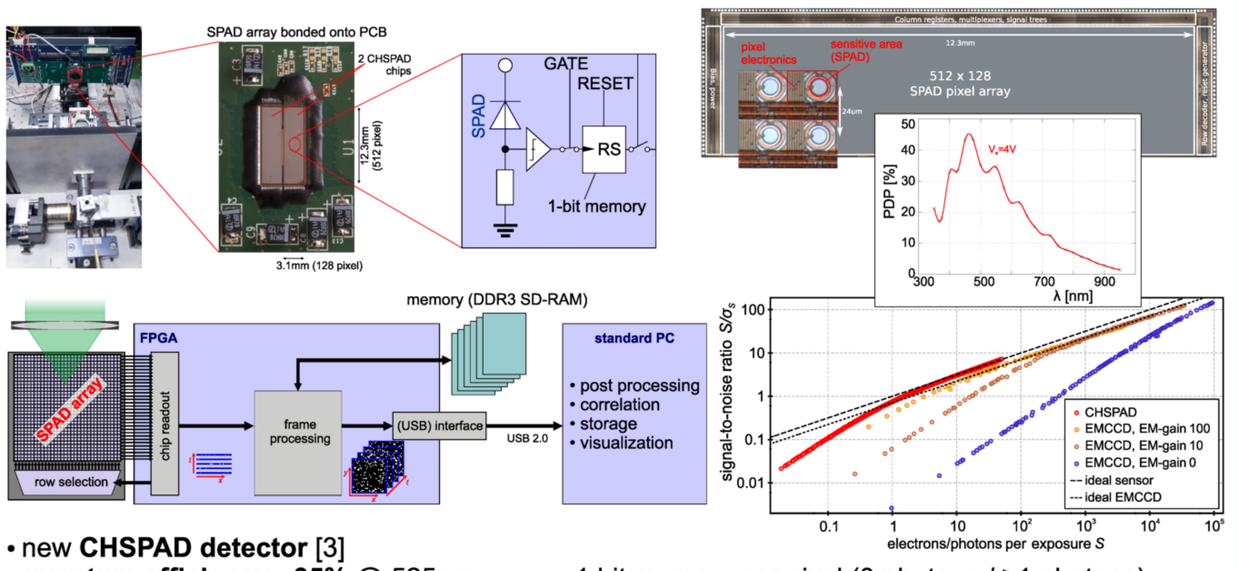


Imaging FCS/FCCS:
- acquire fast image series
  - frame-size: 128x3-128x32 pixels
  - frames: 10^4-10^6
  - temporal resolution: 0.3-1ms
- background and bleach correction
- auto- (ACF) and cross-correlate (CCF) intensity from two color channels for each pixel, or from two separated pixels
- global, non-linear model fit yields spatially resolved:
  - diffusion coefficients (decay times)
  - concentrations (ACF amplitudes)
  - amount of binding (CCF amplitude)
  - flow speeds and directions (2-pixel CCF)
- create 2D maps of these parameters
- parameter statistics, histograms
- parameter scatter/correlations plots

⇨ spatial information
⇨ good per-cell statistics
⇨ data-selection after acquisition



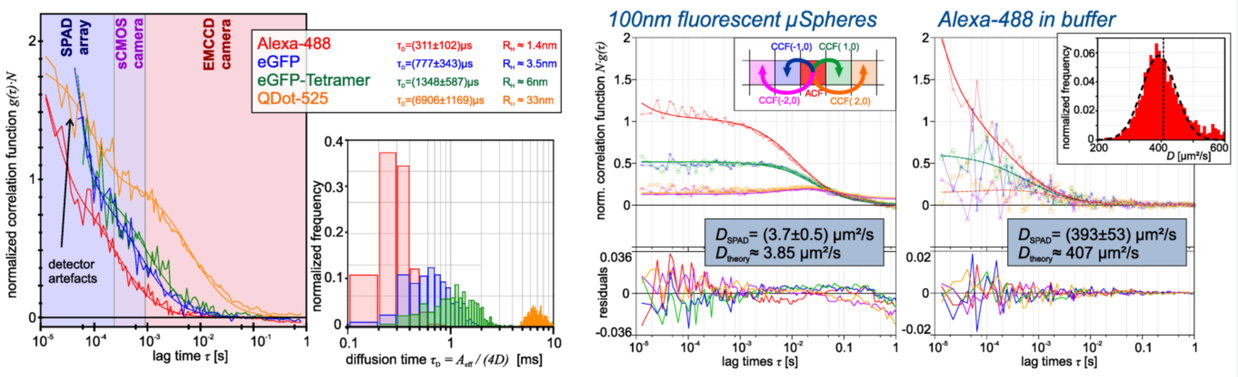
High-Speed Image Sensor: Single Photon Avalanche Diode Array [3]



- new **CHSPAD detector** [3]
- **quantum efficiency ~35%** @ 525nm
- **µLenses** improve fill factor (~8x)
- **512×128 pixels**
- 24×24 µm² pixel size
- 4µm active area
- DCR: ~170Hz

- 1 bit memory per pixel (0 photons / ≥1 photons)
- **full frame rate >120kHz** (faster framerate for sub-regions)
- FPGA-based readout
- correlation in software (CPU/GPU)
- **near-ideal SNR:** better than EM-CCD

In Vitro SPIM-FCS Measurements Using SPAD Arrays

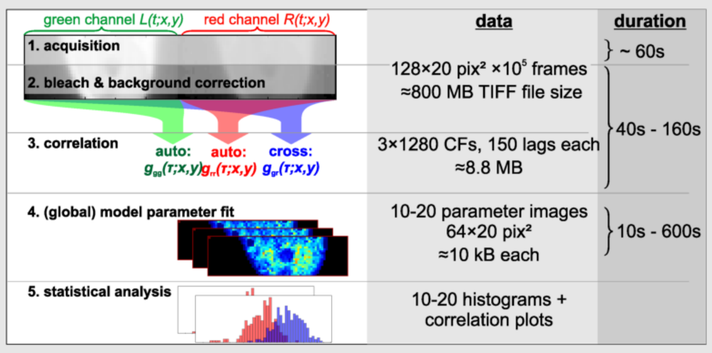


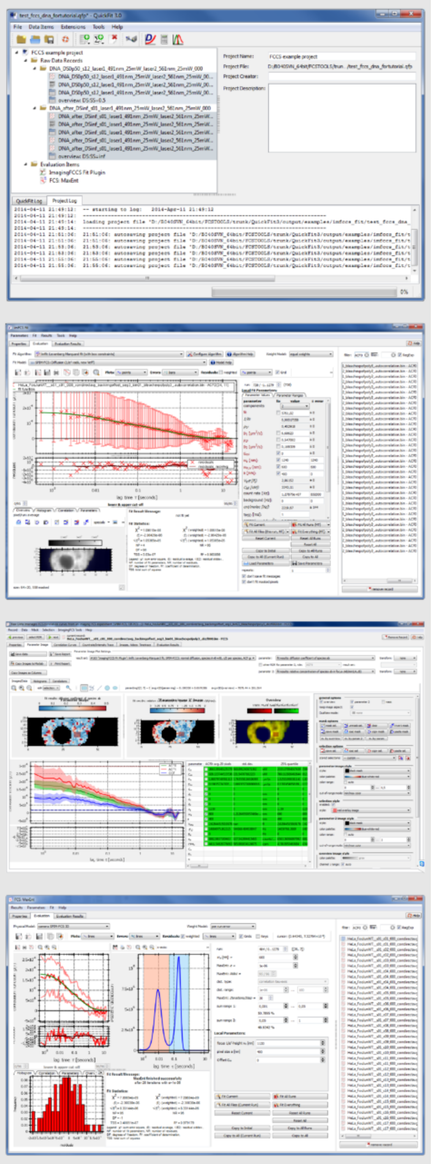
⇨ **fastest and largest imaging FCS sensor**
⇨ time resolution: 10^{-5}-10^{-6}s
⇨ **ACF analysis even for small particles (Alexa-488)**
⇨ But:
  ⇨ Noisy curves due to low photon counts
  ⇨ significant detector artifacts (afterpulsing)

⇨ **2-pixel CCF** analysis with global fits allows for "self-calibrating" measurement of **absolute diffusion coefficients**
⇨ 2-pixel CCF analysis helps to overcome detector artifacts,as they disappear in CCFs

QuickFit 3.0: A Comprehensie Open Source Data Evaluation Package for (imaging) FCS Methods

- contains a **project manager**
- implements all necessary computational methods to perform
  - **confocal FCS/FCCS**
  - camera-based **imaging FCS/FCCS**
  - **>70 fit models** (confocal, SPIM, TIRF, DLS, ...)
- advanced analysis methods:
  - **global fits (2-color/2-pixel FCCS)**
  - **maximum entropy** (MaxEnt)
  - **mean squared displacement** (MSD) evaluations
- implements the complete imaging FCS workflow:
  - lightsheet analysis
  - PSF analysis
  - camera calibration
- can be used to **control microscopes** used for imaging FCS/FCCS (SPIM/TIRF)
- optimized for **speed** (up to 150 fits/second) and **usability** even for **large datasets** (>100,000 correlation curves):



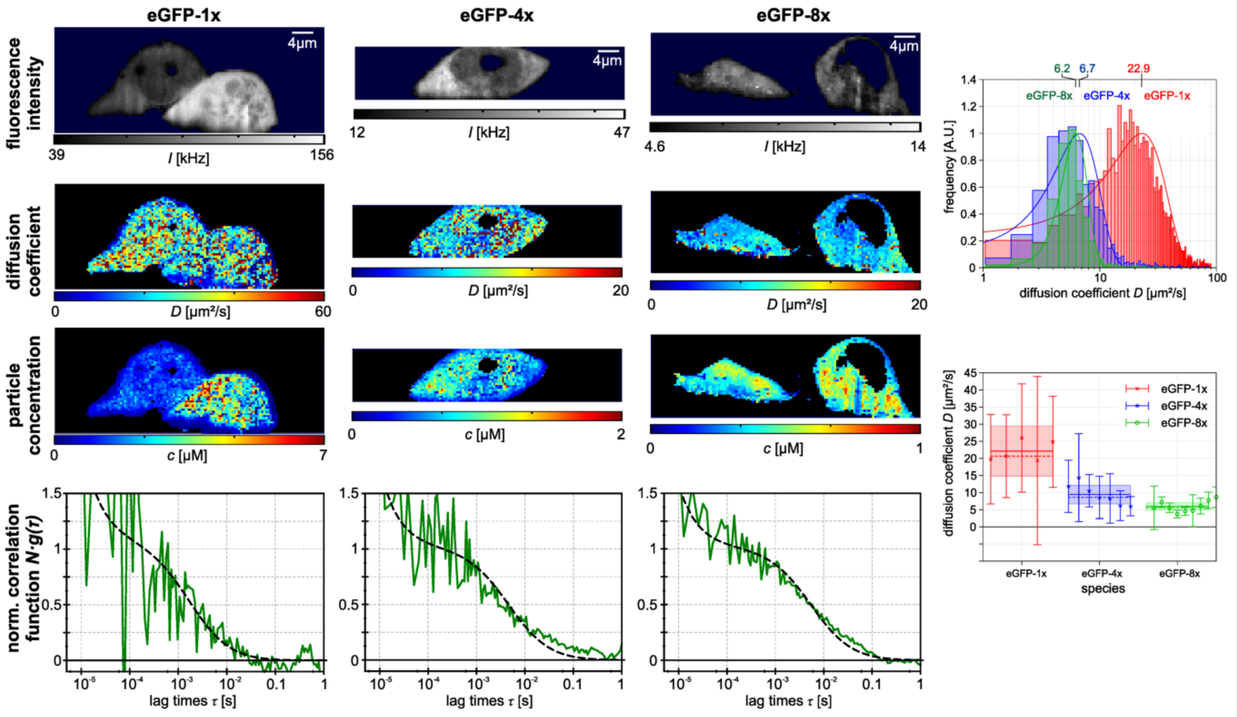


- **high-quality graphics**
- designed for open data-exchange:
  - **data export** to CSV, Excel, Matlab, TIFF, PDF, ...
  - **data import** from TIFF, CSV, ALV, Zeiss Confocor, correlator.com, PicoQuant TCSPC data, ...
- extendable by **plugins** (fit functions/algorithms, file formats, raw data types, evaluations, general extensions)
- written in **C++,** using Qt 5.4 for the user-interface
- **open-source** software (GPL3)

binaries, sources and example-data available for free:
http://www.dkfz.de/Macromol/quickfit/

First High-Speed Imaging FCS Measurements in Living Cells

In Vivo SPAD Array Measurements of eGFP-Oligomers in HeLa Cells



⇨ SPAD arrays are technically applicable to protein diffusion in live-cells
⇨ photo-sensitivity still has to be improved

[1] JW. Krieger, AP. Singh, CS. Garbe, T. Wohland, &J. Langowski.: **„Dual-color fluorescence cross-correlation spectroscopy on a single plane illumination microscope (SPIM-FCCS)"**. *Opt. express*, **22**(3), 2358-2375 (2014)
[2] A.P. Singh, J.W. Krieger, J. Buchholz, E. Charbon, J. Langowski, and T. Wohland, **„The performance of 2D array detectors for light sheet based fluorescence correlation spectroscopy."** *Opt. Express*, **21**, 8652-8668, (2013)
[3] S. Burri, Y. Maruyama, X. Michalet, F. Regazzoni, C. Bruschini, E. Charbon: **„Architecture and applications of a high resolution gated SPAD image sensor"**. *Opt. express*, **22**(14), 17573-17589. (2014)
[4] J. Buchholz, JW. Krieger, G. Mocsár, B. Kreith, E. Charbon, G. Vámosi, U. Kebschull, J. Langowski : **„FPGA implementation of a 32x32 autocorrelator array for analysis of fast image series"**. *Opt. express* **20**(16), 17767-17782 (2012)