Mapping Molecules Quantitatively in Confocal Fluorescence Microscopy

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PicoQuant

Single-molecule fluorescence microscopy has been established in the Life Sciences as an essential tool to study the characteristics and dynamics of individual fluorescent emitters both in vitro as well as in vivo. Still, acquiring quantitative information from the confocal observation volume is a challenging task. The knowledge of the absolute number or concentration of proteins in, e.g., cellular structures can significantly improve our understanding of cell biology towards quantitative microscopy.
Here, a new quantitative analytical tool is presented based on recording coincident photons. The approach, Counting by Photon Statistics (CoPS), relies on a statistical analysis of detected photon coincidences to estimate the number of independent fluorescent labels in the observation volume [1]. Hereby, CoPS exploits the photon antibunching effect: A single photon emitter can only generate one photon at a time.

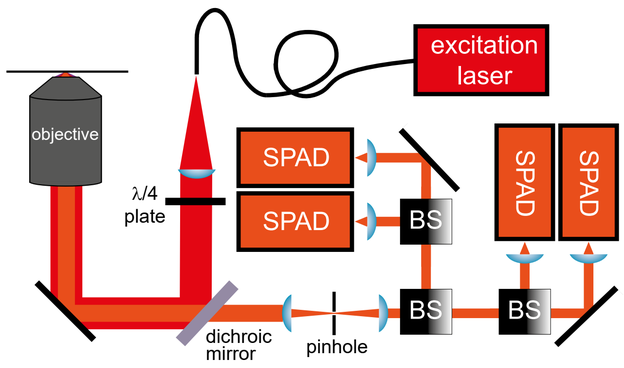
Originally developed for point measurements, CoPS recently has been extended to an imaging scheme [2]. Using a confocal fluorescence microscope setup (MicroTime 200, PicoQuant) with pulsed excitation, four single-photon detectors and parallel time-correlated single photon counting electronics we prove the applicability of the method with artificial model systems (immobilized DNA origami) and present first steps towards biological samples.

[1] Ta, H., Wolfrum, J., Herten, D.-P., An extended scheme for counting fluorescent molecules by photon-antibunching Laser Phys. 20:119 (2010).
[2] Ta, H. et al., Mapping molecules in scanning far-field fluorescence nanoscopy. Nat. Commun. 6:7977 (2015).

We thank Dirk-Peter Herten and Johan Hummert for sharing their expertise and for their valuable input.

Experimental Setup

Confocal time-resolved fluorescence microscope

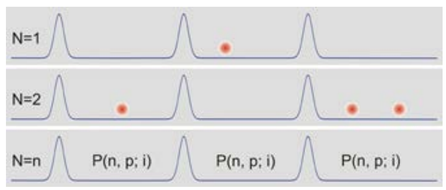


MicroTime 200
- Pulsed excitation laser, HydraHarp 400, four SPAD detectors (Excelitas, SPCM-AQRH-14-TR)
  - BS: 50/50 beam splitter
- Objective: UPlanSApo 100x/1.40 Oil (Olympus)
- Filters (rejection of excitation light and detector afterglow):
  - 640 nm excitation: bandpass BP690/70 in front of every SPAD
  - 485 nm excitation: longpass LP488, shortpass SP750 in front of every SPAD

Method

The principle behind **Counting by Photon Statistics (CoPS)** is similar to antibunching:
A single molecule can only emit one photon at a time.
Method developed by Dirk-Peter Herten, Heidelberg University

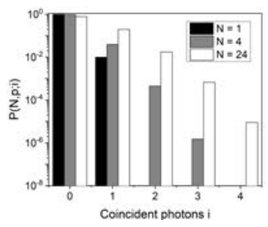
Detection of coincident photons
(photons that arrive after the same laser pulse)



The probability P(N,p;i) for i coincident photons can be calculated recursively for a known number N and brightness p of emitters.

Adapted from Grußmayer et al., Phys. Chem. Chem. Phys., 2017, Suppl.

Measurement of the distribution of multiple photon detection events

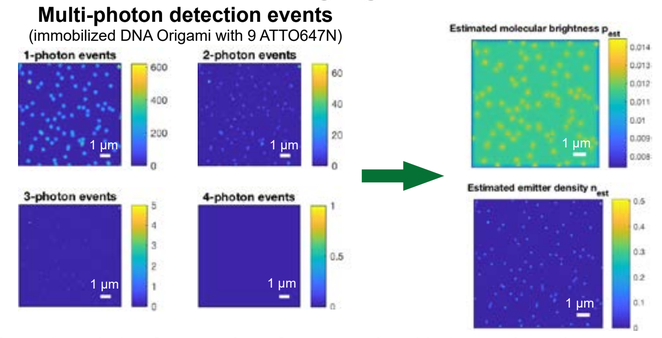


Relative probabilities depend on the number of emitters N, individual brightness p and number of detectors m.

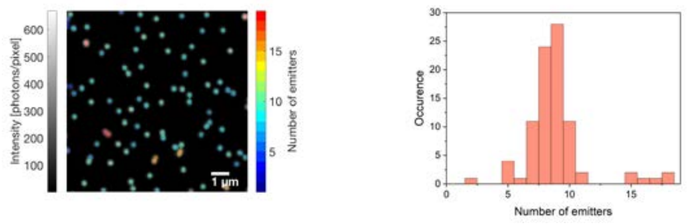
Adapted from K. Grußmayer et al., Phys.Chem.Chem.Phys. 19:8962 (2017)

Imaging

Multi-photon detection events
(immobilized DNA Origami with 9 ATTO647N)



From the relative frequencies of measured multi-photon detection events the molecular brightness p_{est} and emitter density n_{est} are estimated.
By summing over selected pixels, the number of emitters per cluster is calculated and visualized by a color code. The distribution of the number of emitters is shown in a histogram [2].



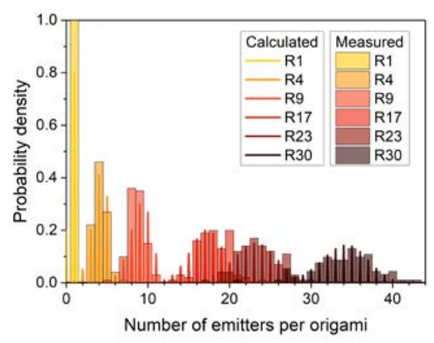
Proof of Principle: Red Origami

Red DNA-Origami with varying number of emitters (GattaQuant)
- 1 ATTO647N
- 4 ATTO647N
- 9 ATTO647N
- 17 ATTO647N
- 23 ATTO647N
- 30 ATTO647N

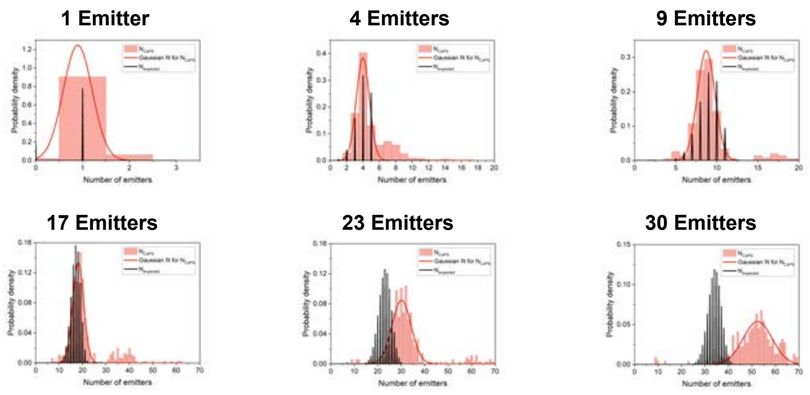
http://www.gattaquant.com/files/gatta-brightness_product_sheet_1.

Expected numbers of emitters per origami:
Calculation assuming binomial distribution with
- n binding sites
- binding probability p

Measured brightness for increasing numbers of emitters per origami:
- Number of detected photons per identified origami in image
- Normalized for one emitter

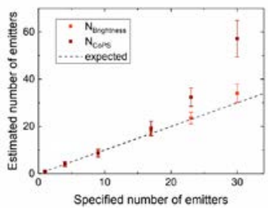


CoPS: Results with Red DNA Origami



CoPS **overestimates** the emitter number for higher numbers per cluster.

Possible issues:
- **Saturation** of detection electronics
- Detector **afterpulsing**
- **Interaction** of fluorophores in DNA origami

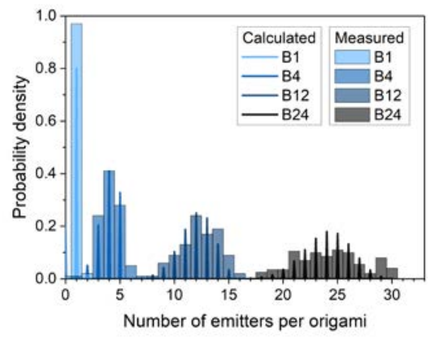


Proof of Principle: Blue/Green Origami

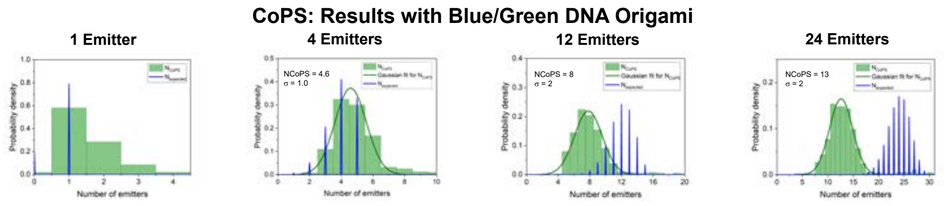
Blue/green DNA-Origami with varying number of emitters (GattaQuant)
- 1 ATTO488
- 4 ATTO488
- 12 ATTO488
- 24 ATTO488

Expected numbers of emitters per origami:
Calculation assuming binomial distribution with
- n binding sites
- binding probability p

Measured brightness for increasing numbers of emitters per origami:
- Number of detected photons per identified origami in image
- Normalized for one emitter



CoPS: Results with Blue/Green DNA Origami

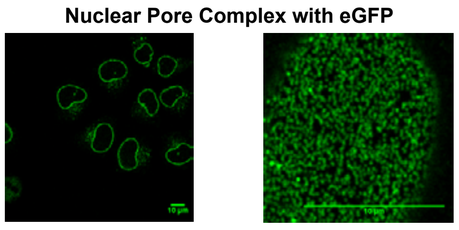


For shorter pixel dwell times and lower laser excitation powers used in the CoPS measurements, the resulting distributions approach the calculated values while the distributions broaden (data not shown).

Biological Samples

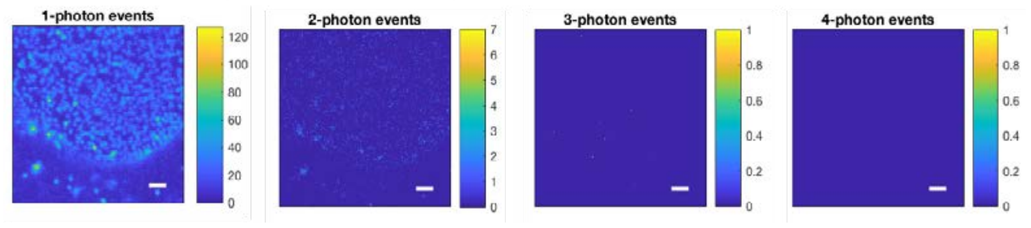
Additional challenges in biological samples
- Higher background
- Dense, overlapping clusters
- Not two-dimensional, differences in z-position of clusters
- Fluorescent proteins not as bright, but slightly more stable than Atto488

Nuclear Pore Complex with eGFP

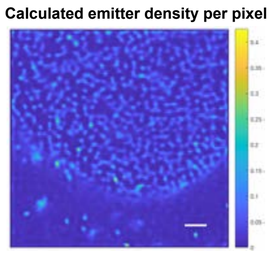


Homozygous cell line NUP214-mEGFP
Sample kindly provided by Arina Rybina, Antonio Politi, Jan Ellenberg, EMBL

Bottom of single interphase cell: Single nuclear pore complexes with 16 emitters each



Calculated emitter density per pixel



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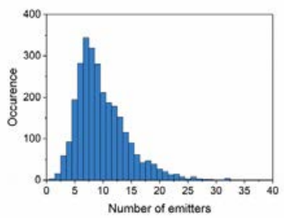
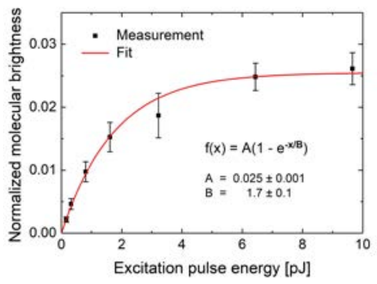
By summing over selected pixels, the number of emitters per cluster is calculated and visualized by a color code. The distribution of the number of emitters is shown in a histogram.

Histogram of emitter numbers per pore



Guidelines/Standard Parameters for CoPS Measurements

Fluorescence saturation curve for the determination of a suitable excitation power



Excitation power: The laser power is determined by a dye-specific saturation curve. To avoid saturating the emitters, the power should be chosen such that the normalized molecular brightness reaches 60% of its maximum.

Normalized molecular brightness
(calculated from images of immobilized DNA origami):
The PSF is fitted by a Gaussian function. Its amplitude is normalized to one emitter and one laser pulse.

Alignment: < 10% difference of SPAD detection efficiencies
Repetition rate: 10 MHz (due to electronics dead times)
Pixel size: 20 nm
Point Spread Function (PSF): Full width at half maximum (FWHM) determined by imaging fluorescent beads or origami

Matlab code (by Haisen Ta) and demo data available by PicoQuant.