Mapping Molecules Quantitatively in Confocal Fluorescence Microscopy

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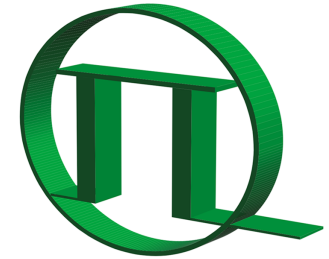
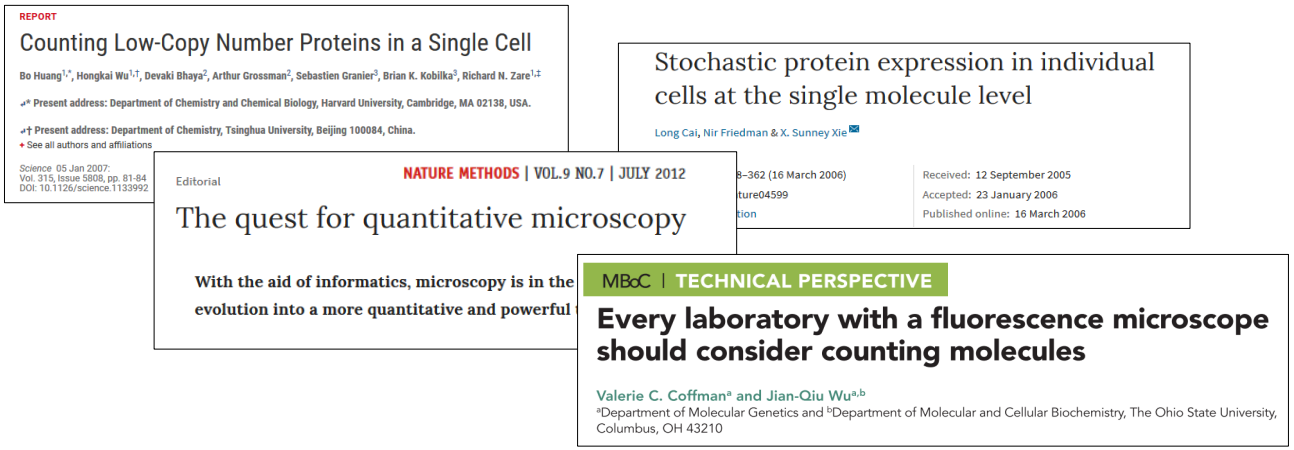
PicoQuant GmbH, Rudower Chaussee 29, 12489, Berlin, Germany



PICOQUANT

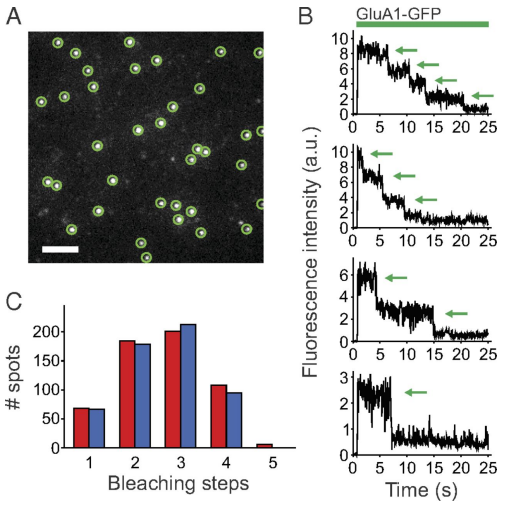
24th International Workshop on
“Single Molecule Spectroscopy and Super-resolution Microscopy in the Life Sciences”
September 12-14, 2018 in Berlin, Germany

Quantification of Molecules



→ Variety of research objectives for counting and mapping of molecules and their concentrations in cells

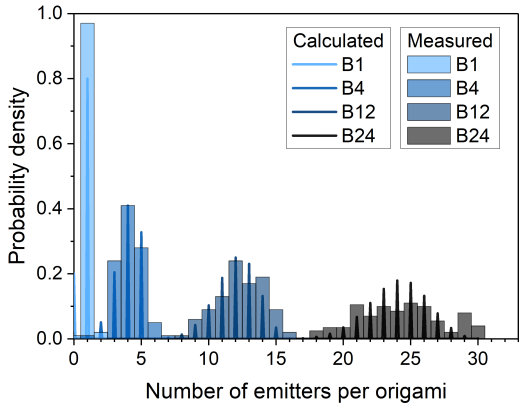
Stepwise Photobleaching



P. Hastie *et al.*, PNAS, 2013

- Limited to small numbers
- Destructive

Comparing Fluorescence Intensity to a Known Standard



V. Coffman *et al.*, Trends in Biochem. Sc., 2012

- Needs calibration measurements

Fluctuation Analysis Based Methods

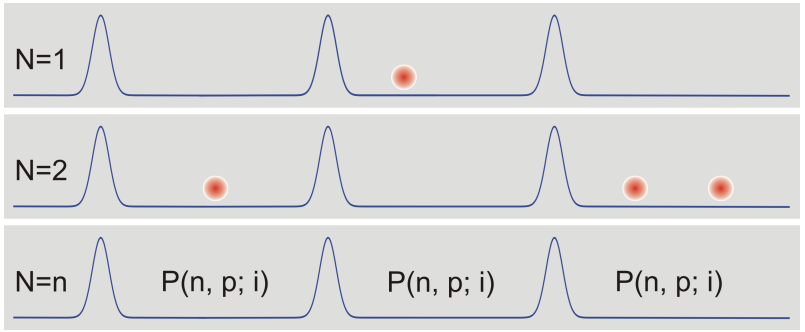
- FCS / FLCS
- PCH
- N&B
- …

Localization Microscopy

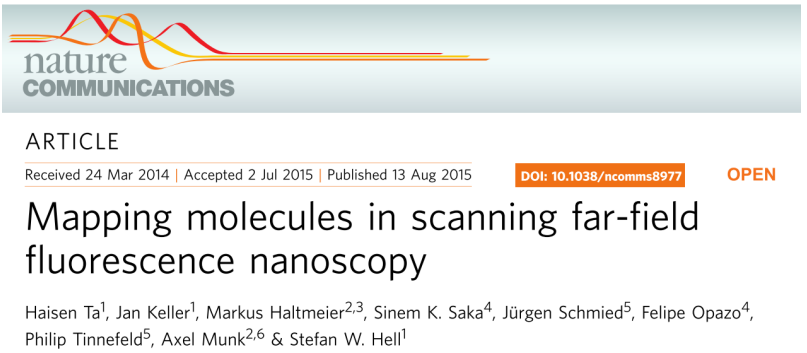
- PALM
- dSTORM
- PAINT
- …

Outline

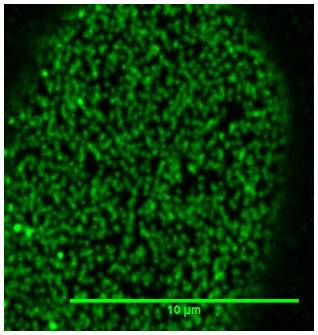
THE METHOD:
Counting by Photon Statistics (CoPS)



PROOF OF PRINCIPLE:
Measurements with Origami



TOWARDS BIOLOGICAL SAMPLES:
First results



Counting by Photon Statistics (CoPS)

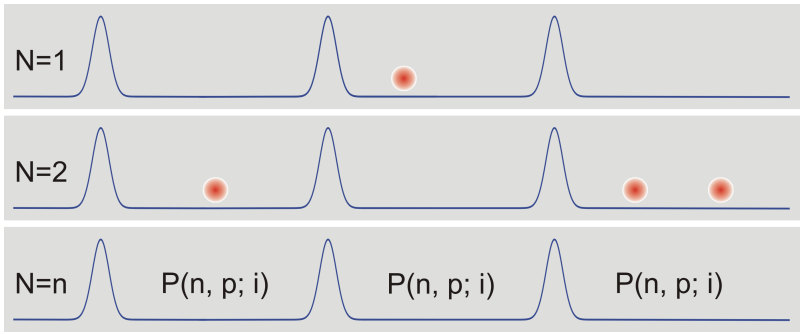
The Principle behind
Counting by Photon Statistics (CoPS)
is similar to antibunching:

A single molecule can only emit one photon at a time.

Method developed by Dirk-Peter Herten, Heidelberg University

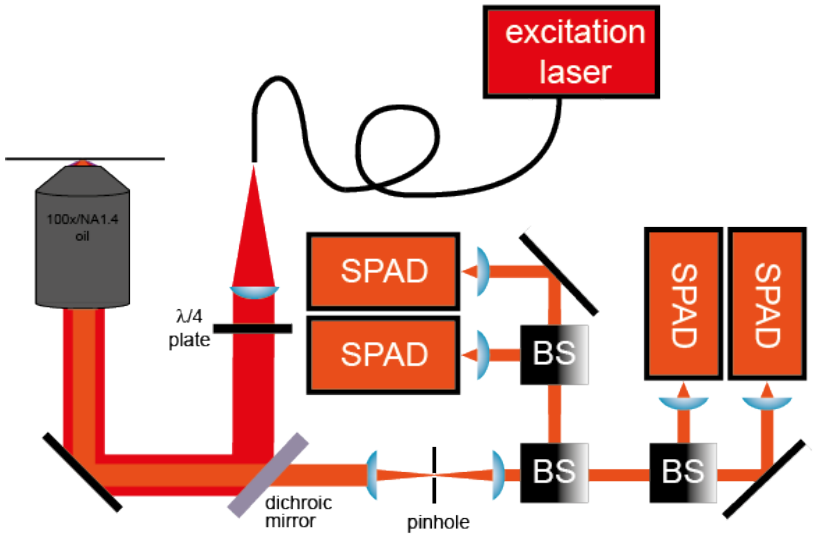
Detection of coincident photons

(photons that arrive after the same laser pulse)



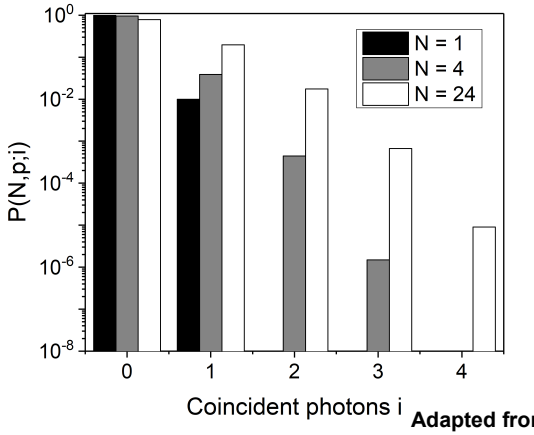
Adapted from Grußmayer et al., Phys. Chem. Chem. Phys., 2017, Suppl.

Confocal microscope with pulsed excitation and four detectors



Measurement of the distribution of multiple photon detection events

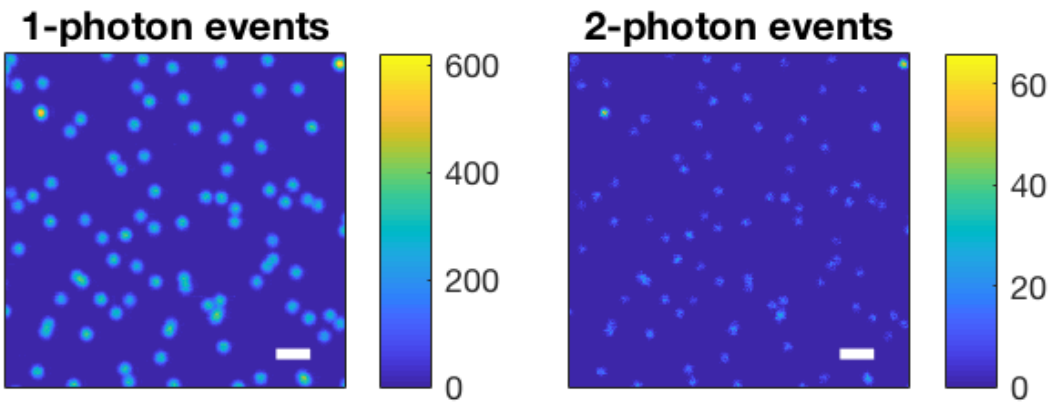
Relative probabilities depend on number of emitters N, individual brightness p and number of detectors m.



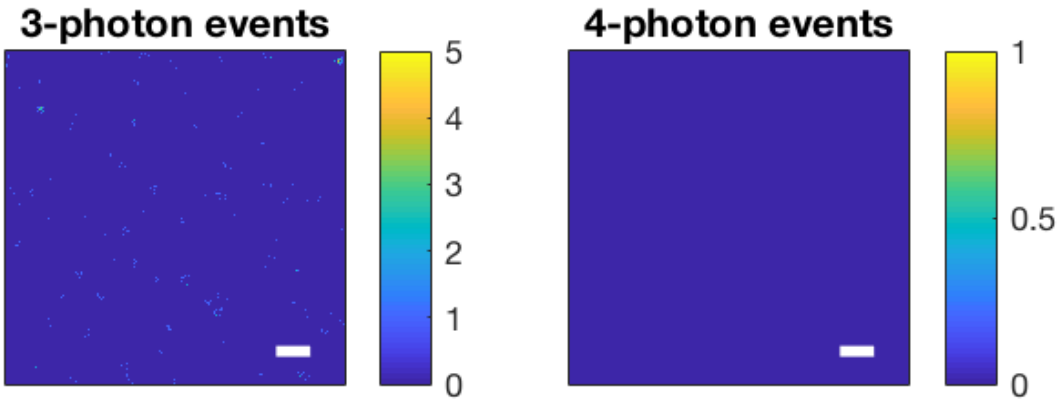
Adapted from Grußmayer et al., Phys. Chem. Chem. Phys., 2017, Suppl.

Mapping Molecules based on Counting by Photon Statistics (CoPS)

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

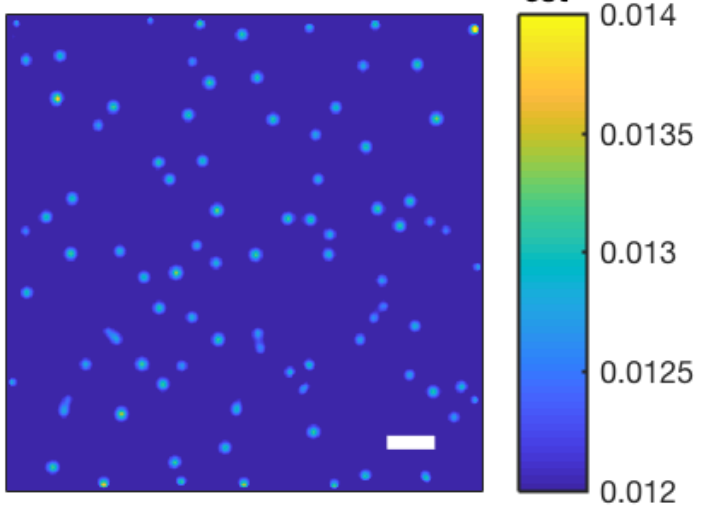


scale bar: 1 µm

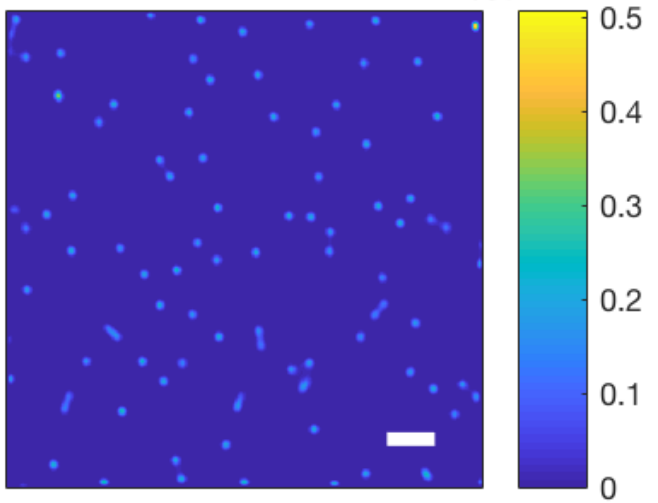


Method published by Haisen Ta *et al.*, Nature Communications, 2015

Estimated molecular brightness p_{est}

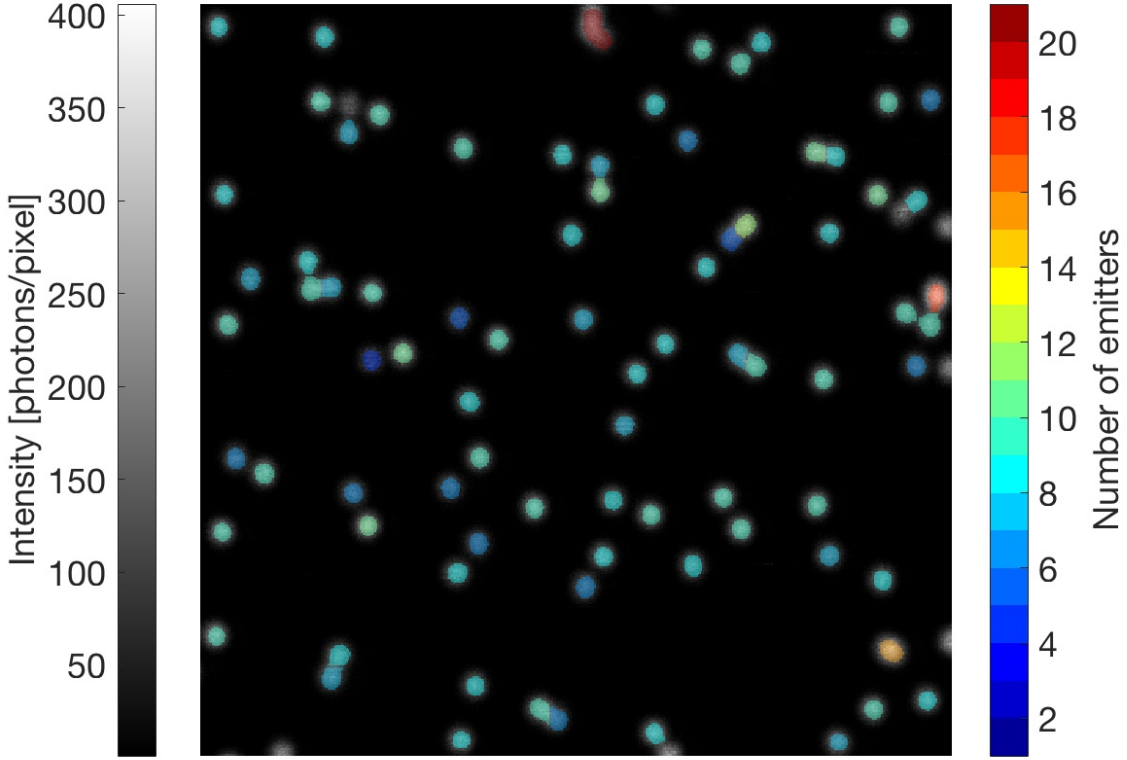


Estimated emitter density n_{est}



Map of Molecule Distributions

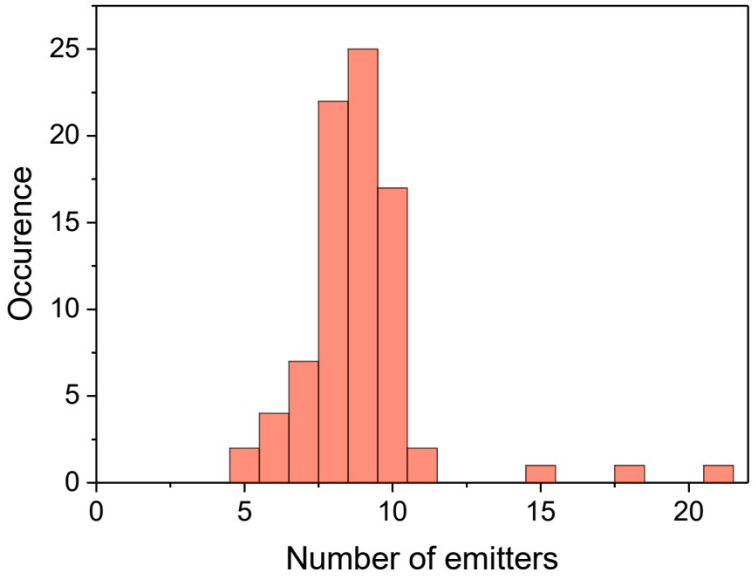
Analysis of multi-photon detection events
(immobilized Origami with 9 ATTO647N)



Grey scalebar: Intensity [photons /pixel];
Color scalebar: Number of emitters per spot (summed up density per pixel)

Parameters:
10 µW excitation, 300 µs px dwell time, 20nm px size, 10 MHz, 500 x 500 px

Histogram of the number of emitters in one origami



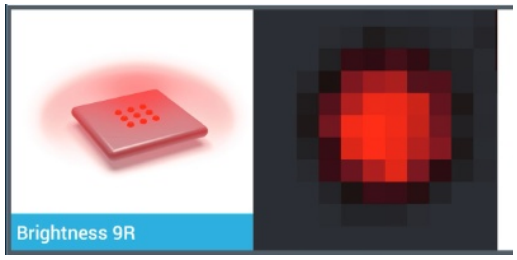
Method published by Haisen Ta *et al.*, Nature Communications, 2015

Analysis Software kindly provided by Haisen Ta

Proof of Principle: Red DNA Origami

Red DNA-Origami with varying number of emitters (GattaQuant)

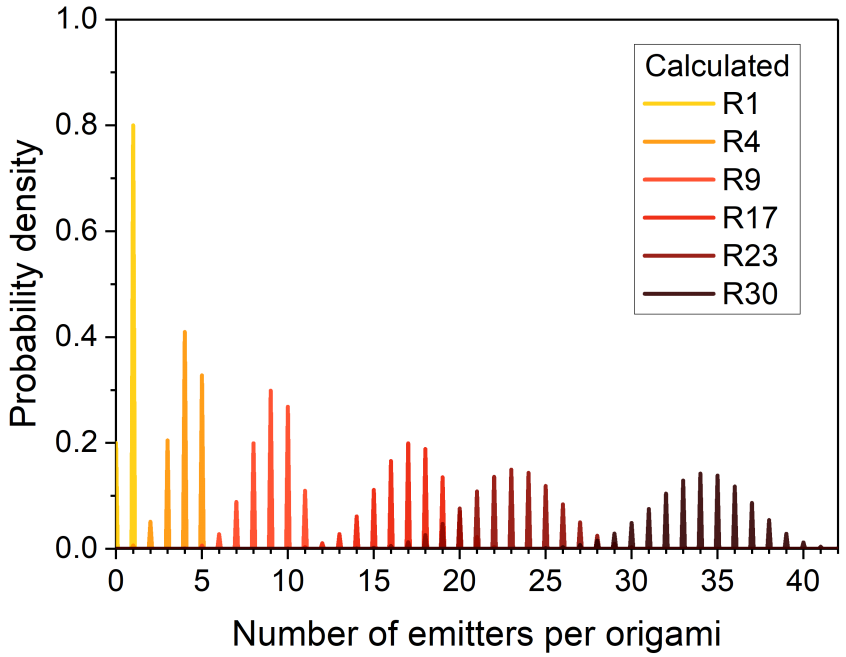
- 1 ATTO647N
- 4 ATTO647N
- 9 ATTO647N
- 17 ATTO647N
- 23 ATTO647N
- 30 ATTO647N



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

Expected numbers of emitters per origami:
Calculation assuming binomial distribution with

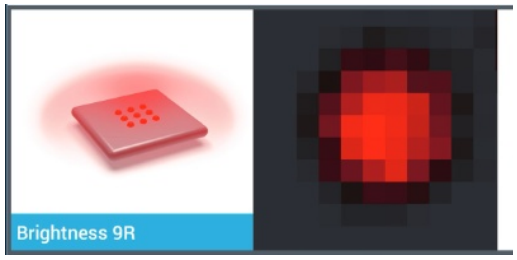
- n binding sites
- binding probability p



Proof of Principle: Red DNA Origami

Red DNA-Origami with varying number of emitters

- 1 ATTO647N
- 4 ATTO647N
- 9 ATTO647N
- 17 ATTO647N
- 23 ATTO647N
- 30 ATTO647N

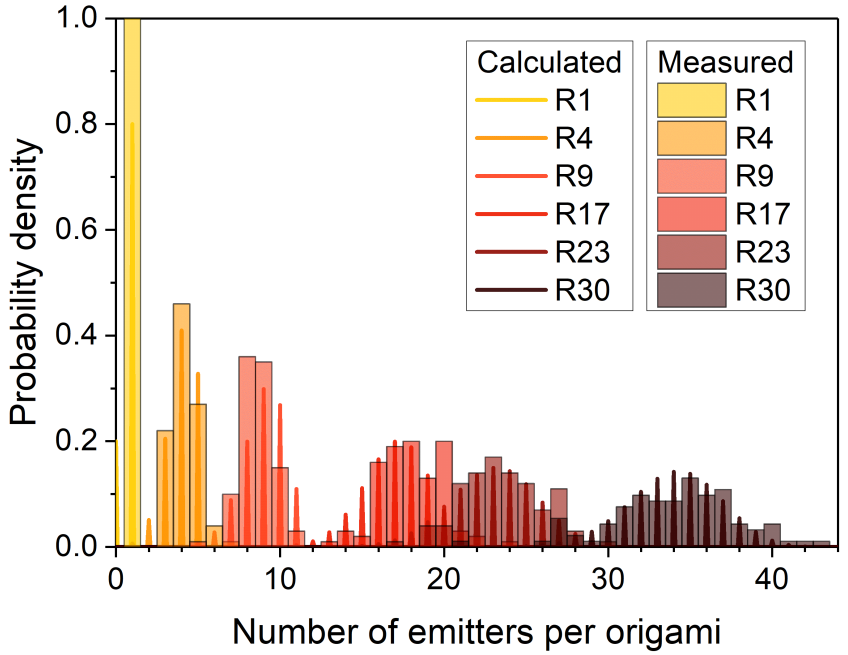


http://www.gattaquant.com/files/gatta-brightness_product_sheet_1.pdf

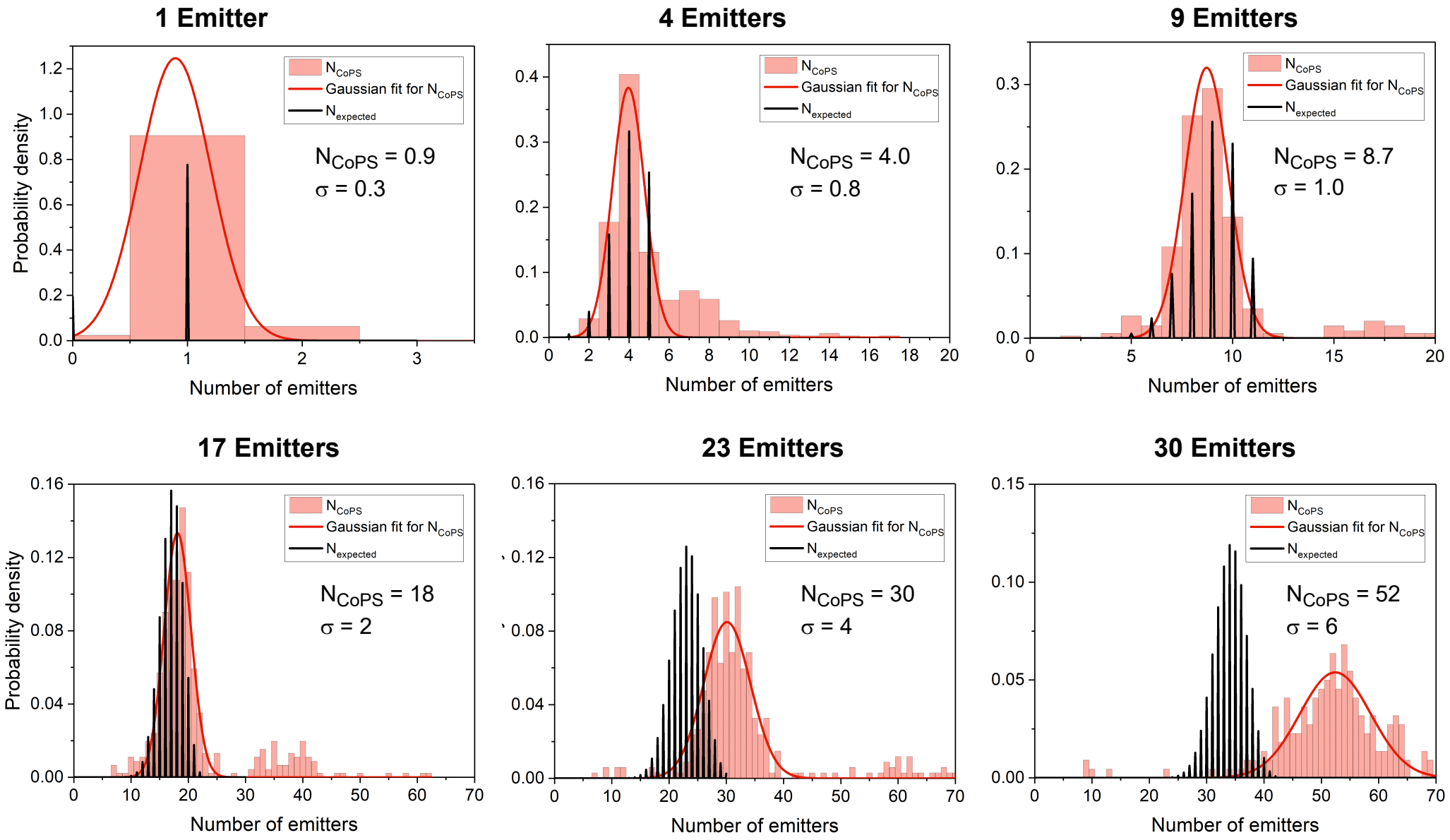
Expected numbers of emitters per origami:
Calculation assuming binomial distribution

Measured brightness for increasing numbers of emitters per origami:

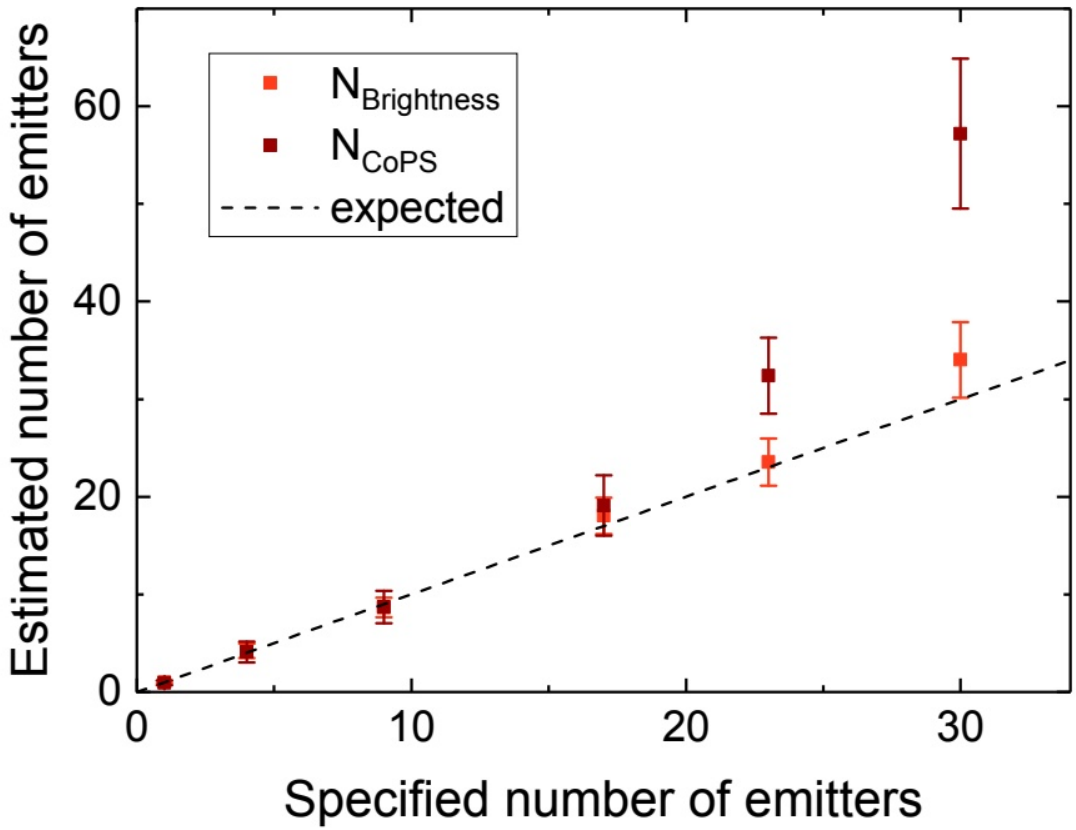
- Number of detected photons per identified origami in image
- Normalized for one emitter



Counting by Photon Statistics: Results with Red DNA Origami



Red DNA Origami: Overestimation for Higher Numbers



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

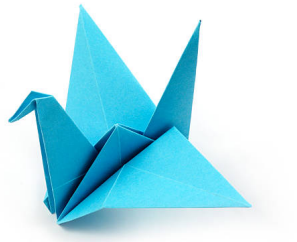
Possible issues:

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

Proof of Principle: Blue/Green DNA Origami

Blue/green DNA-Origami with varying number of emitters (GattaQuant)

- 1 ATTO488
- 4 ATTO488
- 12 ATTO488
- 24 ATTO488

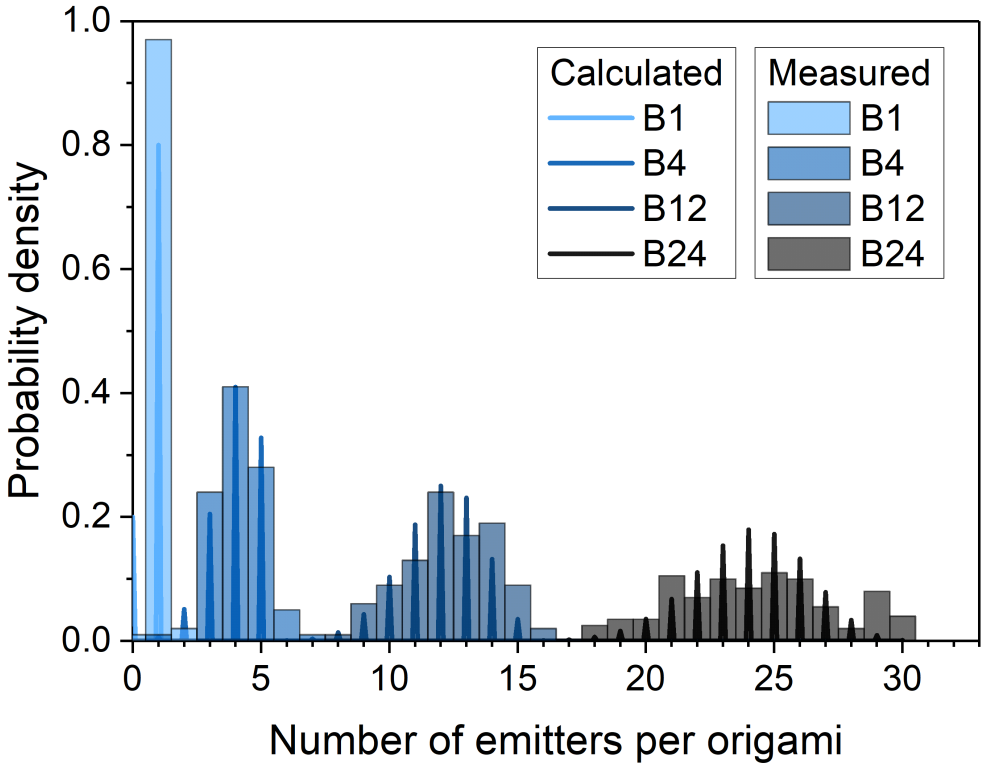


Expected numbers of emitters per origami:
Calculation assuming binomial distribution with

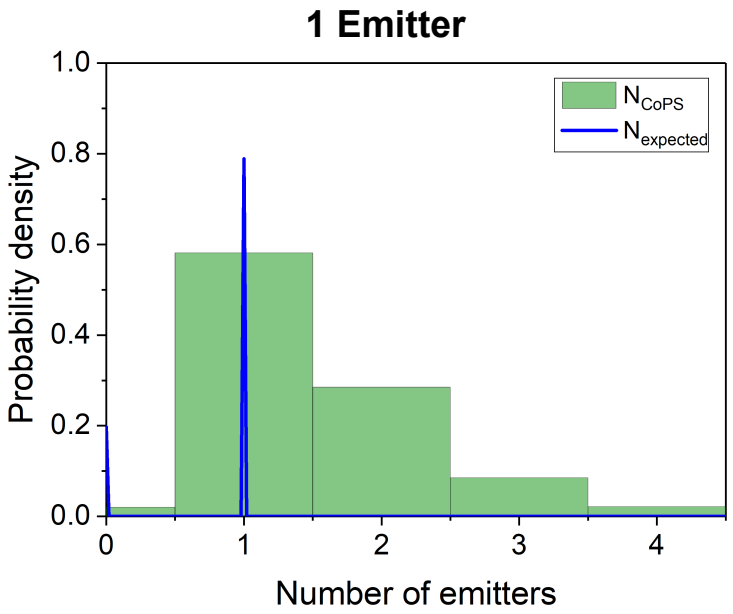
- n binding sites
- binding probability p

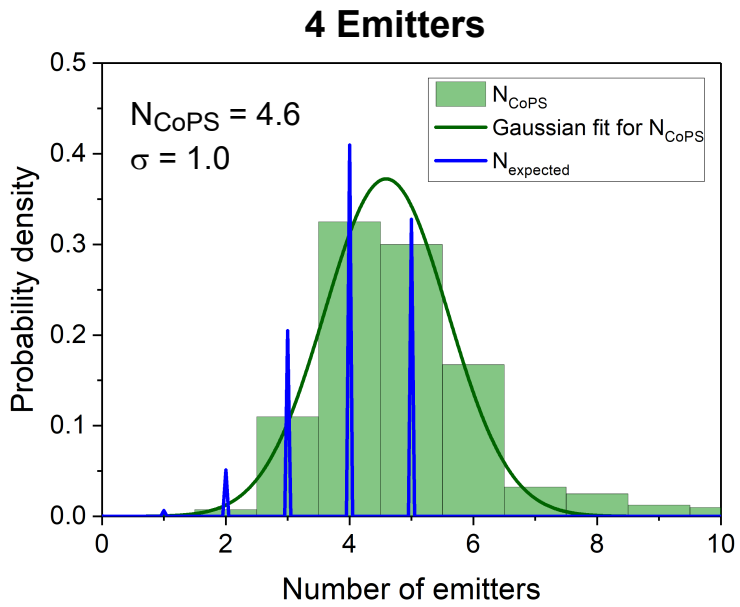
Measured brightness for increasing numbers of emitters per origami:

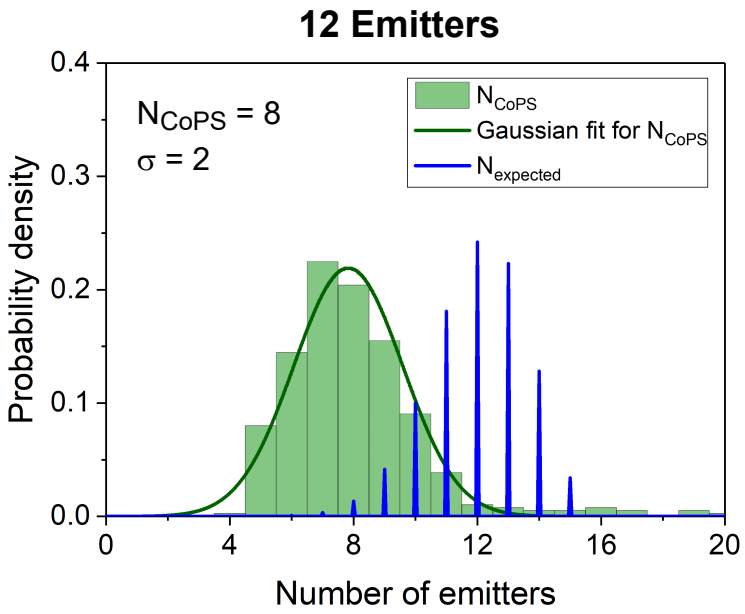
- Number of detected photons per identified origami in image
- Normalized for one emitter

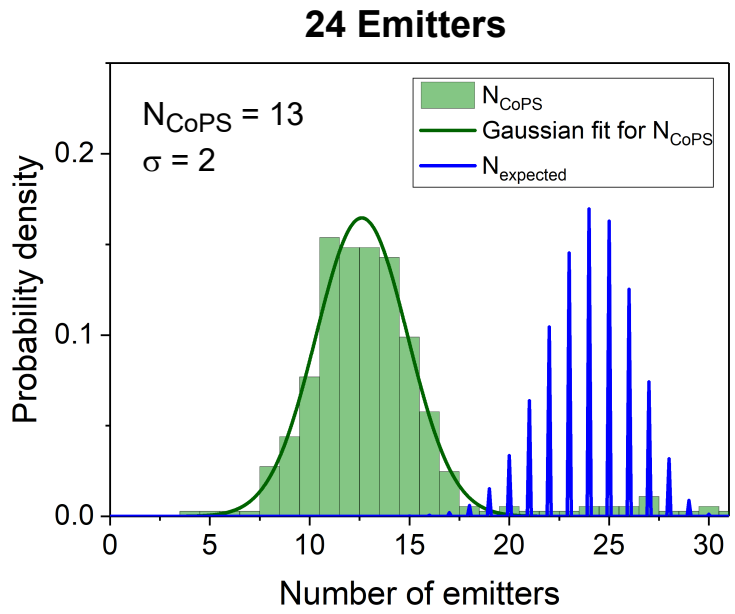


Counting by Photon Statistics: Results with Blue/Green DNA Origami



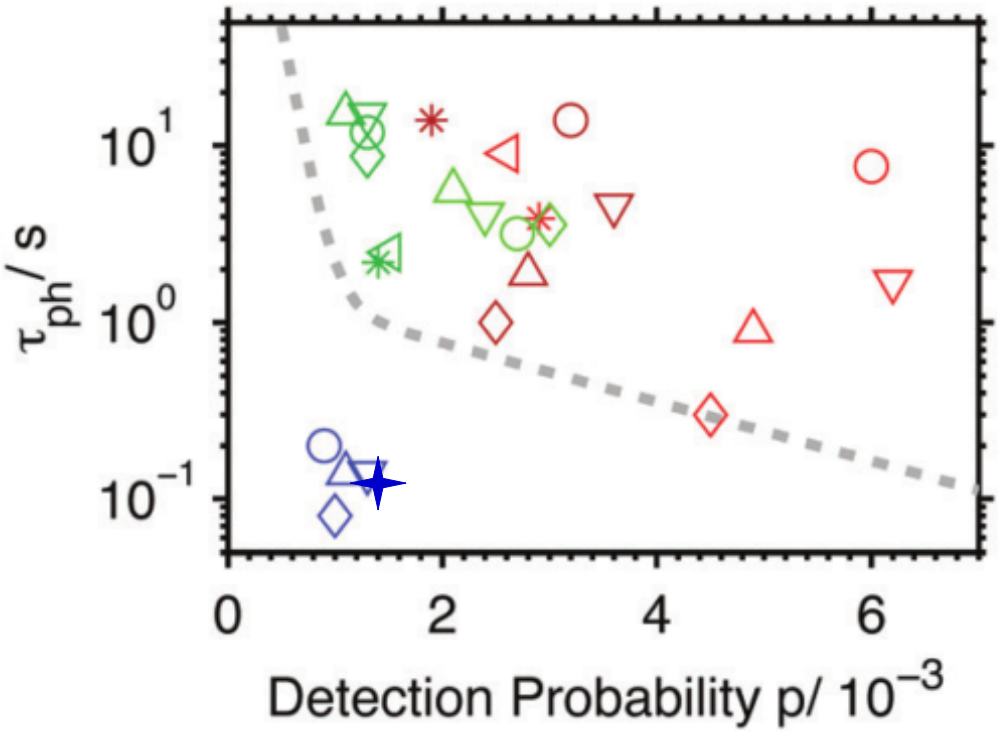






Counting by photon Statistics across the Visible Spectrum

Identification of suitable fluorophores



Photostability time τ_{ph} *versus* detection probability p for

- red (640 nm excitation),
- green (532 nm excitation) and
- blue (470 nm excitation) dyes.

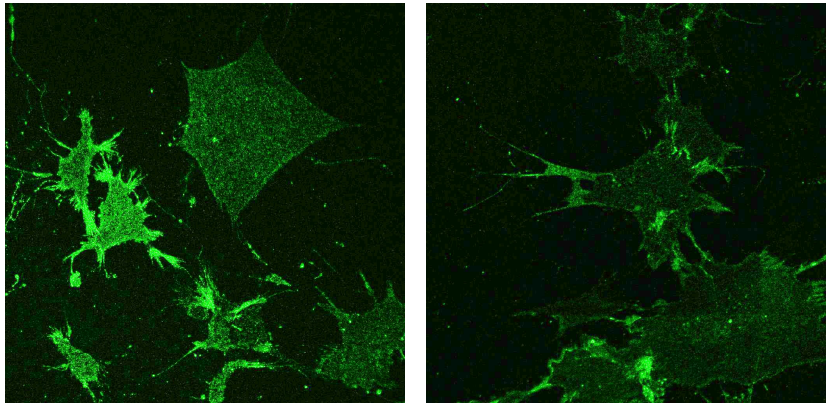
Grey line: Minimum photostability time to retain 90% of all emitters

Published by Grußmayer et al., Phys. Chem. Chem. Phys., 2017

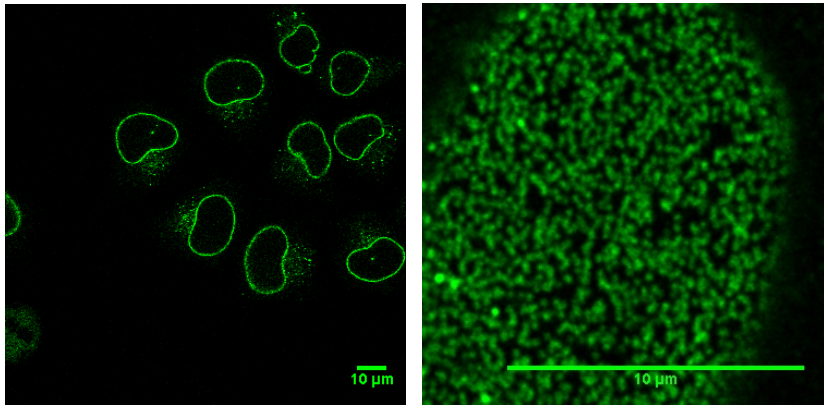
Sample ✦ **:**Measured photostability time τ_{ph} and detection probability p for Origami with 4 ATTO488 (normalized to one emitter)

Biological Samples

Plasma membranes with YFP
S. Munck, KU Leuven



Nuclear Pore Complex with eGFP
A. Rybina, A. Politi, J. Ellenberg, EMBL, Heidelberg

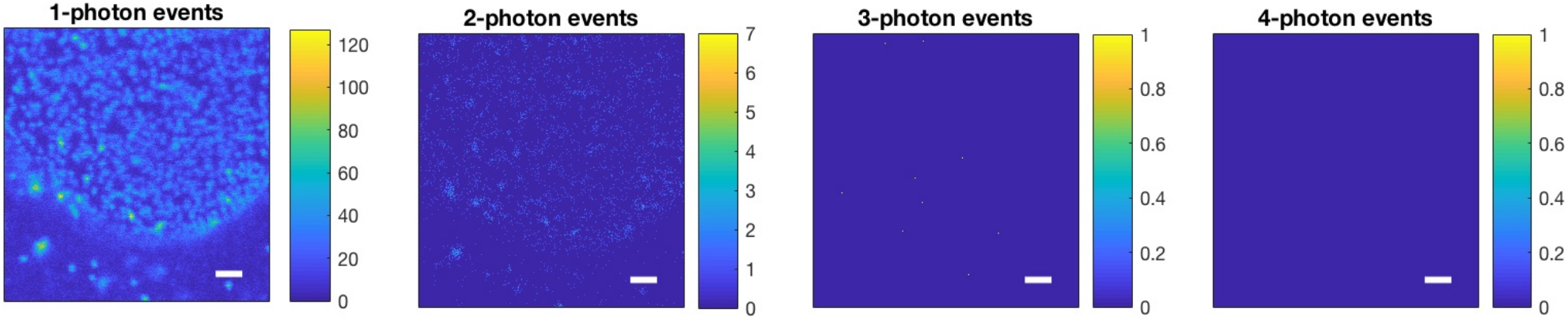


Additional challenges in biological samples

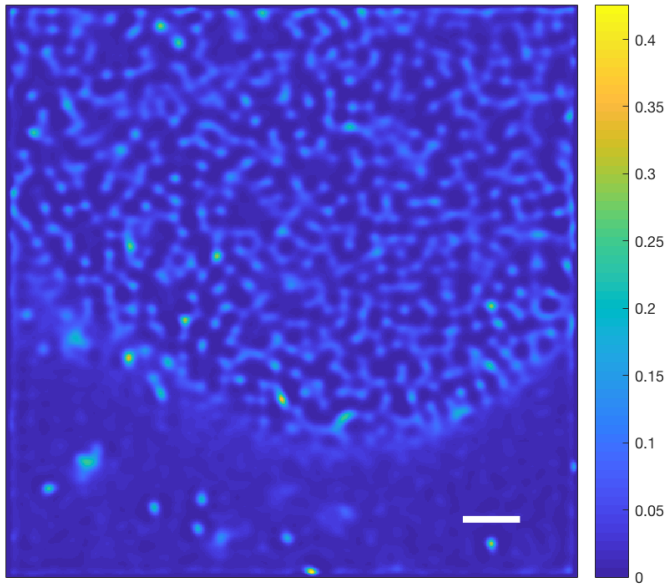
- Even higher background
- Very dense, overlapping clusters
- Not two-dimensional, differences in z-position of clusters
- Fluorescent proteins not as bright, but slightly more stable than Atto488

Biological Samples:
Nuclear Pore Complex (16 Emitters expected, EGFP)

Bottom of single interphase cell: Single pores with 16 emitters each

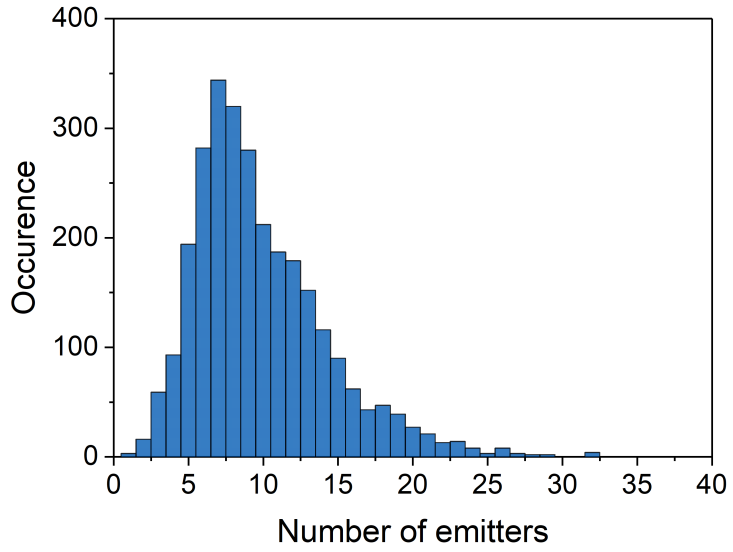


Calculated emitter density per pixel



scale bar: 1 µm

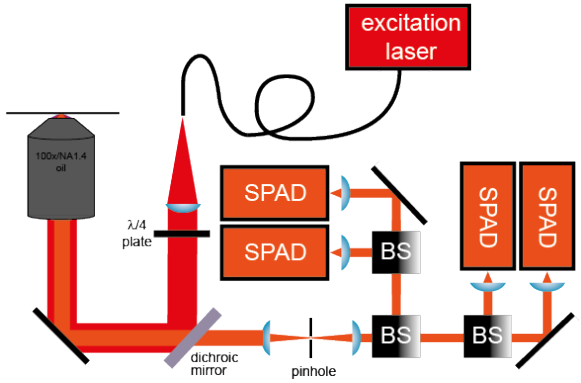
Histogram of emitter numbers per pore



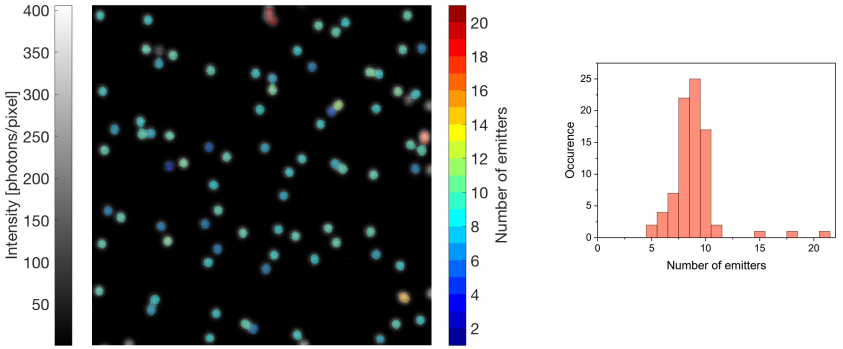
Homozygous cell line NUP214-mEGFP
(Sample kindly provided by Arina Rybina, Antonio Politi, Jan Ellenberg, EMBL)

Summary of What Works So Far...

Data Acquisition with MicroTime200, four SPADs and HydraHarp



Analysis with Matlab-Software from Haisen Ta



Sample Requirements

- Fixed sample with low background
- Bright and stable fluorophores (preferably 640 nm excitation)
- Quantification of single clusters
- Less than 10 Emitters
- Narrow distribution of emitter numbers
- 2D

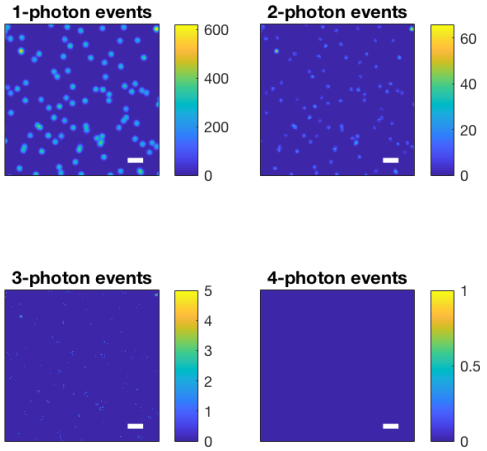
Interested?

Please contact us!

PicoQuant

Acknowledgement

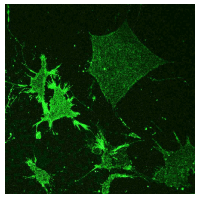
Haisen Ta



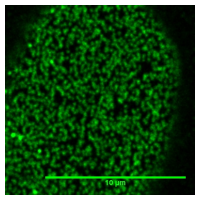
Dirk-Peter Herten and lab members **Johan Hummert** and **Wioleta Chmielewicz**, Heidelberg University

Sample preparation

- **Sebastian Munck**
  KU Leuven



- **Arina Rybina, Antonio Politi, Jan Ellenberg,**
  EMBL, Heidelberg



Caroline Berlage, M.Sc. student
(Supervisor **Oliver Benson**, HUB)

Poster P2: Molecular Counting by Photon Statistics

- Experimental parameters
- Origami with Atto488
- Photobleaching
- Limitations and Outlook