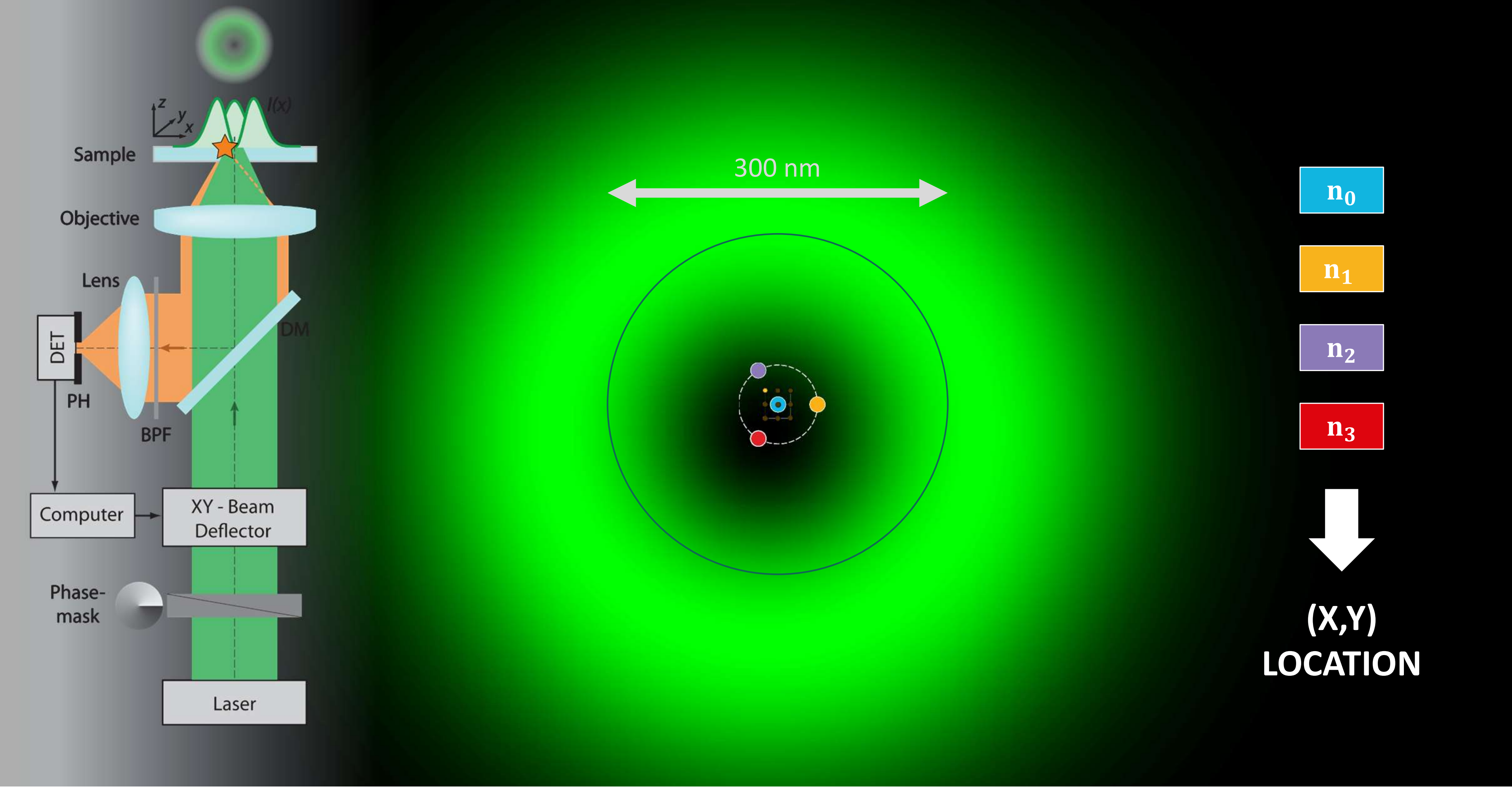
MINFLUX for Super Resolution Microscopy and Single Molecule Tracking

Francisco Balzarotti

2D, 3D MINFLUX, Optical Systems, Iterative MINFLUX
12.07.2022

IMP Research Institute of Molecular Pathology
Part of Vienna BioCenter

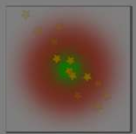
MINFLUX IN 2D



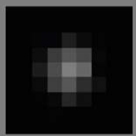
MINFLUX PERFORMANCE

SPATIAL RESOLUTION

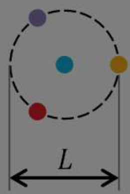
- INDEPENDENT OF WAVELENGTH AND N.A.
- NOT BASED ON PHOTOPHYSICAL SATURATION
- TUNABLE SPATIO-TEMPORAL RESOLUTION



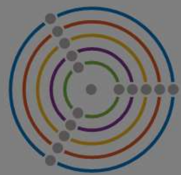
$\sigma_{STED} \propto \frac{\lambda}{NA}\frac{1}{\sqrt{1+I/}}$



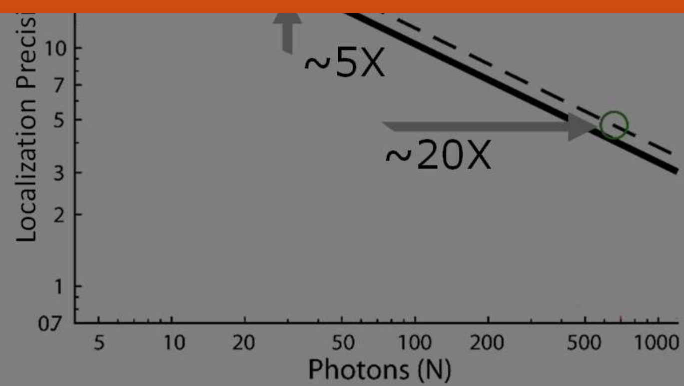
$\sigma_{CAM} \propto \frac{\lambda}{NA}\frac{1}{\sqrt{N}}$



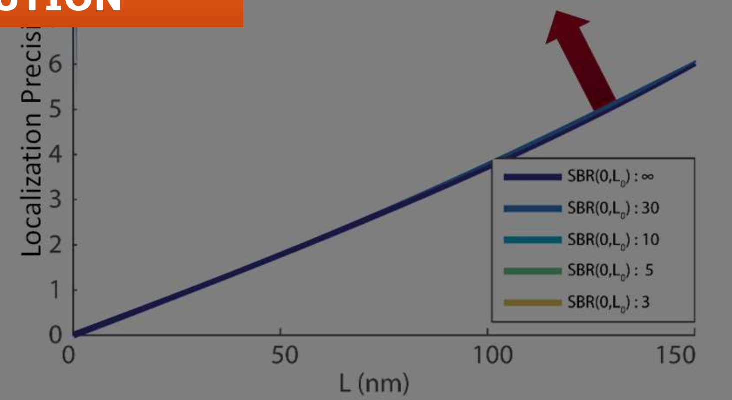
$\sigma_{MF} \propto \frac{L}{\sqrt{N}}$



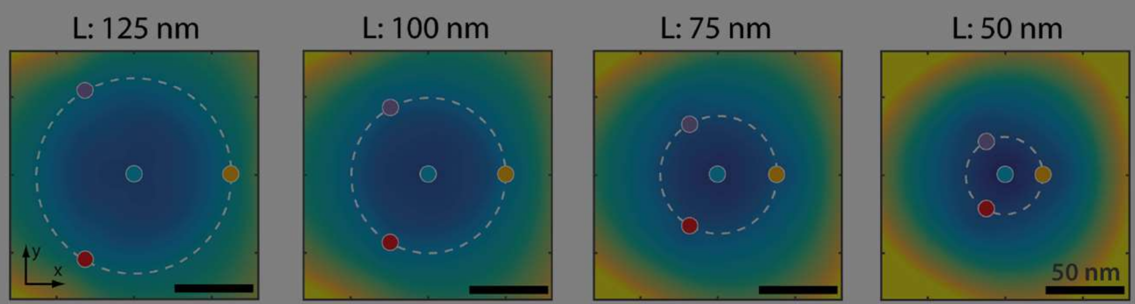
VS PHOTON NUMBER

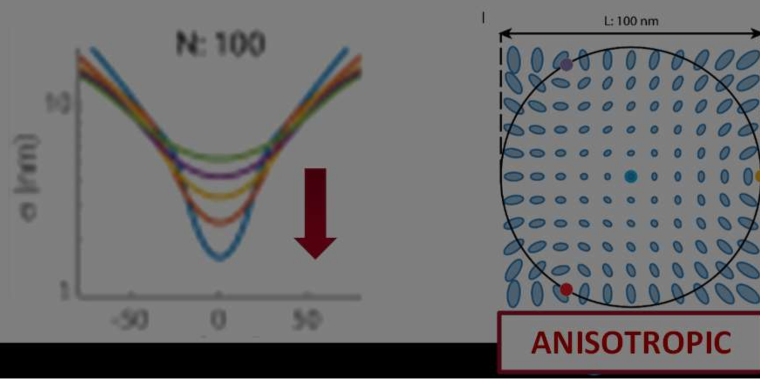


VS RULER SIZE 'L'



VS MOLECULE POSITION

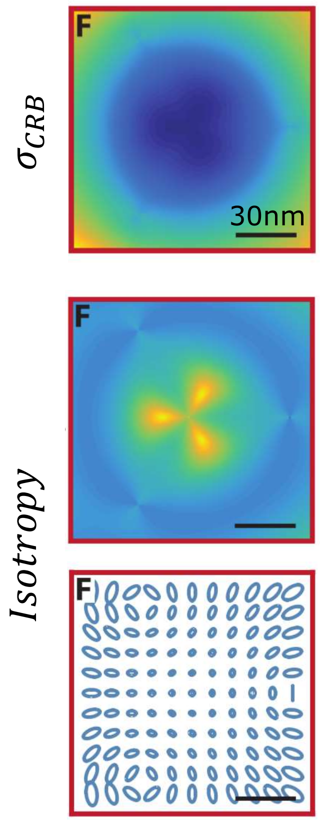


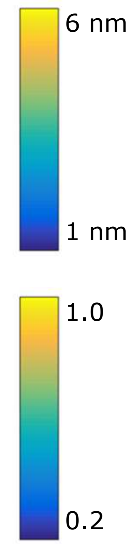


WHAT ABOUT MORE BEAMS?

Number of exposures:

K=4

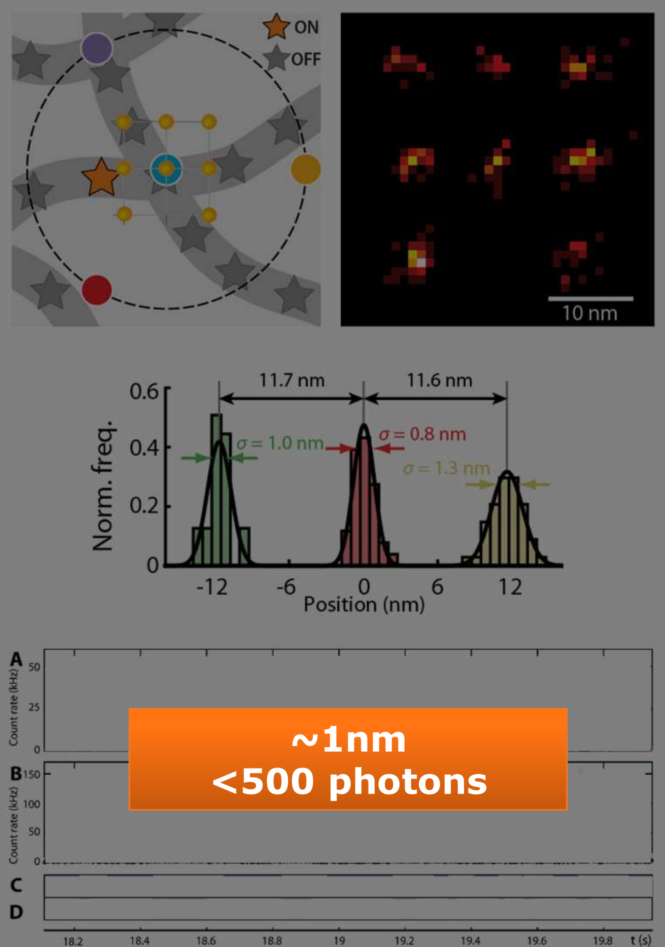




MINFLUX APPLICATIONS

NANOSCOPY

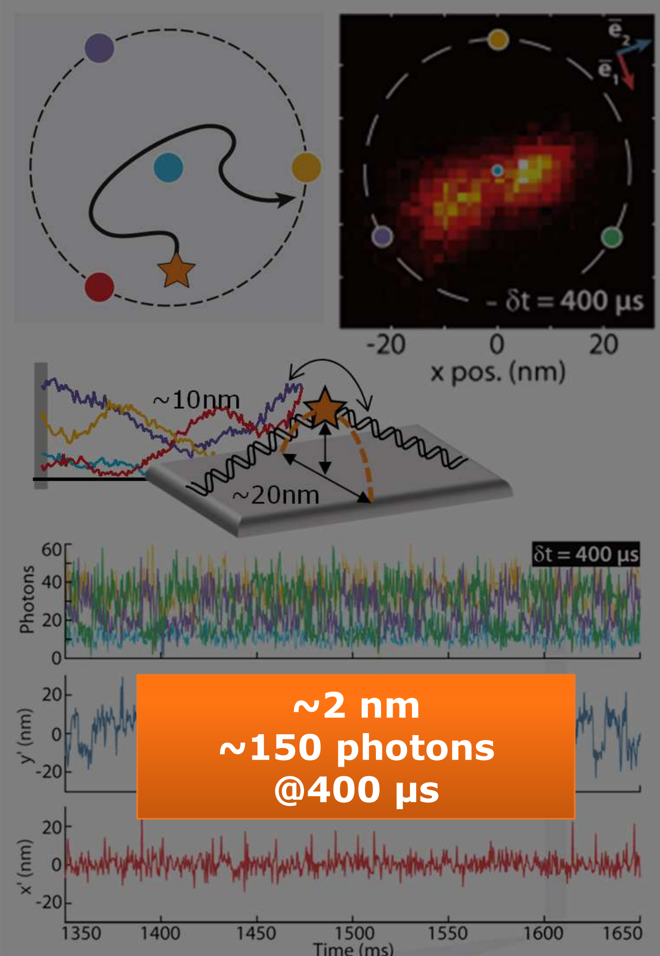
DNA Origami



~1nm
<500 photons

TRACKING ~nm RANGE

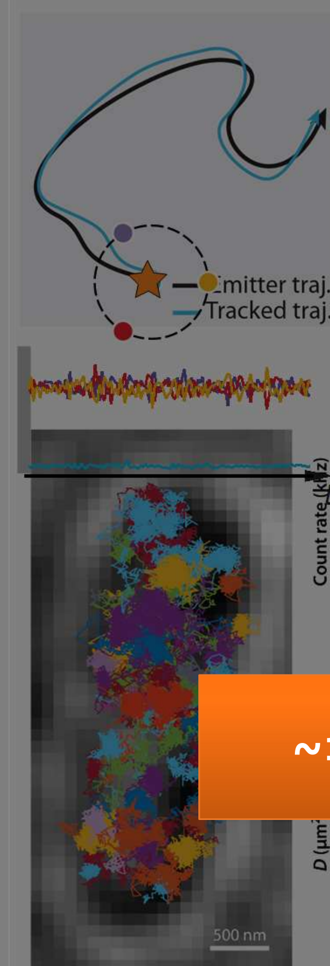
DNA Origami

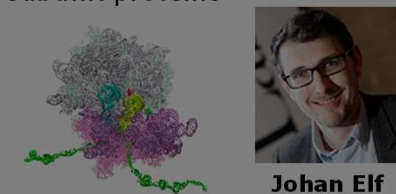


~2 nm
~150 photons
@400 µs

TRACKING ~µm RANGE

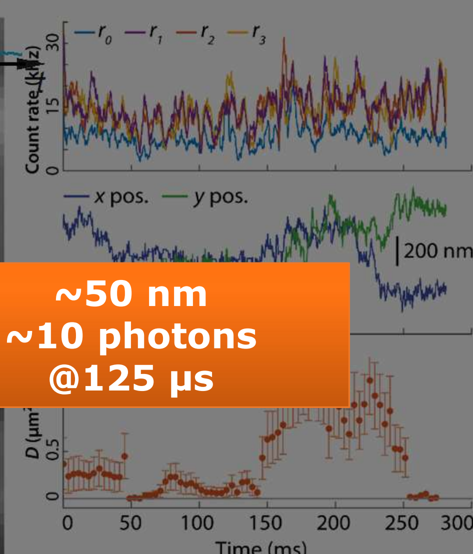
Ribosomal subunit proteins





Johan Elf
Uppsala University

	Camera	MINFLUX
Localizations	4-15	100X 742
Time resolution	10-50 ms	125 µs
Tracking error	20-60 nm	50 nm

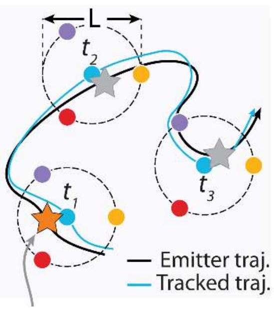


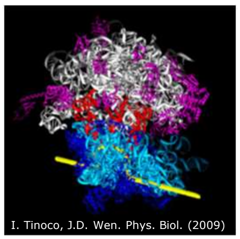
~50 nm
~10 photons
@125 µs

F. Balzarotti, et. al. *Science*, 355(6325), 606-612 (2017)
Y. Eilers, et. al. *PNAS*, 115, 6117-6122 (2018)

RIBOSOME SUBUNIT PROTEIN TRACKING IN E. COLI

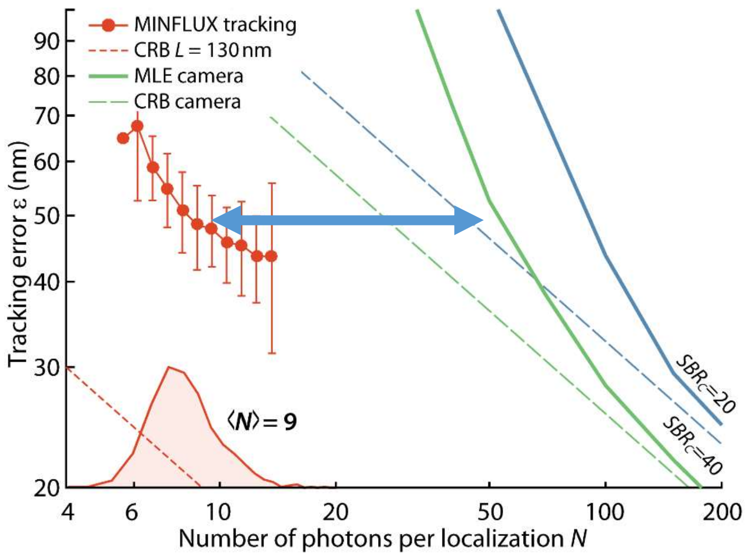
Large range tracking ~ µm

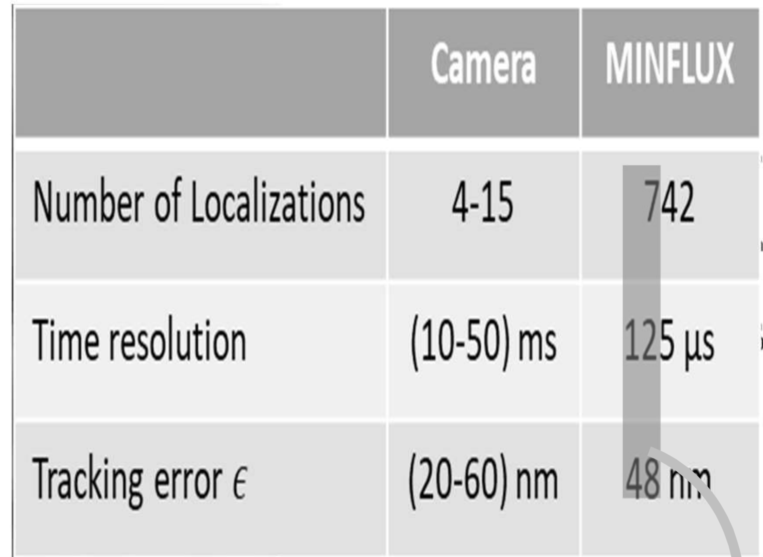




Ribosome complex

Uppsala University





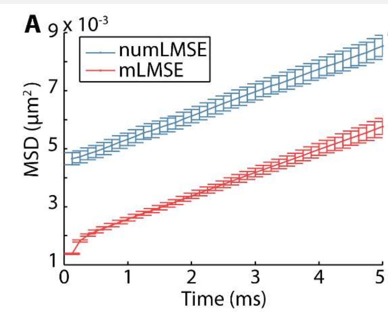
	Camera	MINFLUX
Number of Localizations	4-15	742
Time resolution	(10-50) ms	125 µs
Tracking error ϵ	(20-60) nm	48 nm

- Fast live position estimator **2us in FPGA**
- mEos2 blinking $ON = 2.2\ ms$, $OFF = 0.6\ ms$
- Suitable tracking parameters
  - Live estimator parameter
  - Beam separation
  - Pattern repositioning rate
  - Emission Count rate

- Diffusion
- Blinking
- Brightness

	Camera	MINFLUX
Number of Localizations	4-15 **100X**	742
Time resolution	(10-50) ms	125 µs
Tracking error ϵ	(20-60) nm	48 nm

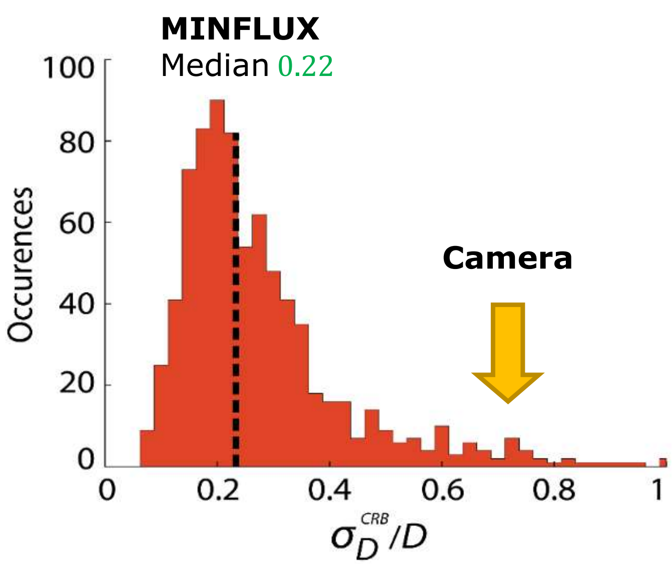
Optimal Least Square Fit of the Mean Squared Displacement

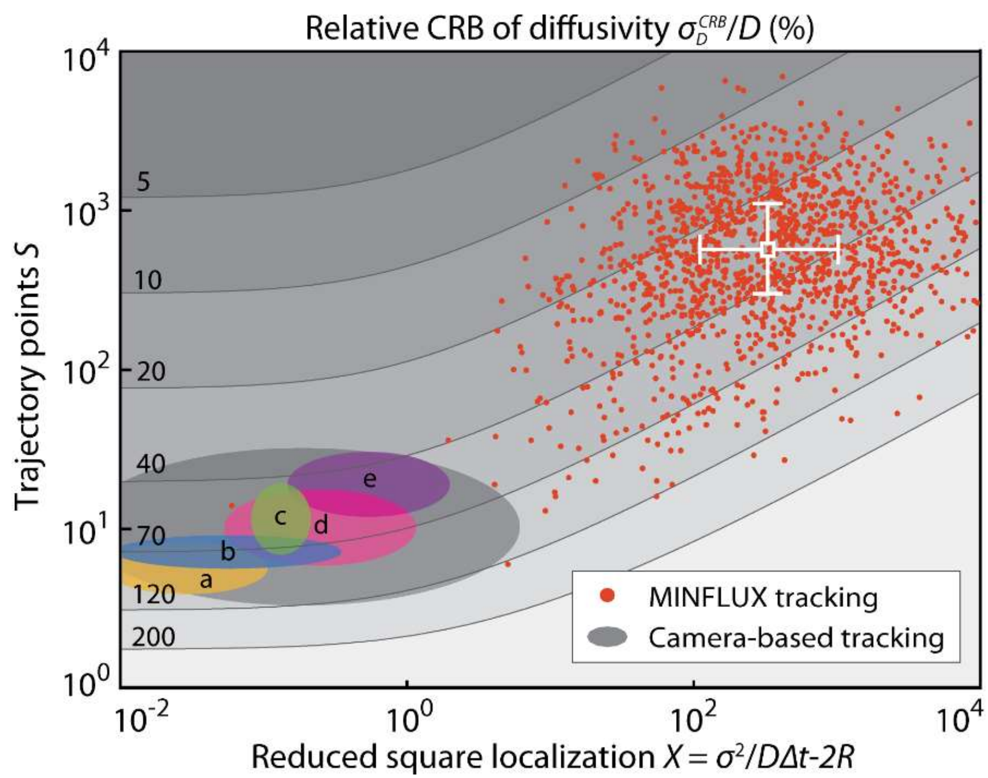


TRACKING METRICS

PHOTONS $\bar{n}$ → LOCALIZATION $\sigma_{\bar{r}}$

TRAJECTORY $\bar{r}(t)$ → DYNAMICS σ_D



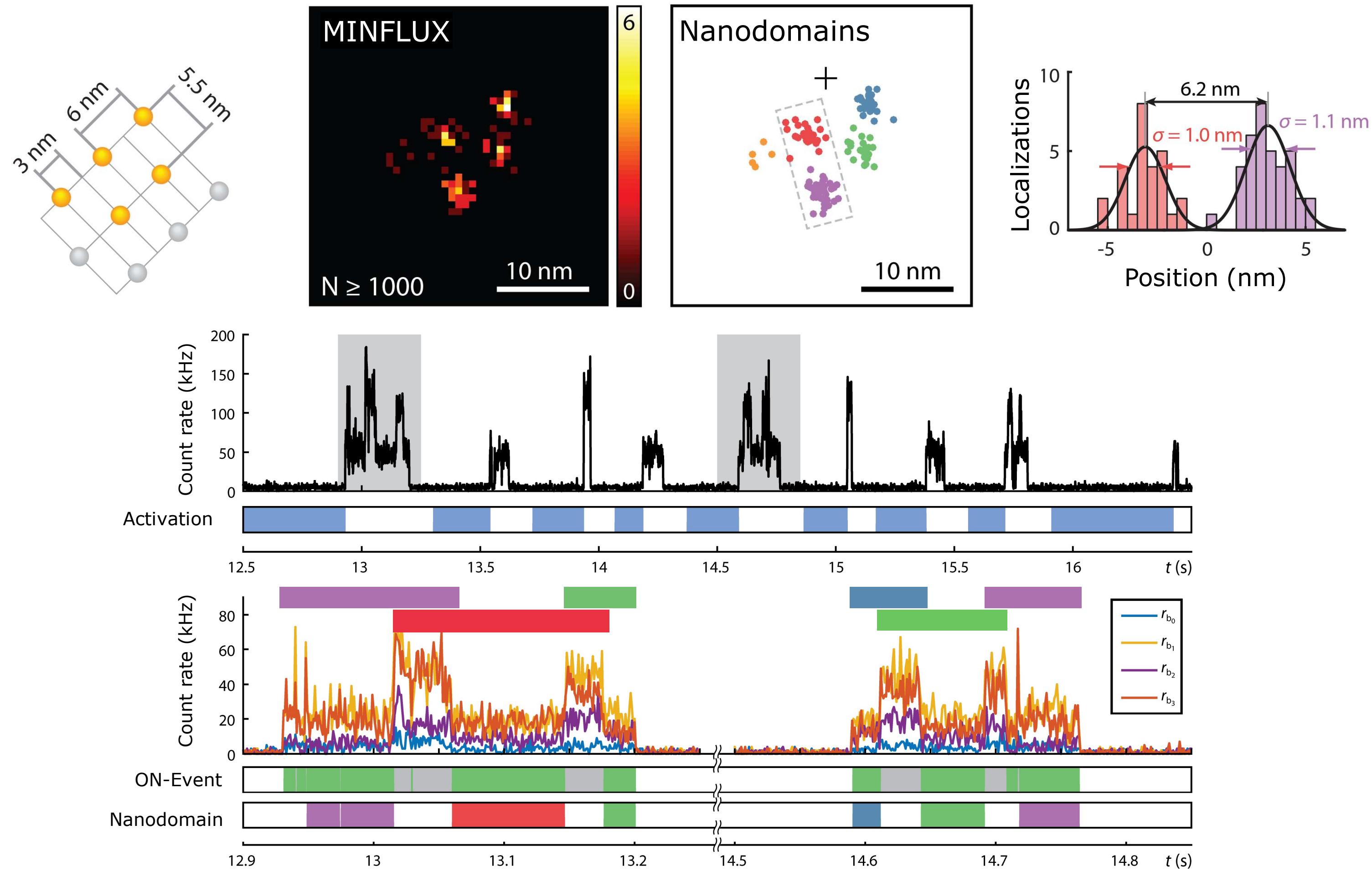


X. Michalet, A.J. Berglund. "Optimal Diffusion Coefficient Estimation in Single-Particle Tracking." Physical Review E (2012)

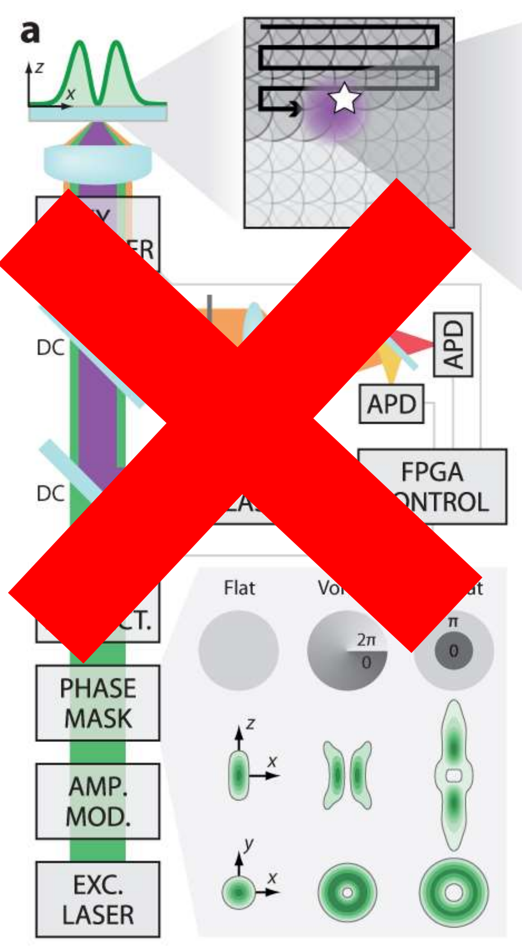
C. L. Vestergaard, "Optimizing experimental parameters for tracking of diffusing particles," Phys Rev E, (2016)

FLUOROPHORE INTERACTIONS

Klaus Gwosch



SYSTEM COMPLEXITY

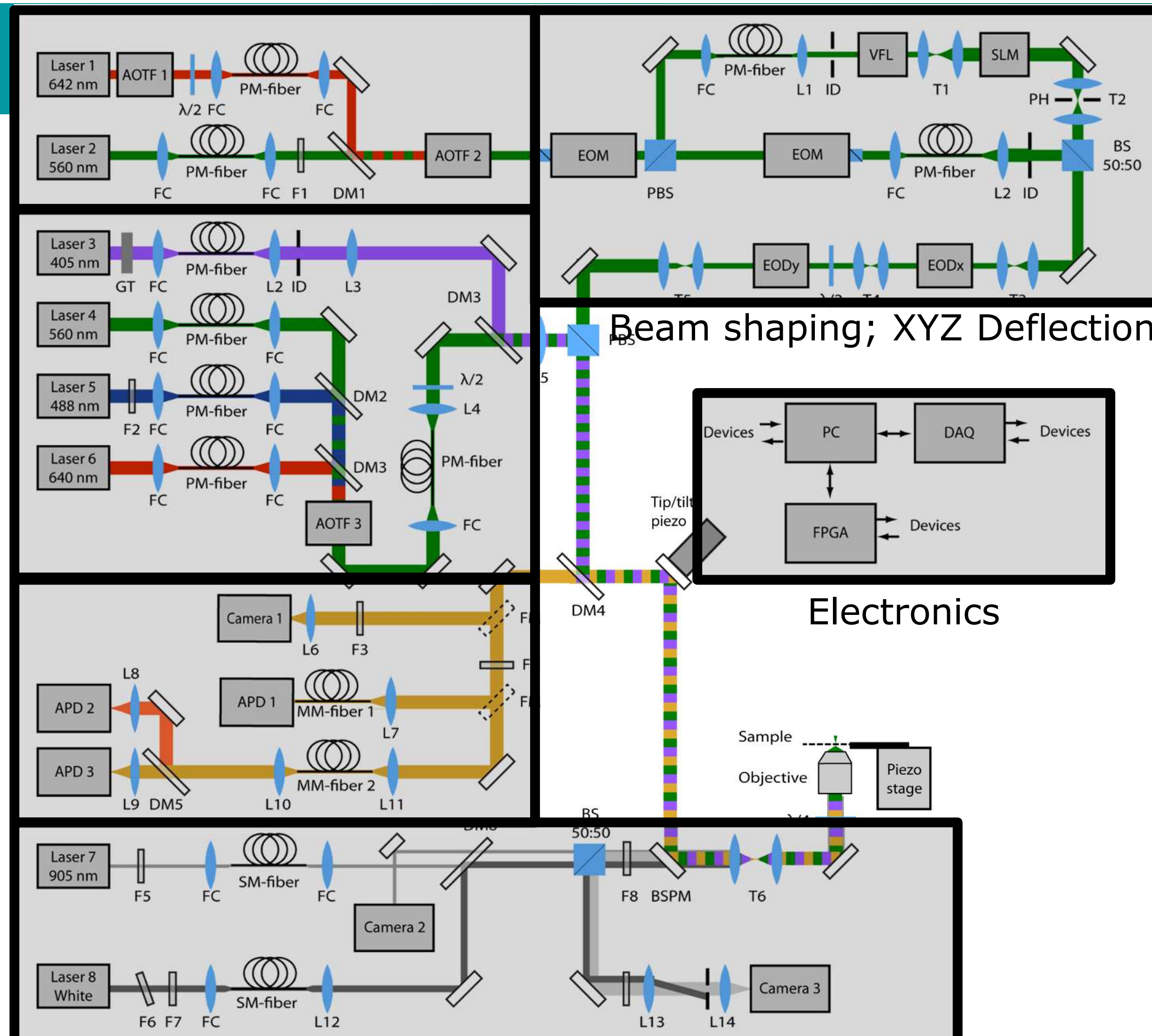


Laser selection

Wide field & activation

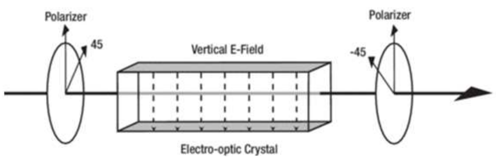
Detection: 2-color APDs EMCCD Camera

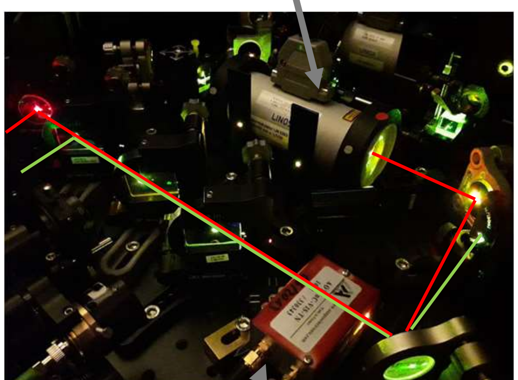
Stabilization

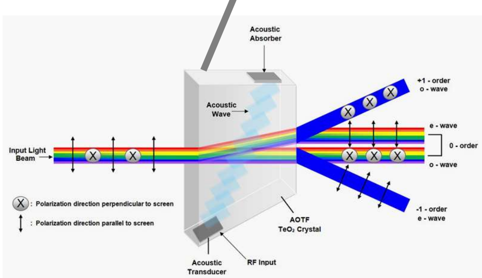


DEVICES TO CONTROL

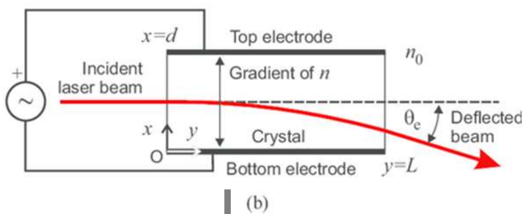
Electro-optical amplitude modulator

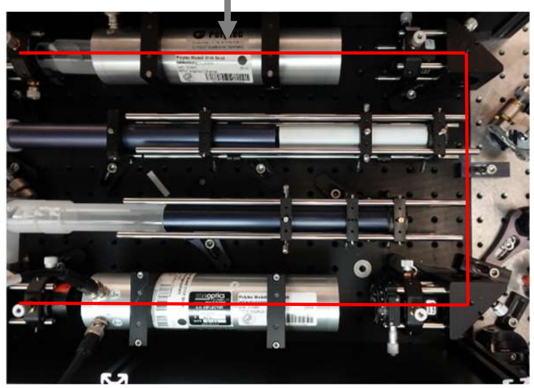


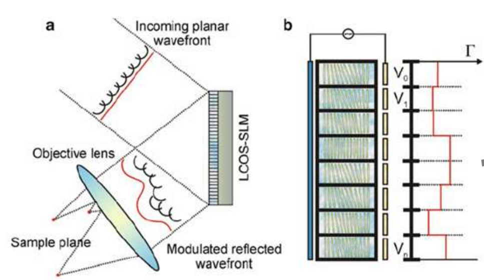




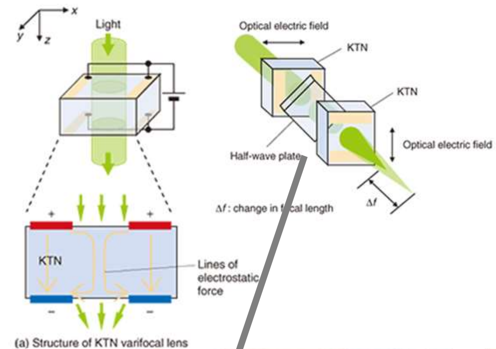
Electro-optical deflector

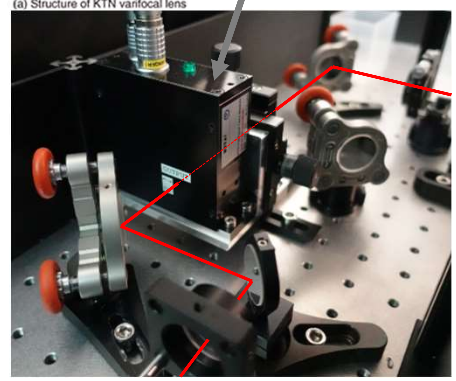


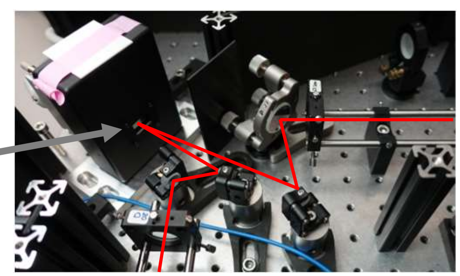




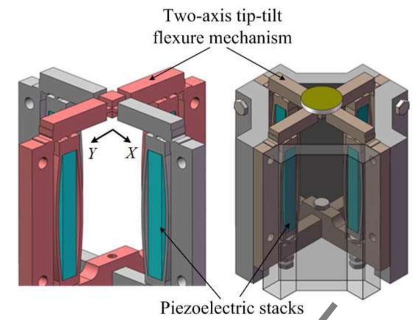
Electro-optical varifocal lens

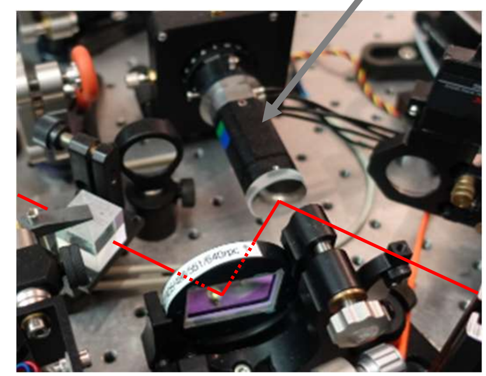






Piezo-electric tip-tilt mirror



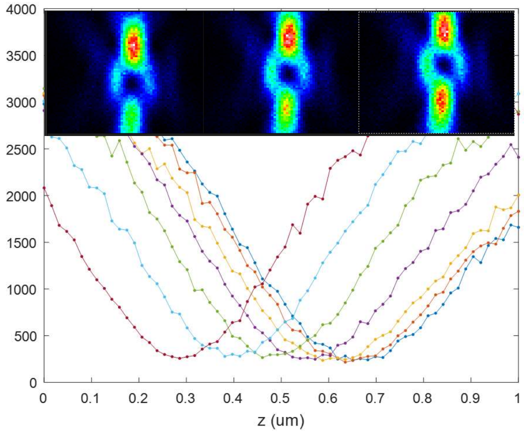


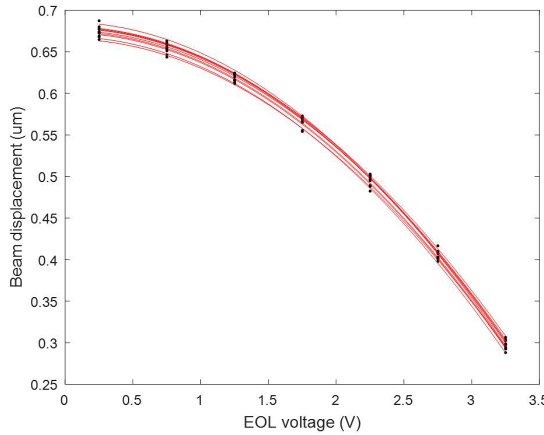
Acousto-optical tunable filter

Spatial light modulator

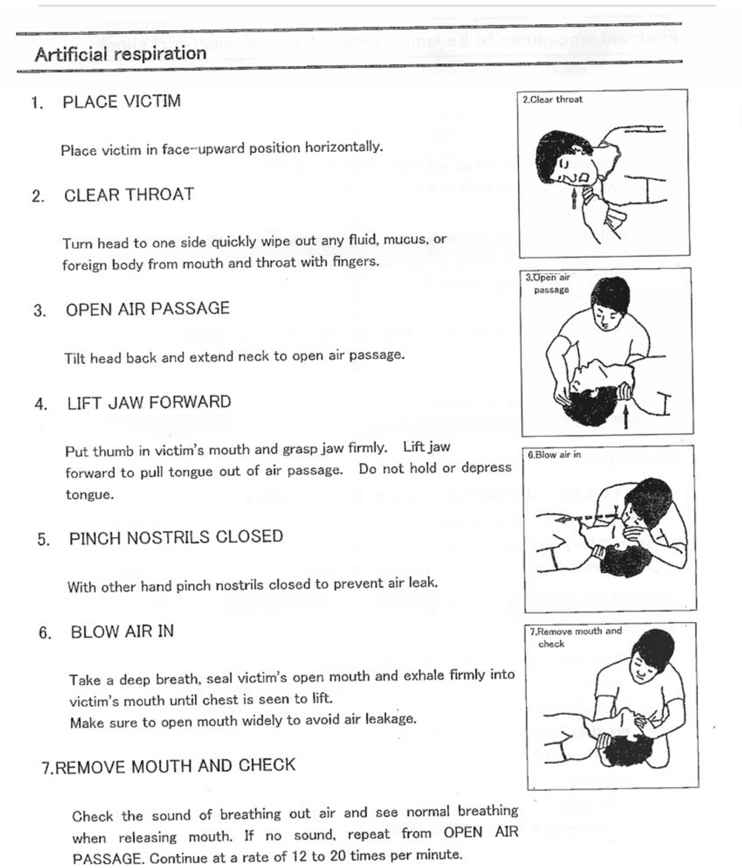
ELECTRO OPTICAL LENS

Calibration





Time response ~15us



Artificial respiration

1. PLACE VICTIM

Place victim in face-upward position horizontally.

2. CLEAR THROAT

Turn head to one side quickly wipe out any fluid, mucus, or foreign body from mouth and throat with fingers.

3. OPEN AIR PASSAGE

Tilt head back and extend neck to open air passage.

4. LIFT JAW FORWARD

Put thumb in victim's mouth and grasp jaw firmly. Lift jaw forward to pull tongue out of air passage. Do not hold or depress tongue.

5. PINCH NOSTRILS CLOSED

With other hand pinch nostrils closed to prevent air leak.

6. BLOW AIR IN

Take a deep breath, seal victim's open mouth and exhale firmly into victim's mouth until chest is seen to lift.
Make sure to open mouth widely to avoid air leakage.

7.REMOVE MOUTH AND CHECK

Check the sound of breathing out air and see normal breathing when releasing mouth. If no sound, repeat from OPEN AIR PASSAGE. Continue at a rate of 12 to 20 times per minute.

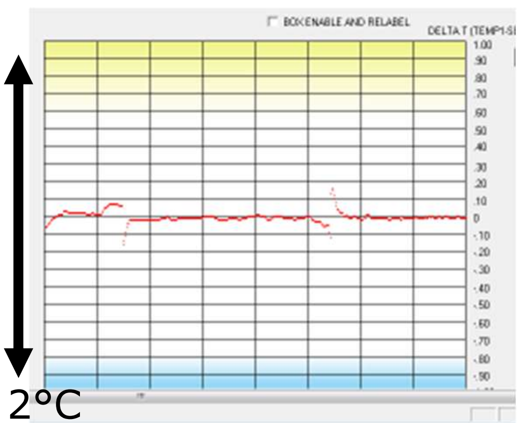
2.Clear throat

3.Open air passage

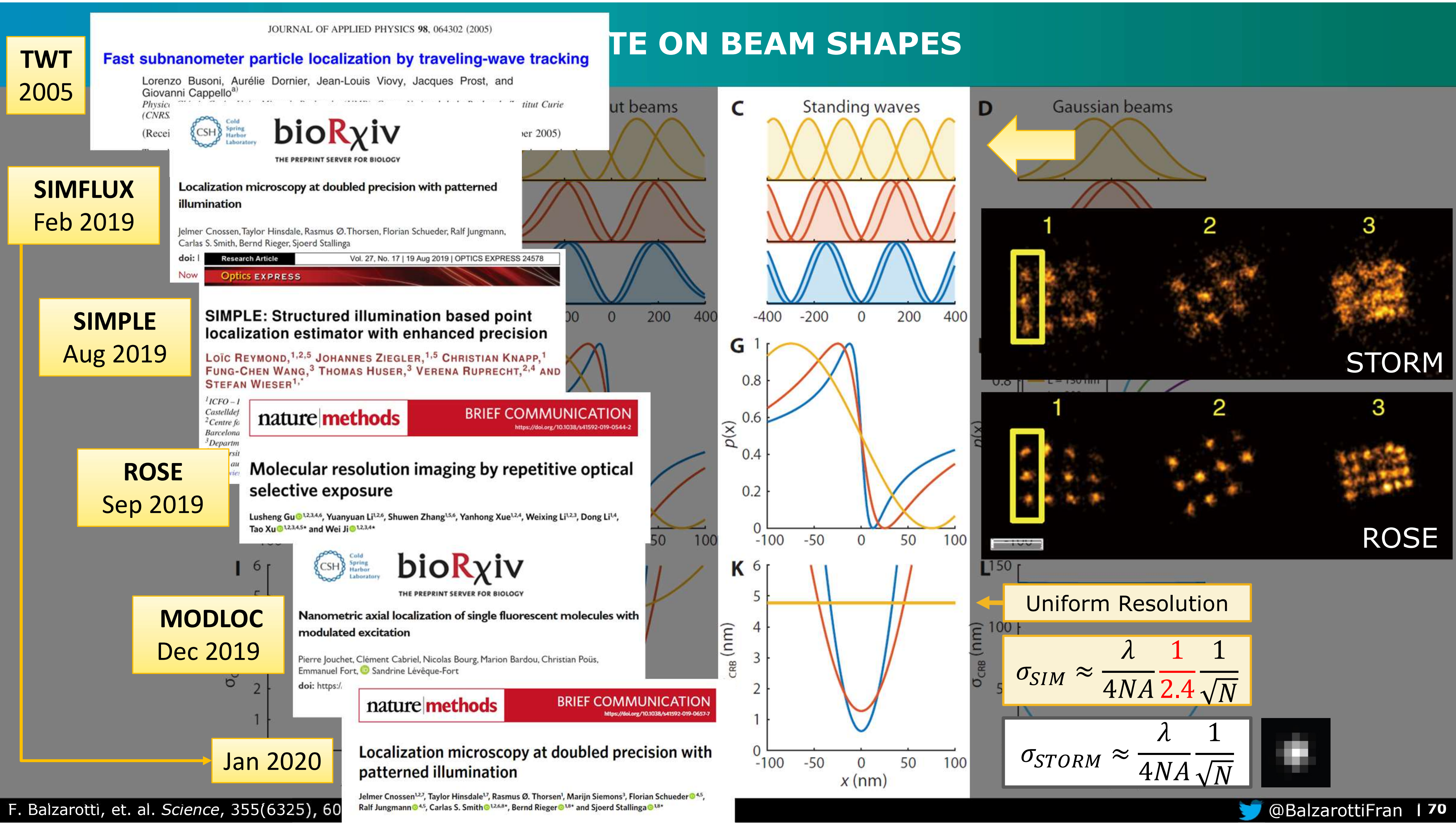
6.Blow air in

7.Remove mouth and check

Temperature stability



- Quadratic calibration. On FPGA!
- Not that fast
- Very sensitive to temperature
- Changes the shape as you use it
- 2kV @ 160kHz Monster Amp!


TE ON BEAM SHAPES
TWT
2005
JOURNAL OF APPLIED PHYSICS 98, 064302 (2005)
Fast subnanometer particle localization by traveling-wave tracking
Lorenzo Busoni, Aurélie Dornier, Jean-Louis Viovy, Jacques Prost, and Giovanni Cappello[a]
SIMFLUX
Feb 2019
bioRχiv
THE PREPRINT SERVER FOR BIOLOGY
Localization microscopy at doubled precision with patterned illumination
Jelmer Cnossen, Taylor Hinsdale, Rasmus Ø. Thorsen, Florian Schueder, Ralf Jungmann, Carlas S. Smith, Bernd Rieger, Sjoerd Stallinga
SIMPLE
Aug 2019
Research Article
Vol. 27, No. 17 | 19 Aug 2019 | OPTICS EXPRESS 24578
Optics EXPRESS
SIMPLE: Structured illumination based point localization estimator with enhanced precision
LOÏC REYMOND,[1,2,5] JOHANNES ZIEGLER,[1,5] CHRISTIAN KNAPP,[1] FUNG-CHEN WANG,[3] THOMAS HUSER,[3] VERENA RUPRECHT,[2,4] AND STEFAN WIESER[1,*]
ROSE
Sep 2019
nature methods
BRIEF COMMUNICATION
Molecular resolution imaging by repetitive optical selective exposure
Lusheng Gu, Yuanyuan Li, Shuwen Zhang, Yanhong Xue, Weixing Li, Dong Li, Tao Xu and Wei Ji
MODLOC
Dec 2019
bioRχiv
THE PREPRINT SERVER FOR BIOLOGY
Nanometric axial localization of single fluorescent molecules with modulated excitation
Pierre Jouchet, Clément Cabriel, Nicolas Bourg, Marion Bardou, Christian Poüs, Emmanuel Fort, Sandrine Lévêque-Fort
Jan 2020
nature methods
BRIEF COMMUNICATION
Localization microscopy at doubled precision with patterned illumination
Jelmer Cnossen, Taylor Hinsdale, Rasmus Ø. Thorsen, Florian Schueder, Ralf Jungmann, Carlas S. Smith, Bernd Rieger and Sjoerd Stallinga
C
Standing waves
D
Gaussian beams
G
K
p(x)
x (nm)
STORM
ROSE
1
2
3
Uniform Resolution
$\sigma_{SIM} \approx \frac{\lambda}{4NA} \frac{1}{2.4} \frac{1}{\sqrt{N}}$
$\sigma_{STORM} \approx \frac{\lambda}{4NA} \frac{1}{\sqrt{N}}$
F. Balzarotti, et. al. Science, 355(6325), 60
@BalzarottiFran
| 70

A NOTE ON BEAM SHAPES

nature photonics
ARTICLES
https://doi.org/10.1038/s41566-021-00774-2
Check for updates

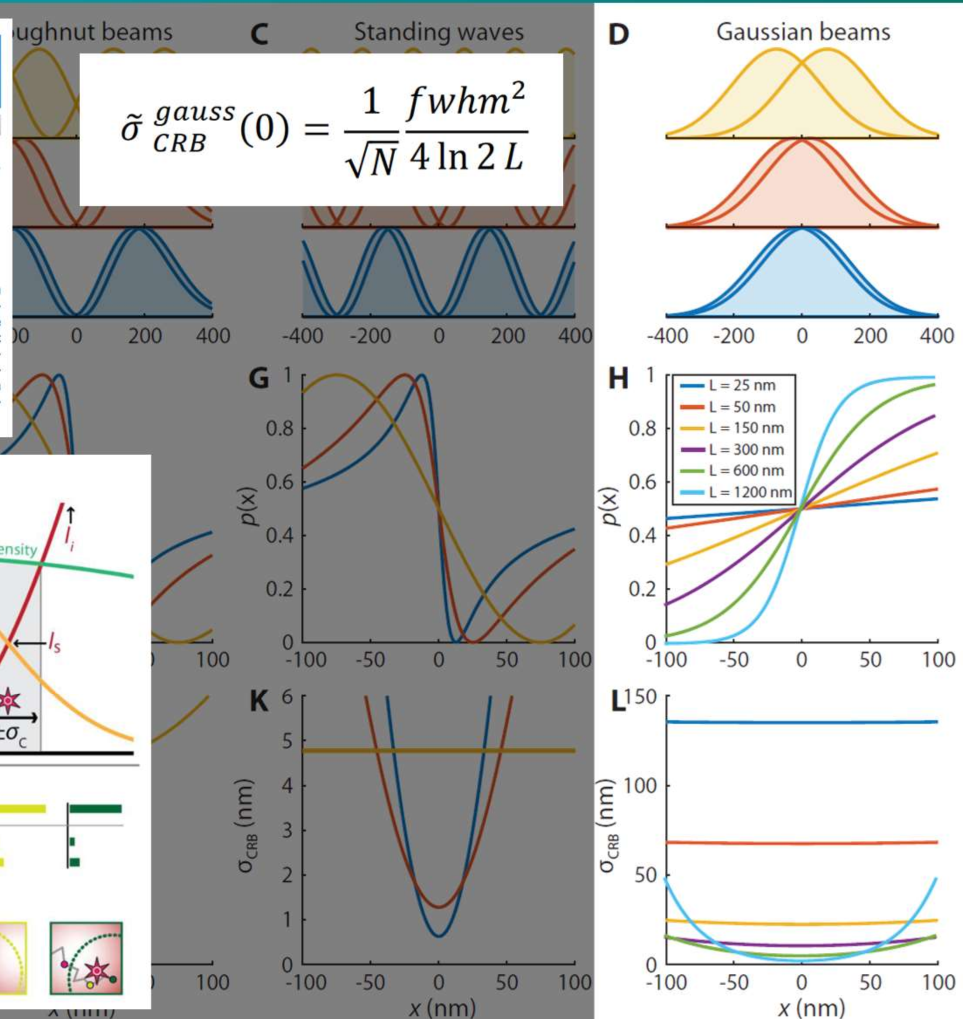
OPEN

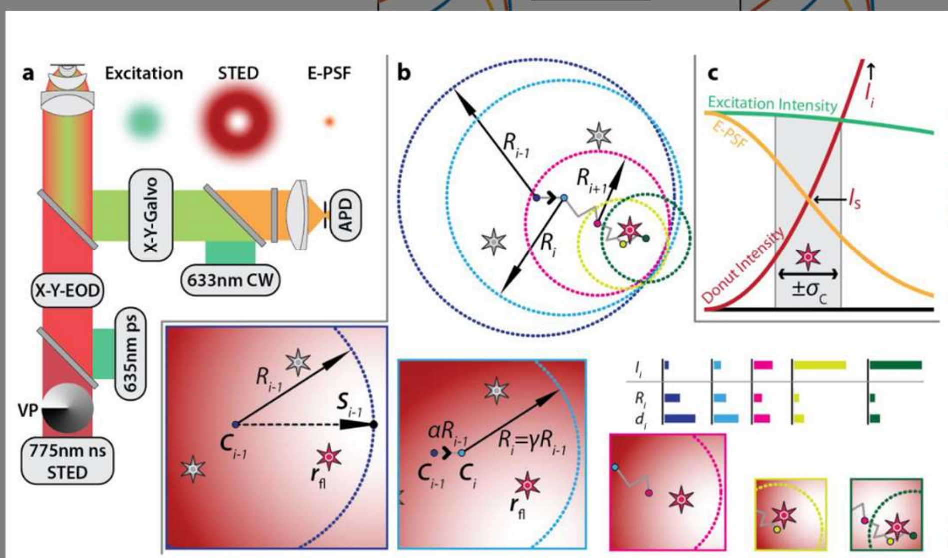
MINSTED fluorescence localization and nanoscopy

Michael Weber[1,4], Marcel Leutenegger[1,4], Stefan Stoldt[1,2], Stefan Jakobs[1,2], Tiberiu S. Mihaila[1], Alexey N. Butkevich[3] and Stefan W. Hell[1,3]

We introduce MINSTED, a fluorophore localization and super-resolution microscopy concept based on stimulated emission depletion (STED) that provides spatial precision and resolution down to the molecular scale. In MINSTED, the intensity minimum of the STED doughnut, and hence the point of minimal STED, serves as a movable reference coordinate for fluorophore localization. As the STED rate, the background and the required number of fluorescence detections are low compared with most other STED microscopy and localization methods, MINSTED entails substantially less fluorophore bleaching. In our implementation, 200–1,000 detections per fluorophore provide a localization precision of 1–3 nm in standard deviation, which in conjunction with independent single fluorophore switching translates to a ~100-fold improvement in far-field microscopy resolution over the diffraction limit. The performance of MINSTED nanoscopy is demonstrated by imaging the distribution of Mic60 proteins in the mitochondrial inner membrane of human cells.

$$\tilde{\sigma}^{gauss}_{CRB}(0) = \frac{1}{\sqrt{N}} \frac{fwhm^2}{4 \ln 2\, L}$$





A BIOLOGIST: “VERY NICE… BUT I DON’T CARE!”

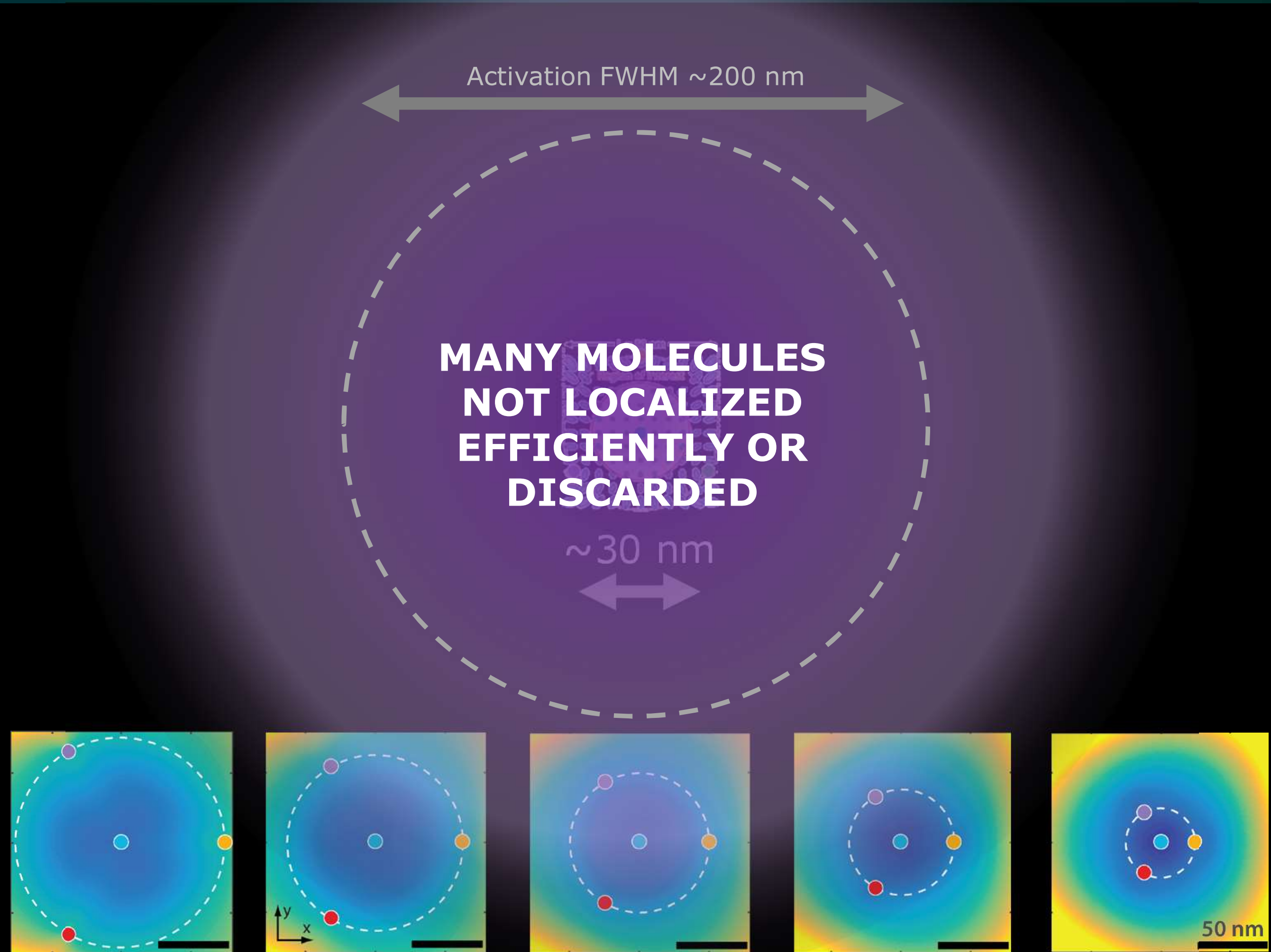
Larger field of view

Work in cells

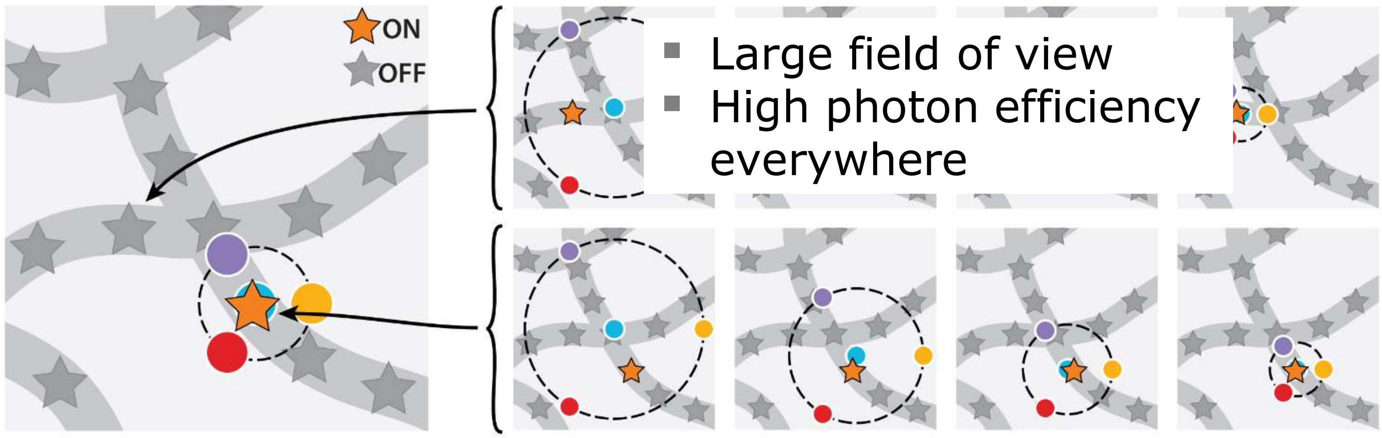
Three dimensions

Multicolor imaging

EXTENDING THE "FIELD OF VIEW"

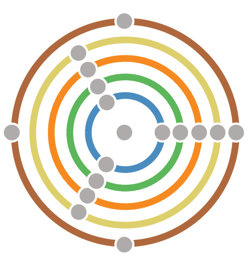


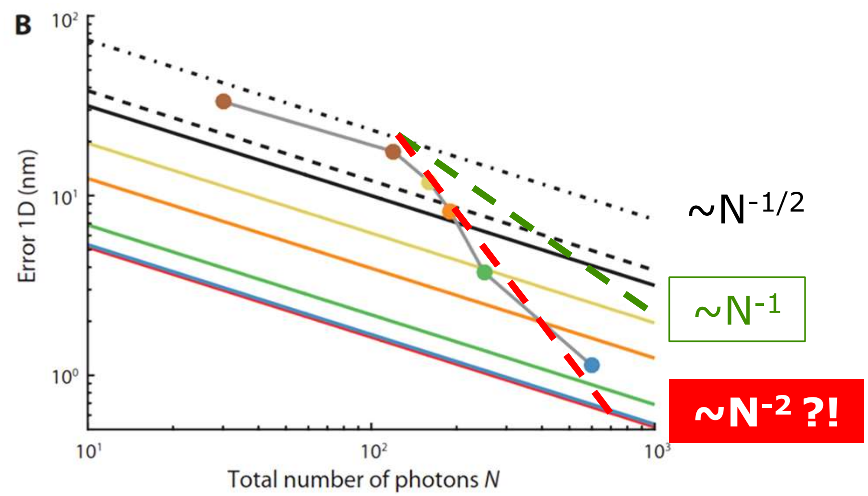
ITERATIVE MINFLUX



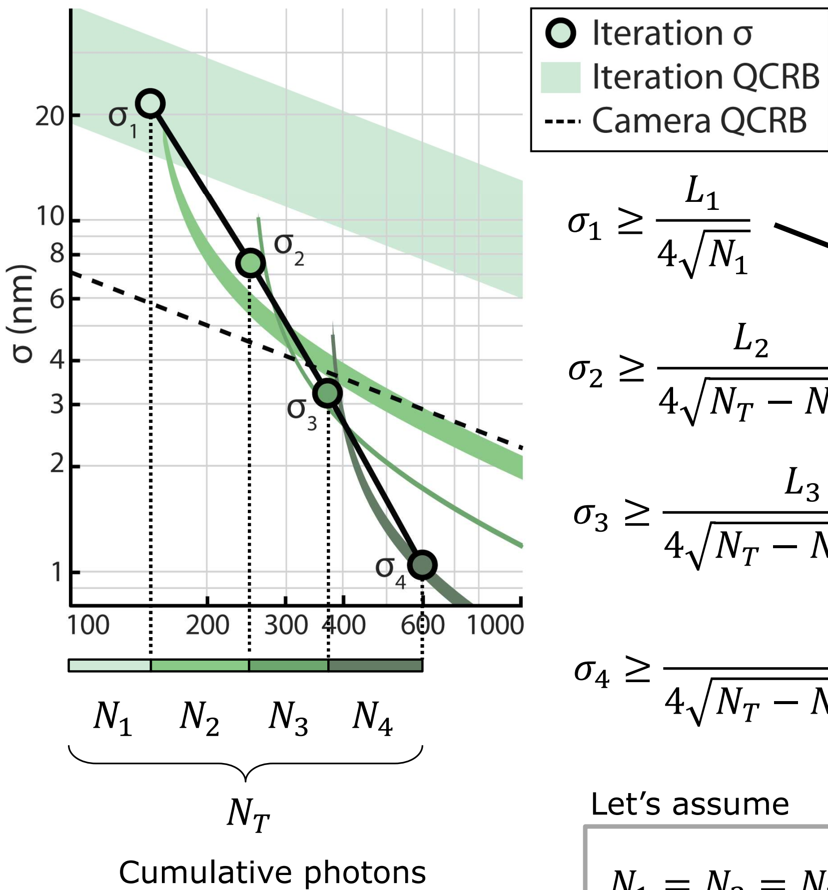
- Large field of view
- High photon efficiency everywhere

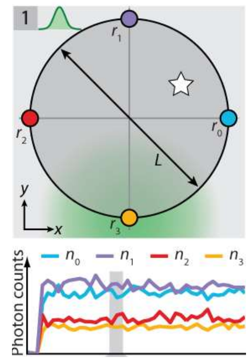
Step	Beam type	Beam separation L_i	N_i	N	Mean error $\langle \mathcal{E} \rangle$
1	Gaussian	300 nm	30	30	(33.3 ± 2.5) nm
2	Gaussian	300 nm	90	120	(17.58 ± 0.16) nm
3	Doughnut	150 nm	40	160	(11.96 ± 0.07) nm
4	Doughnut	100 nm	30	190	(8.21 ± 0.03) nm
5	Doughnut	50 nm	60	250	(3.74 ± 0.02) nm
6	Doughnut	30 nm	350	600	(1.13 ± 0.03) nm





HOW DOES IT WORK?





$$\sigma_1 \geq \frac{L_1}{4\sqrt{N_1}}$$

$$\sigma_2 \geq \frac{L_2}{4\sqrt{N_T - N_1}}$$

$$\sigma_3 \geq \frac{L_3}{4\sqrt{N_T - N_2 - N_1}}$$

$$\sigma_4 \geq \frac{L_4}{4\sqrt{N_T - N_3 - N_2 - N_1}} \propto \frac{L_4}{\sqrt{N}} \triangleq \frac{\sigma_3}{\sqrt{N}} = \frac{1}{\sqrt{N}}\frac{L_3}{\sqrt{N}} \triangleq \frac{\sigma_2}{N} = \frac{L_2}{N^{3/2}} \triangleq \frac{\sigma_1}{N^{3/2}} = \frac{L_1}{N^2}$$!!!

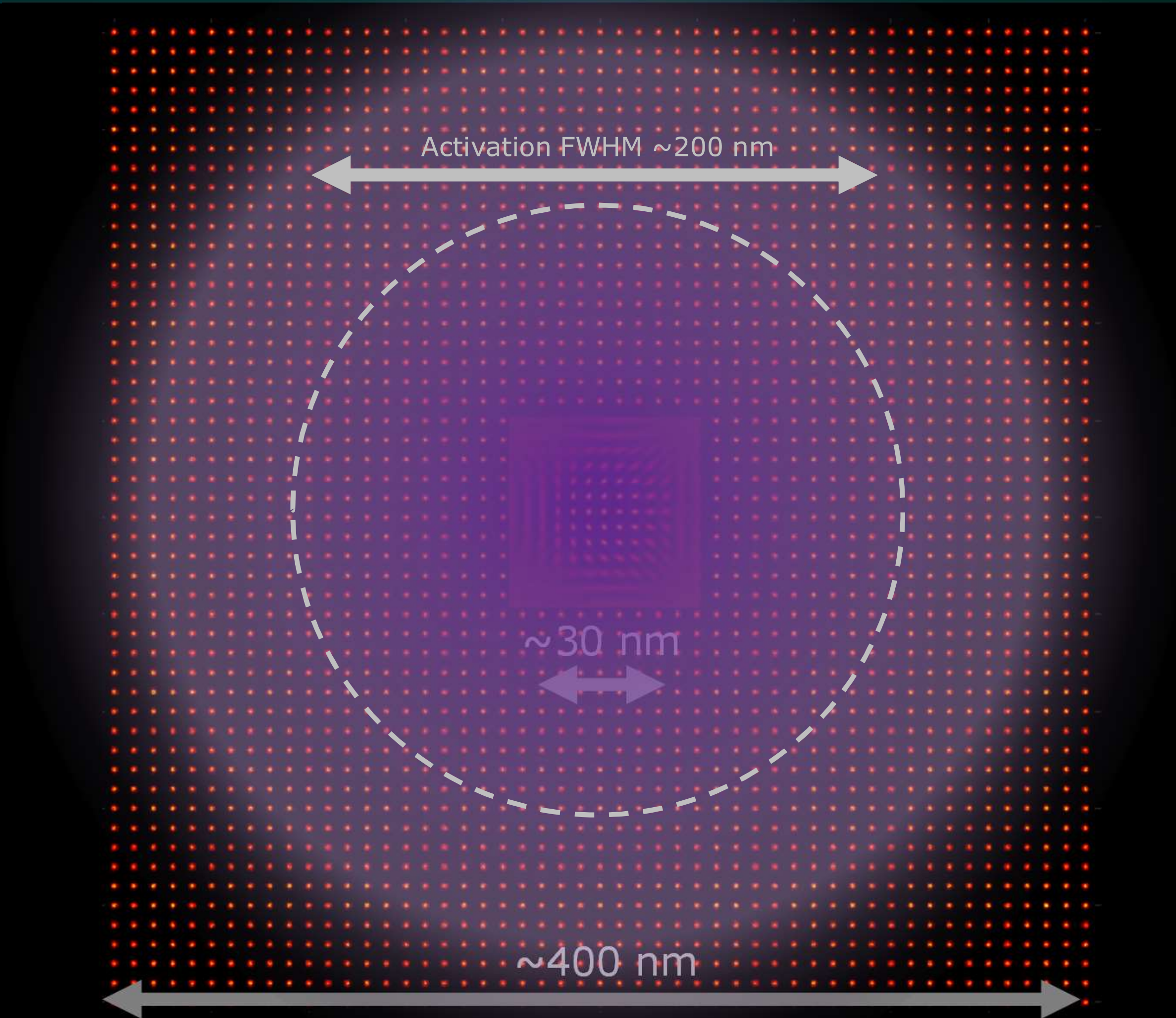
Let's assume

$$N_1 = N_2 = N_3 = N_4 = N = \frac{N_T}{4}$$

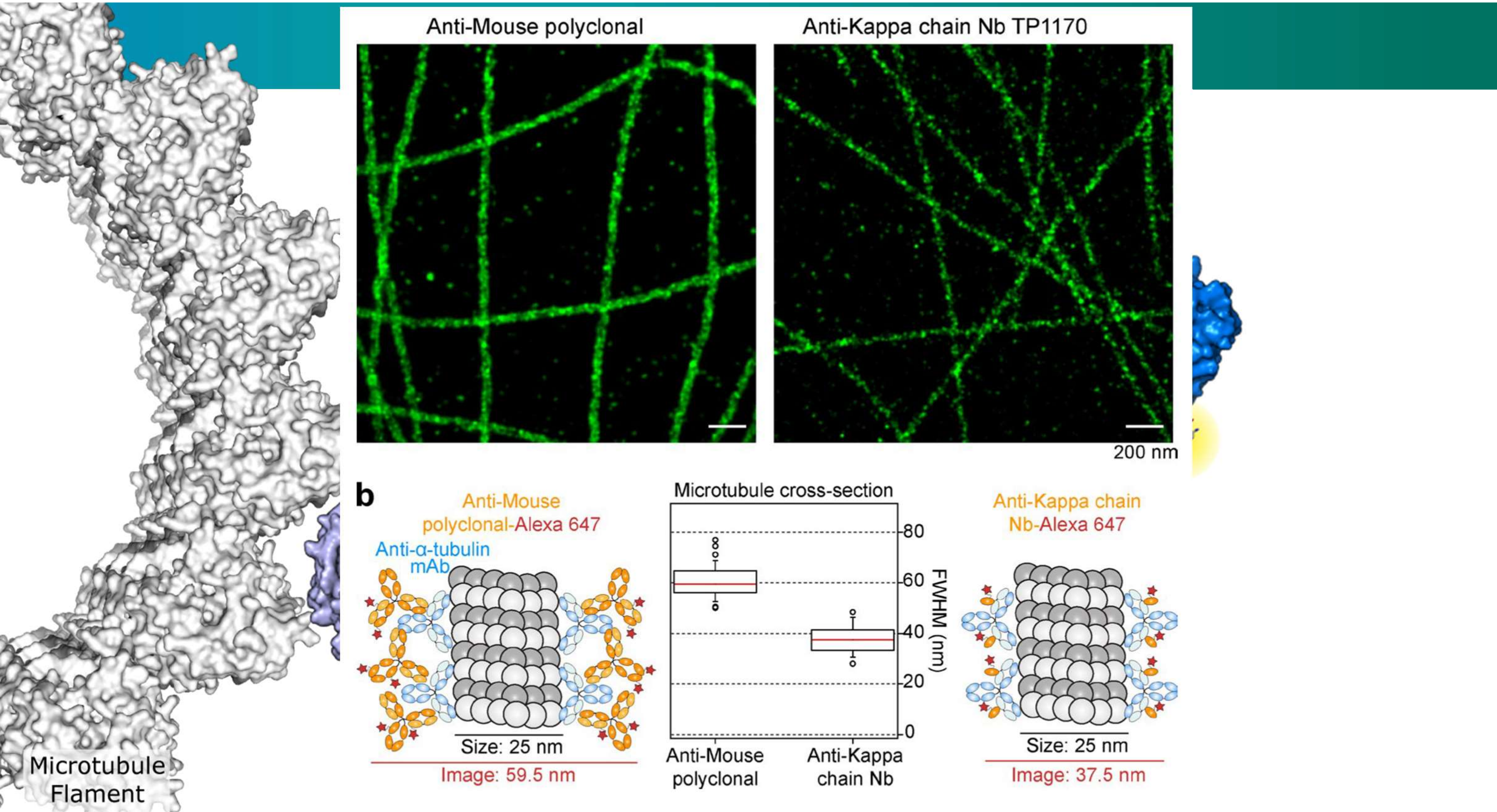
$$\sigma_K \propto \frac{L_1}{N^{k/2}}$$

k iterations

ITERATIVE MINFLUX

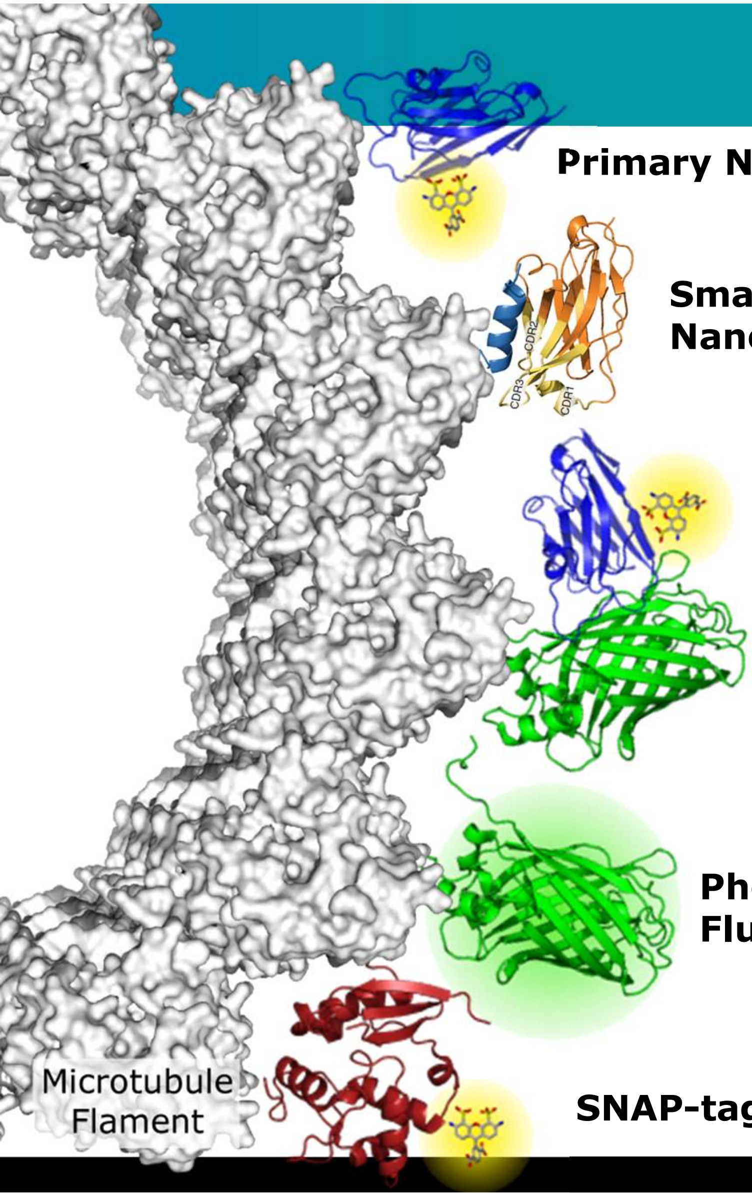


K.C. Gwosch, J.K. Pape, F. Balzarotti et. al *Nature Methods* (2020)



T. Pleiner, et. al. *Journal of Cell Biolog (*2018)

LABELLING



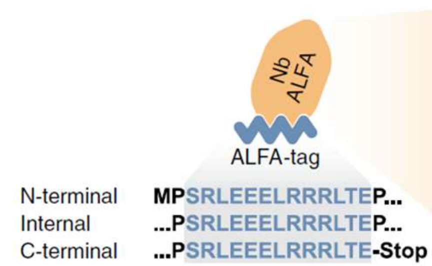
nature COMMUNICATIONS

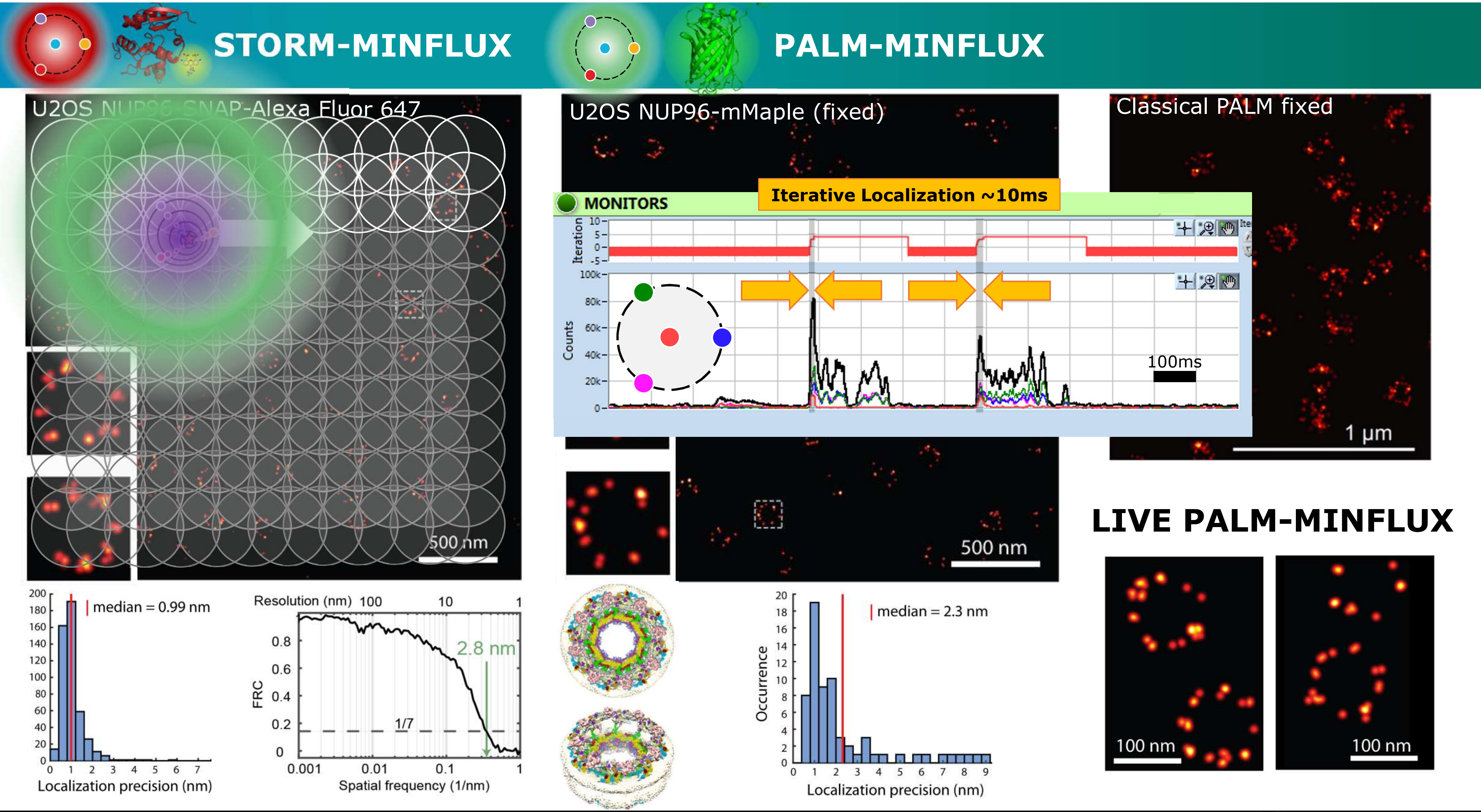
ARTICLE

https://doi.org/10.1038/s41467-019-12301-7 OPEN

The ALFA-tag is a highly versatile tool for nanobody-based bioscience applications

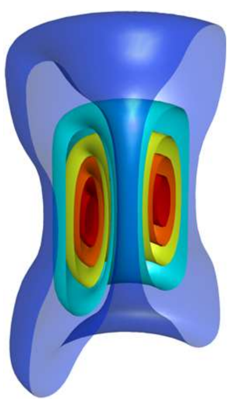
Hansjörg Götzke[1,10], Markus Kilisch[1,2,10], Markel Martínez-Carranza[3,10], Shama Sograte-Idrissi[4,5], Abirami Rajavel[1], Thomas Schlichthaerle[6,7], Niklas Engels[8], Ralf Jungmann[6,7], Pål Stenmark[3,9], Felipe Opazo[1,4,5] & Steffen Frey[1]



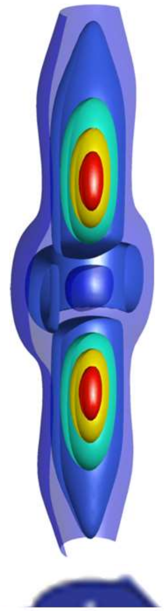

STORM-MINFLUX
PALM-MINFLUX
U2OS NUP96-SNAP-Alexa Fluor 647
500 nm
median = 0.99 nm
Localization precision (nm)
Resolution (nm) 100 10 1
FRC
2.8 nm
1/7
Spatial frequency (1/nm)
U2OS NUP96-mMaple (fixed)
MONITORS
Iterative Localization ~10ms
Iteration
Counts
100ms
500 nm
Occurrence
median = 2.3 nm
Localization precision (nm)
Classical PALM fixed
1 µm
LIVE PALM-MINFLUX
100 nm
100 nm

3D MINFLUX

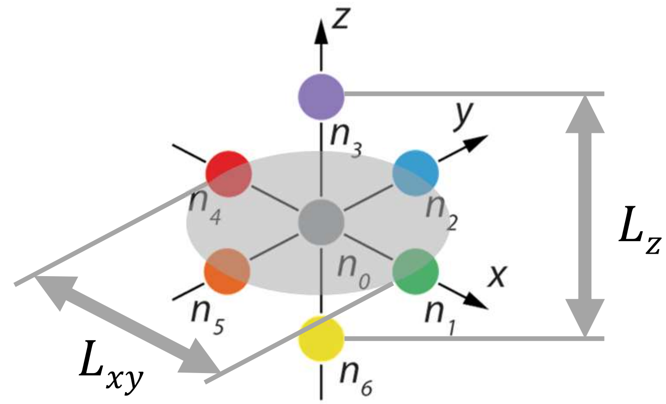
Vortex phase mask



Top-hat phase mask



3D Beam Pattern

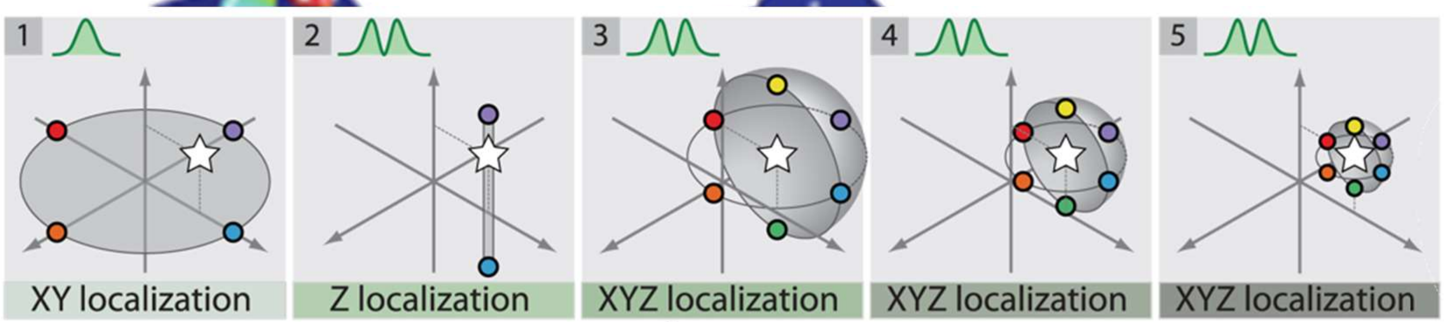


Precision

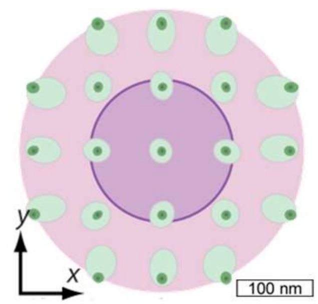
$$\sigma_{xy} \propto \frac{L_{xy}}{\sqrt{N}}$$

$$\sigma_z \propto \frac{L_z}{\sqrt{N}}$$

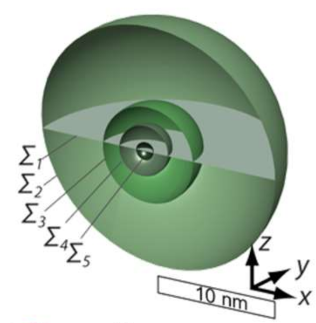
Iterative operation



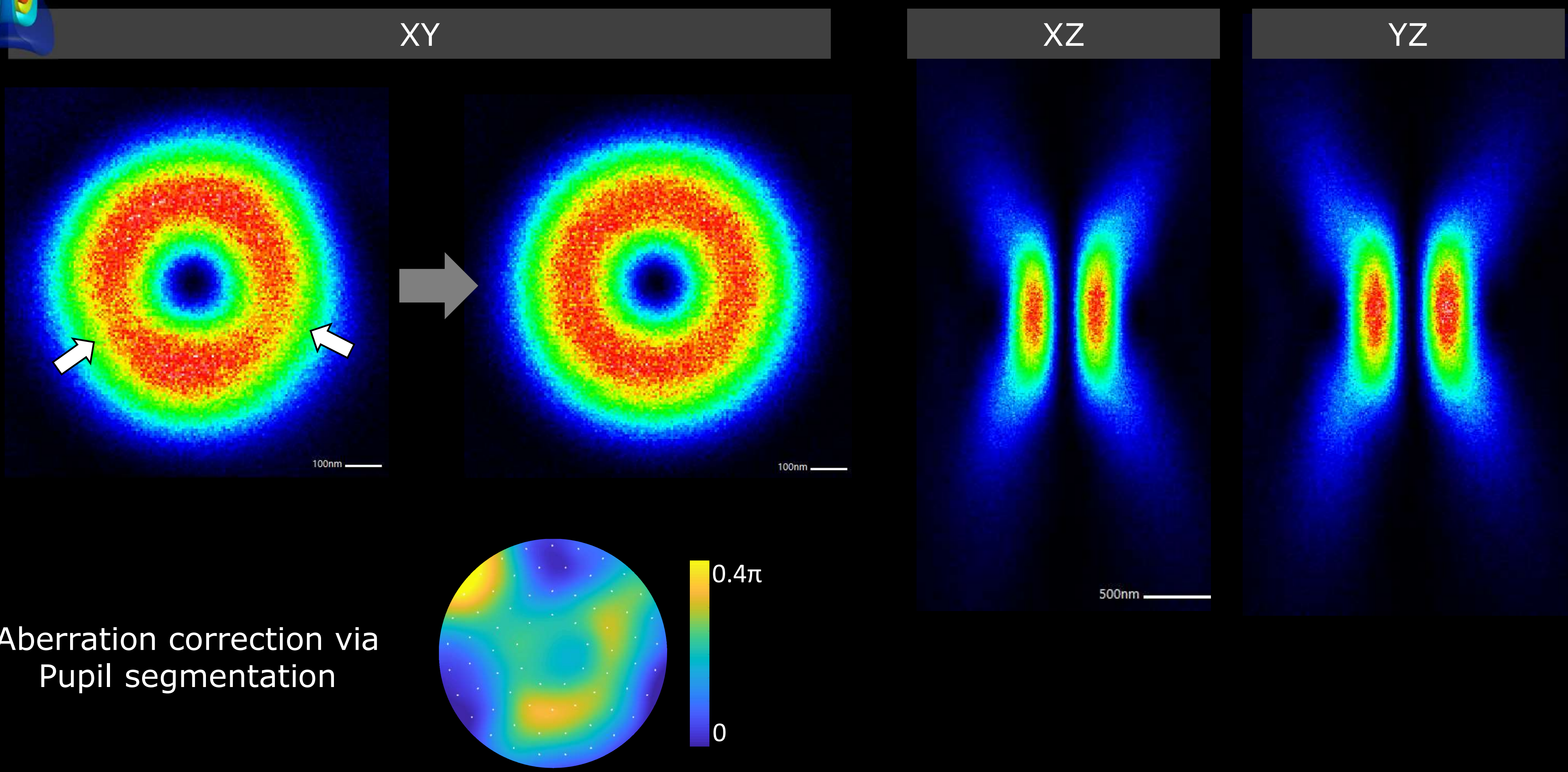
Coverage



Covariance

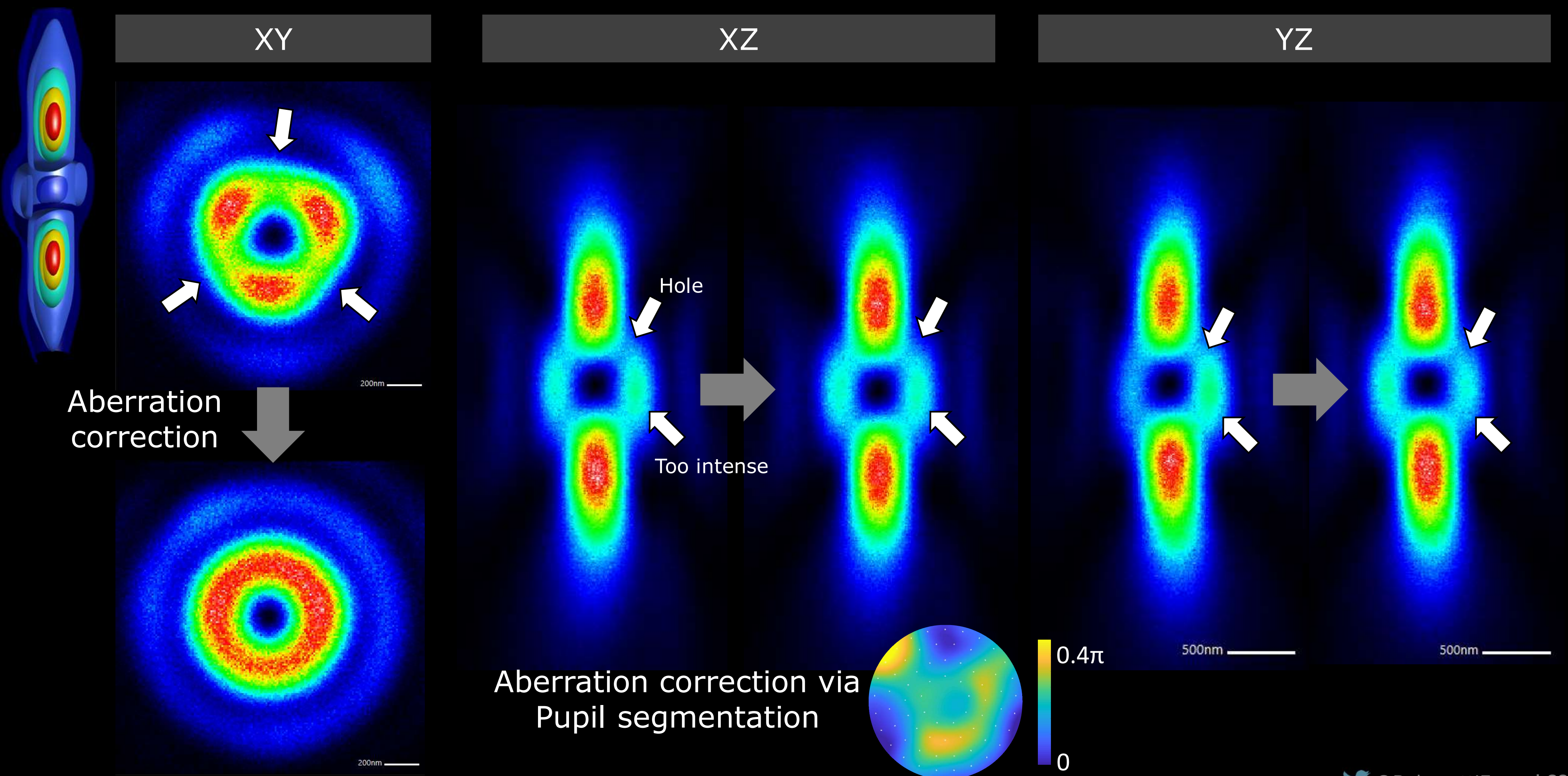


ACTUAL BEAM SHAPES: VORTEX BEAM



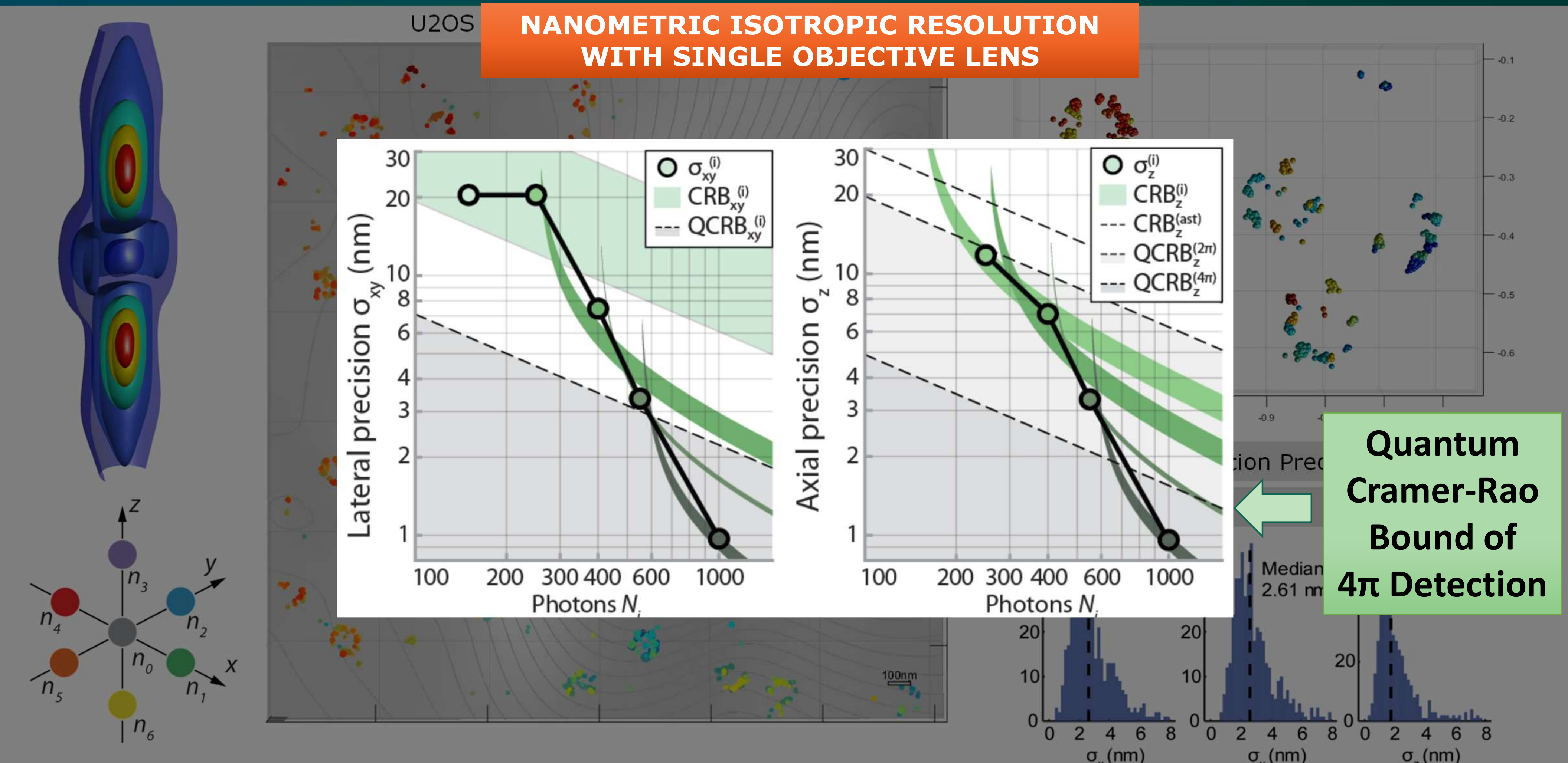
Aberration correction via
Pupil segmentation

ACTUAL BEAM SHAPES: TOPHAT BEAM



3D MINFLUX

NANOMETRIC ISOTROPIC RESOLUTION WITH SINGLE OBJECTIVE LENS

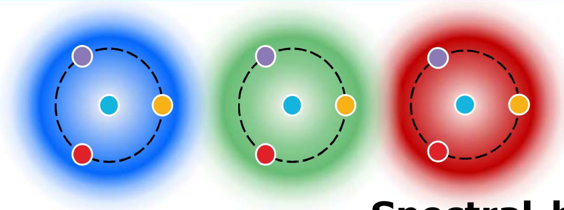


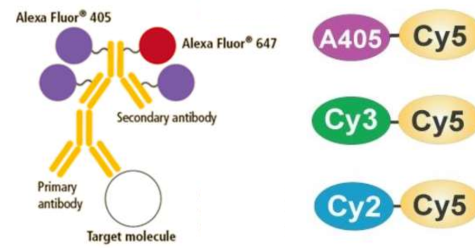
Quantum Cramer-Rao Bound of 4π Detection

MULTICOLOR IMAGING

STRATEGY

- ~~Brute force – Many excitation donuts~~
- Scheme free of chromatic aberrations





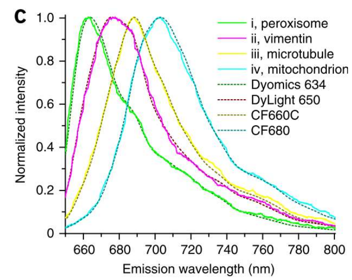
M. Bates et al. *Science* (2007)

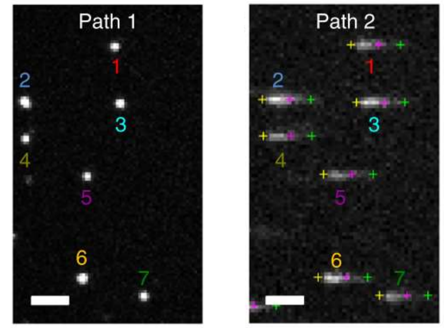
Kinetic-based

- Lifetime kinetics ~ns
- Switching kinetics ~ms
- DNA-PAINT binding/unbinding kinetics ~10ms

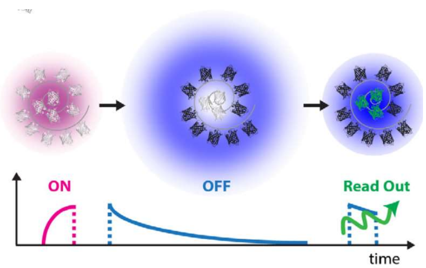
Spectral-based

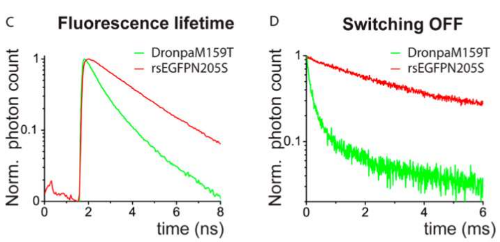
- Activator/reporter
- Slight spectral shift

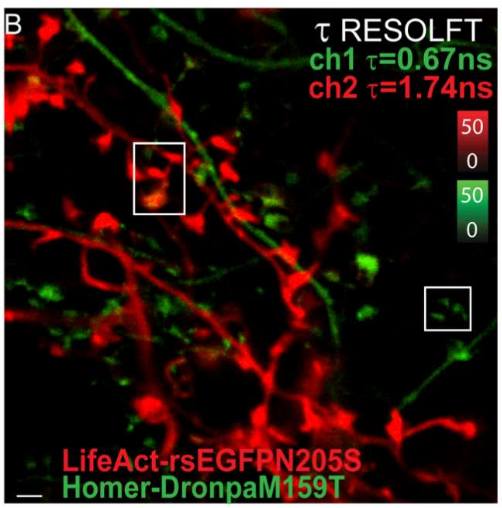


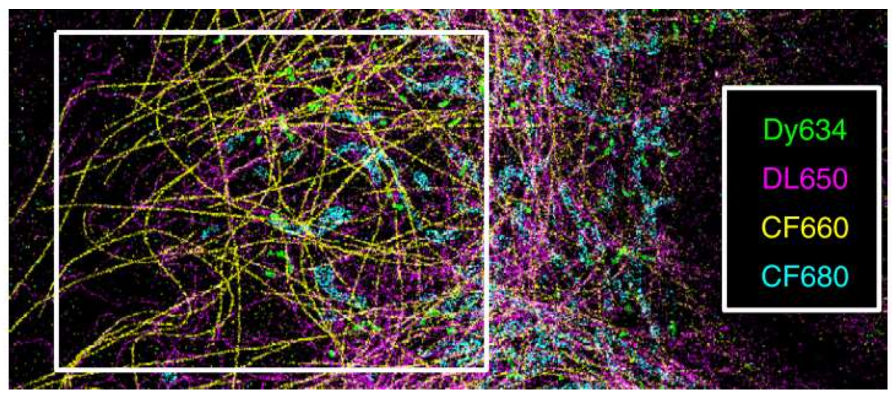


RESOLFT Microscopy







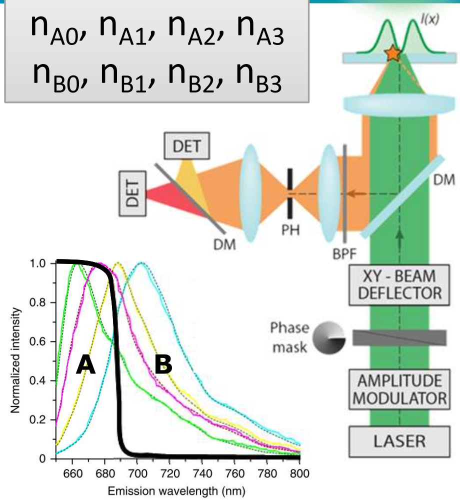


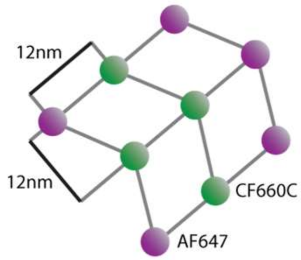
MULTICOLOR 3D IMAGING

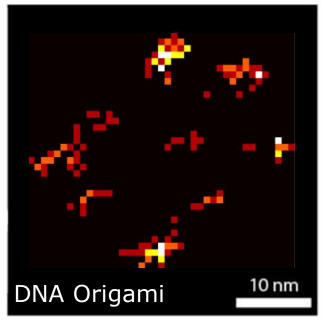
Spectral Splitting

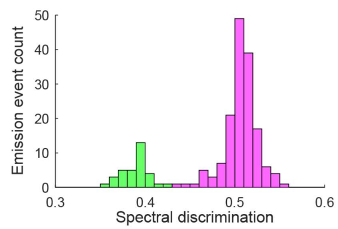
$n_{A0}, n_{A1}, n_{A2}, n_{A3}$

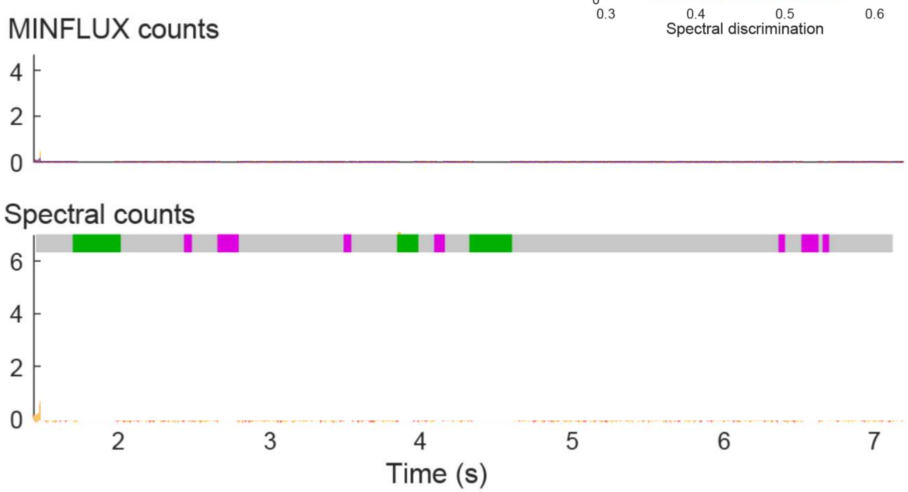
$n_{B0}, n_{B1}, n_{B2}, n_{B3}$

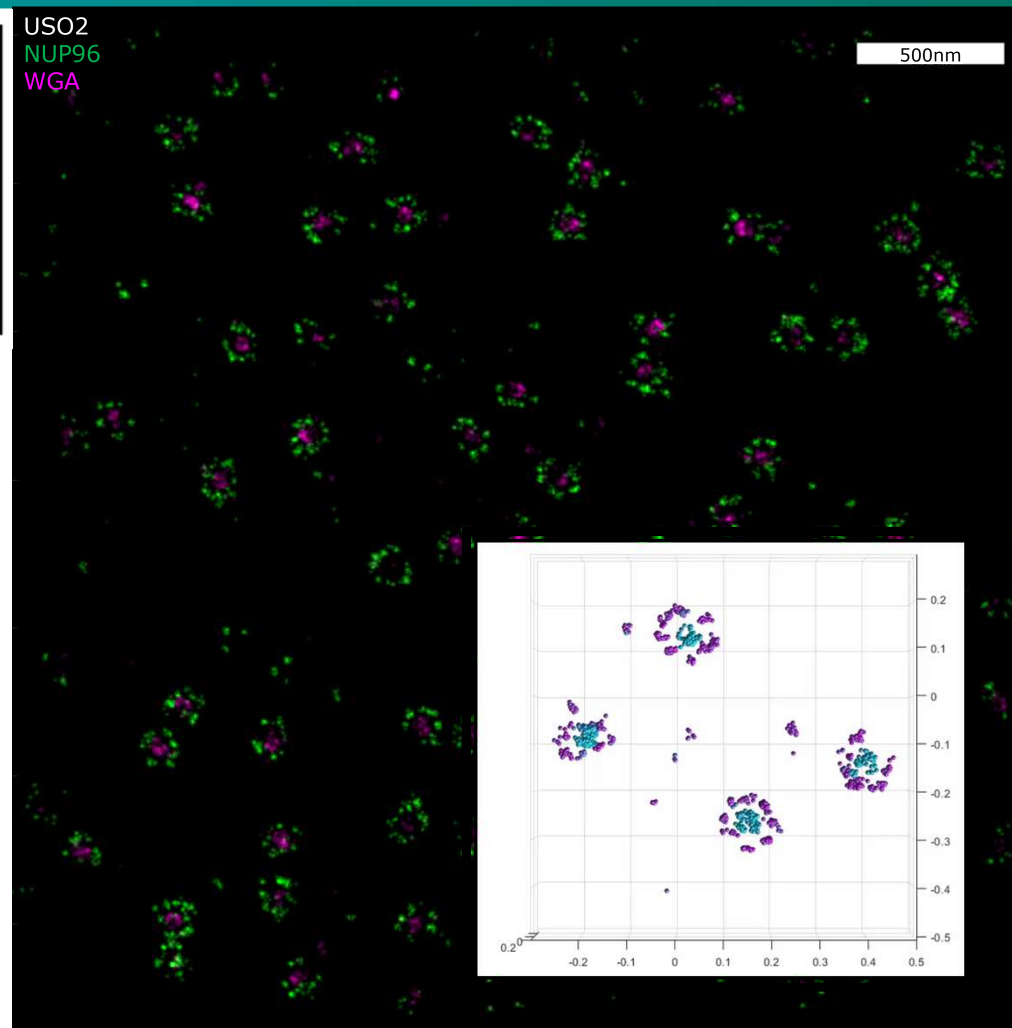




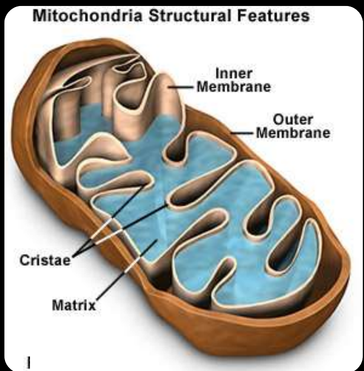




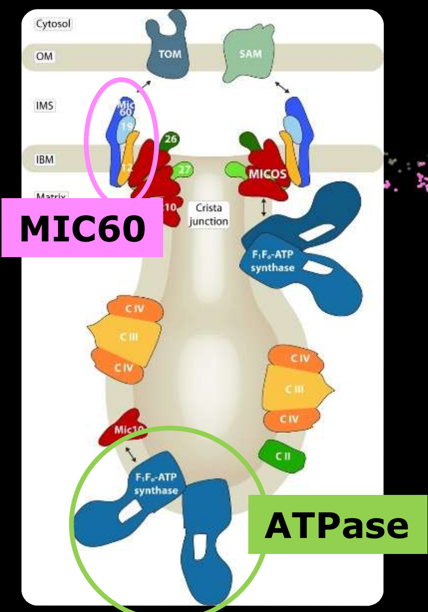




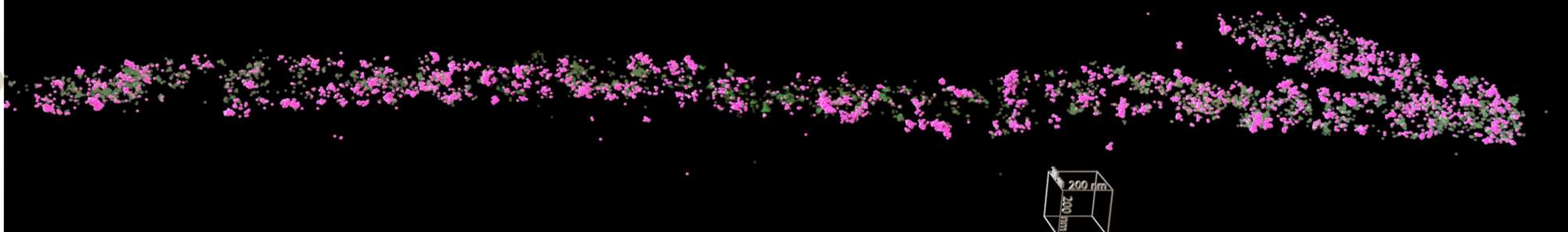
MITOCHONDRIAL PROTEIN DISTRIBUTION



MINFLUX resolves protein distribution on the inner mitochondrial membrane in 3D

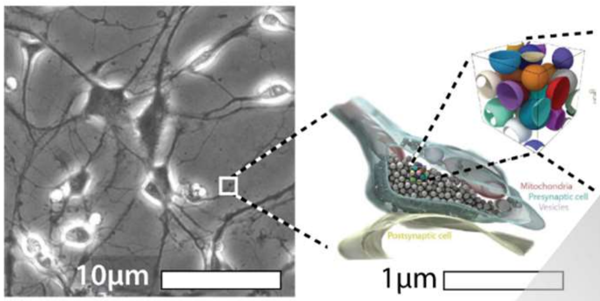


Research Group - Mitochondrial Structure and Dynamics
MPIBPC

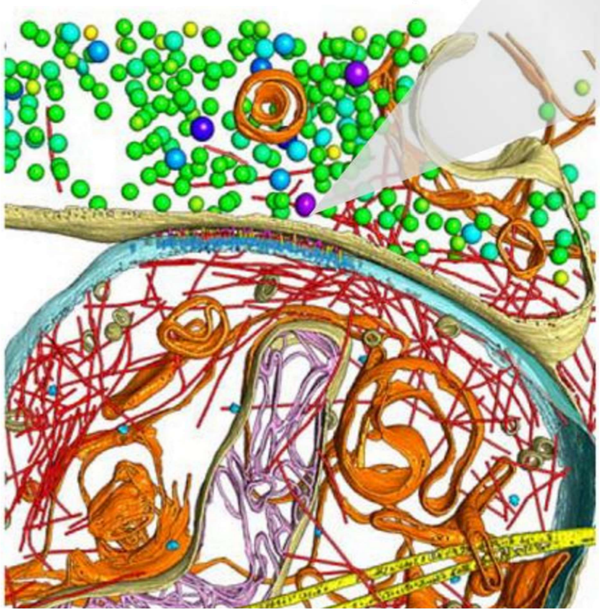


J. K. Pape et al., *PNAS*, 2020; T. Stephan et al., *The EMBO Journal*, p. e104105, 2020

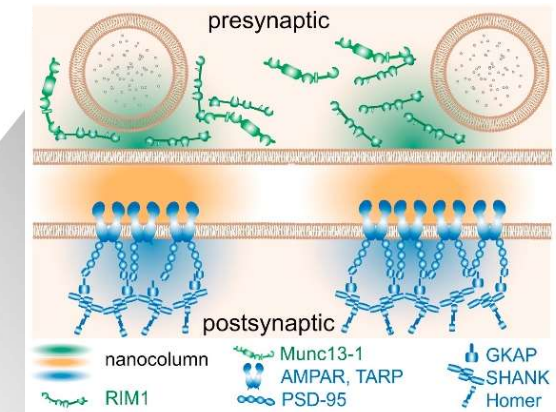
NANOCOLUMNS IN SYNAPSES



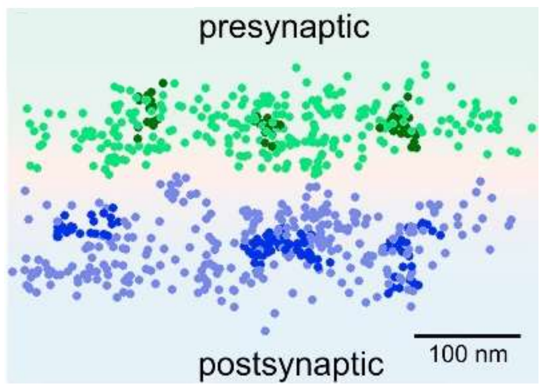
Neurons Synapse



3D structures from EM tomogram



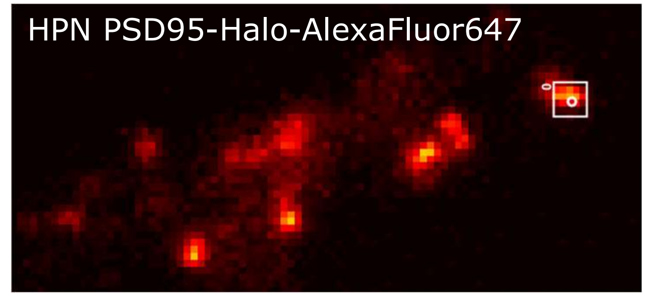
Biederer, et. al. Neuron (2017)

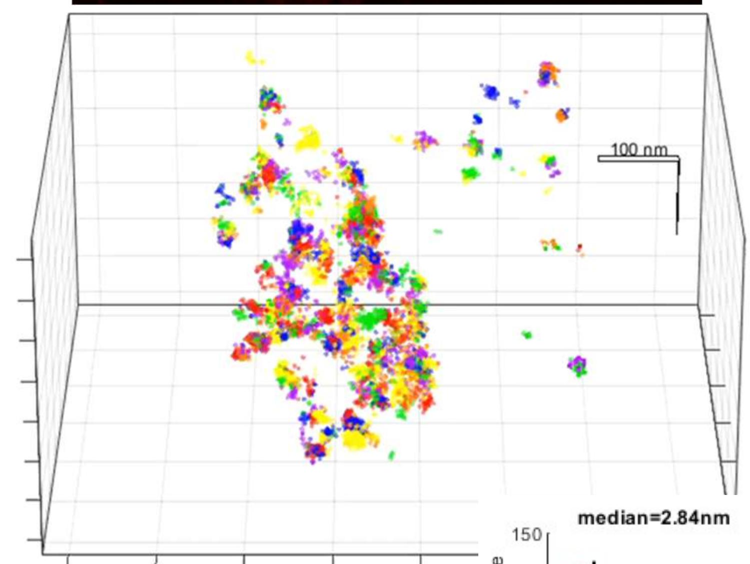


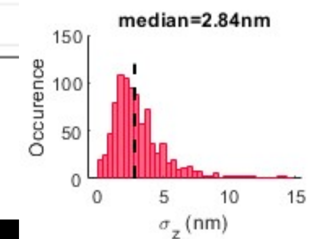
3D STORM 2 Color

RIM1 • within nanocluster • outside nanocluster

PSD-95 • within nanocluster • outside nanocluster





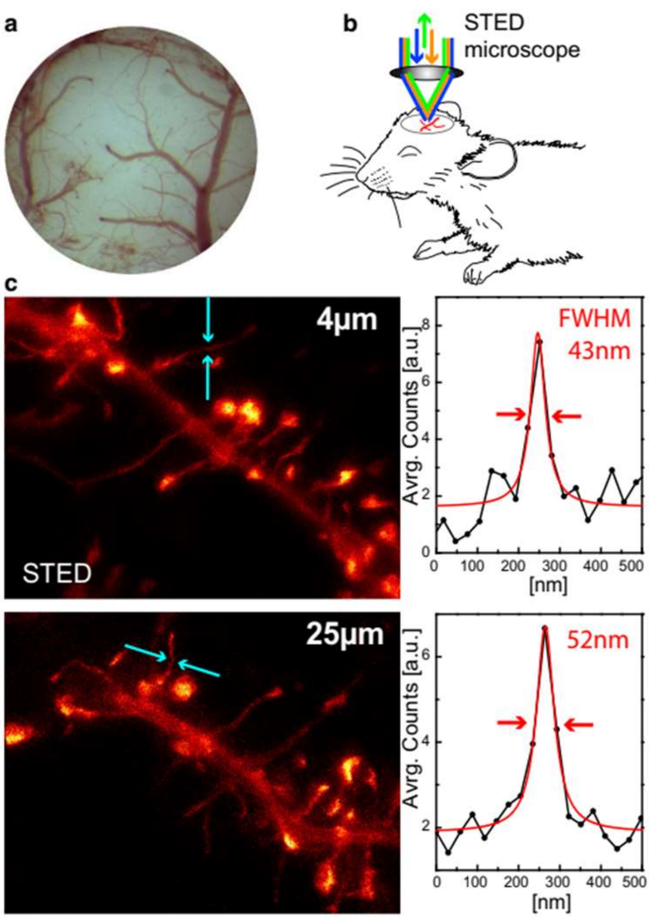


3D MINFLUX 1 Color

Tao et al. J. Neurosci., (2018) Tang, et. al. *Nature* (2016) **PRELIMINARY DATA**

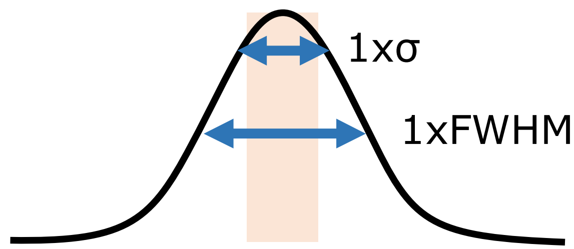
RESOLUTION

Line profiles

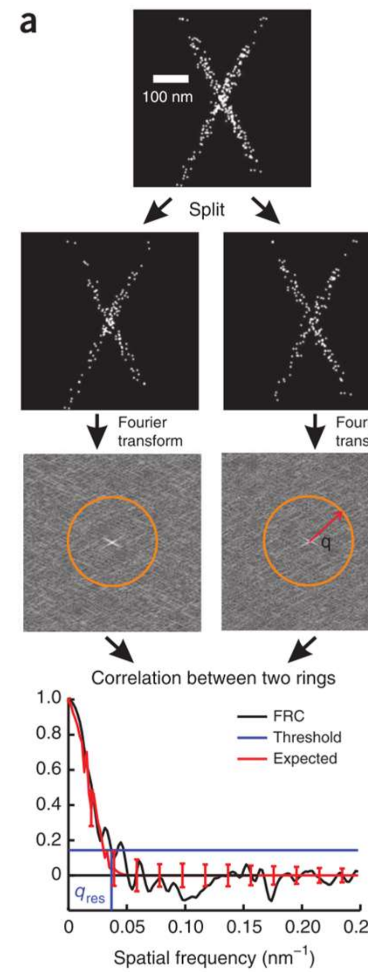


Localization precision

"The *sigma* of the Gaussian"



Fourier Ring Correlation

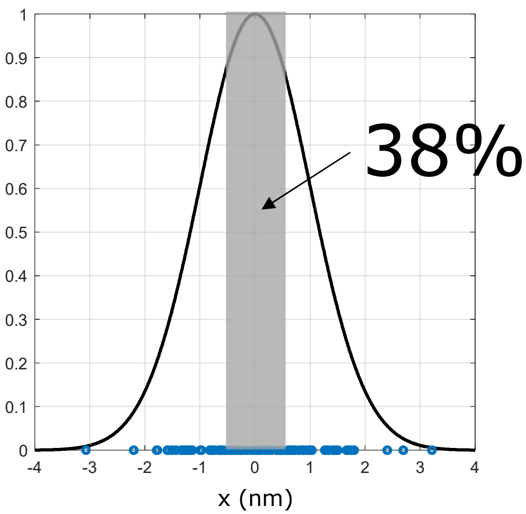


K. I. Willig, et. al. *Biophysical Journal* (2014)

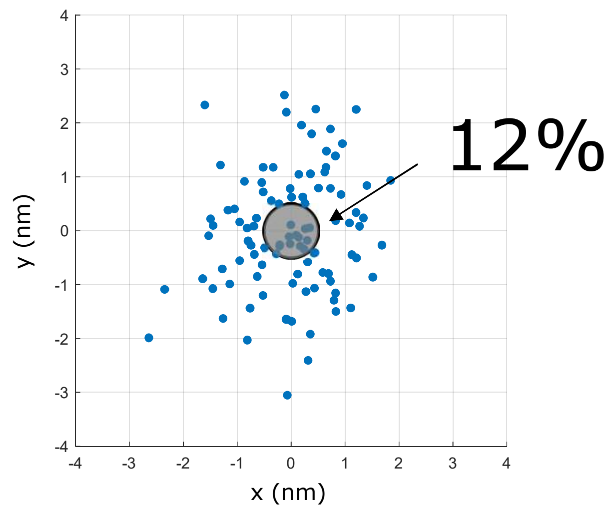
R.P.J. Nieuwenhuizen *et al. Nature Methods*, (2013)

COVERAGE OF 1σ DIAMETER SPHERE

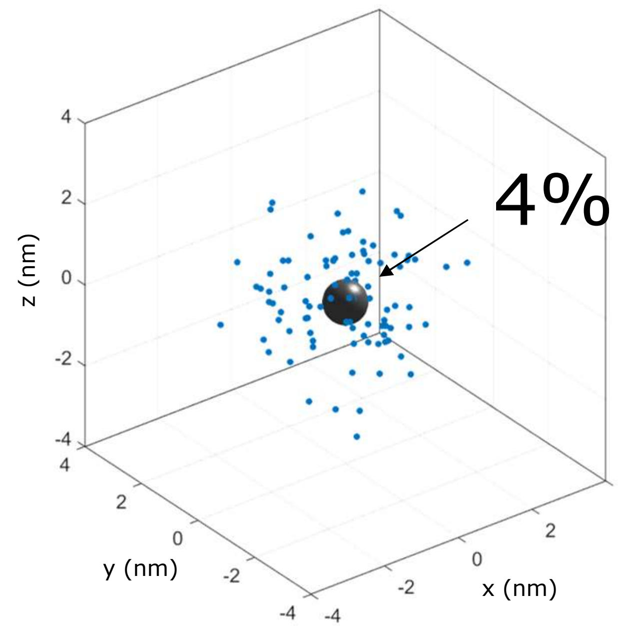
1D



2D



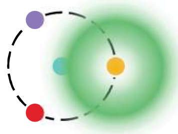
3D



CDF	1D	2D	3D
50%	1.4 σ	2.4 σ	3 σ
90%	2.3 σ	4.3 σ	5 σ

SMLM + SEQUENTIAL STRUCTURED LIGHT

MINFLUX

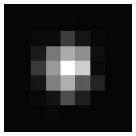


$$\sigma_{MF} \propto \frac{L}{\sqrt{N}}$$

Iterative MINFLUX

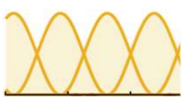
$$\sigma_{iMF} \propto \frac{L}{N^{K/2}}$$

Wide field SMLM



$$\sigma_{CAM} \propto \frac{\lambda}{NA}\frac{1}{\sqrt{N}}$$

SIM + SMLM

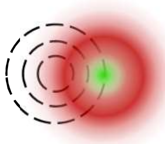


$$\sigma_{SIM} \propto \frac{\lambda}{NA}\frac{1}{\sqrt{N}}\frac{1}{2.4}$$

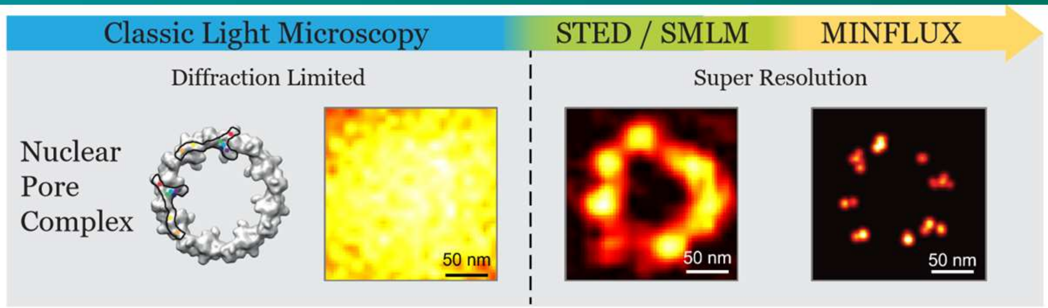
STED

$$\sigma_{STED} \propto \frac{\lambda}{NA}\frac{1}{\sqrt{1+I/I_S}}$$

MINSTED



$$\sigma_{MINSTED} \propto \frac{\lambda}{NA}\frac{1}{\sqrt{1+I/I_S}}\frac{1}{\sqrt{N}}$$



Technology merge and development

- Spectral
- Lifetime
- Polarization
- SPAD arrays
- MINFLUX for dipole orientation
- CLEM
- Combination with cryo-EM

Probe space reevaluation

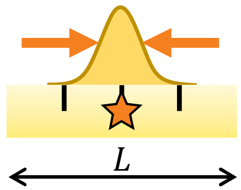
- Photo activatable/convertible fluorescent proteins
- Blinking/photoactivatable dyes
- PAINT scenarios

Input to other pipelines

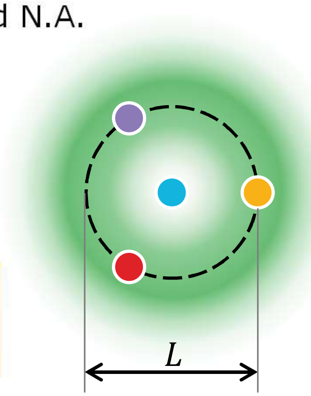
- Particle averaging
- Machine learning reconstructions

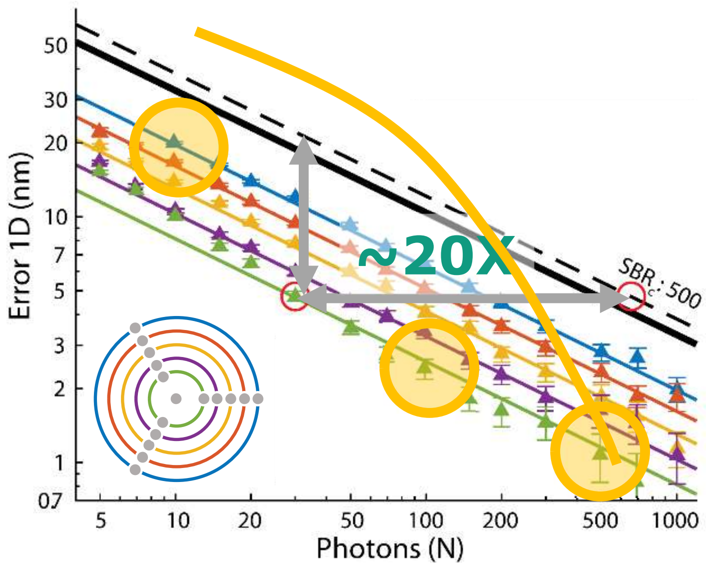
SUMMARY

MINFLUX increases position information
Scheme independent of λ and N.A.



$$\sigma \propto \frac{\lambda}{NA}\frac{1}{\sqrt{N}} \Rightarrow \boldsymbol{\sigma \propto \frac{L}{N^{K/2}}}$$



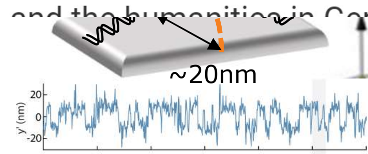


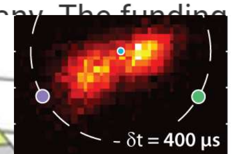
Press Release No. 59 | 10 December 2018

DFG Approves Funding for 13 Optical Microscopes at German Universities

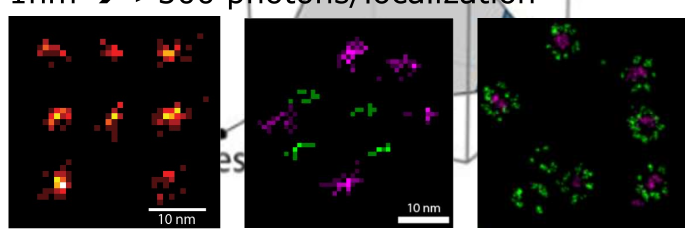
Approximately €14.5 million as part of a thematically focused major instrumentation initiative

The Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) has approved funding of approximately €14.5 million for13 innovative experimental optical microscopes for research. This decision was made in Bonn by the Joint Committee of the largest research funding organisation and central self-governing organisation for science

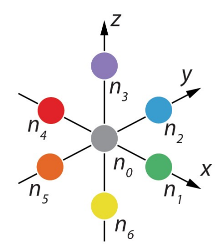


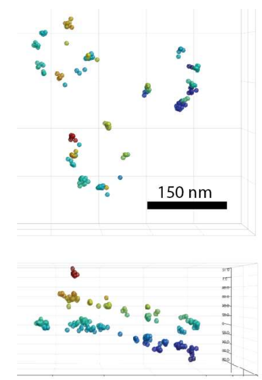


Multicolor imaging
1nm ➔ >500 photons/localization



3D Operation
um-sized images, isotropic resolution





TECHONOLOGY TRANSFER

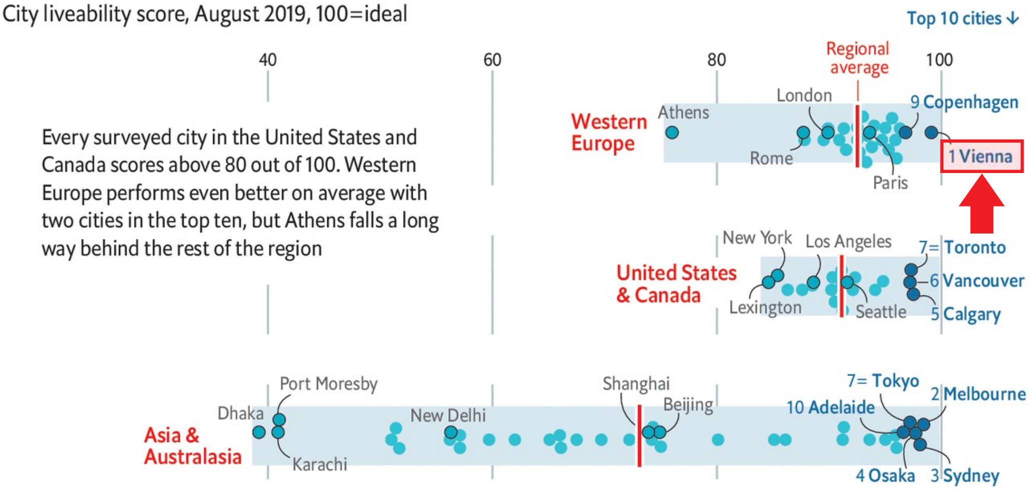
Press Release No. 59 | 10 December 2018

DFG Approves Funding for 13 Optical Microscopes at German Universities

Approximately €14.5 million as part of a thematically focused major instrumentation initiative

Oh, Vienna

City liveability score, August 2019, 100=ideal



Asia & Australasia is the

VIENNA

MOST LIVABLE CITY IN THE WORLD

Top ten positions

City	Location	Rank	Index	Stability	Healthcare	Culture & Environment	Education	Infrastructure
Vienna	Austria	1	99.1	100.0	100.0	96.3	100.0	100.0
Copenhagen	Denmark	2	98.0	100.0	95.8	95.4	100.0	100.0
Zurich	Switzerland	3	96.3	95.0	100.0	96.3	91.7	96.4
Calgary	Canada	3	96.3	95.0	100.0	90.0	100.0	100.0
Vancouver	Canada	5	96.1	90.0	100.0	100.0	100.0	92.9
Geneva	Switzerland	6	95.9	95.0	100.0	94.9	91.7	96.4
Frankfurt	Germany	7	95.7	90.0	100.0	96.3	91.7	100.0
Toronto	Canada	8	95.4	95.0	100.0	95.4	100.0	89.3
Amsterdam	Netherlands	9	95.3	90.0	100.0	97.2	91.7	96.4
Osaka	Japan	10	95.1	100.0	100.0	83.1	100.0	96.4
Melbourne	Australia	10	95.1	95.0	83.3	98.6	100.0	100.0

Source: EIU.






Source: Economist Intelligence Unit

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