Single-Molecule Localization Microscopy: Theoretical Basis and Practical Guide

“Widening the Lens” Microscopy Education program
Vanderbilt University, Nashville, TN, USA

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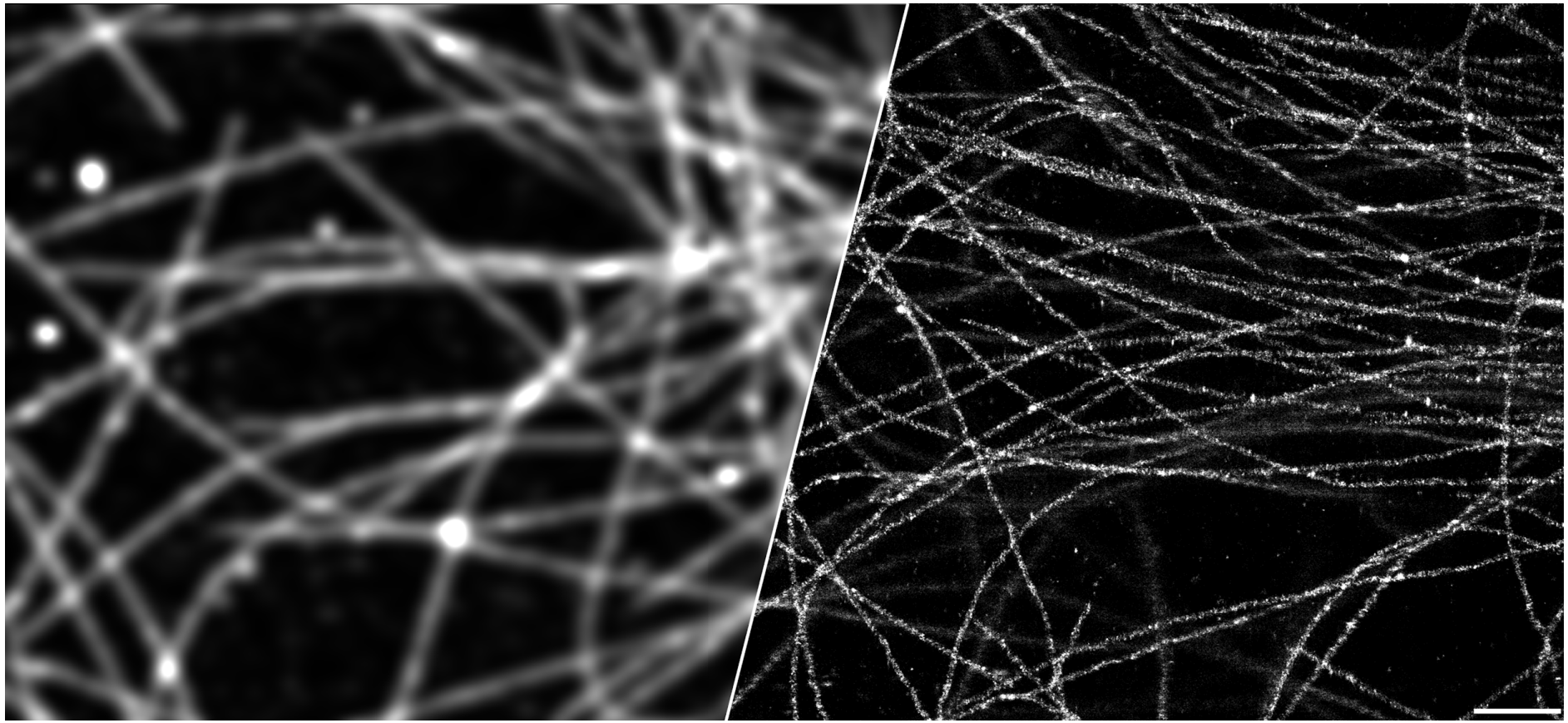
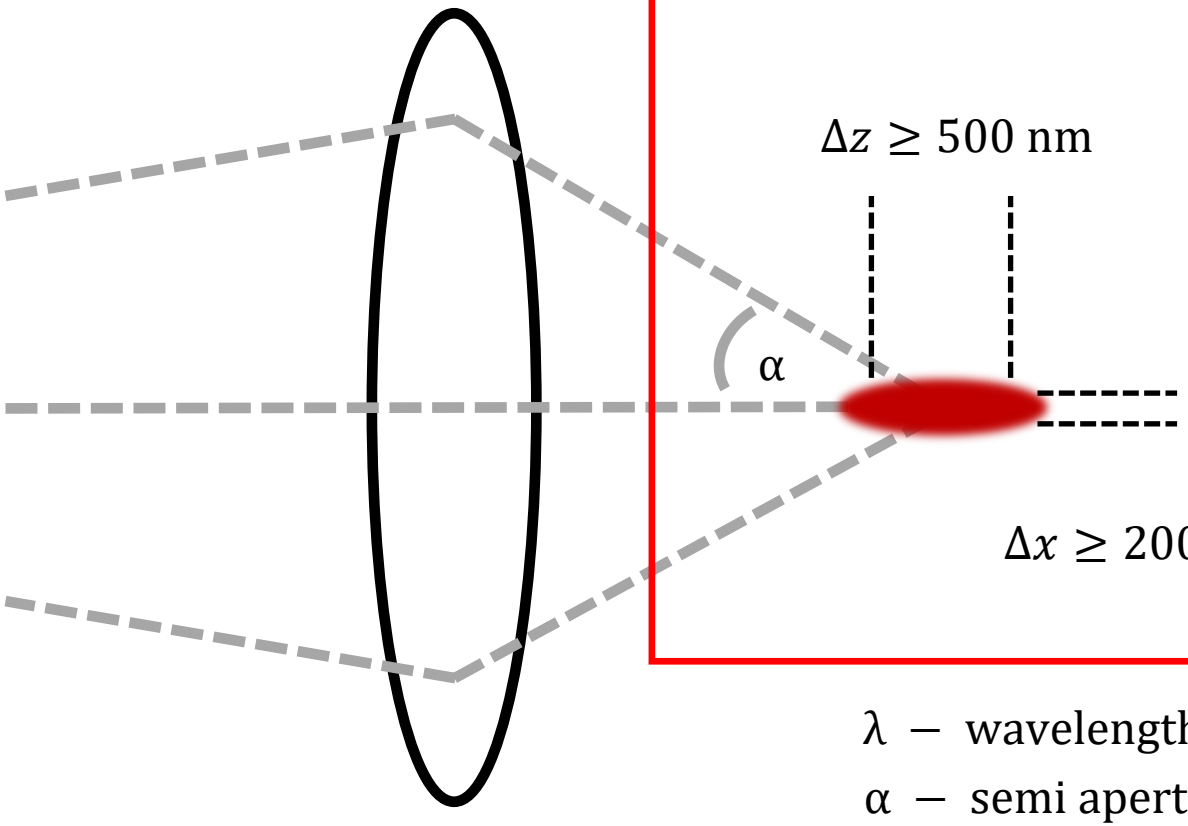
Diffraction Limited vs. Diffraction Unlimited



Microtubules, scale bar: 1 um

The Diffraction Limit

Point Spread Function (PSF)



$\Delta z \geq 500$ nm

$\Delta x \geq 200$ nm

Lateral resolution

$$\Delta x \approx \frac{\lambda}{2\mathrm{NA}}$$

Abbe Equation

Axial resolution

$$\Delta z \approx \frac{2\lambda n}{\mathrm{NA}^2}$$

λ – wavelength

α – semi aperture angle

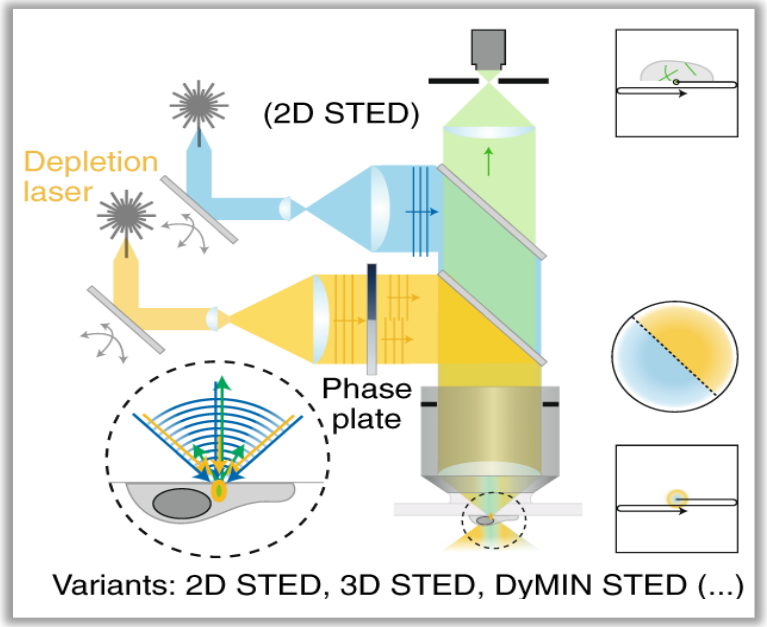
n – refractive index

NA – refractive index

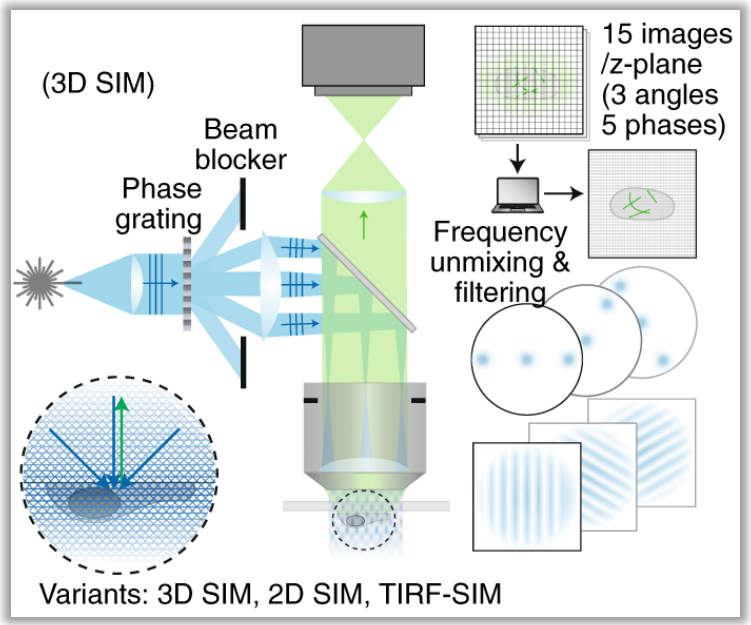
$\mathrm{NA} = n \sin\alpha$

Abbe et al. *Arch. Mikroskop.* (1873)

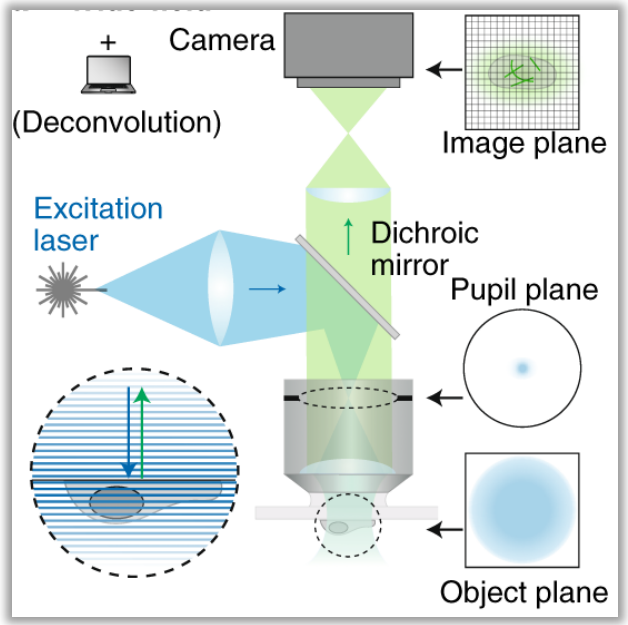
Circumventing the diffraction limit with…



STED



SIM

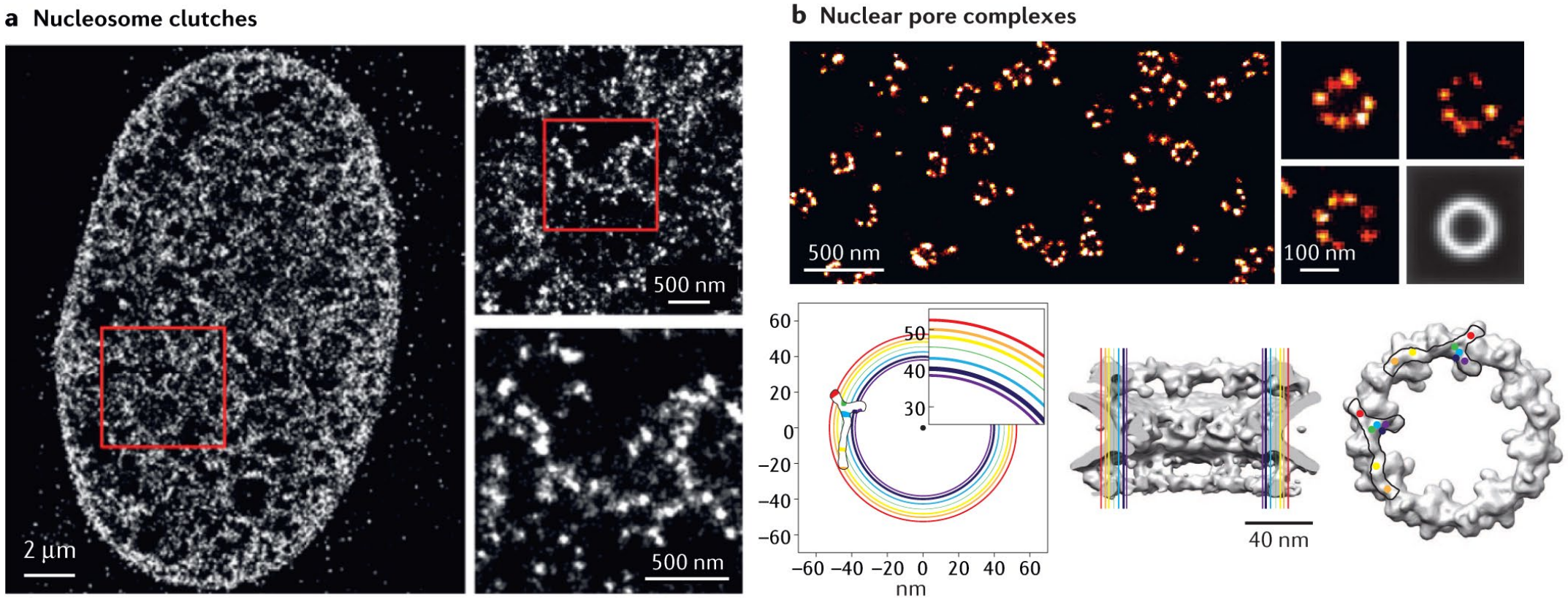


SMLM

This lecture's focus

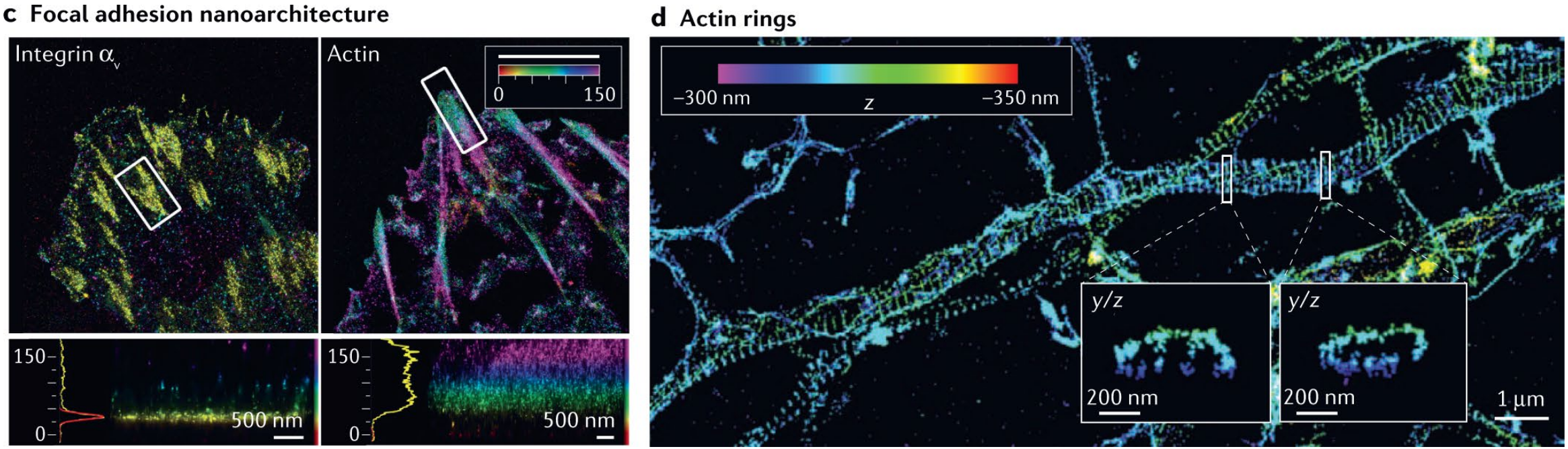
Schermelleh et al. *Nature Cell Biology* (2019)

Major discoveries enabled by SMLM



Lelek et al. *Nature Reviews Methods Primer* (2021)

Major discoveries enabled by SMLM



Lelek et al. *Nature Reviews Methods Primer* (2021)

Overview

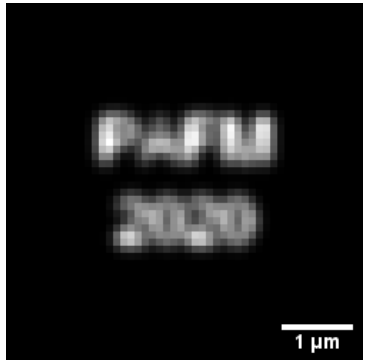
- Basic principles 2D SMLM
- Hardware for SMLM
- Practical considerations: sample preparation, suitable dyes, linkage errors, and buffers
- Processing, quantification, and interpretation of SMLM data
- 3D SMLM
- Summary

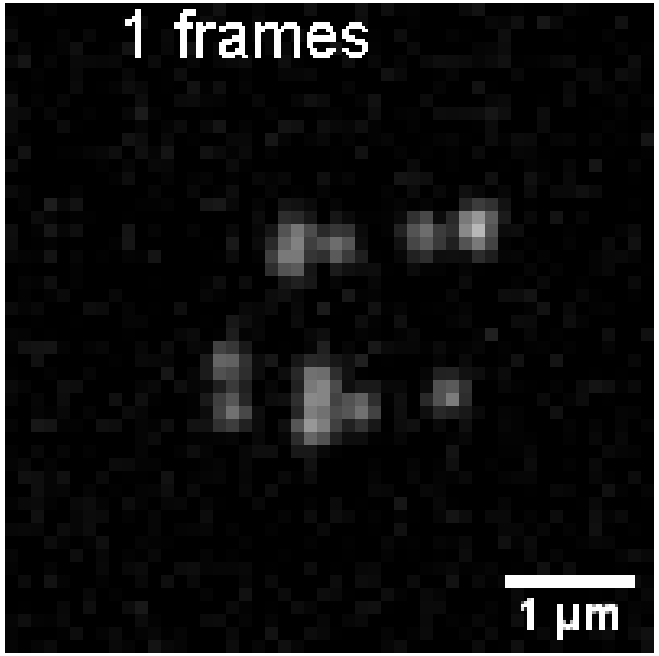
- Extra: New directions in SMLM
- Extra: SRM as a multidimensional challenge

Basic principles of 2D SMLM

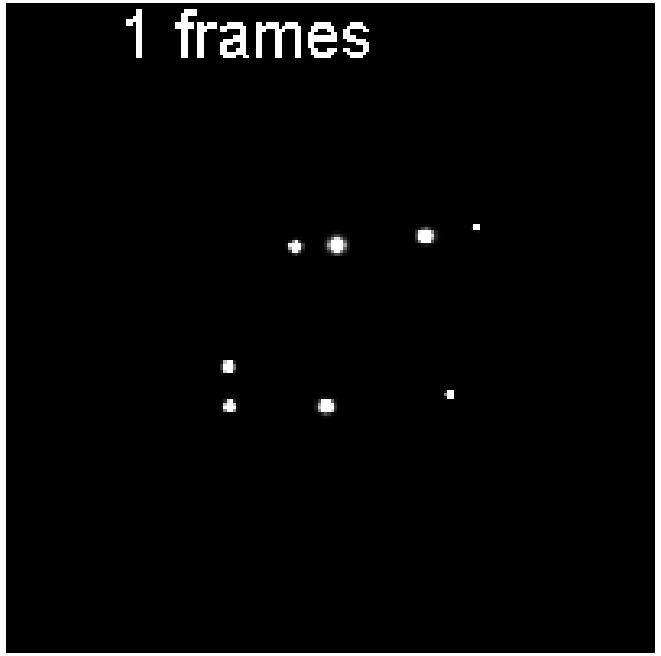
What is Single Molecule Localization Microscopy (SMLM)?

Widefield image





Sequence of widefield images



Accumulated localizations

Superresolution image

Betzig et al. *Science* (2006), Hess et al. *Biophy. J.* (2006), Rust et al. *Nature Methods* (2006)

What is Single Molecule Localization Microscopy (SMLM)?

Sample Preparation

Widefield image

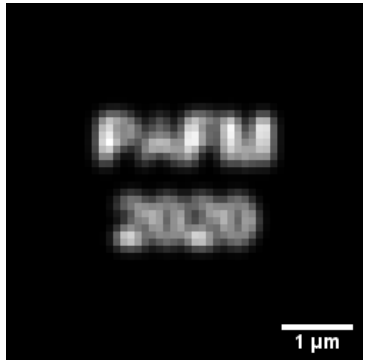


Image Acquisition

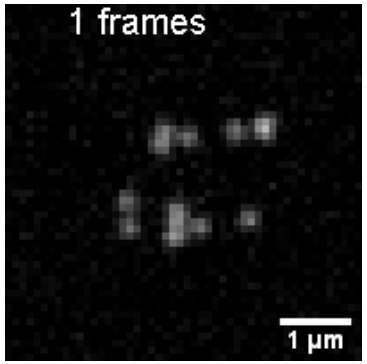


Image at t_1

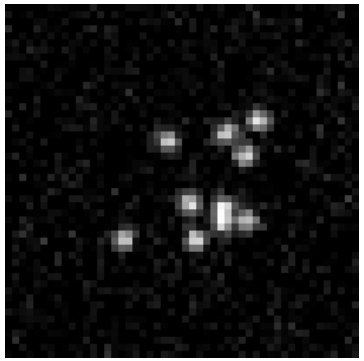


Image at t_2

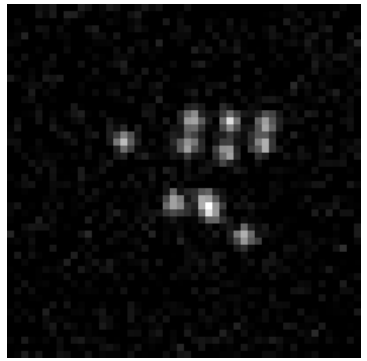
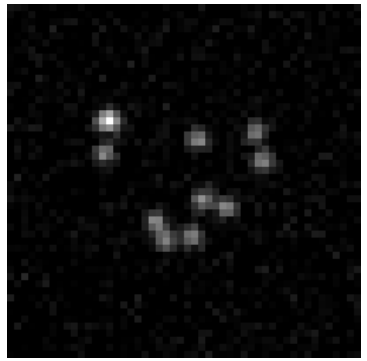


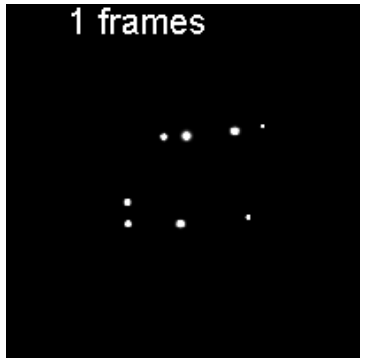
Image at t_3

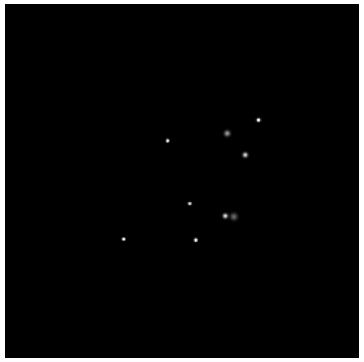


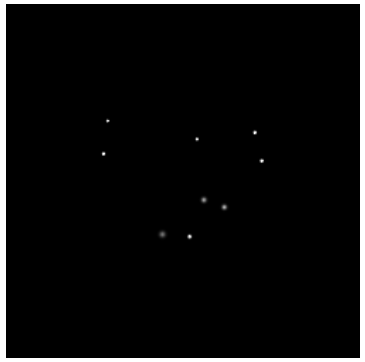
Processing

Superresolution image

Hardware







$t_1 < t_2 < t_3$

Betzig et al. *Science* (2006), Hess et al. *Biophy. J.* (2006), Rust et al. *Nature Methods* (2006)

What is Single Molecule Localization Microscopy (SMLM)?

- In SMLM small subsets of individual emitters are randomly activated or switched ON/OFF in consecutive acquisitions.
- If sparse enough to be identified as single molecule switching events, signals become spatiotemporally separated and are collected over several thousands of camera frames.
- Raw data are computationally processed to detect single molecules and determine their center positions with nanometer precision dependent on the number of photons detected per individual emitter.
- These are finally assembled through superimposition into a single-plane image.

Betzig et al. *Science* (2006), Hess et al. *Biophy. J.* (2006), Rust et al. *Nat Methods* (2006)

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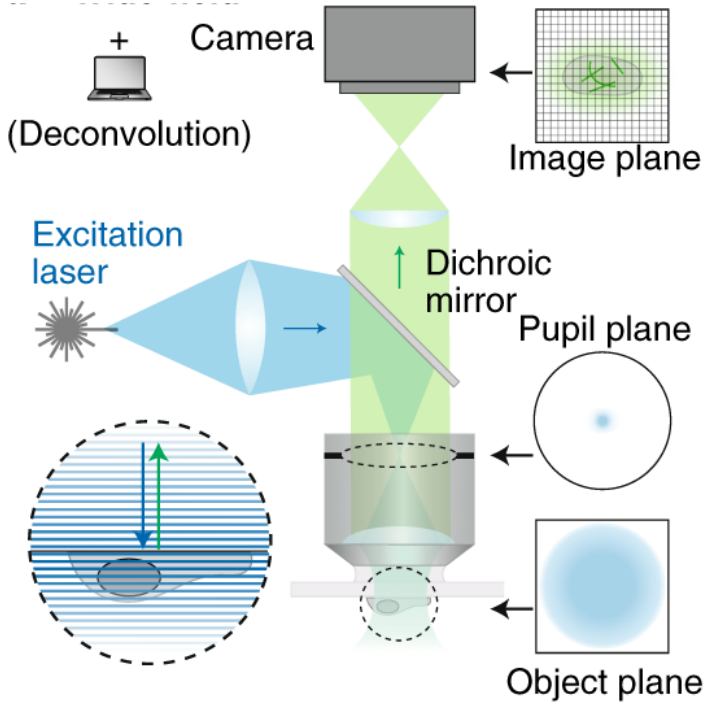
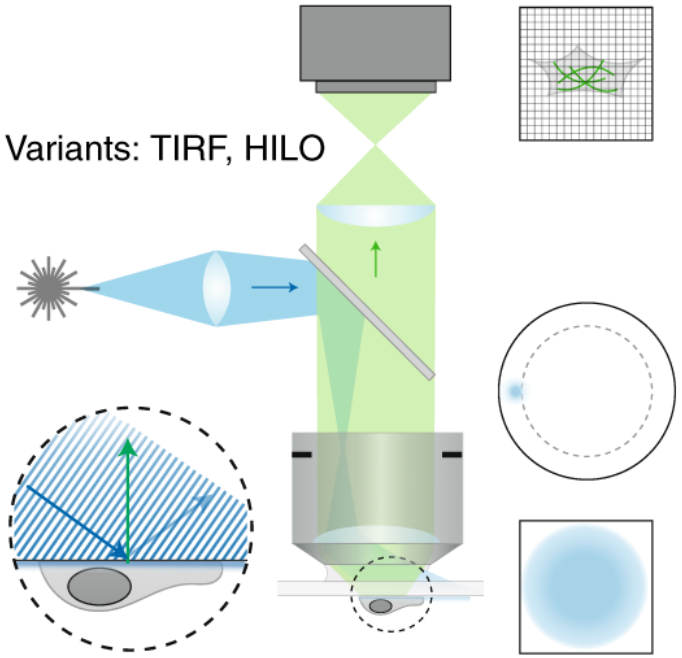
Hardware for SMLM

Hardware for SMLM



Wide-field



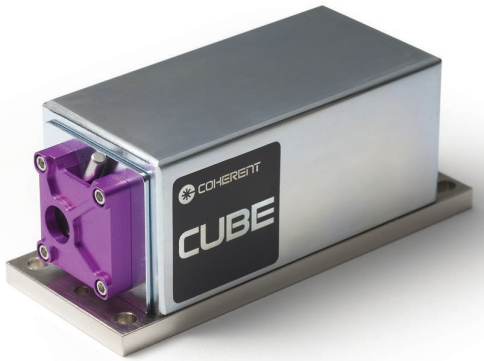
TIRF

Schermelleh et al. *Nature Cell Biology* (2019)

Java Tutorial: Evanescent Field Penetration Depth

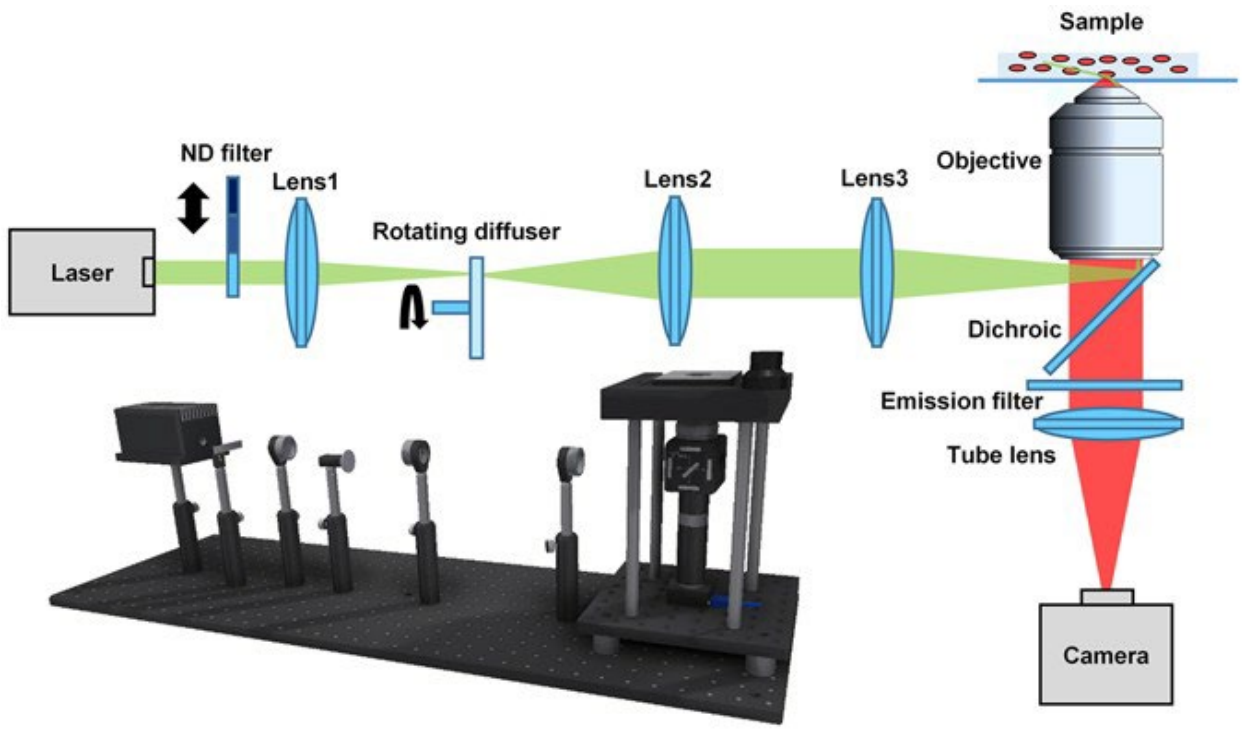
Hardware for SMLM

- Wide-field or TIRF with high NA objectives (15k-20k)
- High power lasers (~25k)
- Sensitive EMCCD (~35k) or sCMOS (~16k) cameras
- Additional optics for 3D information



System upgrade:
~$80,000

Hardware for SMLM



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SCIENTIFIC REPORTS

OPEN

A simple and cost-effective setup for super-resolution localization microscopy

Hongqiang Ma[1], Rao Fu[1,2], Jianquan Xu[1] & Yang Liu[1,3]

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Published online: 08 May 2017

Single molecule localization microscopy (SMLM) has become a powerful imaging tool for biomedical research, but it is mostly available in imaging facilities and a small number of laboratories due to its high cost. Here, we evaluate the possibility of replacing high-cost components on standard SMLM with appropriate low-cost alternatives and build a simple but high-performance super-resolution SMLM setup. Through numerical simulation and biological experiments, we demonstrate that our low-cost SMLM setup can yield similar localization precision and spatial resolution compared to the standard SMLM equipped with state-of-the-art components, but at a small fraction of their cost. Our low-cost SMLM setup can potentially serve as a routine laboratory microscope with high-performance super-resolution imaging capability.

In the past few decades, fluorescence microscopy has significantly expanded our ability to study biological processes at the cellular and subcellular level. However, due to the diffraction-limited spatial resolution, conventional fluorescence microscopy cannot visualize biological structures smaller than ~200 nm. Until the recent decade, a number of super-resolution techniques have been developed to break the diffraction barrier. These super-resolution techniques can generally be divided into two categories: the technique based on the nonlinear effects of the fluorescence that sharpens the point spread function (PSF) using modulated light, including stimulated emission depletion (STED)[1] and structured-illumination microscopy (SIM)[2,3] and the technique based on localization of sparsely excited single fluorescent emitters, including (fluorescence) photo-activated localization microscopy ((f) PALM)[4,5] and (direct) stochastic optical reconstruction microscopy ((d) STORM)[6,7]. These super-resolution techniques have improved the spatial resolution by approximately an order of magnitude. In particular, single molecule localization microscopy (SMLM), which provides impressive spatial resolution down to 20 nm with relatively simple hardware compared to SIM and STED, has a great potential as a common laboratory tool for biomedical research. However, despite that SMLM has been commercialized for almost a decade, it remains a high-end microscopy instrument only available in the imaging facilities at major academic institutions and a small number of laboratories. Its high cost limits its widespread use as a routine microscopy system as conventional fluorescence microscope.

The key technical requirement for SMLM includes (1) a powerful laser to photo-bleach most fluorophores and activate only a small portion of sparsely distributed single fluorophores; (2) high numerical aperture (NA) objective lens to efficiently collect the limited number of photons emitted by single molecule; and (3) scientific cameras with high quantum efficiency and low noise to record the image from individual fluorescent emitters at a high signal to noise ratio (SNR). To meet these technical requirements and achieve the best performance, it is a common belief that the high-end optical and optoelectronic components have to be used, including high-power single-mode laser, high-end objectives, nano-positioning mechanical system and highly sensitive cameras, which easily drive the total cost to exceed $100,000.

To lower the cost of light source (one of the most expensive items), the industry-grade laser was proposed to replace the scientific grade laser, which has shown a decent performance in SMLM[8,9]. Unlike SIM and STED, the imaging quality of SMLM is not sensitive to the slight degradation on the stability of the laser output and spectral linewidth. In contrast, the imaging quality of SMLM is more likely to be affected by the objective lens and cameras. Low-cost objective lens usually have lower NA, which not only degrades the spatial resolution, but also

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Total cost: $4,100.00

Ma et al. *Scientific Reports* (2017)

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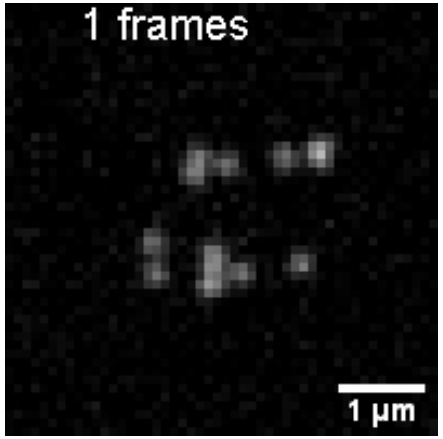
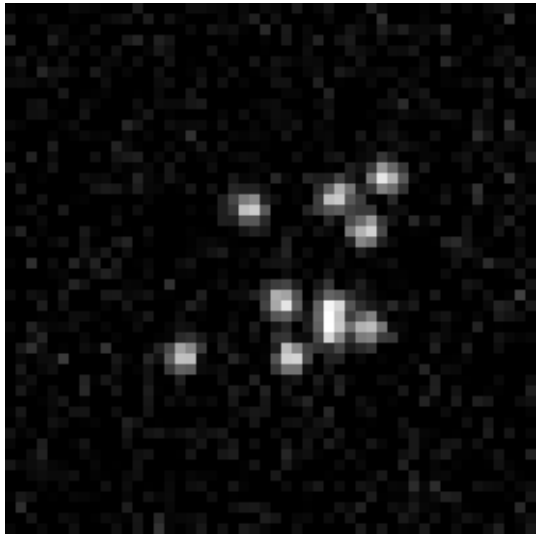
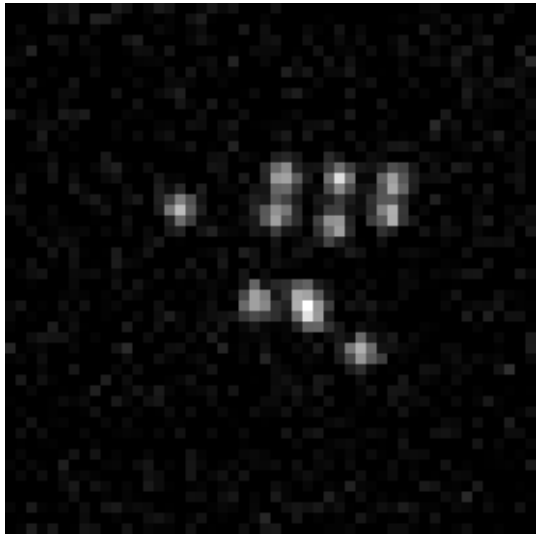
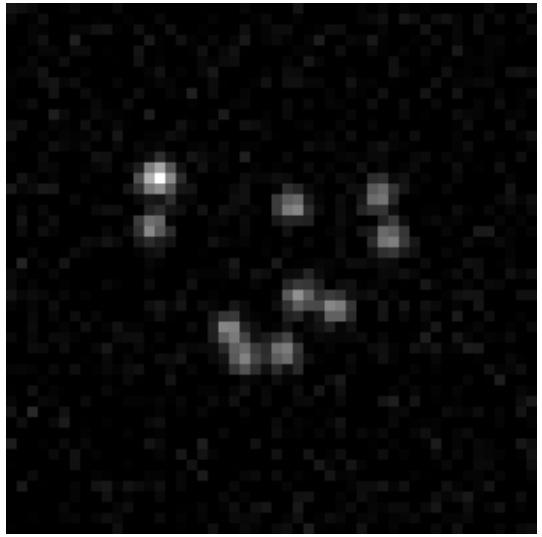
Photoswitching – ON/OFF switching

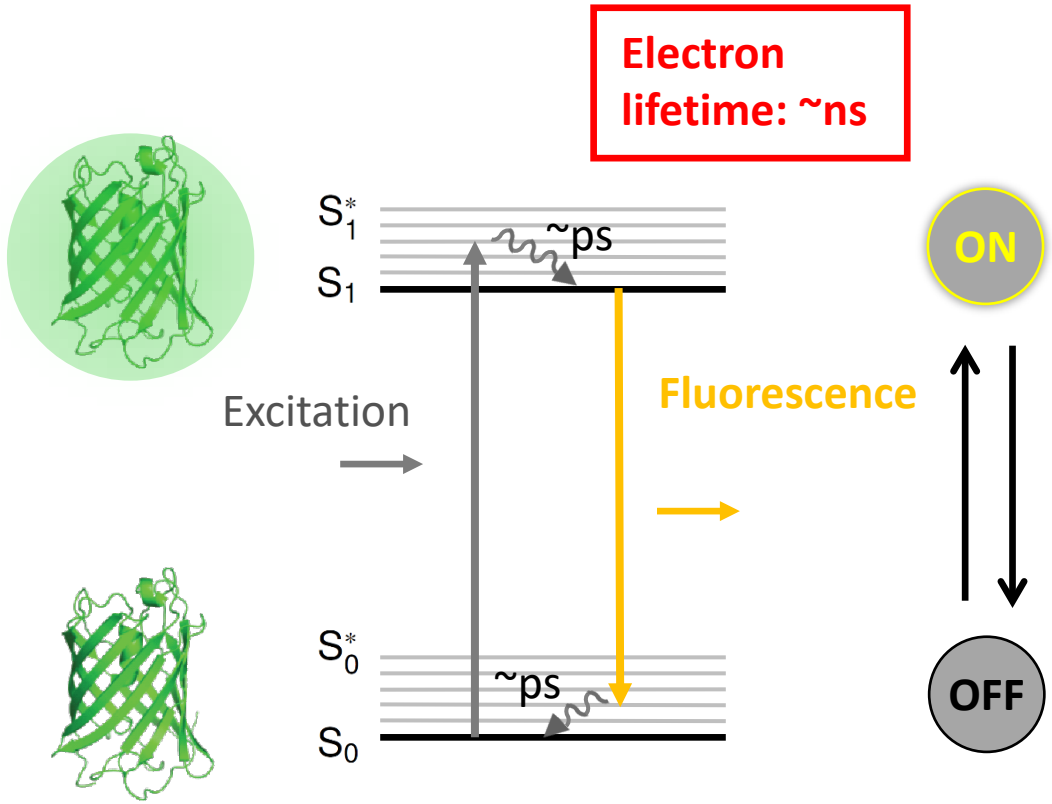


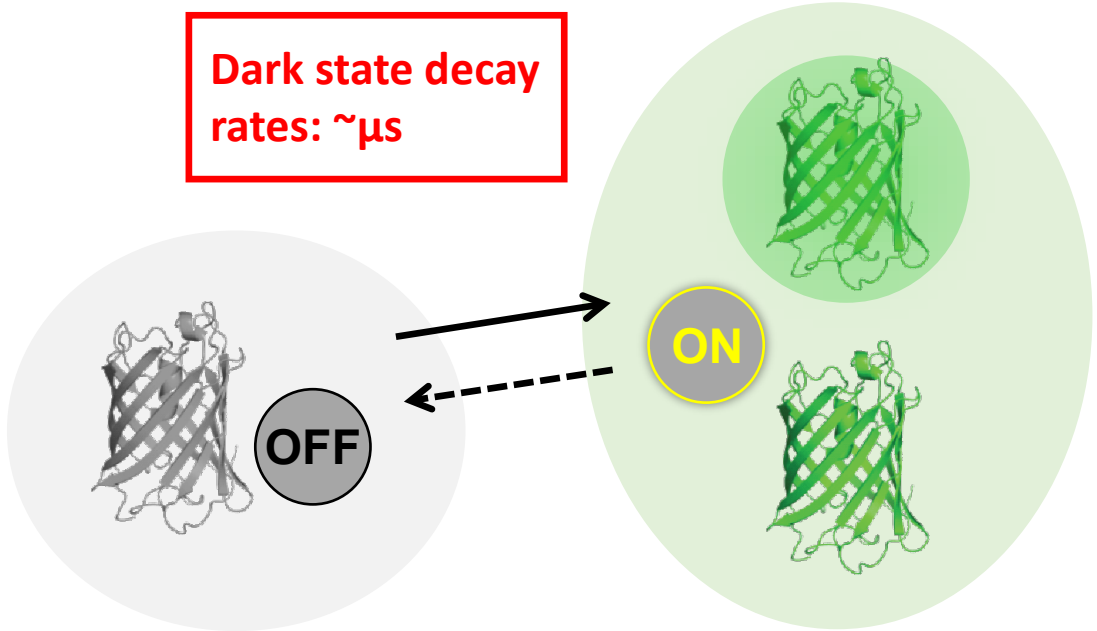






Photoswitching – ON/OFF switching





Conventional dyes

Temporal separation not possible

Typical SMLM dyes

“Dark” state allows temporal separation for SMLM

A good SMLM dye has…

Long ON-time to allow the collection of many photons, but not too long such that the data acquisition is slowed down.

Complete dark OFF-state that is long enough to prevent too many fluorophores from being ON simultaneously.

High absorption coefficient and quantum yield to produce bright events.

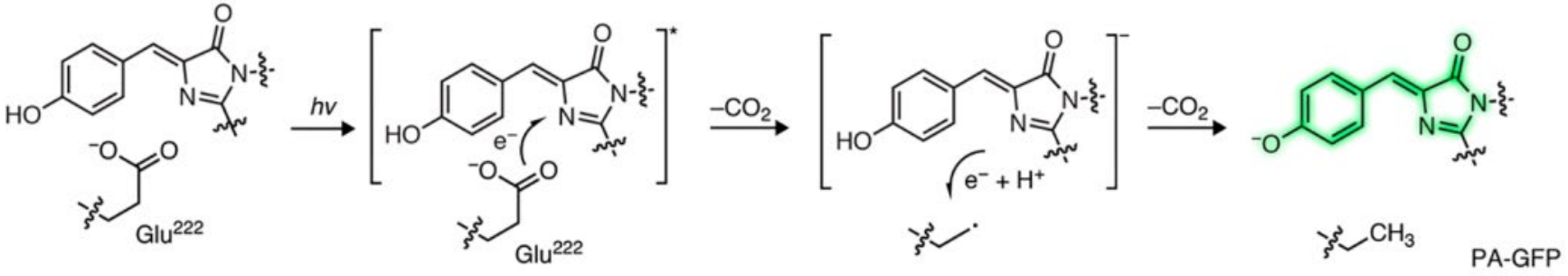
Exceptional resistance to bleaching since tens of thousands of images are collected at high laser power.

Bleaching, brightness, ON-time, and ON/OFF duty cycle are all strongly affected by laser power and buffer composition.

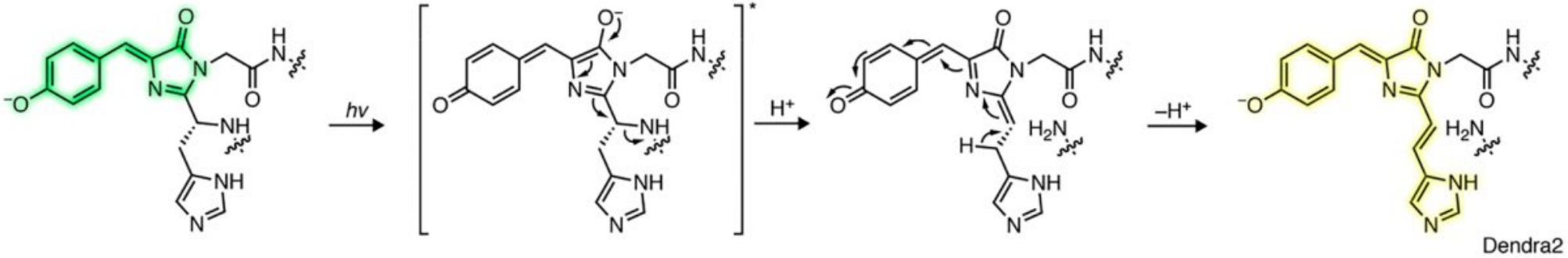
Nahidiazar et al. *PLOS ONE* (2016)

Photoswitchable Fluorescent Proteins

Photoactivation by decarboxylation (PA-GFP, PA-mKate, PA-mCherry)



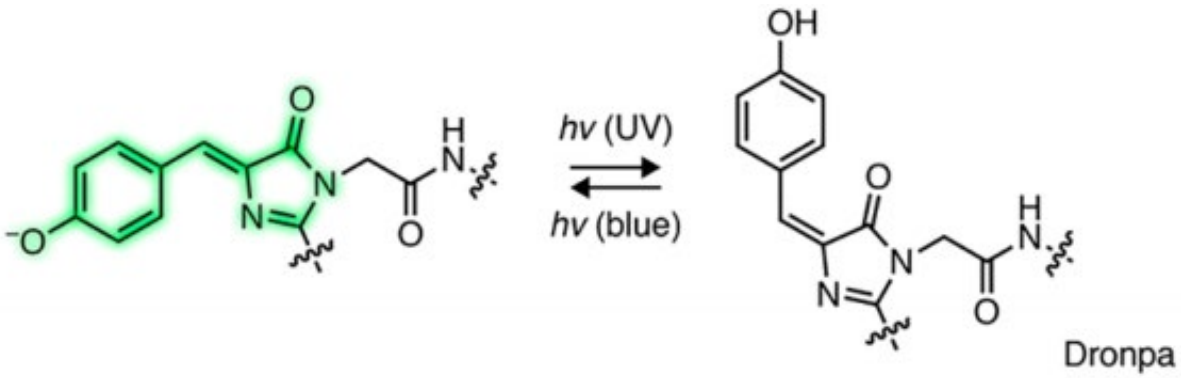
Photoswitching by beta-elimination (Dendra, mEos, mClavGR2, mIrisFP, mMaple)



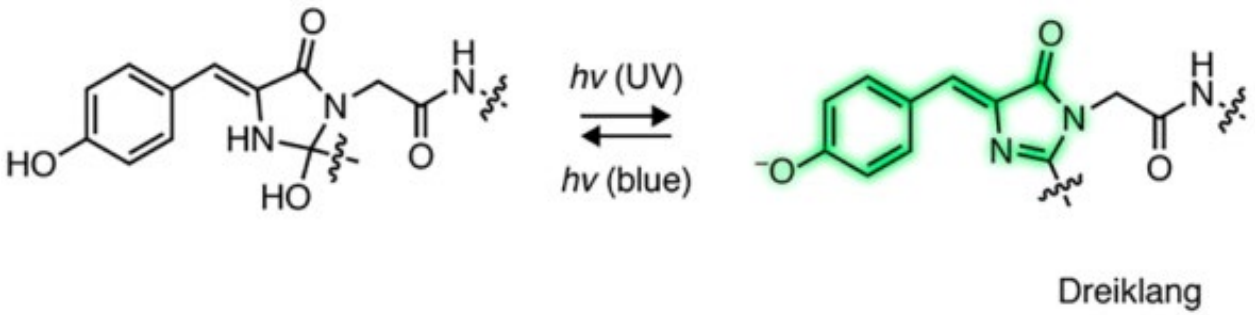
Jradi et al. *ACS Chem. Biol.* (2019)

Photoswitchable Fluorescent Proteins

Photoconversion by *cis-trans* isomerization (Dronpa)



Photoconversion by hydration/dehydration (Dreiklang)



Jradi et al. *ACS Chem. Biol.* (2019)

Photoswitchable Fluorescent Proteins

Name	λ_{ex} / λ_{em} $\xrightarrow{h\nu}$ λ_{ex} / λ_{em}	$\varepsilon \times 10^4$ ($M^{-1}cm^{-1}$)	Φ	F_{on}/F_{off}	$t_{1/2}M^b$ (min)	$t_{1/2}on^c$ (s)	$t_{1/2}PB^c$ (s)	N	σ (nm)
Photoactivatable									
PA-GFP	$\xrightarrow{405}$ 488 / 517	1.74	0.79	60	<10	1.8	150	313 ($\bar{x}$)	41 ($\bar{x}$)
PAmCherry1	$\xrightarrow{405}$ 561 / 595	1.80	0.46	4000	23	2.5	100	724 ($\bar{x}$) 413 (M)	18 ($\bar{x}$) 17 (M)
PATagRFP	$\xrightarrow{405}$ 561 / 595	6.60	0.38	550	75	4.5	180	251–906 ($\bar{x}$) 150 (M)	39 ($\bar{x}$) 31 (M)
PAmKate	$\xrightarrow{405}$ 561 / 628	2.50	0.18	100	19	6.3	350	NR	15 ($\bar{x}$)
Photoswitchable									
PS-CFP2	405 / 468 $\xrightarrow{405}$ 488 / 511	4.30; 4.70	0.20; 0.23	1000	NR	NR	NR	262–930 ($\bar{x}$) 250 (M)	29 ($\bar{x}$) 27 (M)
Dendra2	488 / 507 $\xrightarrow[488]{405}$ 561 / 573	4.50; 3.50	0.50; 0.55	300	90	NR	260; 2420	131–686 ($\bar{x}$)	23 ($\bar{x}$)
mEos3.2	488 / 516 $\xrightarrow{405}$ 561 / 580	6.34; 3.22	0.84; 0.55	NR	20	NR	13; 48	482–809 ($\bar{x}$) 264 (M)	12 ($\bar{x}$) 10 (M)
mClavGR2	488 / 504 $\xrightarrow{405}$ 561 / 583	1.90; 3.20	0.77; 0.53	200	27	NR	233; 3644	379 ($\bar{x}$)	<30($\bar{x}$)
mMaple3	488 / 505 $\xrightarrow{405}$ 561 / 583	1.50; 0.74	3.0; 0.56	400	49	NR	9.4;133	675 ($\bar{x}$)	NR
PSmOrange	561 / 565 $\xrightarrow{405}$ 640 / 662	11.3; 3.3	0.51; 0.28	550	96	NR	15; 49	337 ($\bar{x}$)	45 ($\bar{x}$)

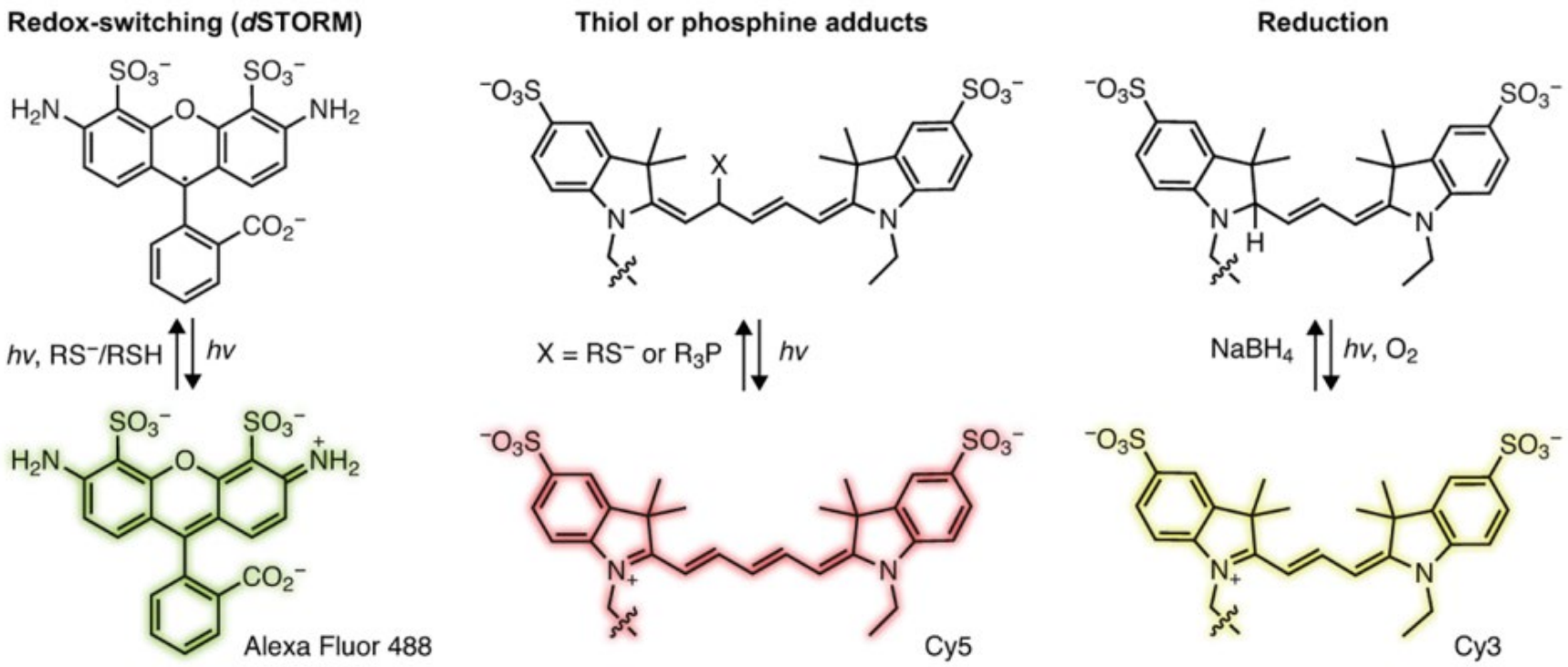
Jradi et al. *ACS Chem. Biol.* (2019)

Name	λ_{ex} / λ_{em} $\overset{hv}{\underset{hv}{\rightleftarrows}}$ λ_{ex} / λ_{em}	$\varepsilon \times 10^4$ ($M^{-1}cm^{-1}$)	*FR*	*Φ*	F_{on}/F_{off}	$t_{1/2}M^b$ (min)	$t_{1/2}on^c$ (s)	$t_{1/2}off^c$ (s)	$t_{1/2}PB^c$ (s)	*N*	σ (nm)
Photochromic											
bsDronpa	488 / 504 $\overset{488}{\underset{405}{\rightleftarrows}}$	4.5	100	0.50	20	NR	0.04	1.25	12.5	560 ($\bar{x}$)	<45($\bar{x}$)
rsEGFP	488 / 510 $\overset{488}{\underset{405}{\rightleftarrows}}$	4.7	1200	0.36	65	120	0.00002	0.001	800	<60 ($\bar{x}$)	70 ($\bar{x}$)
Skylan-S	488 / 511 $\overset{488}{\underset{405}{\rightleftarrows}}$	15.2	7000	0.64	26	NR	0.073	4.77	NR	NR	20 ($\bar{x}$)
mGeos-M	488 / 514 $\overset{488}{\underset{405}{\rightleftarrows}}$	5.2	8	0.85	20	<10	NR	21.03	20	458 ($\bar{x}$) 387 (M)	14 ($\bar{x}$) 12 (M)
Dronpa	488 / 518 $\overset{488}{\underset{405}{\rightleftarrows}}$	9.5	4	0.85	20	25	0.12	115	2	299 ($\bar{x}$) 269 (M)	17($\bar{x}$) 15 (M)
rsFastlime	488 / 518 $\overset{488}{\underset{405}{\rightleftarrows}}$	4.6	35	0.60	65	NR	0.03	2.60	65	<60 ($\bar{x}$)	40 ($\bar{x}$)
Padron	$\overset{488}{\underset{405}{\rightleftarrows}}$ 488 / 522	4.3	15	0.64	140	NR	5.6	0.06	40	NR	NR
Drieklang	515 / 529 $\overset{405}{\underset{365}{\rightleftarrows}}$	8.3	160	0.41	20	120	1	3	21	720 ($\bar{x}$)	15 ($\bar{x}$)
rsTagRFP	561 / 585 $\overset{561}{\underset{445}{\rightleftarrows}}$	3.7	75	0.11	20	43	0.0028	0.18	NR	NR	NR
rsCherry	$\overset{561}{\underset{450}{\rightleftarrows}}$ 561 / 610	8.0	50	0.02	10	NR	3.0	0.05	NR	359 ($\bar{x}$) 288 (M)	24 ($\bar{x}$) 22 (M)
rsCherryRev	561 / 610 $\overset{561}{\underset{450}{\rightleftarrows}}$	8.4	400	0.005	10	42	0.05	0.7	NR	401 ($\bar{x}$) 264 (M)	25 ($\bar{x}$) 23 (M)

Jradi et al. *ACS Chem. Biol.* (2019)

Photoswitchable Dyes

Conventional fluorophores (Alexa Fluor 488, Cy5, Cy3)

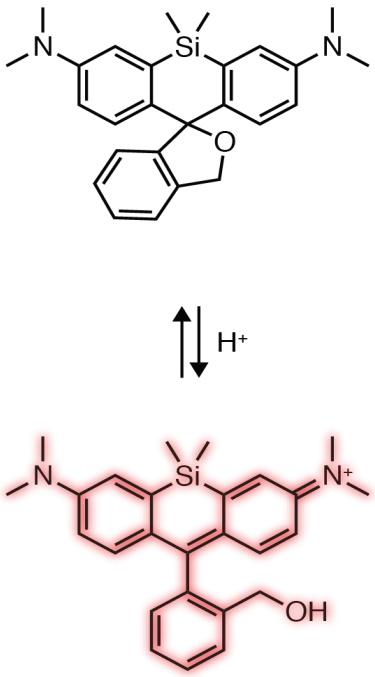


Jradi et al. *ACS Chem. Biol.* (2019)

Photoswitchable Dyes

Spontaneously blinking (HM-SiR)

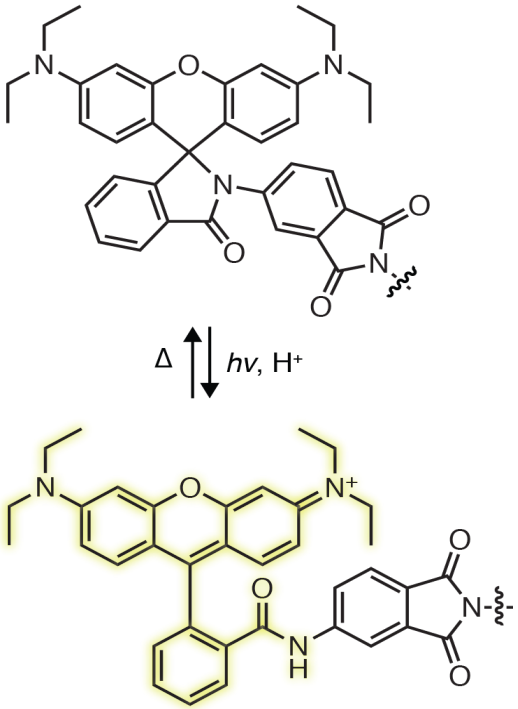
Hydroxymethyl Rhodamines



HM-SiR

Photochromic rhodamines

Rhodamine lactams



Rh_B phthalmide

Jradi et al. *ACS Chem. Biol.* (2019)

Photoswitchable Dyes

Name	λ_{ex} / λ_{em} $\overset{hv}{\underset{hv}{\rightleftharpoons}}$ λ_{ex} / λ_{em}	$\varepsilon \times 10^4$ ($M^{-1}cm^{-1}$)	Φ	$t_{1/2}$on (s)	$t_{1/2}$off (s)	N	σ (nm)
Activator-reporter pairs							
Cy3-Cy5	532 $\rightleftharpoons$ 532; 640 / 670	25	0.28	1.0	1.0	3000 ($\bar{x}$)	NR

Name	λ_{ex} / λ_{em} $\overset{hv}{\underset{hv}{\rightleftharpoons}}$ λ_{ex} / λ_{em}	$\varepsilon \times 10^4$ ($M^{-1}cm^{-1}$)	Φ	*on/off* $\times 10^{-5}$	*SC*	*SF*	N^b	σ (nm)
Activator free								
ATTO 488	405 $\rightleftharpoons$ 488; 488 / 523	9	0.80	220	49	0.99	1110 ($\bar{x}$)	29
Cy3B	405 $\rightleftharpoons$ 561; 561 / 570	13	0.67	40	5	0.89	2057 ($\bar{x}$)	22
Alexa Fluor 647	405 $\rightleftharpoons$ 640; 640 / 665	24	0.33	120	26	0.73	5202 ($\bar{x}$)	17
DyLight750	405 $\rightleftharpoons$ 752; 752 / 778	22	NR	20	6	0.58	749 ($\bar{x}$)	30

Jradi et al. *ACS Chem. Biol.* (2019)

Name	λ_{ex} / λ_{em} $\xrightarrow{h\nu}$ λ_{ex} / λ_{em}	$\varepsilon \times 10^4$ ($M^{-1}cm^{-1}$)	Φ	on/off $\times 10^{-5}$	Φ_{PA}	N	σ (nm)
Photoactivatable/Caged							
Rhodol-NN	405 → 561/563	9	0.3	NR	0.16	NR	36
PA-JF_{549}	405 → 561/570	9	0.78	10	0.022	637 ($\bar{x}$)	14
PA-JF_{646}	405 → 640/665	12	0.47	0.16	NR	760 ($\bar{x}$)	21
N_3-DCDHF	488 → 594/627	5	0.03–0.4[c]	NR	0.095	1100 ($\bar{x}$)	18
$NVOC_2$-Rh_Q	405 → 532/558	9.9	0.83	NR		540 ($\bar{x}$)	NR
$NVOC_2$-Rh_{110}	405 → 488/520	7.6	0.88[d]	NR	0.0013	3488 ($\bar{x}$)	16
$NVOC_2$-OG	405 → 488/526	8.2	0.97	NR		235 ($\bar{x}$)	NR
$NVOC_2$-$SiRh_Q$	405 → 640/654	8	0.38	NR		14944 ($\bar{x}$)	5
Photochromic							
OA-2	405 → 561/641	8	0.09	NR	0.02	600 ($\bar{x}$)	70
Rh_B phthalimide	375 → 532/580	10	0.31	NR	NR	900 ($\bar{x}$)	55
Rh_B stilbene	405 → 561/582	10	0.31	NR	0.009	3800 ($\bar{x}$)	13-17
DAE-sulfone	375 ⇄ 488 (488/588)	5.4	0.34	NR	0.0065	200–350 ($\bar{x}$)	70
Spontanuously blinking							
HMSiR	Δ ⇄ 640/665	10	0.39	NR	NR	2600 ($\bar{x}$)	52

Jradi et al. *ACS Chem. Biol.* (2019)

Current Approaches in SMLM

They differ primarily in how ON/OFF switching is achieved:

(f)PALM – photoactivation

STORM – photoswitching of activator and reporter dye-pair

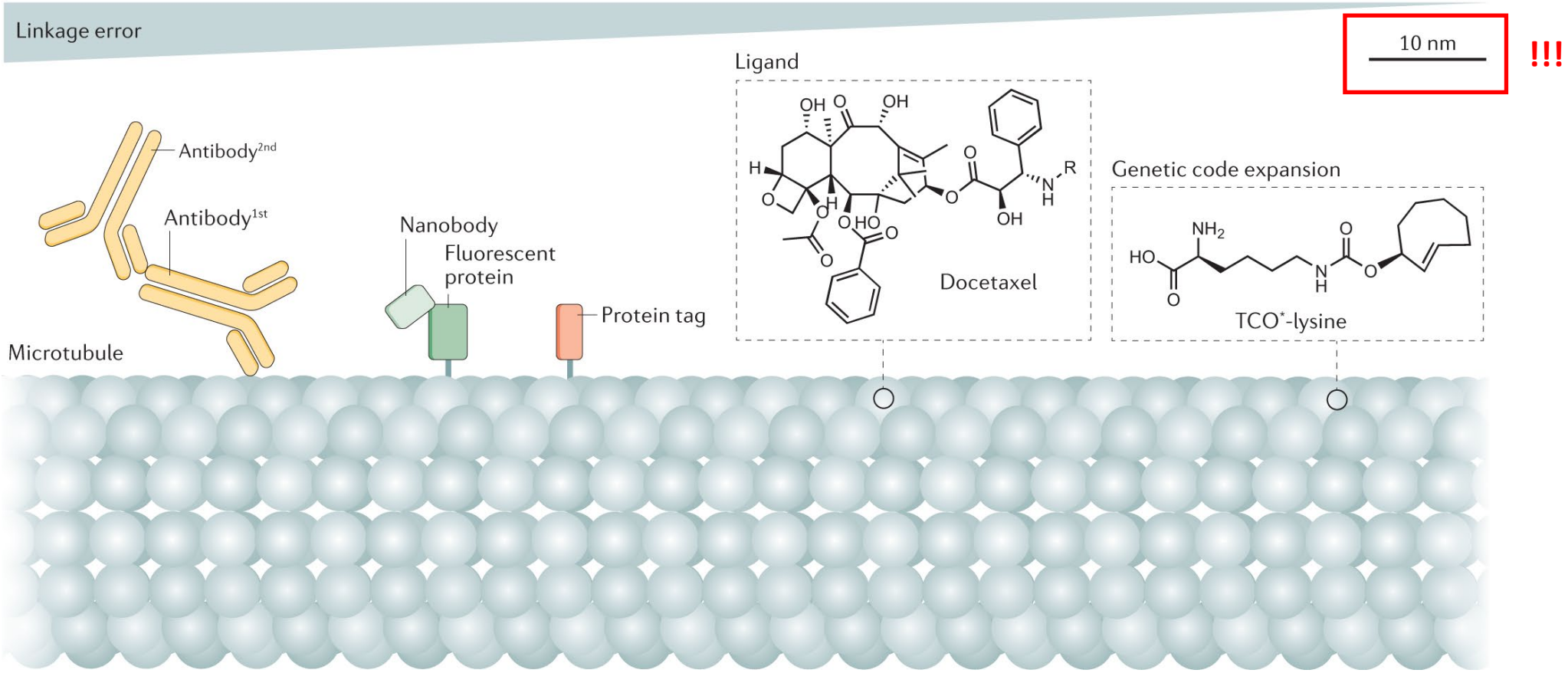
dSTORM – conventional fluorescent probes in the presence of thiols
transfer dyes to a long-lived OFF state

(f)BALM – binding and fluorescence activation of specific dyes

DNA-PAINT/Exchange-PAINT – transient oligonucleotide hybridization

Betzig et al. *Science* (2006), Hess et al. *Biophy. J.* (2006), Rust et al. *Nature Methods.* (2006), Heilemann et al. *Angew Chem Int Ed Engl.* (2008), Schoen et al. *Nano Lett.* (2011), Szczurek et al. *Nucleic Acids Res.* (2017), Jungmann et al. *Nano Lett.* (2010), Jungmann et al. *Nat. Methods* (2010), Jungmann et al. *Nat. Methods* (2014)

Linkage errors of various fluorescent labelling approaches

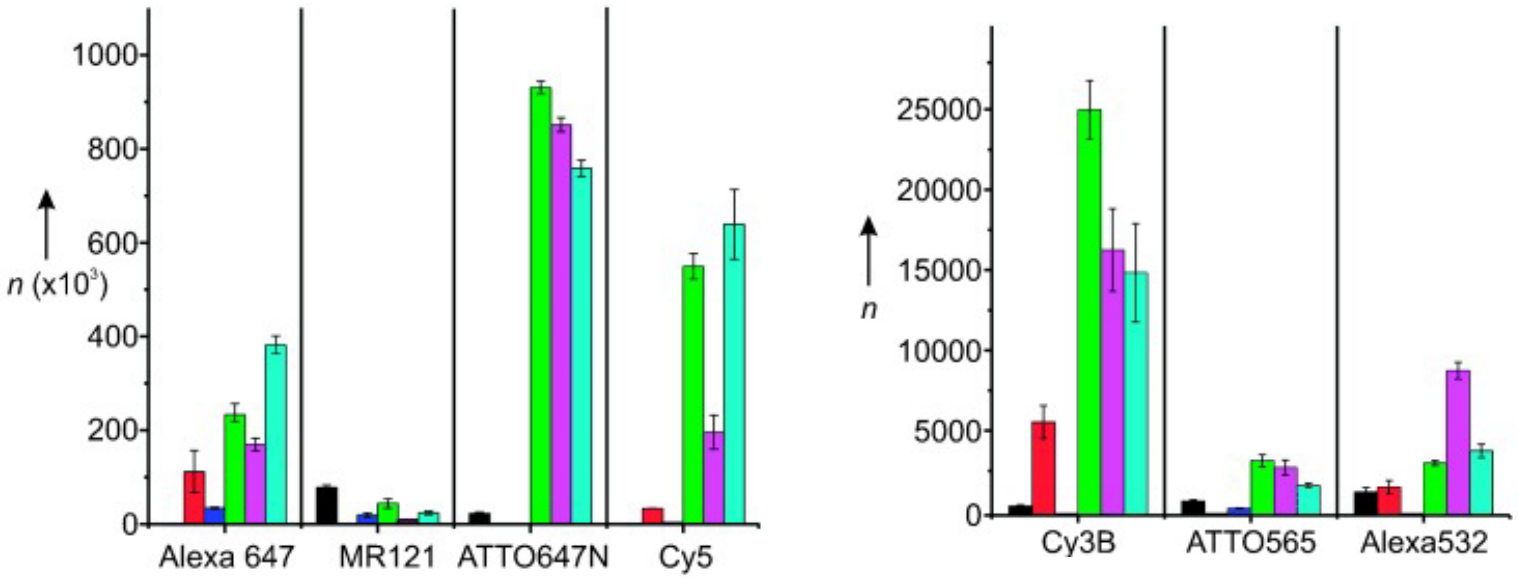


Lelek et al. *Nature Reviews Methods Primer* (2021)

Buffers in SMLM

Different dyes blink optimally in different imaging buffers. Therefore, the acquisition of good quality 2- and 3-color images can be challenging.

Average photon counts per molecule in different buffers

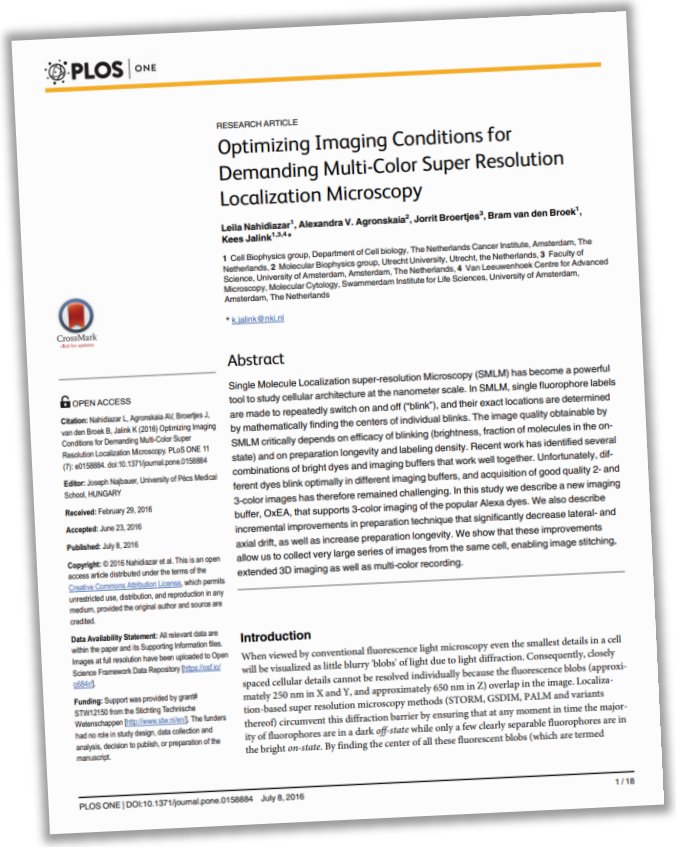


Vogelsang et al. *Angew Chem Int Ed Engl.* (2008)

Buffers in SMLM

<u>Advice:</u> Once you have picked a dye for your SMLM experiment depending on your microscope (available lasers) and biological constraints, make yourself familiar with which buffers are most suitable and test & optimize them.

Buffers in SMLM



PLOS | ONE

RESEARCH ARTICLE

Optimizing Imaging Conditions for Demanding Multi-Color Super Resolution Localization Microscopy

Leila Nahidiazar[1], Alexandra V. Agronskaia[2], Jorrit Broertjes[3], Bram van den Broek[1], Kees Jalink[1,3,4]*

1 Cell Biophysics group, Department of Cell biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands, **2** Molecular Biophysics group, Utrecht University, Utrecht, the Netherlands, **3** Faculty of Science, University of Amsterdam, Amsterdam, The Netherlands, **4** Van Leeuwenhoek Centre for Advanced Microscopy, Molecular Cytology, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands

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OPEN ACCESS

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files. Images at full resolution have been uploaded to Open Science Framework Data Repository [https://osf.io/q684r].

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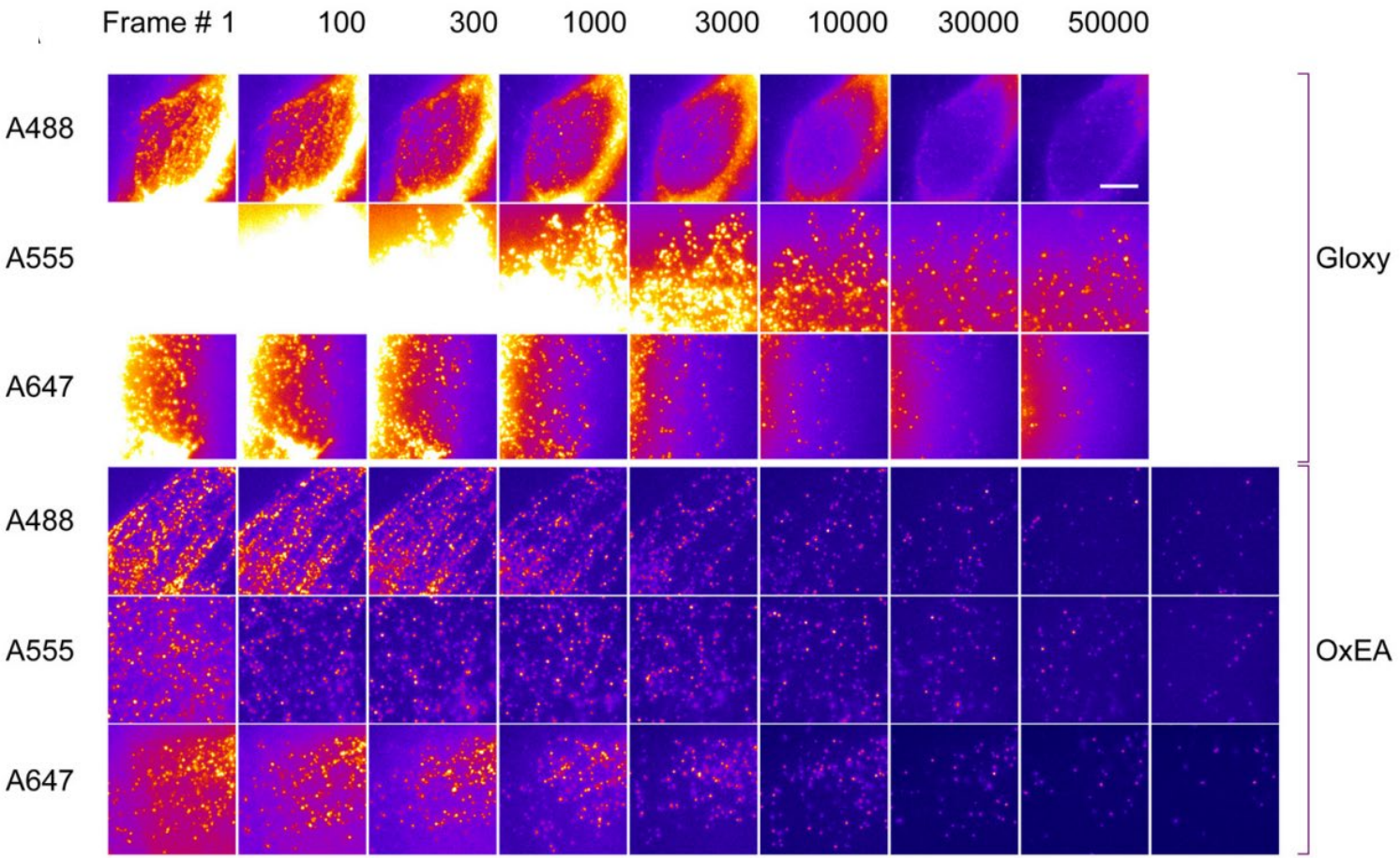
Abstract

Single Molecule Localization super-resolution Microscopy (SMLM) has become a powerful tool to study cellular architecture at the nanometer scale. In SMLM, single fluorophore labels are made to repeatedly switch on and off ("blink"), and their exact locations are determined by mathematically finding the centers of individual blinks. The image quality obtainable by SMLM critically depends on efficacy of blinking (brightness, fraction of molecules in the on-state) and on preparation longevity and labeling density. Recent work has identified several combinations of bright dyes and imaging buffers that work well together. Unfortunately, different dyes blink optimally in different imaging buffers, and acquisition of good quality 2- and 3-color images has therefore remained challenging. In this study we describe a new imaging buffer, OxEA, that supports 3-color imaging of the popular Alexa dyes. We also describe incremental improvements in preparation technique that significantly decrease lateral- and axial drift, as well as increase preparation longevity. We show that these improvements allow us to collect very large series of images from the same cell, enabling image stitching, extended 3D imaging as well as multi-color recording.

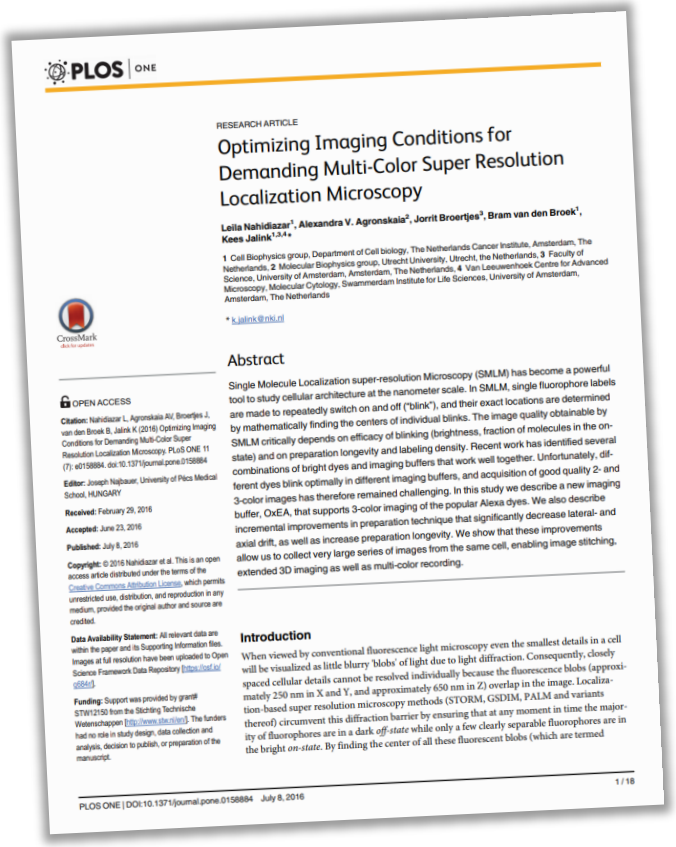
Introduction

When viewed by conventional fluorescence light microscopy even the smallest details in a cell will be visualized as little blurry 'blobs' of light due to light diffraction. Consequently, closely spaced cellular details cannot be resolved individually because the fluorescence blobs (approximately 250 nm in X and Y, and approximately 650 nm in Z) overlap in the image. Localization-based super resolution microscopy methods (STORM, GSDIM, PALM and variants thereof) circumvent this diffraction barrier by ensuring that at any moment in time the majority of fluorophores are in a dark *off-state* while only a few clearly separable fluorophores are in the bright *on-state*. By finding the center of all these fluorescent blobs (which are termed



Nahidiazar et al. *PLOS ONE* (2016)

Buffers in SMLM



PLOS | ONE

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Single Molecule Localization super-resolution Microscopy (SMLM) has become a powerful tool to study cellular architecture at the nanometer scale. In SMLM, single fluorophore labels are made to repeatedly switch on and off ("blink"), and their exact locations are determined by mathematically finding the centers of individual blinks. The image quality obtainable by SMLM critically depends on efficacy of blinking (brightness, fraction of molecules in the on-state) and on preparation longevity and labeling density. Recent work has identified several combinations of bright dyes and imaging buffers that work well together. Unfortunately, different dyes blink optimally in different imaging buffers, and acquisition of good quality 2- and 3-color images has therefore remained challenging. In this study we describe a new imaging buffer, OxEA, that supports 3-color imaging of the popular Alexa dyes. We also describe incremental improvements in preparation technique that significantly decrease lateral- and axial drift, as well as increase preparation longevity. We show that these improvements allow us to collect very large series of images from the same cell, enabling image stitching, extended 3D imaging as well as multi-color recording.

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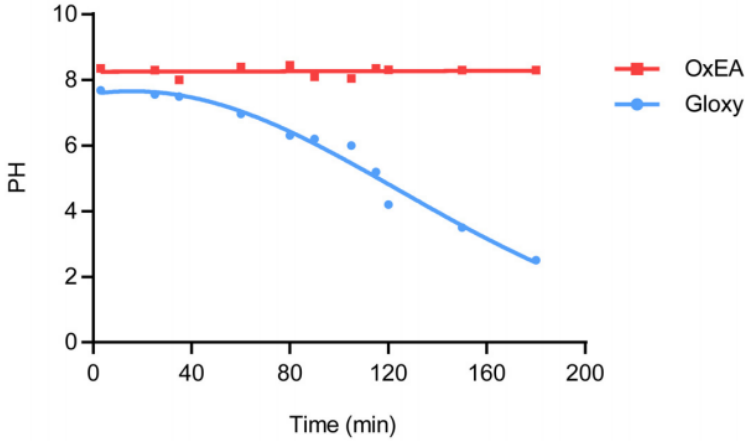
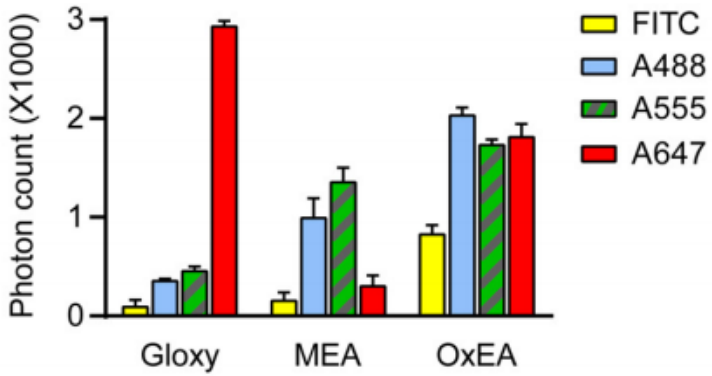
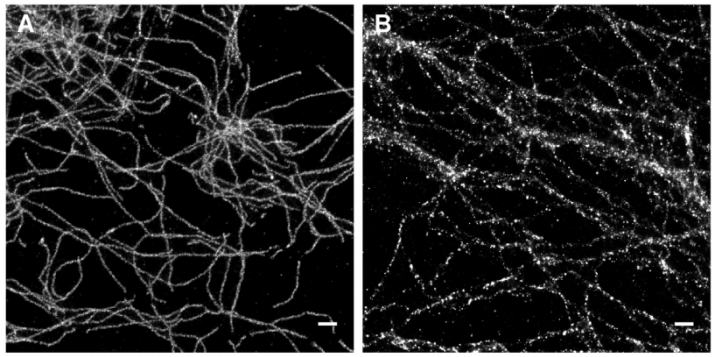






Comparison of image quality in ageing Gloxy buffer (B) to that in OxEA buffer (A).

<u>Advice:</u> Always prepare your imaging buffer right before experiments & adjust your imaging buffer choice for multicolor experiments.

Nahidiazar et al. *PLOS ONE* (2016)

Overview

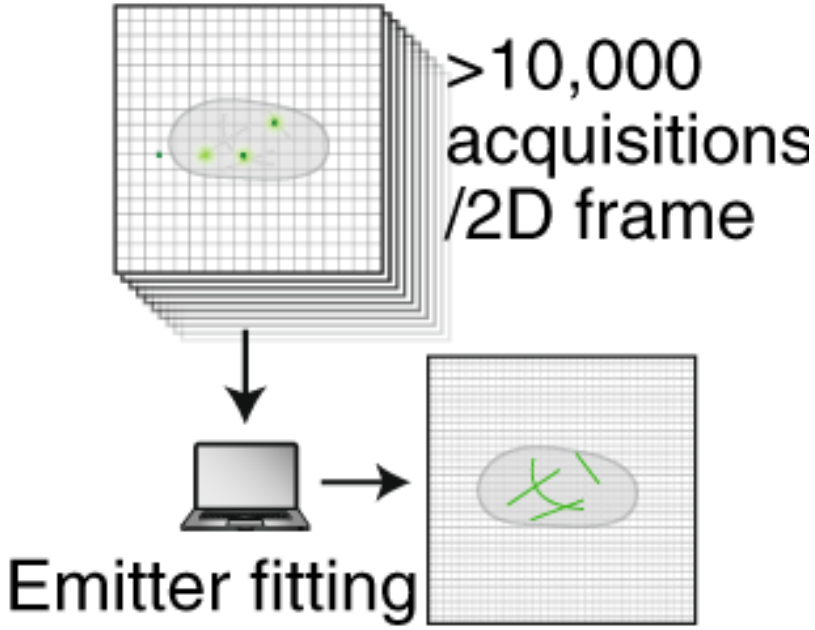
- Basic principles 2D SMLM ✓
- Hardware for SMLM ✓
- Practical considerations: sample preparation, suitable dyes, linkage errors, and buffers ✓
- Processing, quantification, and interpretation of SMLM data
- 3D SMLM
- Summary

- Extra: New directions in SMLM
- Extra: SRM as a multidimensional challenge

Processing, quantification, and interpretation of SMLM data

How large is an SMLM dataset?

50,000 frames taken with a 16-bit camera (1024 x 1024 pixel) lead to…



Total number of pixel on the detector:

1024 x 1024 = 1,048,576 pixel

Total bits:

1,048,576 x 16 bits = 16,777,216 bits

Conversion into bytes:

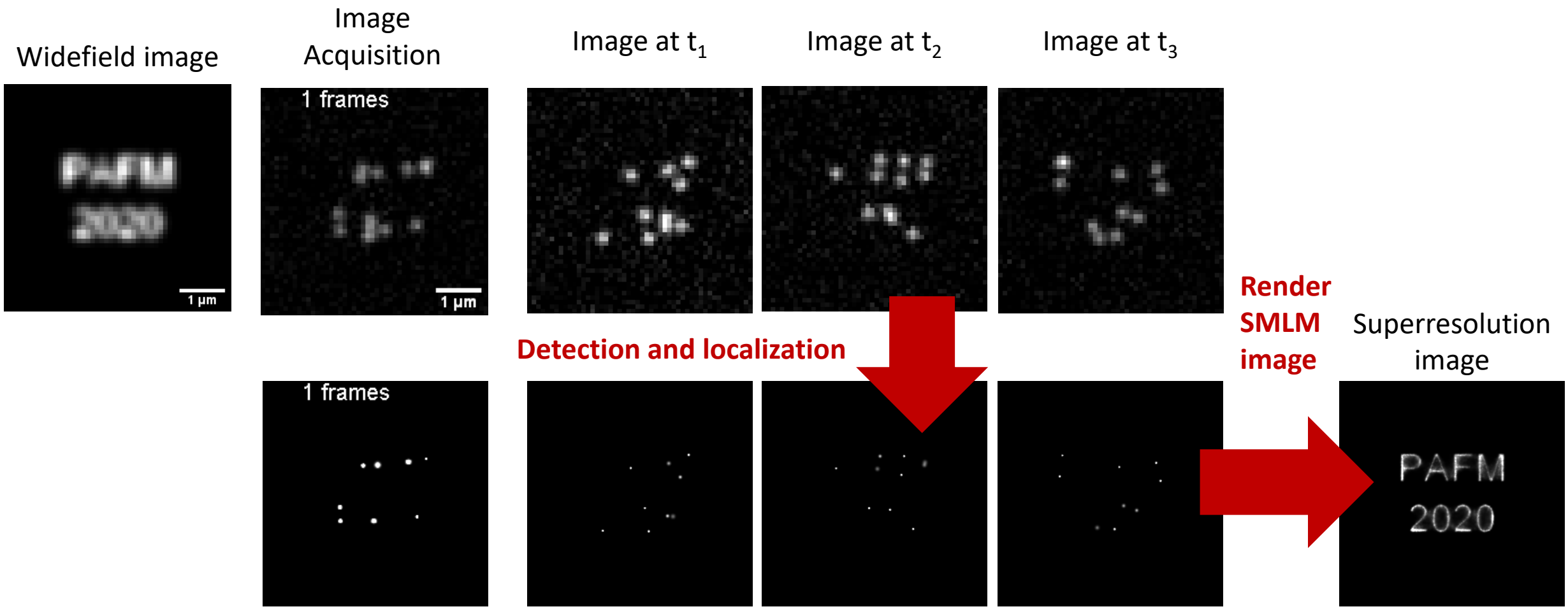
16,777,216 bits / 8 = 2,097,152 bytes
~ 2 MB (one frame)

Upscaled to 50,000 frames:

2 MB x 50,000 **~ 100 GB**

That is a lot of data that needs to be handled…

From raw SMLM data to reconstructed super-resolved image

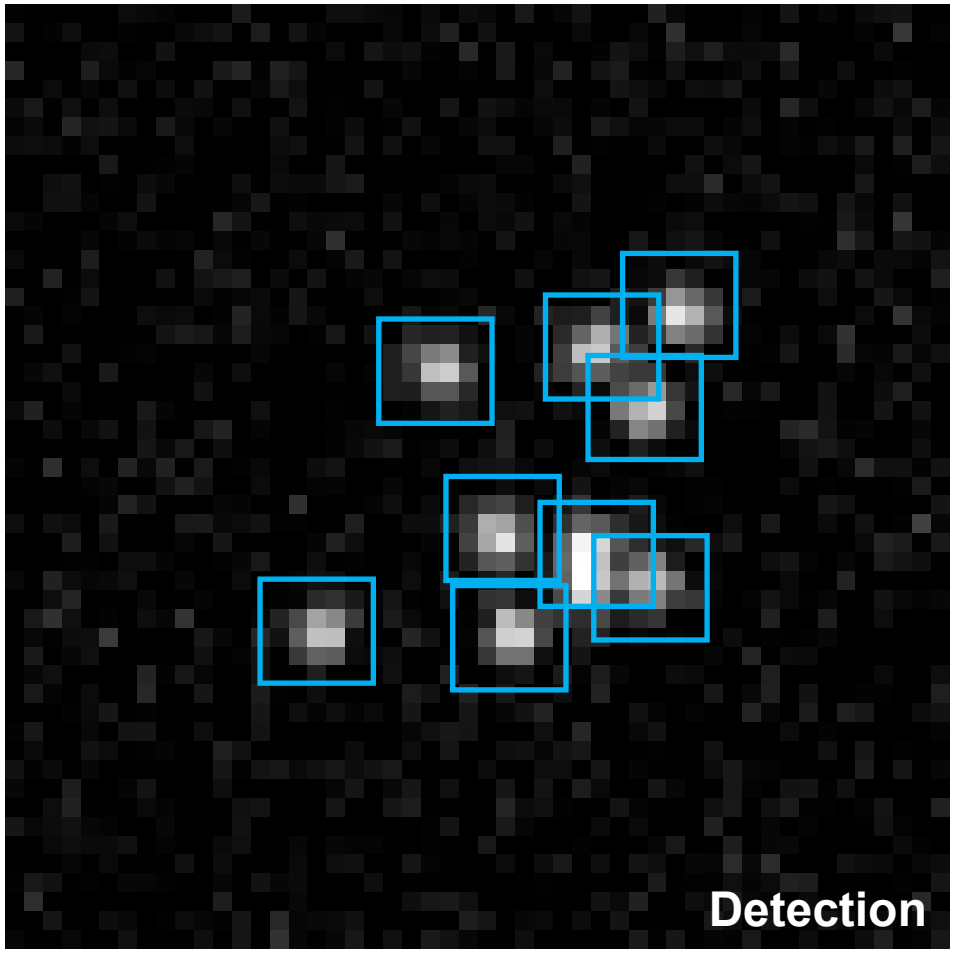


$t_1 < t_2 < t_3$

Betzig et al. *Science* (2006), Hess et al. *Biophy. J.* (2006), Rust et al. *Nat Methods* (2006)

Detection

- Approximate molecule location = Detection

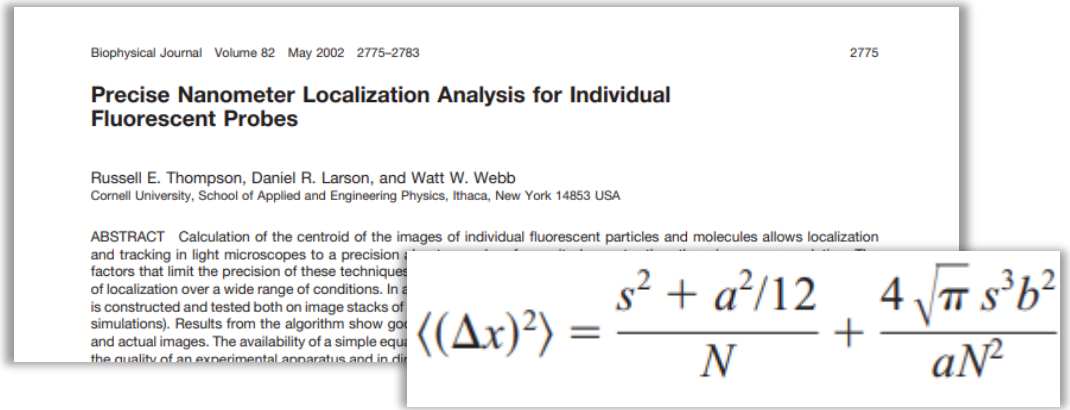


Detection and localization

$$\Delta x \approx \frac{\lambda}{2\mathrm{NA}}$$ Abbe Equation

vs.

- Approximate molecule location = Detection
- Accurate determination of the molecule center = Localization

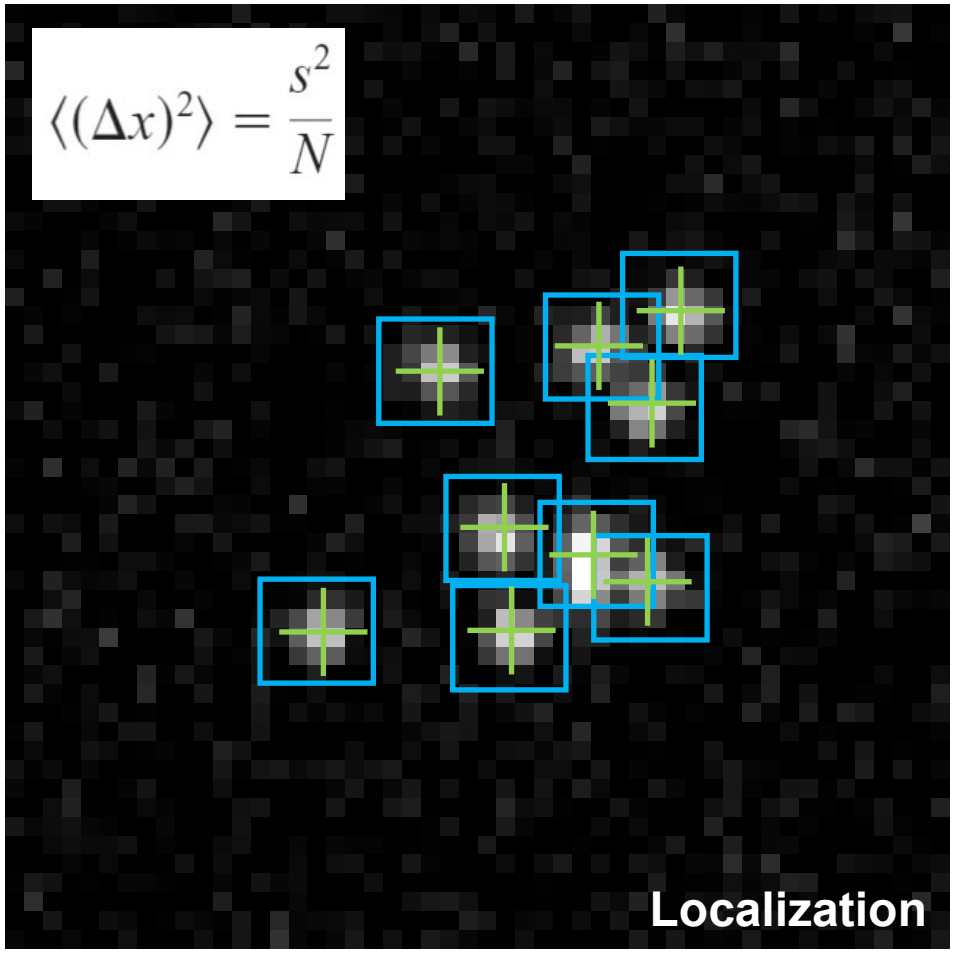

Biophysical Journal Volume 82 May 2002 2775–2783 2775

Precise Nanometer Localization Analysis for Individual Fluorescent Probes

Russell E. Thompson, Daniel R. Larson, and Watt W. Webb
Cornell University, School of Applied and Engineering Physics, Ithaca, New York 14853 USA

ABSTRACT Calculation of the centroid of the images of individual fluorescent particles and molecules allows localization and tracking in light microscopes to a precision
factors that limit the precision of these techniques
of localization over a wide range of conditions. In
is constructed and tested both on image stacks of
simulations). Results from the algorithm show go
and actual images. The availability of a simple equa

$$\langle(\Delta x)^2\rangle = \frac{s^2 + a^2/12}{N} + \frac{4\sqrt{\pi}\, s^3 b^2}{aN^2}$$



Thompson et al. *Biophysical Journal* (2002)

Error in localization – Equations

$$\langle (\Delta x)^2 \rangle = \frac{s^2}{N}$$

Δx	Error in localization
s	Standard deviation of the PSF
N	Number of photons collected

The modified equation to account for photon-counting noise, pixelation noise and background noise:

$$\langle (\Delta x)^2 \rangle = \frac{s^2 + a^2/12}{N} + \frac{4\sqrt{\pi}\, s^3 b^2}{a N^2}$$

a	Size of the pixel
b	Background noise

Thompson et al. *Biophysical Journal* (2002)

Detection and localization

- Approximate molecule location
  = Detection

- Accurate determination of the molecule center
  = Localization

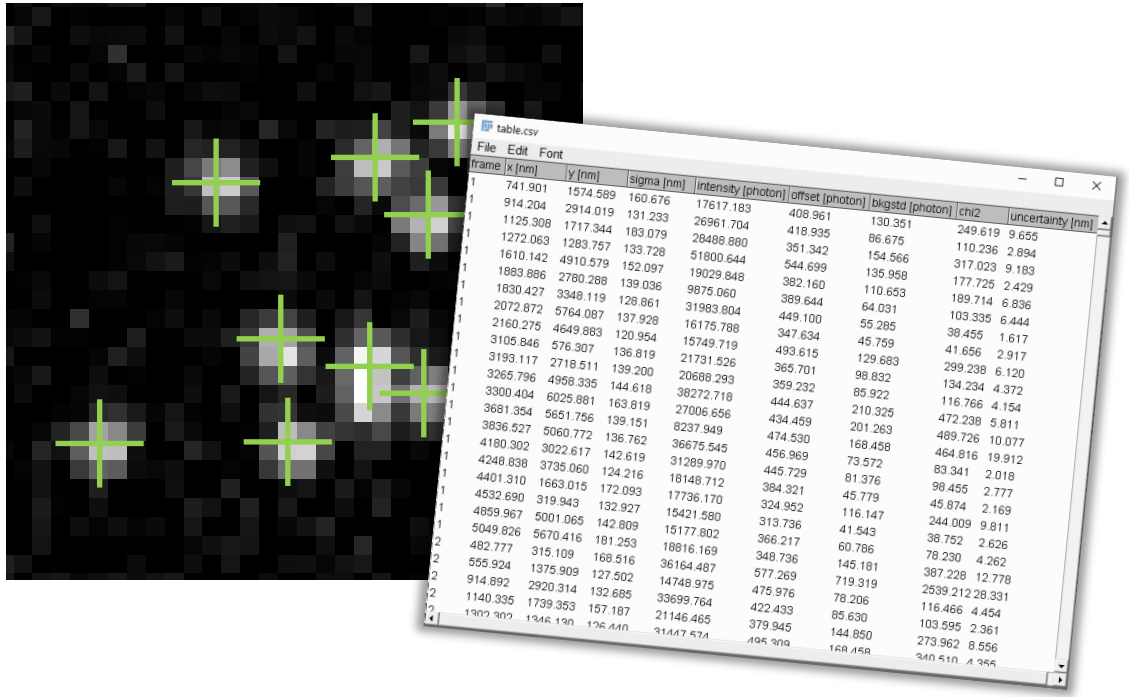
- Result: List of localizations (x,y coordinates, localization errors / uncertainties & add. parameters)

Pixel data vs. point clouds

In contrast to standard microscopy imaging that produces 2D pixel or 3D voxel grid data, SMLM generates big data of 2D or 3D point clouds with millions of localizations and associated uncertainties.

Therefore, processing, quantification, analysis and interpretation of SMLM must be approaches differently.

To this day, the SMLM data quantification and interpretation methods have yet to keep pace with the rapid advancement of SMLM imaging.



frame	x [nm]	y [nm]	sigma [nm]	intensity [photon]	offset [photon]	bkgstd [photon]	chi2	uncertainty [nm]
1	741.901	1574.589	160.676	17617.183	408.961	130.351	249.619	9.655
1	914.204	2914.019	131.233	26961.704	418.935	86.675	110.236	2.894
1	1125.308	1717.344	183.079	28488.880	351.342	154.566	317.023	9.183
1	1272.063	1283.757	133.728	51800.644	544.699	135.958	177.725	2.429
1	1610.142	4910.579	152.097	19029.848	382.160	110.653	189.714	6.836
1	1883.886	2780.288	139.036	9875.060	389.644	64.031	103.335	6.444
1	1830.427	3348.119	128.861	31983.804	449.100	55.285	38.455	1.617
1	2072.872	5764.087	137.928	16175.788	347.634	45.759	41.656	2.917
1	2160.275	4649.883	120.954	15749.719	493.615	129.683	299.238	6.120
1	3105.846	576.307	136.819	21731.526	365.701	98.832	134.234	4.372
1	3193.117	2718.511	139.200	20688.293	359.232	85.922	116.766	4.154
1	3265.796	4958.335	144.618	38272.718	444.637	210.325	472.238	5.811
1	3300.404	6025.881	163.819	27006.656	434.459	201.263	489.726	10.077
1	3681.354	5651.756	139.151	8237.949	474.530	168.458	464.816	19.912
1	3836.527	5060.772	136.762	36675.545	456.969	73.572	83.341	2.018
1	4180.302	3022.617	142.619	31289.970	445.729	81.376	98.455	2.777
1	4248.838	3735.060	124.216	18148.712	384.321	45.779	45.874	2.169
1	4401.310	1663.015	172.093	17736.170	324.952	116.147	244.009	9.811
1	4532.690	319.943	132.927	15421.580	313.736	41.543	38.752	2.626
1	4859.967	5001.065	142.809	15177.802	366.217	60.786	78.230	4.262
1	5049.826	5670.416	181.253	18816.169	348.736	145.181	387.228	12.778
2	482.777	315.109	168.516	36164.487	577.269	719.319	2539.212	28.331
2	555.924	1375.909	127.502	14748.975	475.976	78.206	116.466	4.454
2	914.892	2920.314	132.685	33699.764	422.433	85.630	103.595	2.361
2	1140.335	1739.353	157.187	21146.465	379.945	144.850	273.962	8.556
2	1302.302	1346.130	126.440	31447.574	495.309	168.458	340.510	4.355

Khater et al. *Biophysical Journal* (2002)

List of localizations

table.csv

File Edit Font

frame	x [nm]	y [nm]	sigma [nm]	intensity [photon]	offset [photon]	bkgstd [photon]	chi2	uncertainty [nm]
1	741.901	1574.589	160.676	17617.183	408.961	130.351	249.619	9.655
1	914.204	2914.019	131.233	26961.704	418.935	86.675	110.236	2.894
1	1125.308	1717.344	183.079	28488.880	351.342	154.566	317.023	9.183
1	1272.063	1283.757	133.728	51800.644	544.699	135.958	177.725	2.429
1	1610.142	4910.579	152.097	19029.848	382.160	110.653	189.714	6.836
1	1883.886	2780.288	139.036	9875.060	389.644	64.031	103.335	6.444
1	1830.427	3348.119	128.861	31983.804	449.100	55.285	38.455	1.617
1	2072.872	5764.087	137.928	16175.788	347.634	45.759	41.656	2.917
1	2160.275	4649.883	120.954	15749.719	493.615	129.683	299.238	6.120
1	3105.846	576.307	136.819	21731.526	365.701	98.832	134.234	4.372
1	3193.117	2718.511	139.200	20688.293	359.232	85.922	116.766	4.154
1	3265.796	4958.335	144.618	38272.718	444.637	210.325	472.238	5.811
1	3300.404	6025.881	163.819	27006.656	434.459	201.263	489.726	10.077
1	3681.354	5651.756	139.151	8237.949	474.530	168.458	464.816	19.912
1	3836.527	5060.772	136.762	36675.545	456.969	73.572	83.341	2.018
1	4180.302	3022.617	142.619	31289.970	445.729	81.376	98.455	2.777
1	4248.838	3735.060	124.216	18148.712	384.321	45.779	45.874	2.169
1	4401.310	1663.015	172.093	17736.170	324.952	116.147	244.009	9.811
1	4532.690	319.943	132.927	15421.580	313.736	41.543	38.752	2.626
1	4859.967	5001.065	142.809	15177.802	366.217	60.786	78.230	4.262
1	5049.826	5670.416	181.253	18816.169	348.736	145.181	387.228	12.778
2	482.777	315.109	168.516	36164.487	577.269	719.319	2539.212	28.331
2	555.924	1375.909	127.502	14748.975	475.976	78.206	116.466	4.454
2	914.892	2920.314	132.685	33699.764	422.433	85.630	103.595	2.361
2	1140.335	1739.353	157.187	21146.465	379.945	144.850	273.962	8.556
2	1302.302	1346.130	126.440	31447.574	495.309	168.458	340.510	4.355

x,y – central coordinate

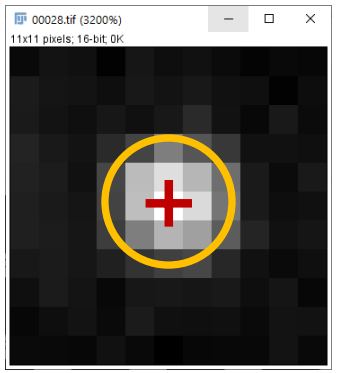
sigma - Gaussian width

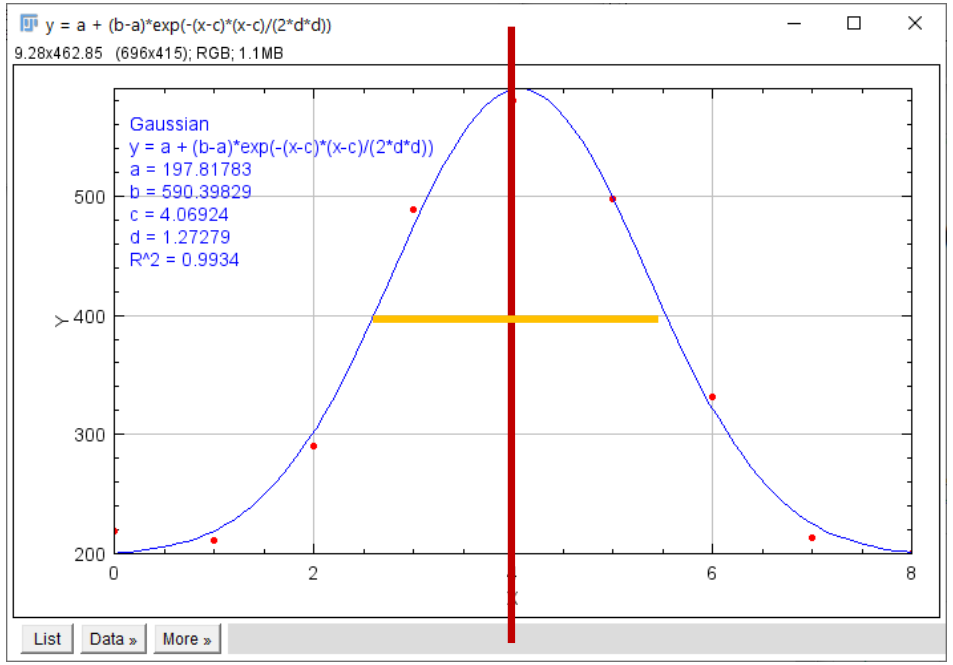
Gaussian amplitude / offset

χ2 – goodness-of-fit

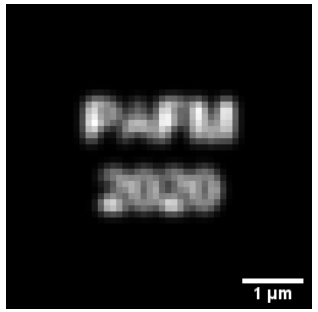
Uncertainty

$$\langle(\Delta x)^2\rangle = \frac{s^2 + a^2/12}{N} + \frac{4\sqrt{\pi}\, s^3 b^2}{a N^2}$$





Sequence of SMLM processing and analysis steps



Raw camera image (typ. 128x128x10,000)

(1) Detection & localization

List of localizations (typ. 200,000)

(2) Sieving / Filtering

List of localizations (typ. 100,000)

(3) De-drifting and Grouping

List of localizations (typ. 80,000)

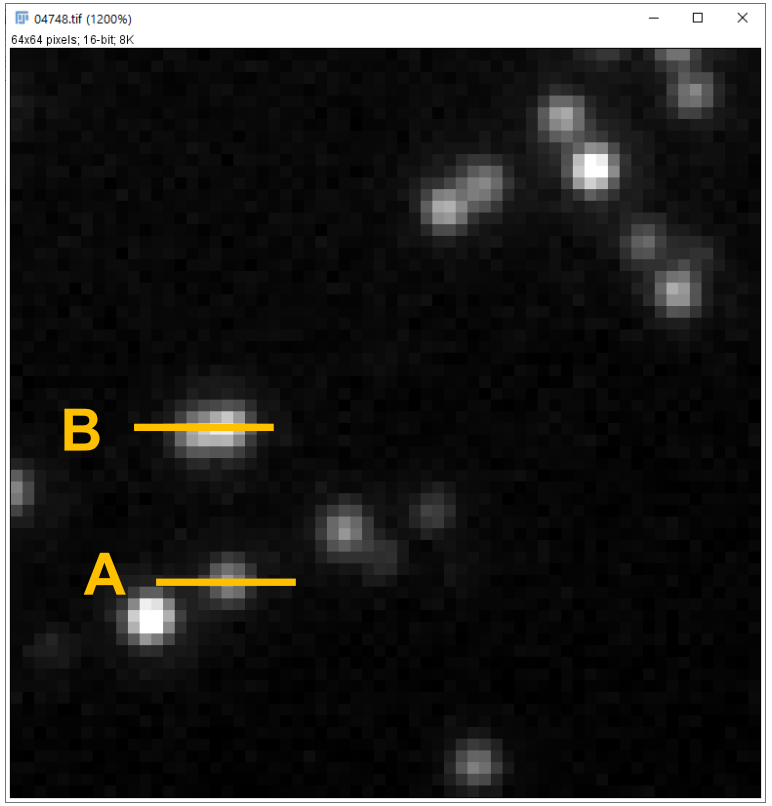
(4) Rendering

Super-resolved image (typ. 1028x1028)

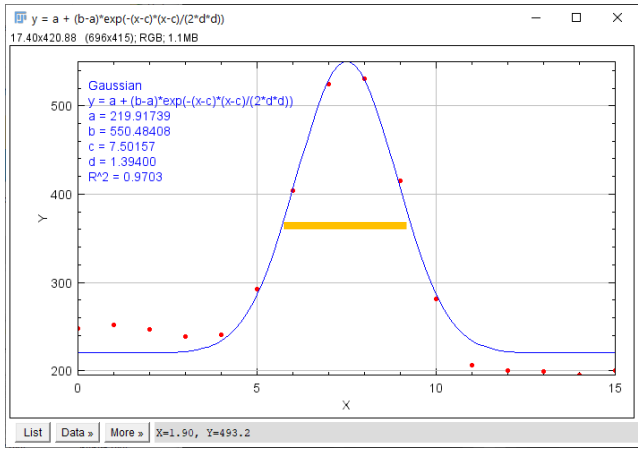
(5) Quantification

Size (nm), Clusters, Stoichiometry

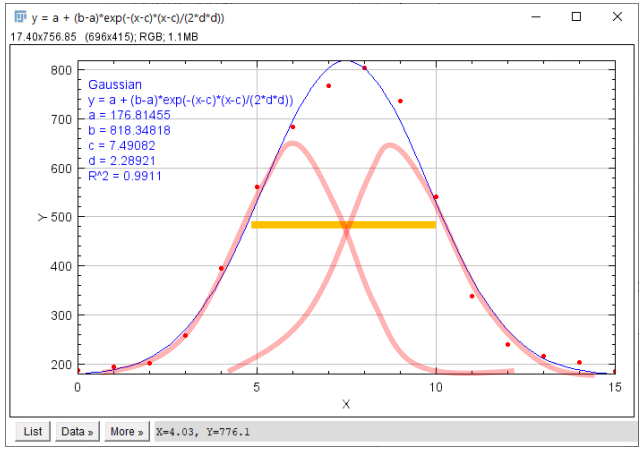
High-density data / multiple emitters



A

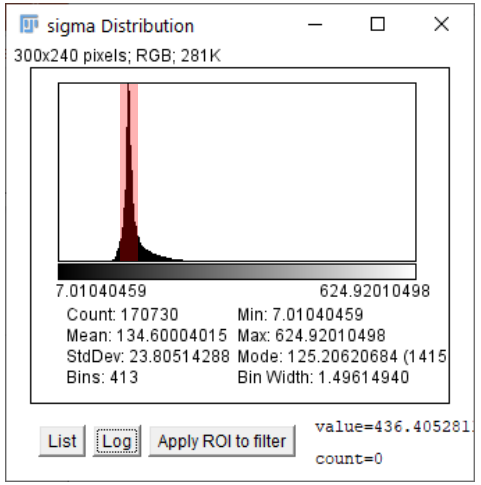


B



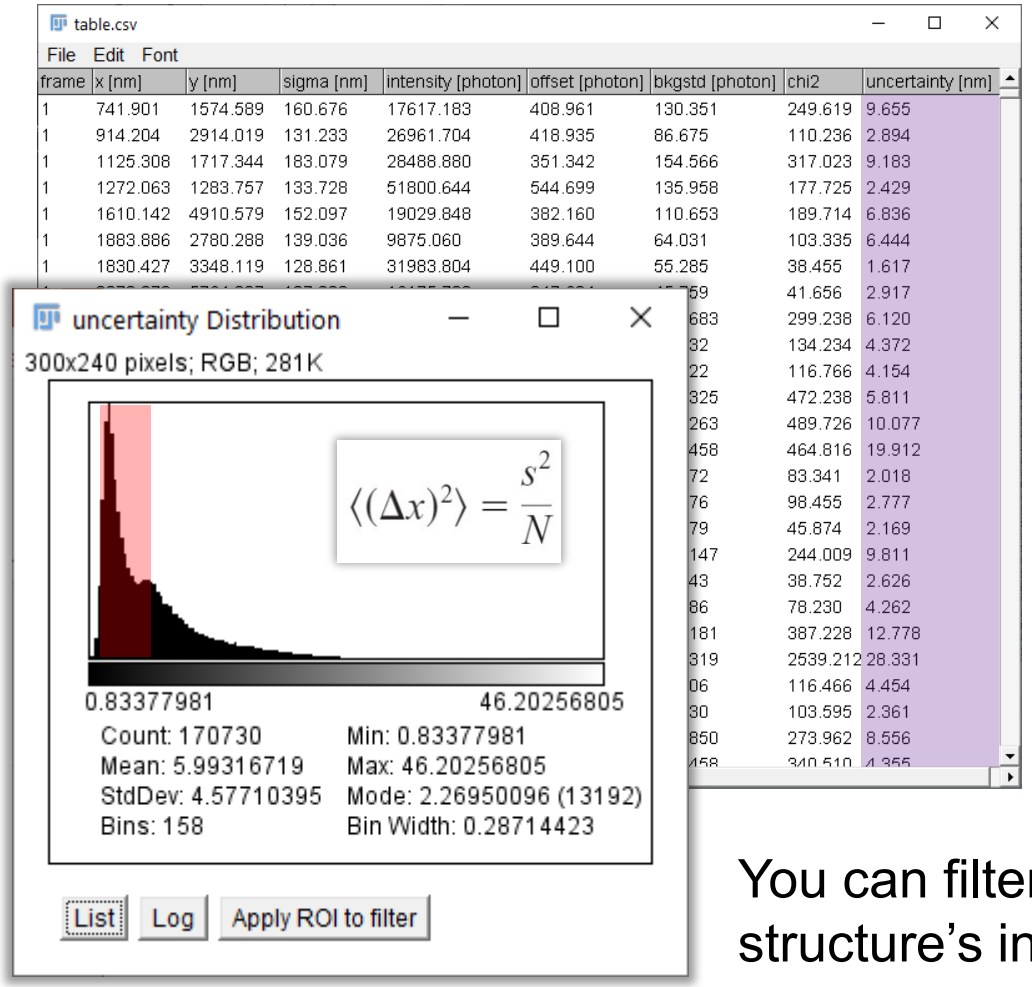
Choosing a suitable multi-emitter fitting method/algorithm can help to deal with high-density data.

Alternatively, you can filter/sieve your data to handle invalid localizations.



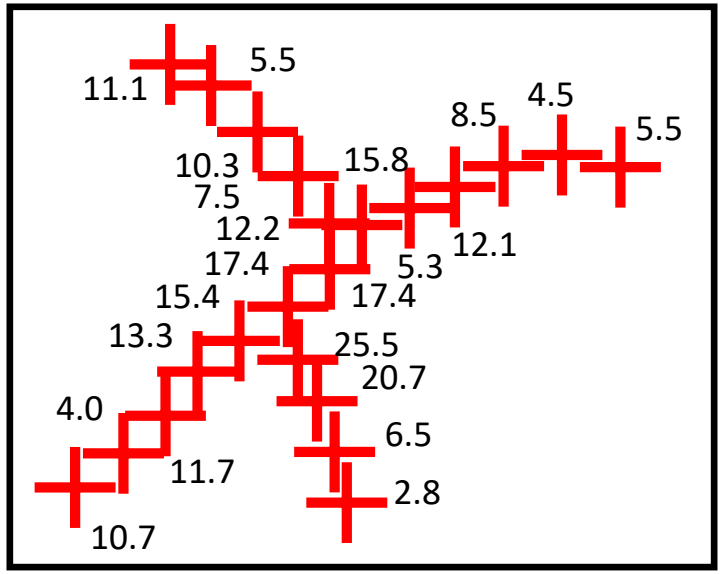
<u>Advice:</u> Choose suitable filtering parameters with the histogram of the respective fitting parameter.

SMLM's resolution

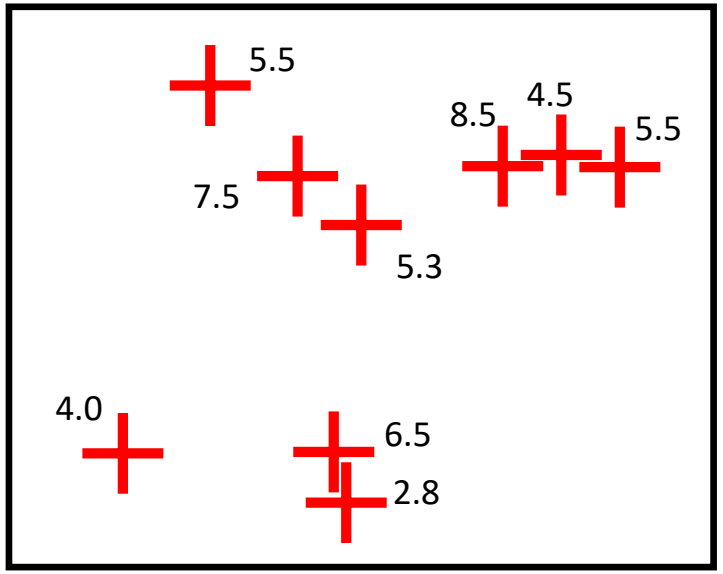


frame	x [nm]	y [nm]	sigma [nm]	intensity [photon]	offset [photon]	bkgstd [photon]	chi2	uncertainty [nm]
1	741.901	1574.589	160.676	17617.183	408.961	130.351	249.619	9.655
1	914.204	2914.019	131.233	26961.704	418.935	86.675	110.236	2.894
1	1125.308	1717.344	183.079	28488.880	351.342	154.566	317.023	9.183
1	1272.063	1283.757	133.728	51800.644	544.699	135.958	177.725	2.429
1	1610.142	4910.579	152.097	19029.848	382.160	110.653	189.714	6.836
1	1883.886	2780.288	139.036	9875.060	389.644	64.031	103.335	6.444
1	1830.427	3348.119	128.861	31983.804	449.100	55.285	38.455	1.617
						59	41.656	2.917
						683	299.238	6.120
						32	134.234	4.372
						22	116.766	4.154
						325	472.238	5.811
						263	489.726	10.077
						458	464.816	19.912
						72	83.341	2.018
						76	98.455	2.777
						79	45.874	2.169
						147	244.009	9.811
						43	38.752	2.626
						86	78.230	4.262
						181	387.228	12.778
						319	2539.212	28.331
						06	116.466	4.454
						30	103.595	2.361
						850	273.962	8.556
						468	340.510	4.355

So, when you filter/sieve out high uncertainties, you can reach insanely high resolutions, right? **No, not really!**



Uncertainty < 25 nm

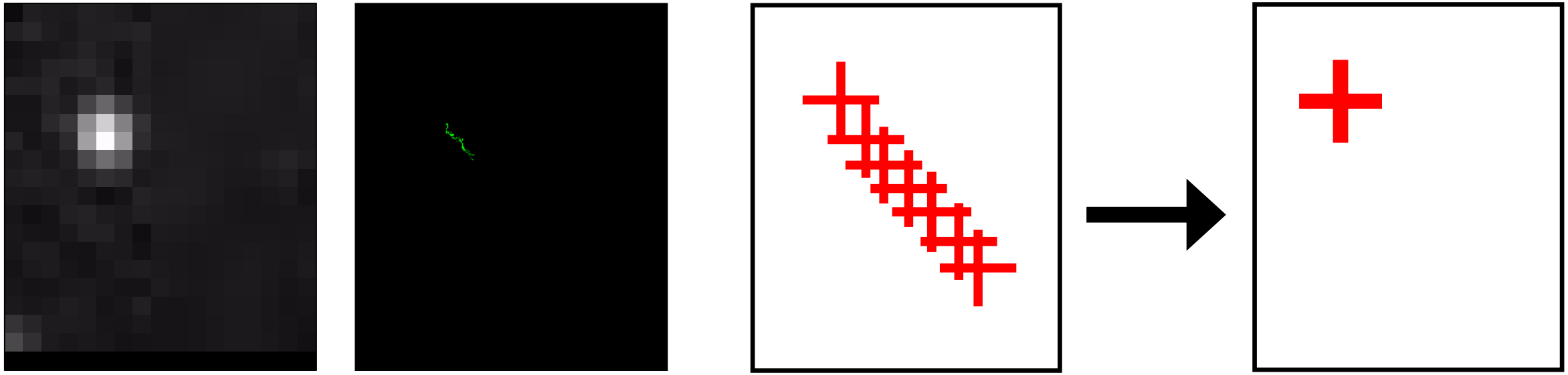


Uncertainty < 10nm

You can filter high uncertainties, but at a certain point you'll lose the structure's integrity. The measure of resolution would be meaningless.

Drift-correction

4 min movie

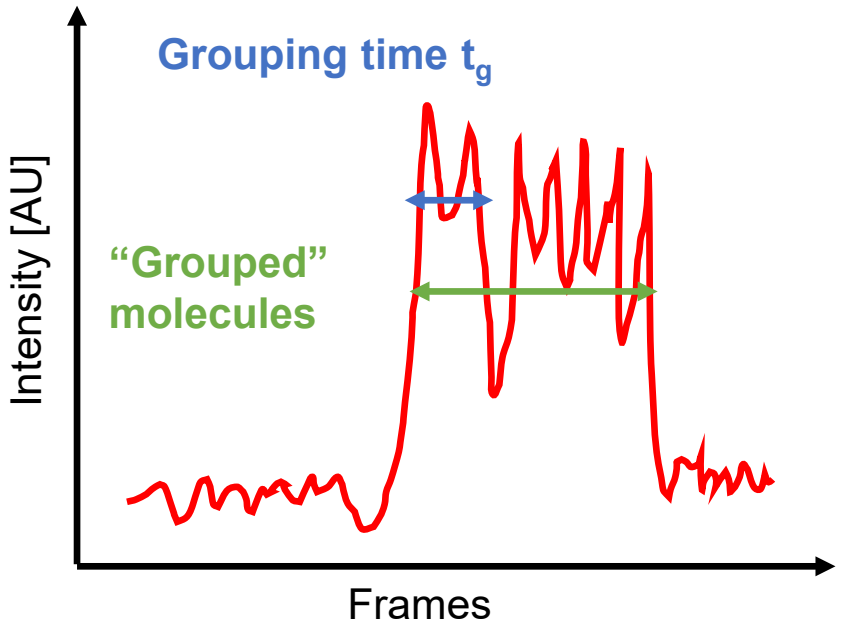


0.5 µm

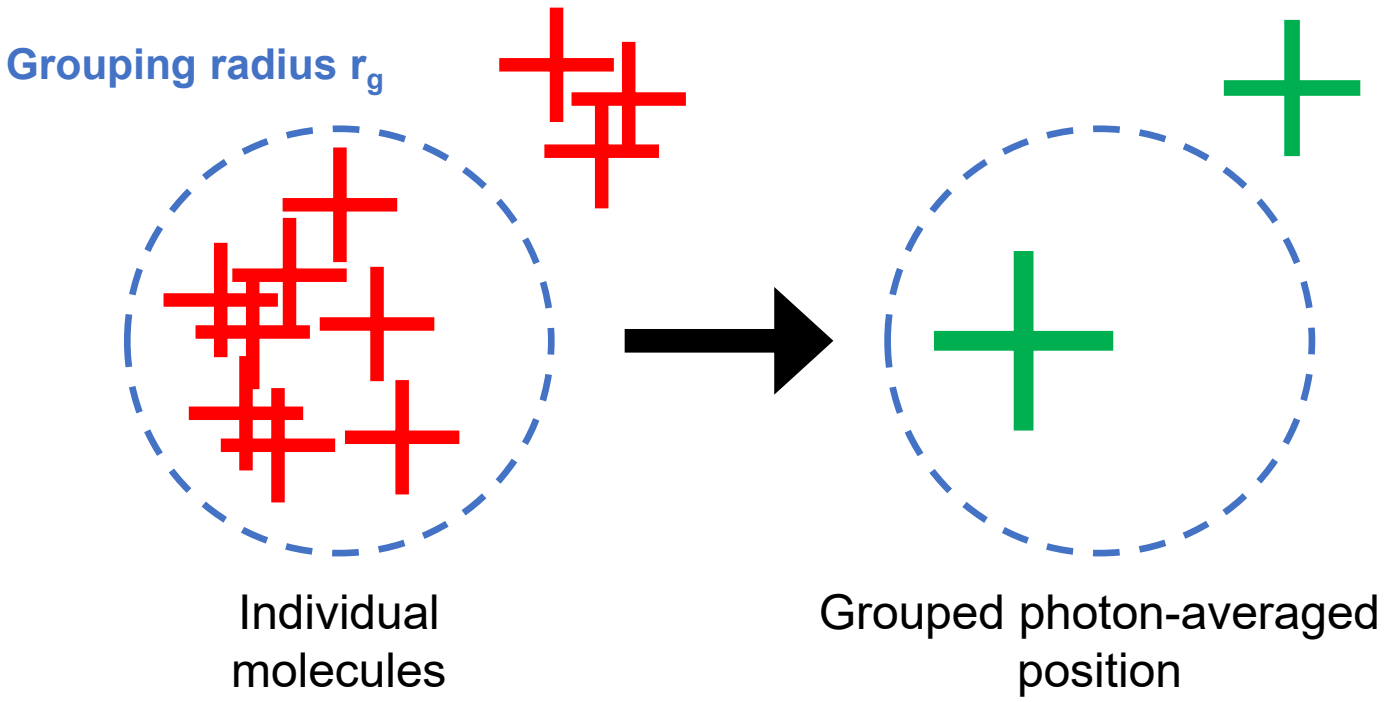
Drift becomes a limiting factor in achieving high resolution. Fortunately, it can be corrected for by fiducial-based or image-based (cross-correlation) strategies.

Grouping / Blink-correction

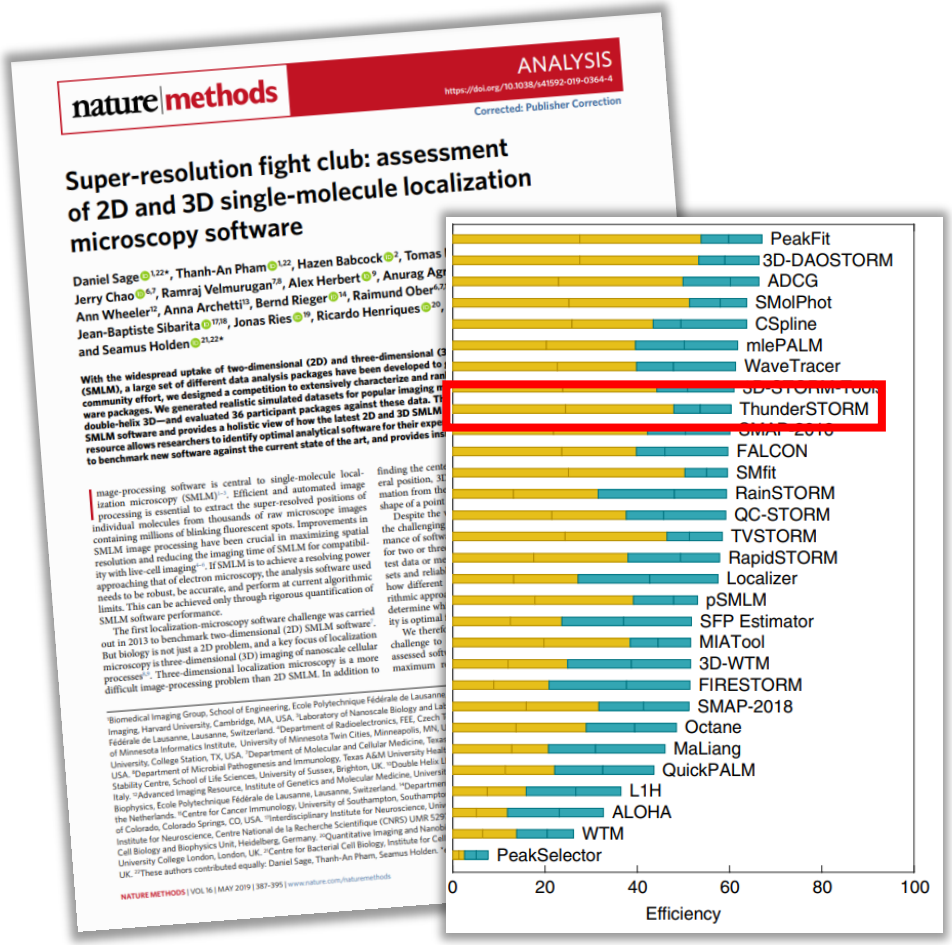
According to time



According to space



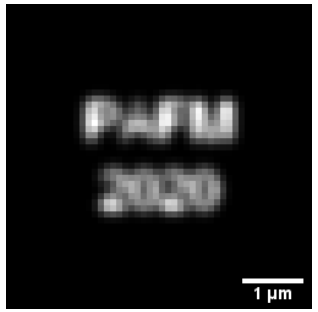
Software for SMLM



Software	Molecule detection	PSF	Method	Platform	Acc.
3D-DAOSTORM[28]	Adaptive threshold—update on residual images	Gauss	LS	Python	+
a-livePALM[29]	Denoising, SNR threshold, adaptive histogram equalization	Gauss	MLE	Matlab	+
Auto-Bayes	Generalized minimum-error threshold (GMET), local maximum	Gauss, Weibull	LS	Stand-alone	+
B-recs	Detection: n/a; fit: Bayesian inference framework	Arbitrary	MMSE, MAP	Stand-alone	–
CSSTORM[30]	No explicit localization; convex optimization problem (HD)	Gauss	Compressed sensing	Matlab	+
DAOSTORM[31]	Gaussian filtering, local maximum (HD)	Measured,	LS	Python	+
FacePALM[32]	No explicit localization; background estimation	arbitrary	–	Python	–
FALCON[33]	Deconvolution with sparsity prior, local maximum (HD)	Taylor approx.	ADMM	Matlab	+
Fast-ML-HD[34]	Sparsity constraint, concave-convex procedure (HD)	Gauss	MLE	Matlab	–
FPGA[35]	Adaptive threshold	Gauss	MLE, CoMass	Stand-alone	–
Gauss2DCirc[36]	Fixed SNR threshold	Gauss	REG	Matlab	+
GPUgaussMLE[37]	Simple (unspecified) methods to select subregions	Gauss	MLE	Matlab	+
GraspJ[38]	Peak finding: fixed threshold value	Gauss	MLE	ImageJ	+
Insight3	Low-pass filtering, local maximum	Arbitrary	LS	Stand-alone	–
L1H[39]	No explicit localization; L1 homotopy, FIST deconvolution	Gauss, arbitrary	Compressed sensing	Python	+
M2LE[40]	Adaptive threshold	Gauss	MLE	ImageJ	+
Maliang[41]	Annular averaging filters, denoising by convolution	Gauss	MLE	ImageJ	+
Micro-Manager LM	Adaptive threshold	Gauss	LS	ImageJ	+
MrSE[42]	Band-pass filtering, local maximum	Radial	CoSym	Stand-alone	–
Octane[43]	Watershed maximum	Gauss	LS	ImageJ	+
PeakFit	Band-pass filtering, local maximum	Gauss	LS	ImageJ	+
PeakSelector[44]	Time-domain filtering, adaptive threshold	Gauss	LS	IDL, Matlab	–
PYME[27]	Wiener filtering, adaptive threshold	Arbitrary	LS	Python	+
QuickPALM[45]	Band-pass filtering, fixed SNR threshold	Gauss	CoMass	ImageJ	+
RadialSymmetry[46]	Filtering, local max., minimal distance to gradient	Radial	CoSym	Matlab	+
rapidSTORM[12]	Low-pass filtering, local maximum	Gauss	LS, MLE	Stand-alone	+
SimplePALM[47]	Variance stabilization denoising, DoG, probabilistic threshold	n/a	Mean-shift	Stand-alone	–
simpleSTORM[14]	Self-calibration, noise normalize, background subtraction, *P* value	Gauss, measured	Interpolation	Stand-alone	+
SNSMIL	Gaussian filtering, fixed contrast threshold	Gauss	LS	Stand-alone	+
SOSplugin	Wavelet transform, local maximum, Gaussian mixture	Gauss	LS	ImageJ	+
ThunderSTORM[15]	Extensive collection of methods, preview, filtering, local maximum	Gauss	LS, MLE	ImageJ	+
W-fluoroBancroft[48]	Wavelet, adaptive threshold	Gauss	fB	Matlab	+
WaveTracer[49]	Wavelet, watershed maximum	Gauss	LS	Metamorph	–
WTM[50]	Wedge template matching (HD)	Wedge	Match.	Stand-alone	–

Sage et al. *Nature Methods* (2019), Sage et al. *Nature Methods* (2015)

Sequence of SMLM processing and analysis steps



Raw camera image (typ. 128x128x10.000)

(1) Detection & localization

List of localizations (typ. 200.000)

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List of localizations (typ. 100.000)

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List of localizations (typ. 80.000)

(4) Rendering

Super-resolved image (typ. 1028x1028)

(5) Quantification

Size (nm), Clusters, Stoichiometry

With ThunderSTORM you can…

Ovesny et al. *Bioinformatics* (2014)

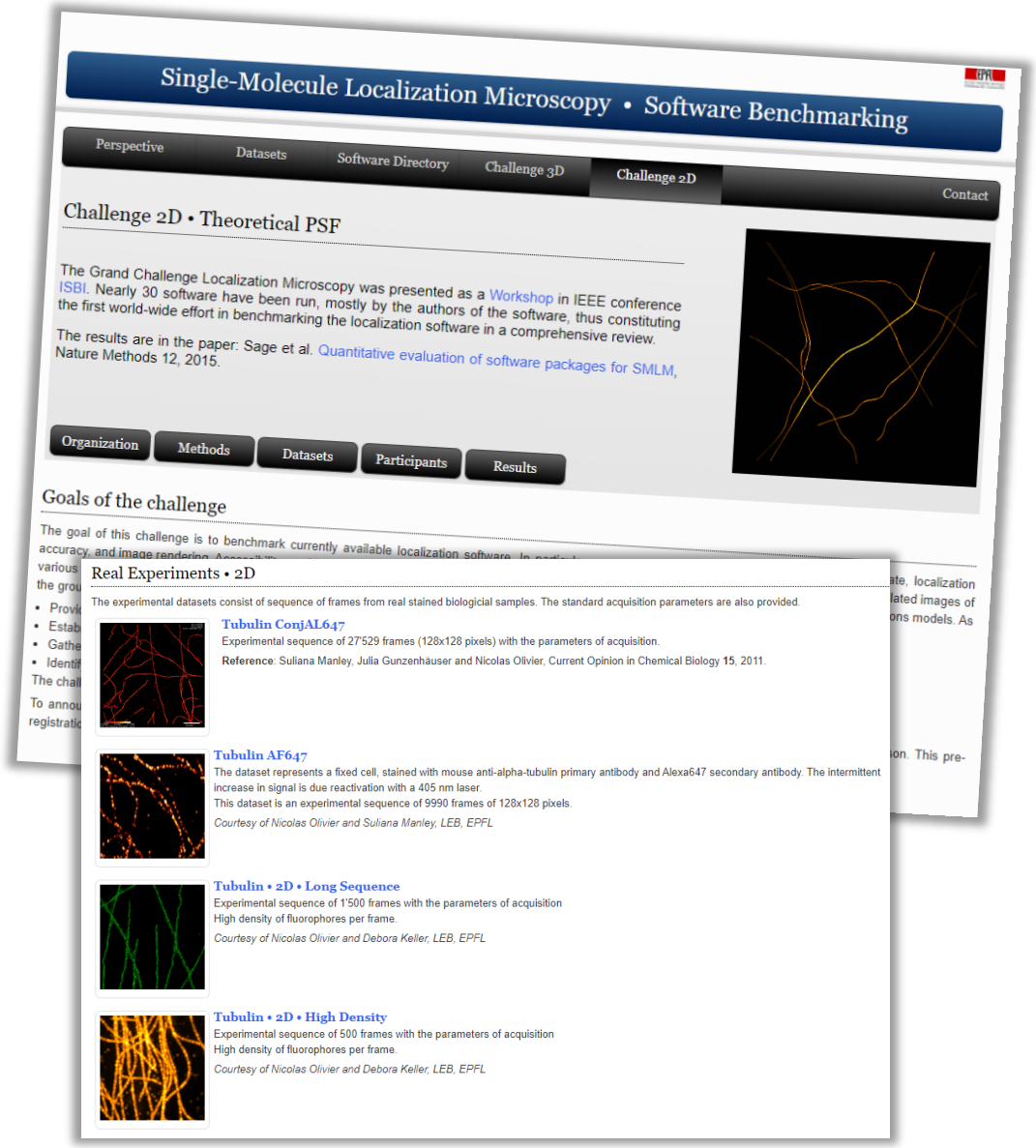
Feel free to test it…

Use the Fiji plugin ThunderSTORM to analysis the Tubulin 2D Long Sequence Dataset

Software: Fiji plugin ThunderSTORM
Plugin can be downloaded here:
https://github.com/zitmen/thunderstorm/releases/tag/v1.3
and will be located here: Plugins > ThunderSTORM

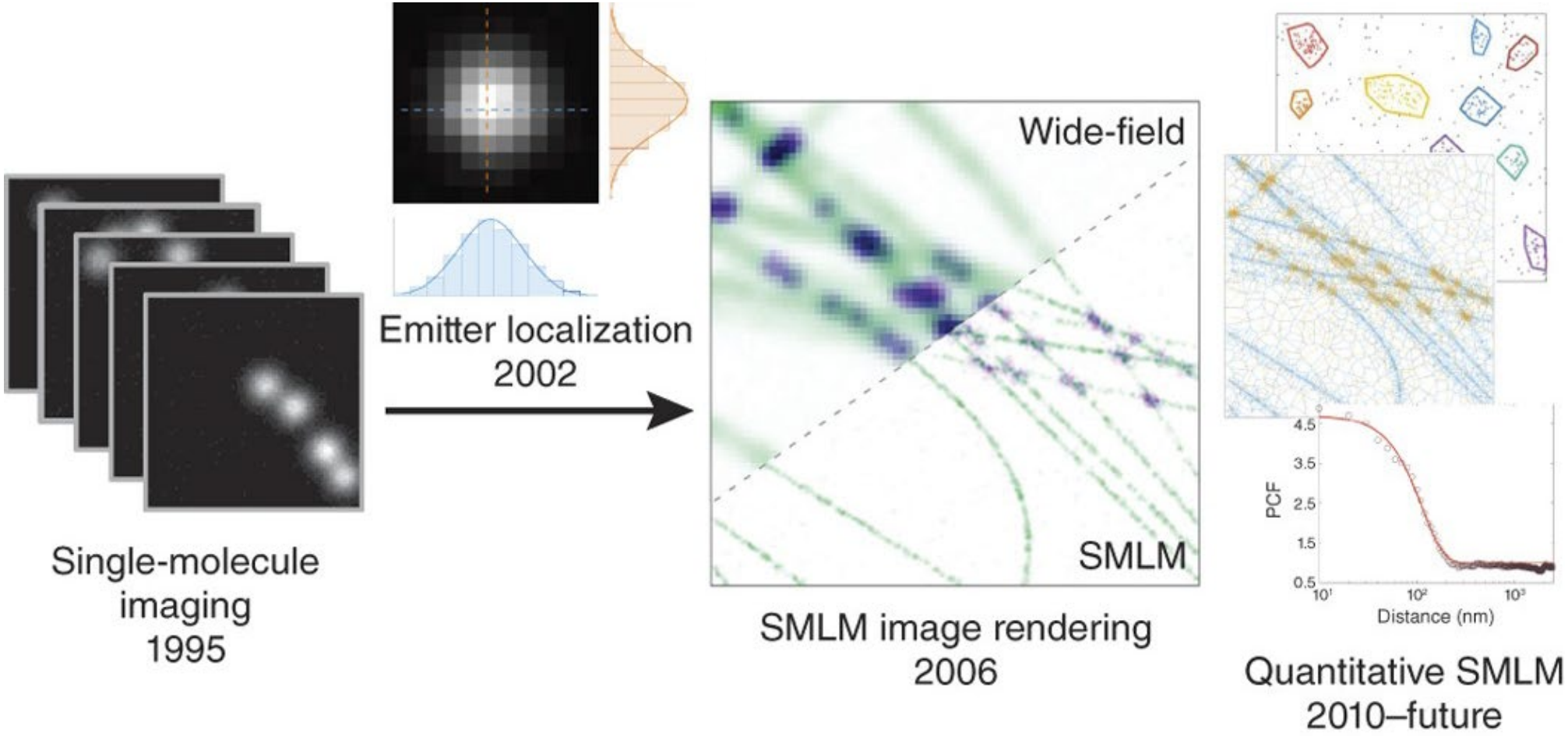
Online manual:
https://github.com/zitmen/thunderstorm/wiki/Tutorials

Dataset: Tubulin 2D Long Sequence
http://bigwww.epfl.ch/smlm/datasets/index.html?p=../challenge2013/datasets/Real_Long_Sequence



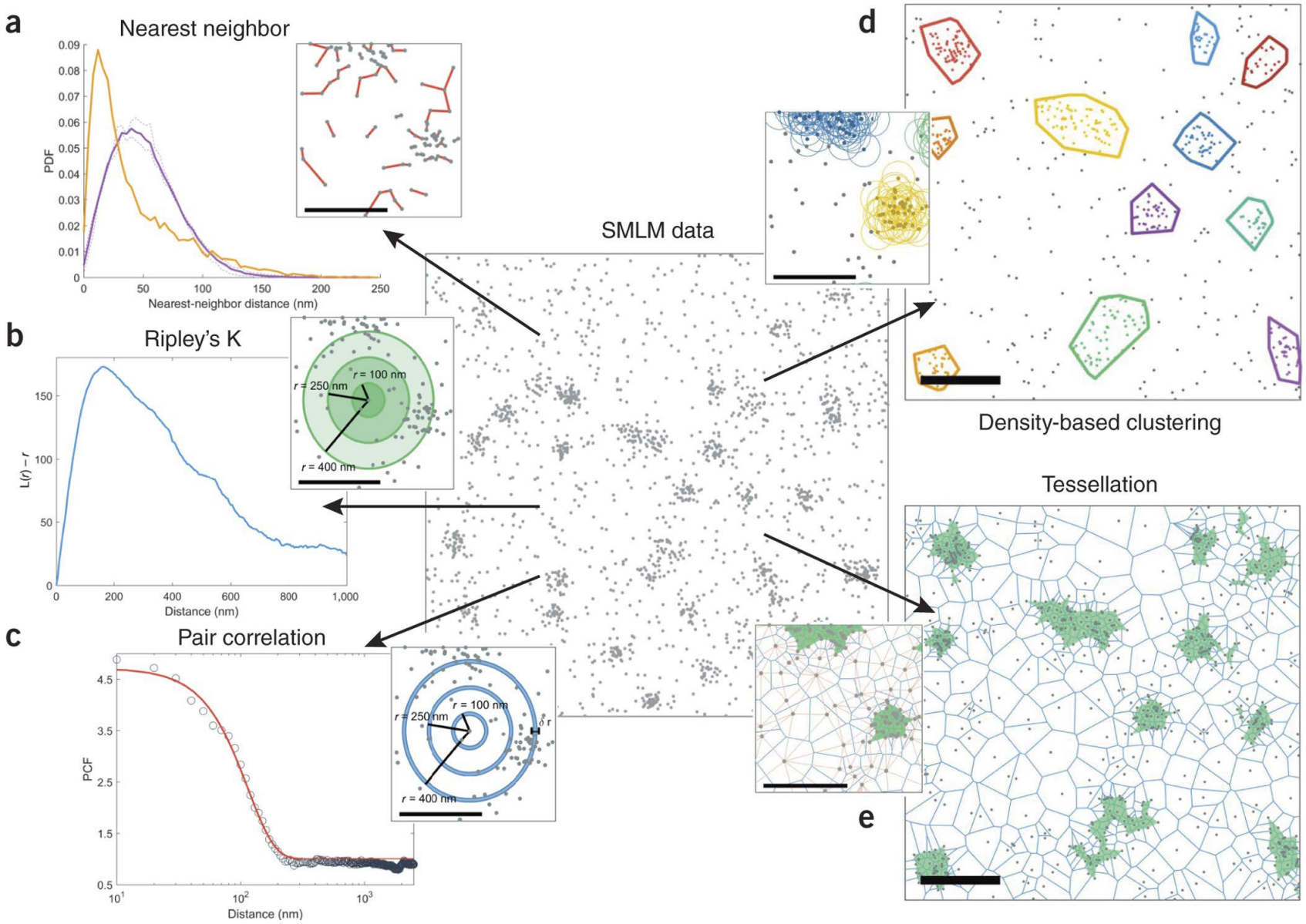
Ovesny et al. *Bioinformatics* (2014)

Timeline of SMLM developments



Nicovich et al. *Nature Protocols* (2017)

Spatial analysis approaches



Nicovich et al. *Nature Protocols* (2017)

Fiji plugins for SMLM post-processing

Name	Description	Year
bUnwarpJ	2D image registration; channel alignment by elastic transformation, deformations are represented by cubic B-splines	2006
ClearVolume	Multi-channel visualization package; rendering of image stacks for 3D and multi-colour representation	2015
FIRE	Resolution estimation; first ImageJ plugin for FRC analysis	2013
GDSC SMLM	Collection of plugins; many features such as drift correction, local density analysis, pair correlation analysis, and FRC	
MosaicIA	Cluster analysis; calculates an interaction potential that is most likely to generate the observed object distribution	2013
NanoJ-core	Drift correction and multi-colour channel alignment	2015
NanoJ-SQUIRREL	Benchmarking SMLM images; generates error map and FRC map, uncovers local differences in resolution	2018
QuASIMoDOH	Cluster analysis; divides images into tiles (tessellation), pattern analysis done by analysing the distribution of tile areas	2016
TRABI	Intensity analysis; macro that determines spot intensities in SMLM data by temporal analysis; can extract 3D information from 2D data	2017

Van der Linde et al. *Journal of Physics D: Applied Physics* (2019)

Overview

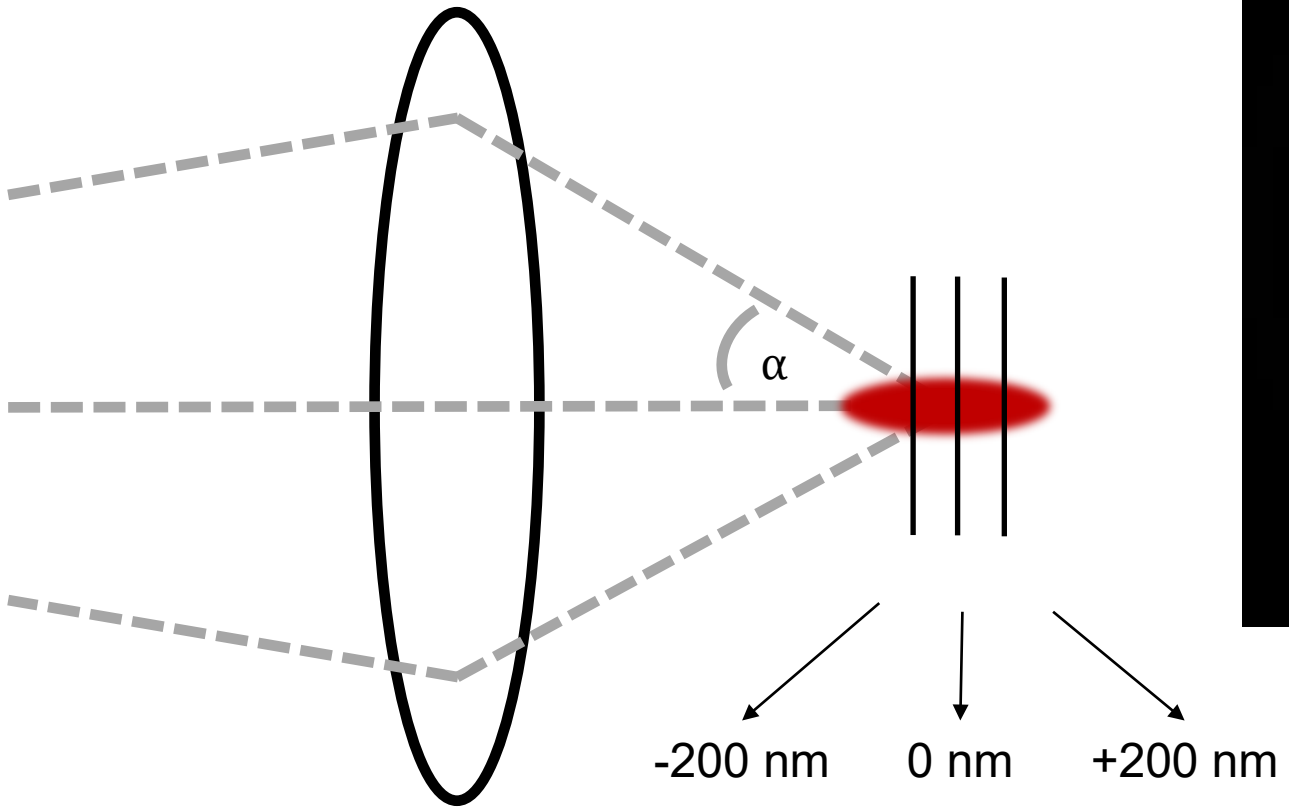
- Basic principles 2D SMLM ✓
- Hardware for SMLM ✓
- Practical considerations: sample preparation, suitable dyes, linkage errors, and buffers ✓
- Processing, quantification, and interpretation of SMLM data ✓
- 3D SMLM
- Summary

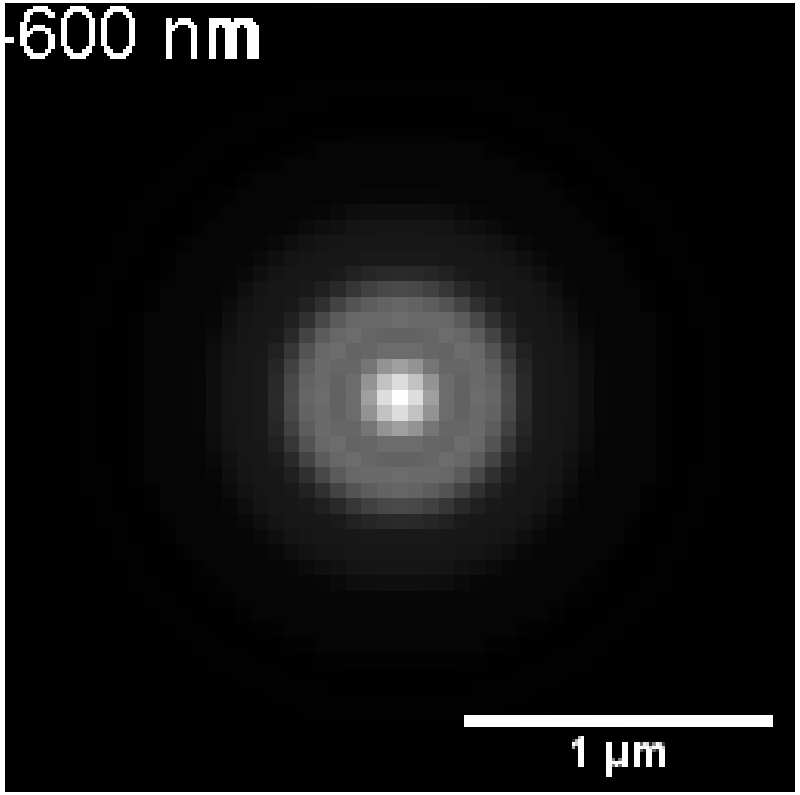
- Extra: New directions in SMLM
- Extra: SRM as a multidimensional challenge

3D SMLM

Can we localize SM in the axial direction?

A microscope's PSF is symmetric in the axial direction, which complicates single molecules' localization along the axial axes.

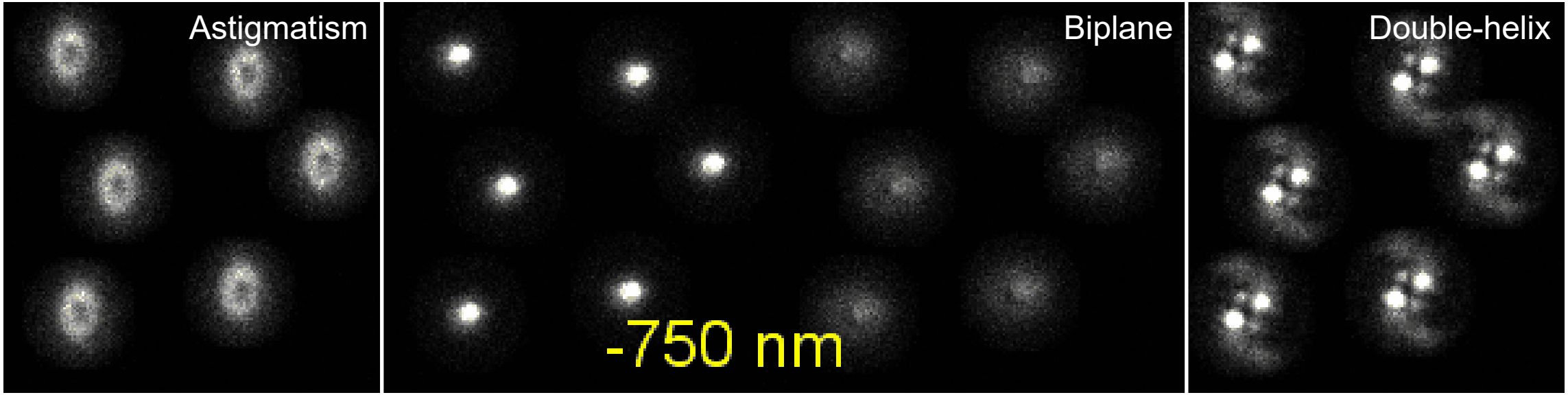




Simulated PSF

Decoding the axial information

Breaking the symmetry of the PSF enables us to decode axial information.



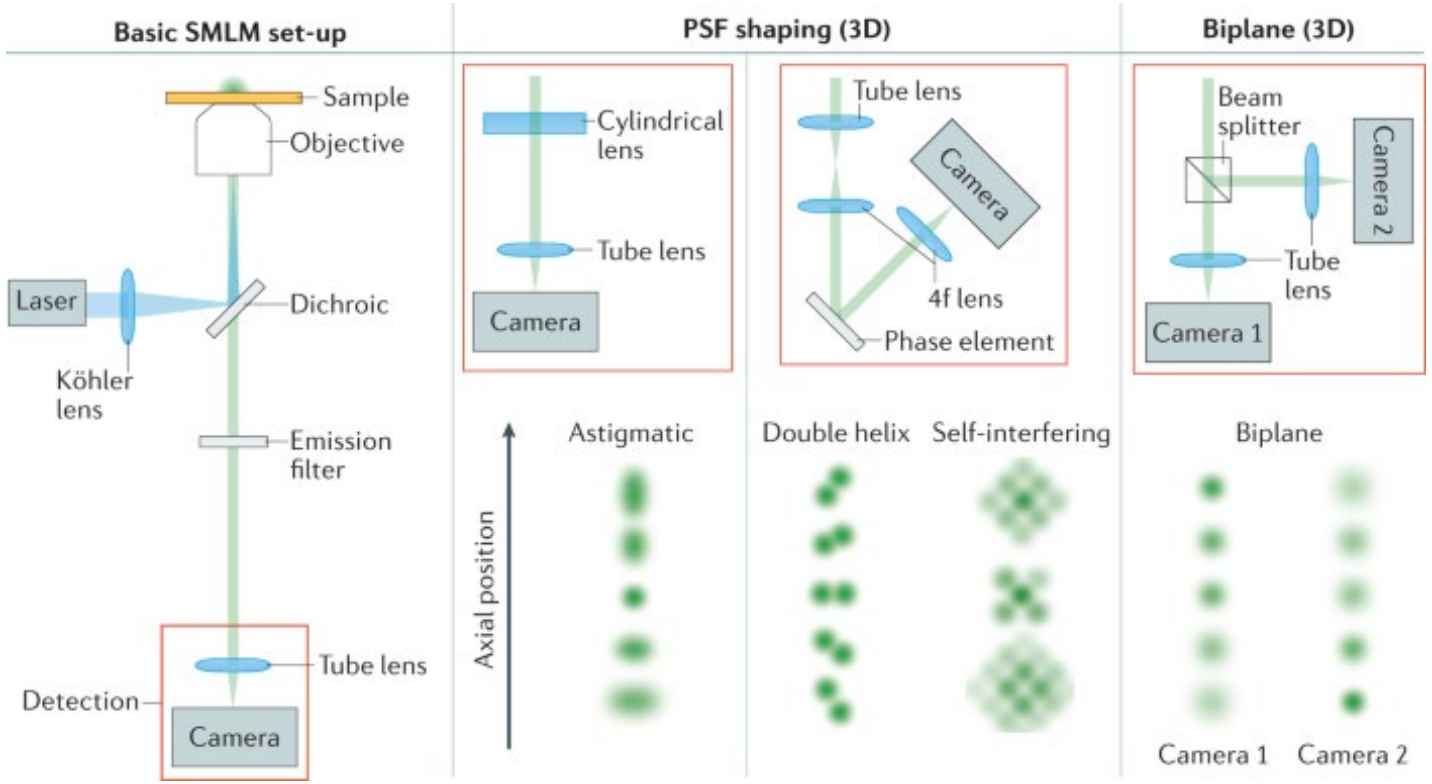
Cylindrical lens in detection path

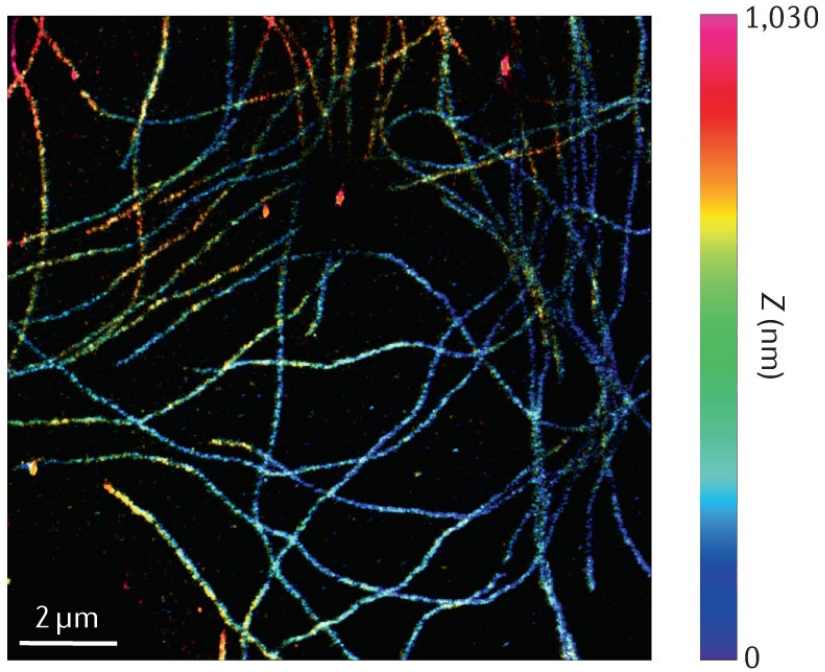
Emitted light detected onto two cameras (or two halves of one camera) defocused relative to one another

Spatial light modulator / phase mask in detection path

Huang et al. *Science* (2008), Juette et al. *Nature Methods* (2008), Pavani et al. *Optics Express* (2008)

Decoding the axial information



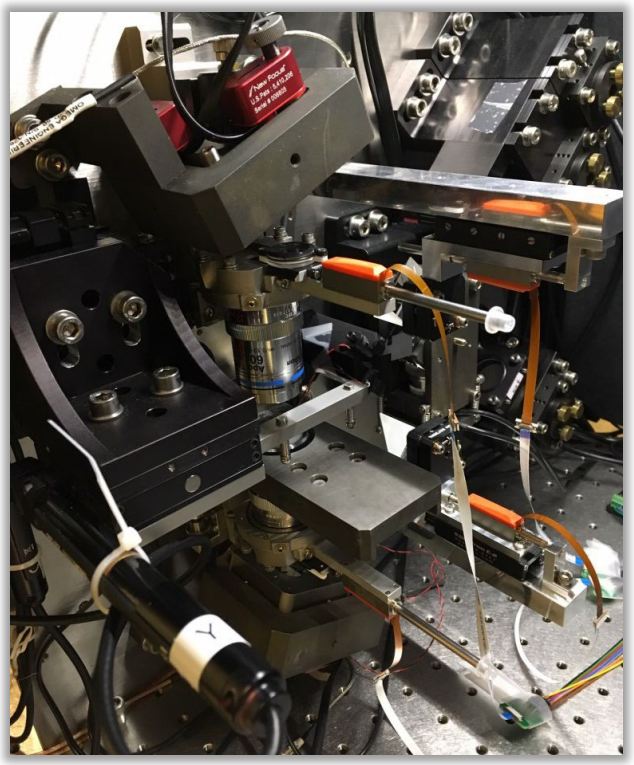


Lelek et al. *Nature Reviews Methods Primer* (2021)

Decoding the axial information

Breaking the symmetry of the PSF enables us to decode axial information.

Resolution: 10-20 nm in all three dimensions



iPALM

Shtengel et al. *PNAS* (2009)

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Summary

Let’s wrap it up…

Merits

- Very high resolution
- Single molecule detection
- Relative simple microscope setup
- Can be combined with TIRF and inclined illumination (HILO)
- Quantification of protein numbers
- Upgrade solution for exciting setups to enable extended 3D localization using PSF engineering (e.g., Double Helix)

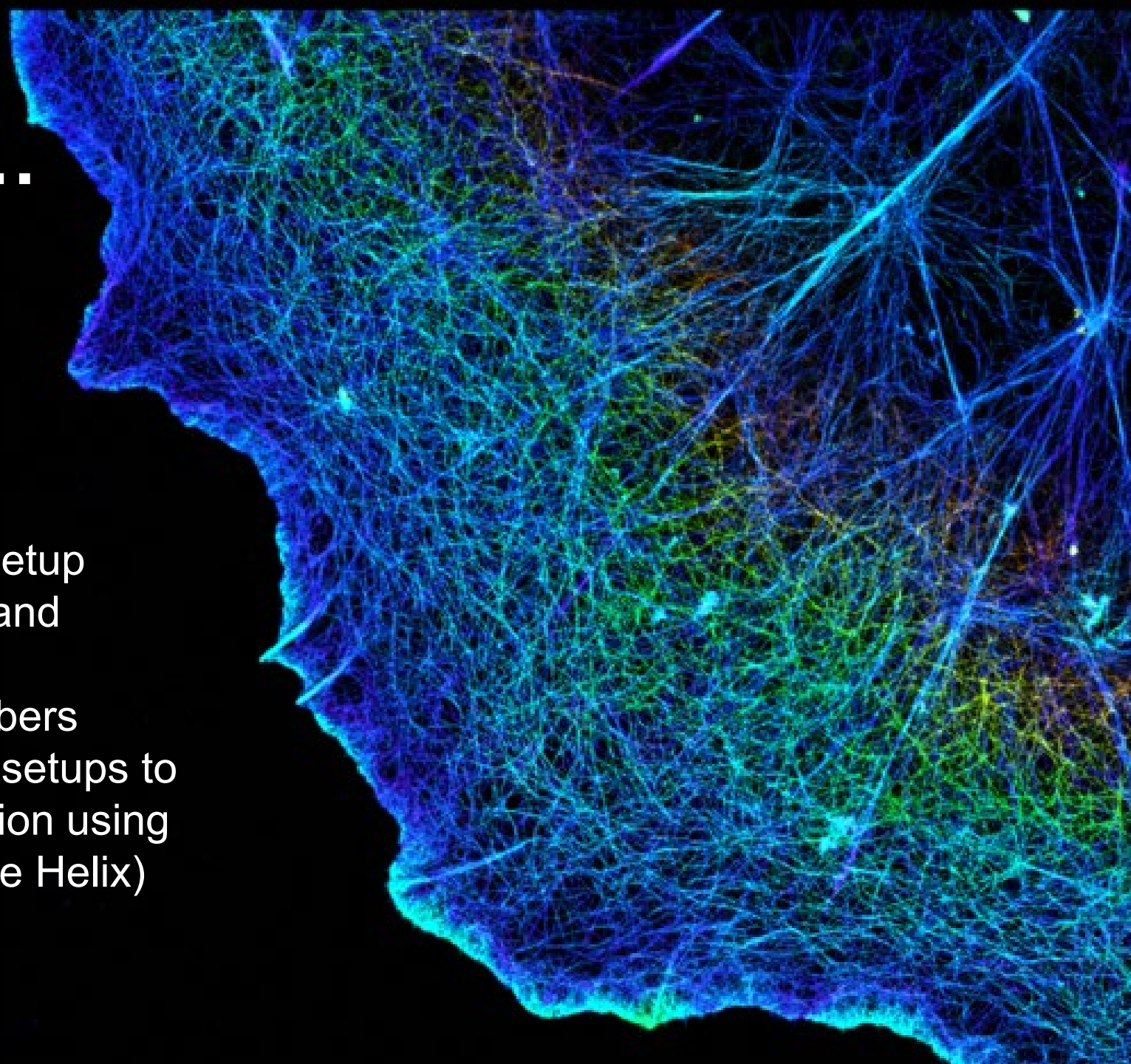

Image: Xu et al. *Nature Methods* (2012)

Let’s wrap it up…

Disadvantages

- Special buffers/probes required
- Not for thick samples (< 10 um)
- Slow acquisition
- Limited 3D (no sectioning)
- Advanced post-processing needed
- Prone to reconstruction artifacts
- Structural resolution labeling density-dependent

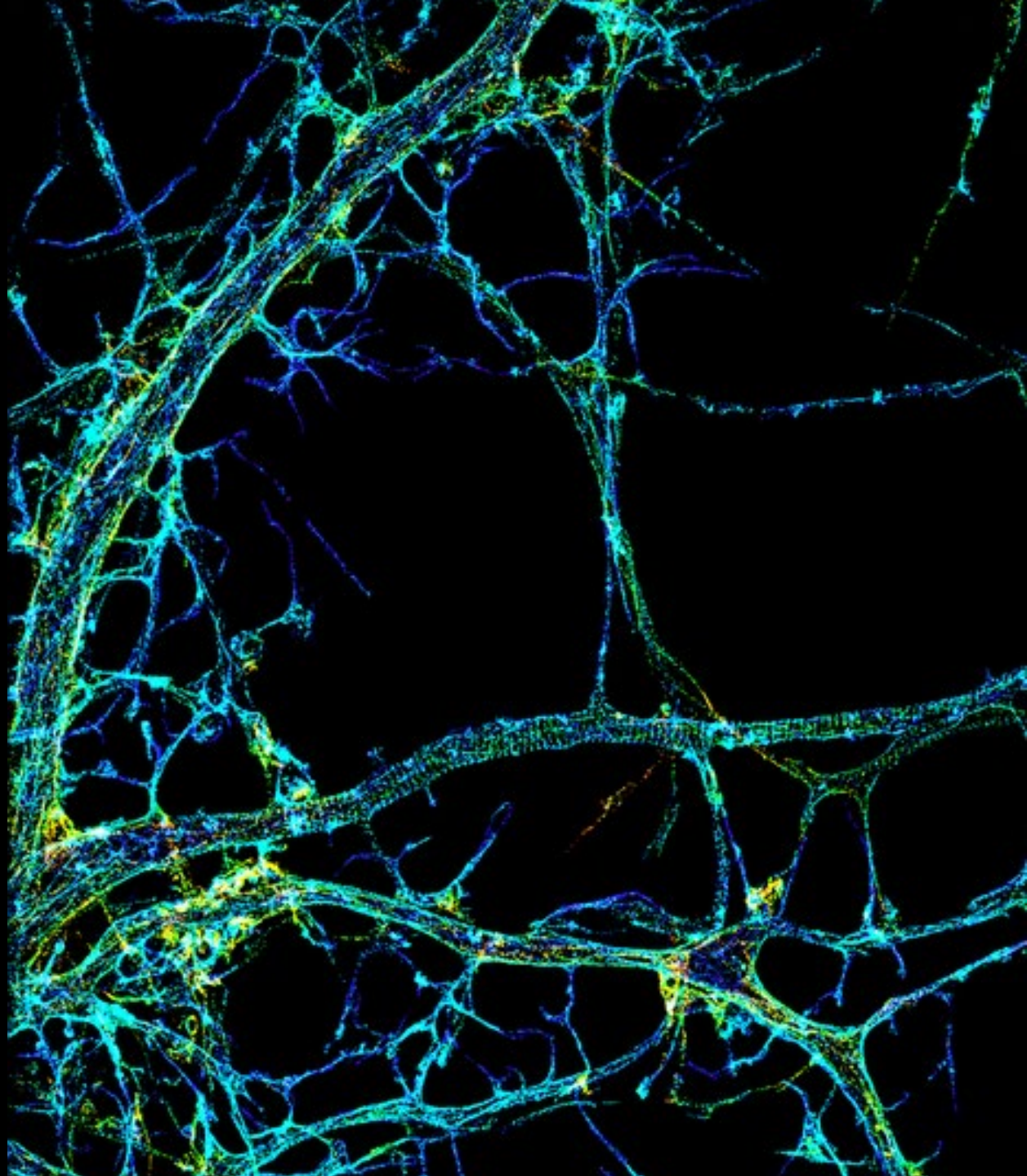

Image: Xu et al. *Science* (2013)

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Thank you! Questions?

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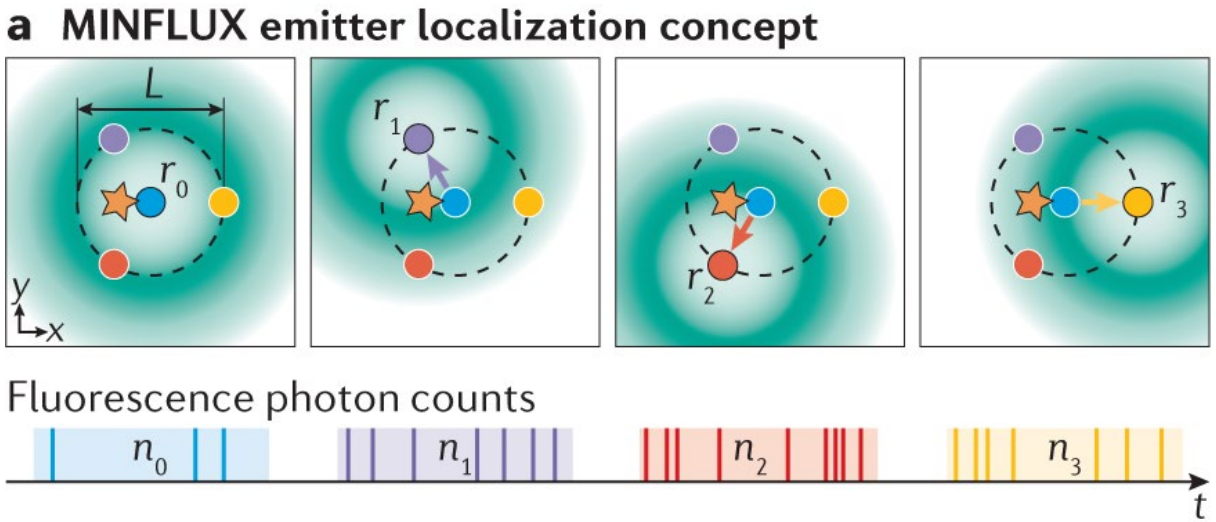
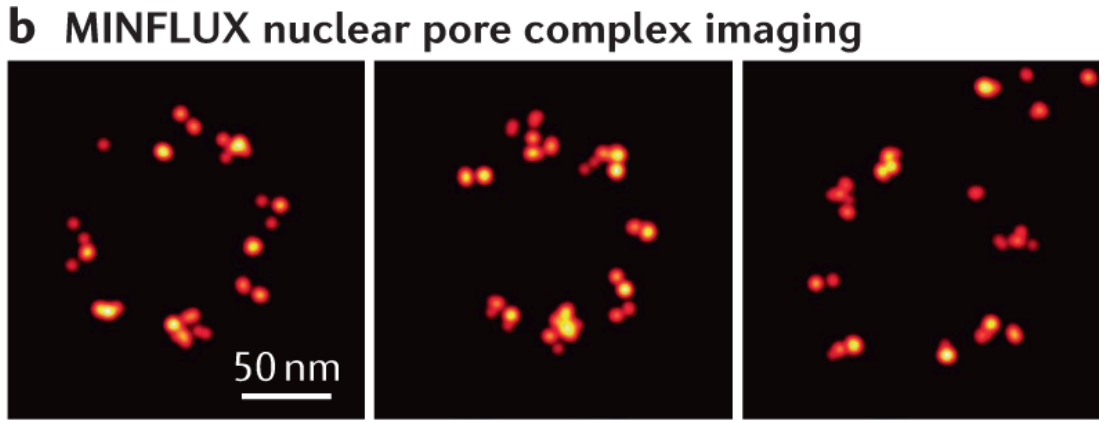
Photo: Martin Duckek

New dimensions in SMLM

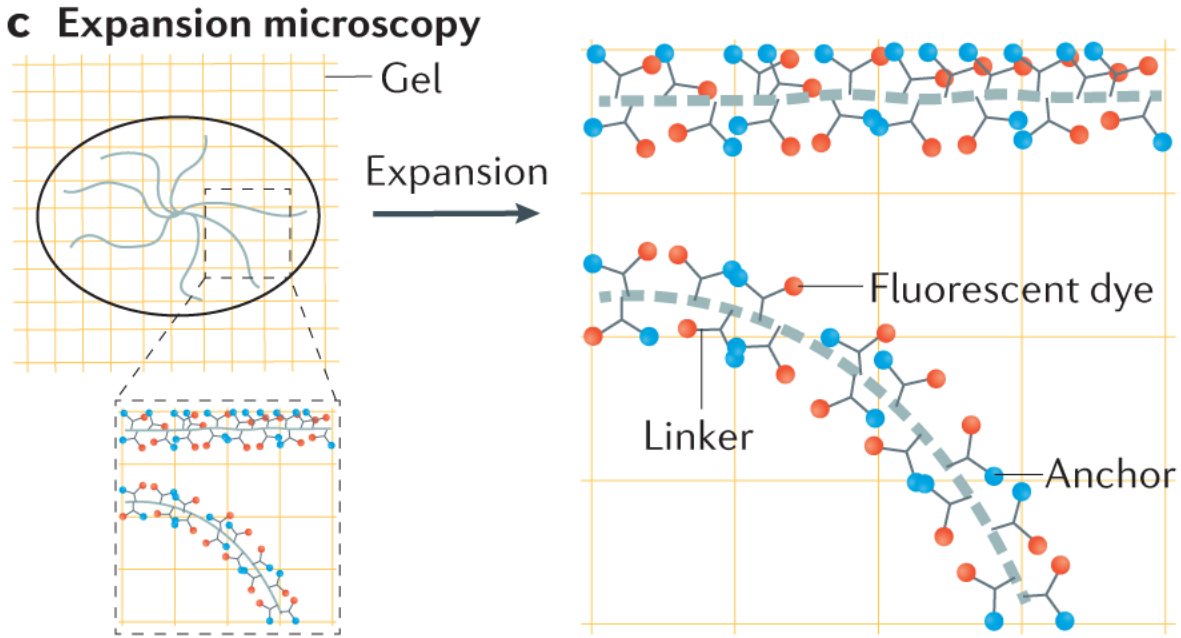
MINFLUX emitter localization concept

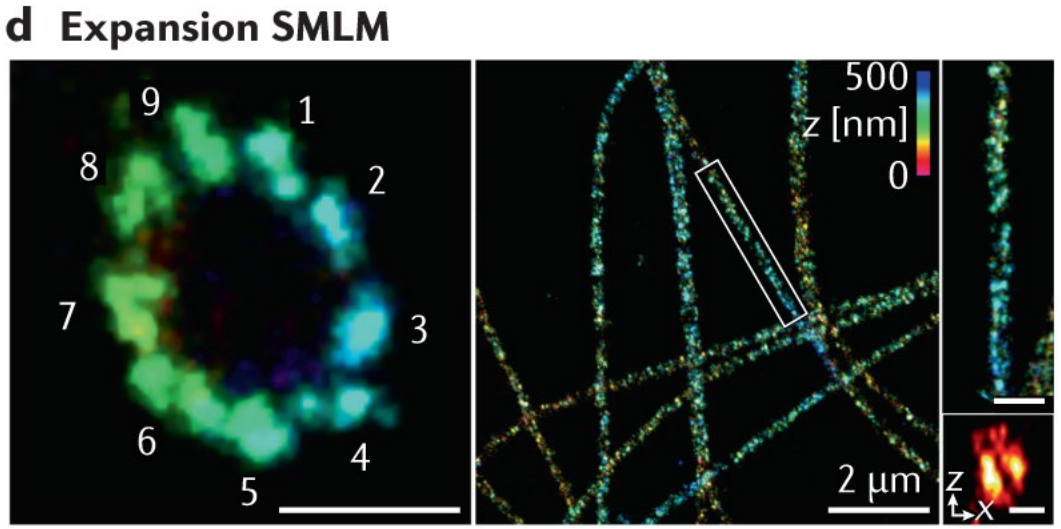




Lelek et al. *Nature Reviews Methods Primer* (2021)

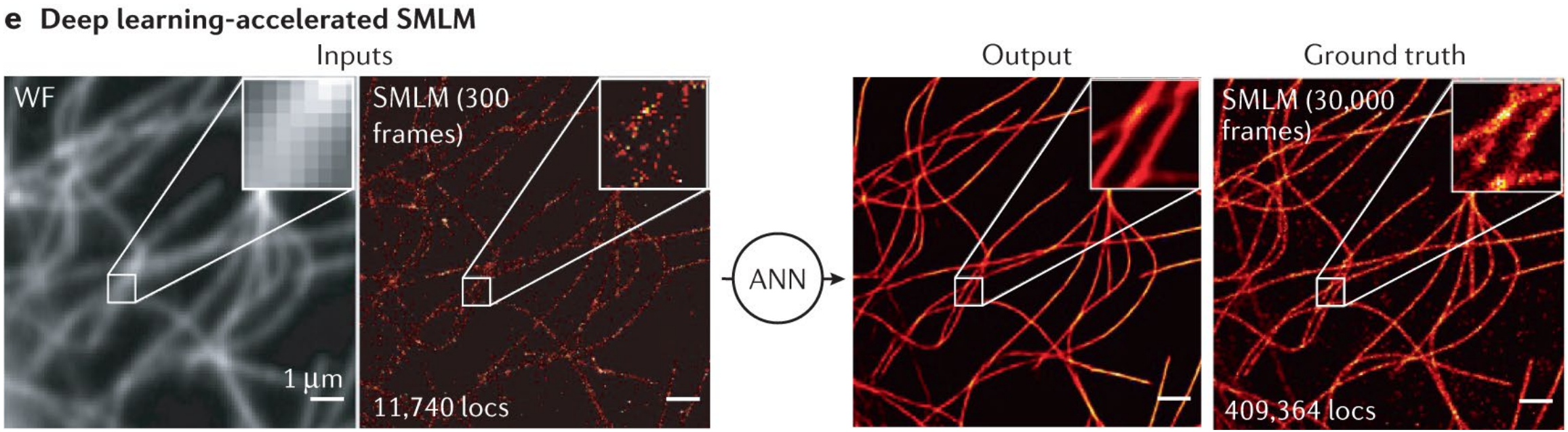
Expansion SMLM





Lelek et al. *Nature Reviews Methods Primer* (2021)

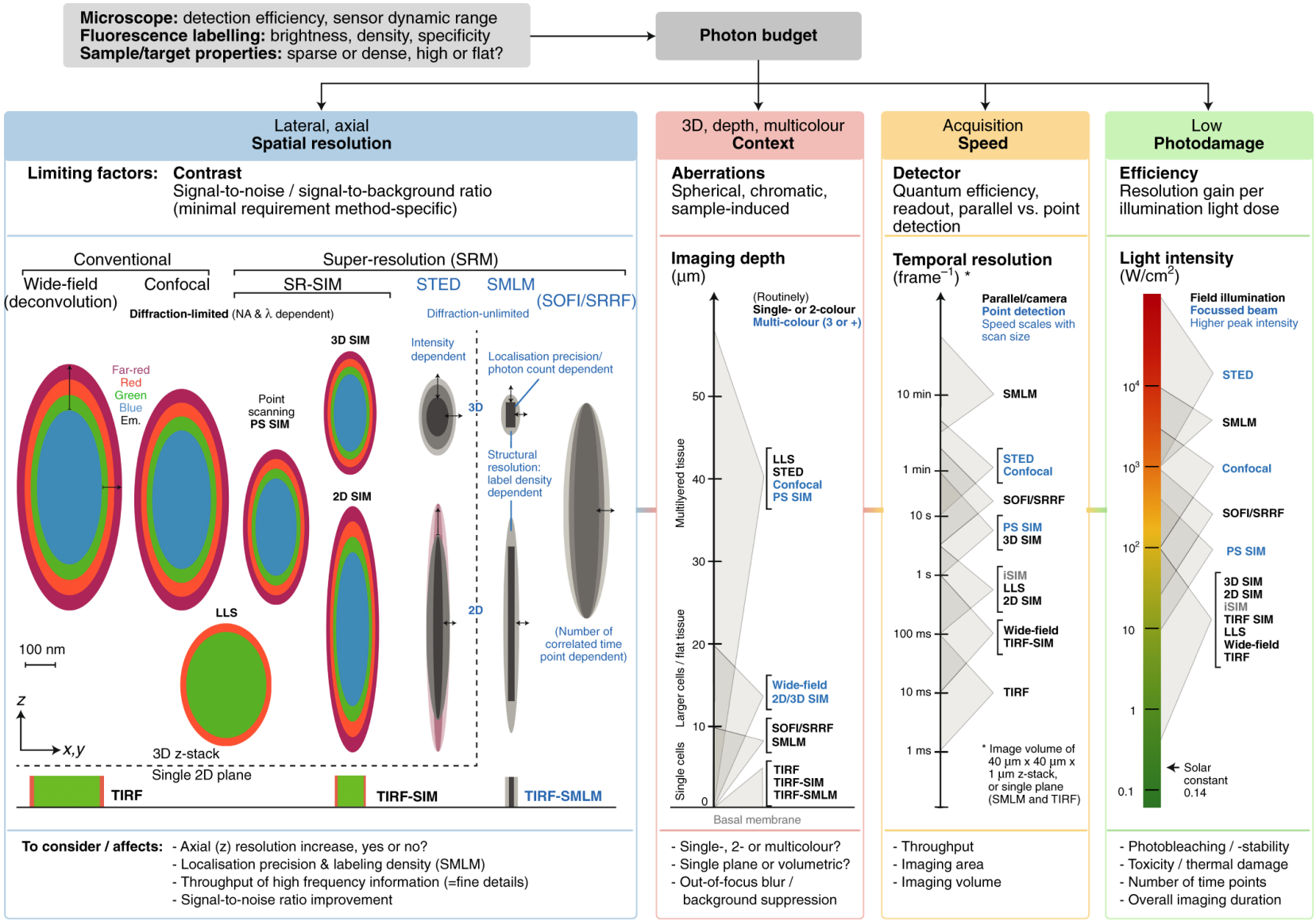
Deep learning-accelerated SMLM



Lelek et al. *Nature Reviews Methods Primer* (2021)

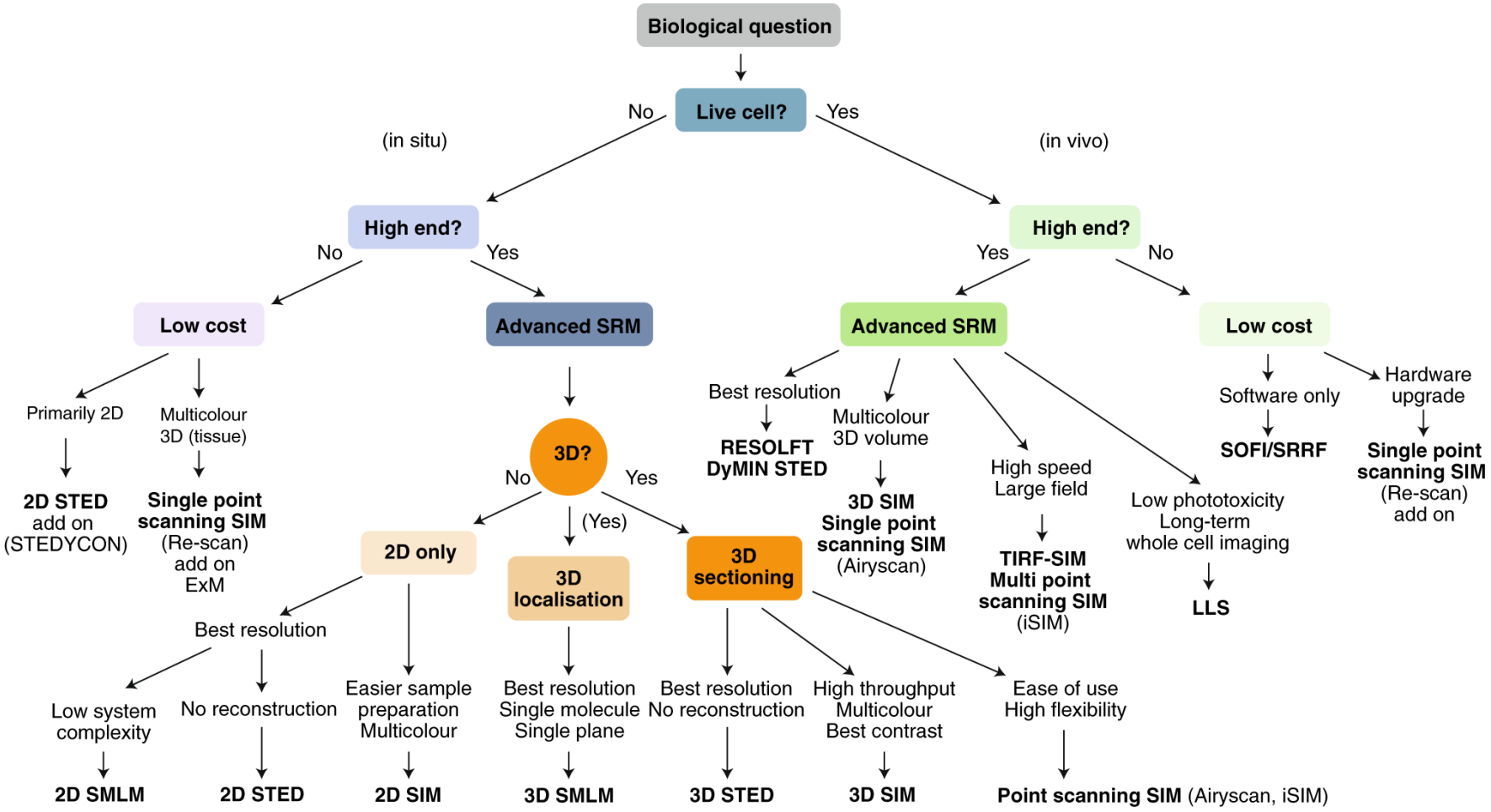
SRM as a multidimensional challenge

Inherent trade-offs in SRM



Schermelleh et al. *Nature Cell Biology* (2019)

Decision tree for selecting SRM techniques



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Appendix

Switching buffers

Buffer class	Buffer base	Compounds	Organic dyes used in switching buffer / description
Reducer - O_2	PBS/TRIS pH 7.4 - 9 - O_2	10 - 100 mM MEA	AF 750 [96], CF 680 [63], CF 647 [63], AF 647 and Cy5 [10], CF 568 [63], AF 532 [106], ATTO 520 [103]
		0.5 - 1% BME	AF 750 [112], CF 680 [98], Cy5 readout pairs [9, 58], AF 647 [92]
		10 - 100 mM GSH	AF 647 [106], TMR [106]
Reducer only	PBS/TRIS pH 7.4 - 9	10 - 100 mM MEA	AF 647 [54], ATTO 655 [100], AF 568 [54], ATTO 520 [111], AF 532 [105], AF 488 [111]
		0.5 - 1% BME	ATTO 655 [100]
		50 µM AA	ATTO 655 [169]
		10 - 100 mM GSH	ATTO 655 [52], ATTO 520 [52]
		50 mM TCEP + 2 mM COT	AF 750 [97], AF 647 [97]
Oxygen removal (- O_2)	PBS/TRIS pH 7.4 - 9	GLOX*	AF 568 [121], ATTO 520 [121], AF 488 [121]
		PCD/PCA*	
		POC*	
		100 mM MEA + 1 µM MB	Cy5 [170]

Turkowyd et al. *Anal. Bioanal. Chem.* (2016)

Switching buffers

Switching ROXS reducer and oxidizer - O_2	PBS/TRIS pH 7.4 - 9 - O_2	500 µM AA + 25 µM MV	ATTO 655 [171]
		1 mM AA + 1 mM MV + 25 mM TCEP, pH 9	AF 750 [172], AF 647 [172], Cy5 [172]
Switching mount	Vectashield	20% Vectashield + 80% (95% glycerol 50 mM TRIS)	AF 647 [110], CF 647 [110]
	Mowiol	0.5% Mowiol + 50 mM DTT	SiR [101]
	Resin	100% dehydration + EM resin embedding	
	PVA	1% in PBS, spin coat	Oregon Green [41], AF 488 [41]
Live-cell media	DMEM, modified to not contain phenol red	None	SiR [102], TMR [55, 102]
		100 mM GSH + GLOX	AF 647 [106], TMR [106]
		25 mM TCEP	AF 647 [172], Cy5 [172]

Turkowyd et al. *Anal. Bioanal. Chem.* (2016)

Multicolor SMLM

	Name	Dual color combinations	Triple color combinations
Fluorescent proteins	mEos2 (G)	AF 647 [90-92], ATTO 655 [53], Caged SiRhQ [57], Dronpa [56], psCFP2 [56]	
	mEos2 (R)		
	PAmCherry1	paGFP [59]	eYFP + NileRed [93], PAmKate + Dendra2 [60]
	Dendra2 (G)		PAmKate + PAmCherry1 [60]
	Dendra2 (R)		
	paGFP	PAmCherry [59]	PAmKate + PAmCherry1 [60], PAtagRFP + ATTO 655 [95]

Turkowyd et al. *Anal. Bioanal. Chem.* (2016)

	Name	Dual color combinations	Triple color combinations
Organic dyes	**Alexa Fluor 750**	AF 647 [97]	
	CF 680	AF 647 (#) [62, 98, 99]	CF 660C + DyLight 650 + Dy 634 (#) [62], CF 647 + CF 568 (#) [63]
	ATTO 655 (§)	ATTO 520 [52], mEos2 [53]	PAtagRFP + paGFP [95]
	SiR (§)	mEos2 [57]	TMR + paGFP [102]
	Alexa Fluor 647	ATTO 520 [103], AF 532 [104, 105], ATTO 532 [54], AF 546 [54], AF 568 [54], TMR [106], mEos2 [90-92], AF 488 [91], psCFP2 [56], mMaple [99], ATTO 488 [107], CF 680 (#) [62,98, 99], AF 700 (#) [61], AF 750 (#) [97], Dy678 (#) [108], Dronpa [109]	AF 568 + ATTO 488 [107]
	CF 647		CF 680 (#) + CF 568 [63]
	Cy5		
	Alexa Fluor 568	AF 647 [54]	AF 647 + ATTO 488 [107]
	CF 568		CF 680 + CF 647 [63]
	TMR (§)	AF 647 [106], Citrine [55]	SiR + paGFP [102]
	Alexa Fluor 532	AF 647 [104, 105]	
	ATTO 520	AF 647 [103], ATTO 655 [52]	
	Alexa Fluor 488 (§)	AF 647 [91]	AF 647 + AF 568 [107], Cy3 + ATTO 532 [55], Rhodamine 3C + AF 514 [55]
	Cy5/AF 647 readout dye pairs	Reporter: AF 750 [112] Activator: Cy3 + AF 405 [112]	Reporters: Cy7 + Cy5.5 [58] Activators: Cy3 + Cy2 + AF 405 [58]

Turkowyd et al. *Anal. Bioanal. Chem.* (2016)