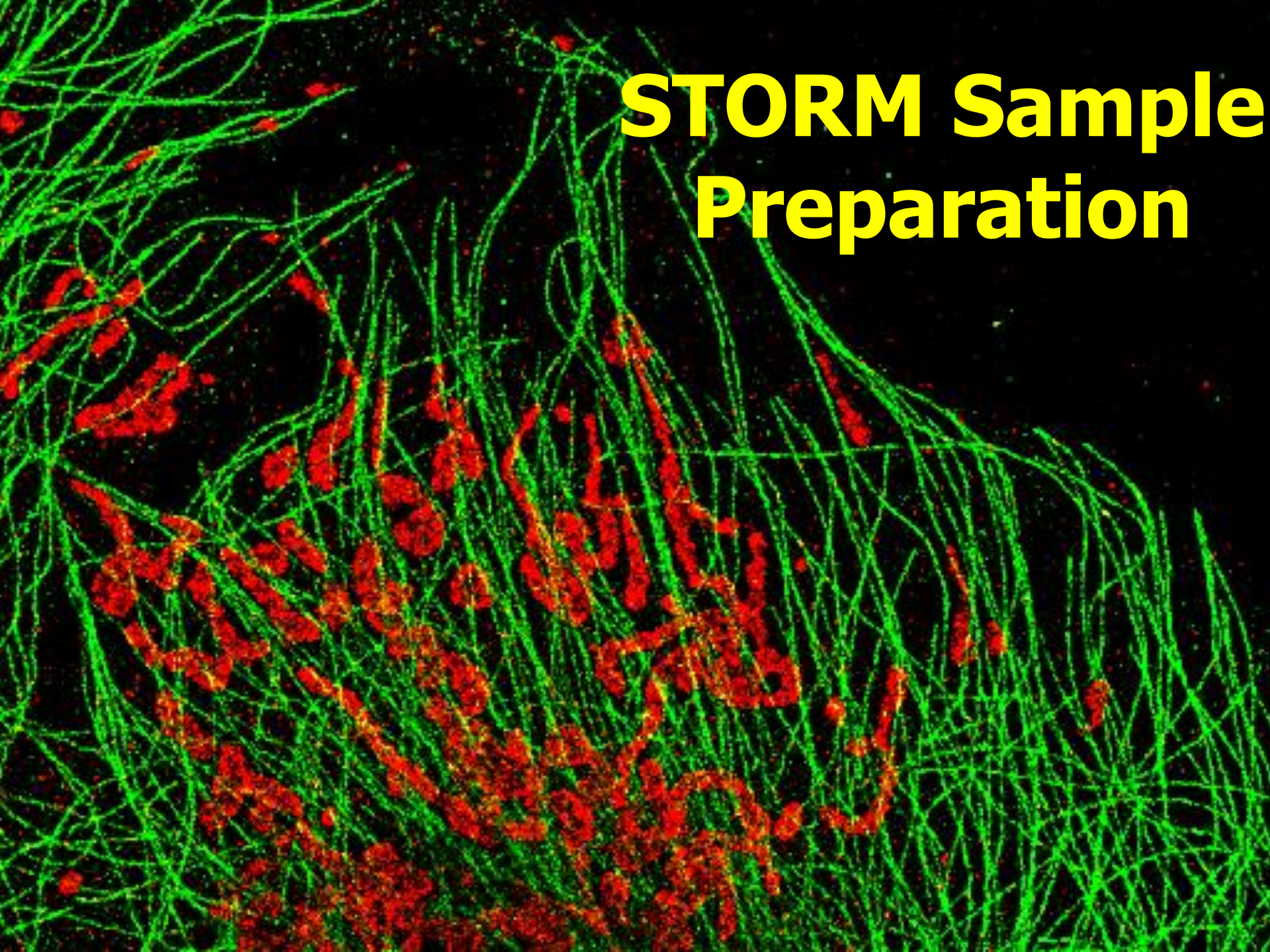
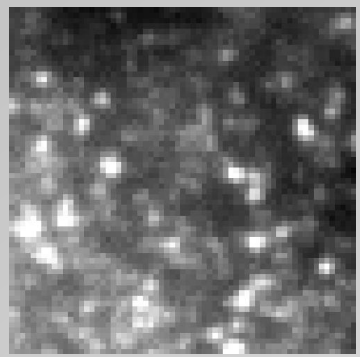

STORM Sample
Preparation

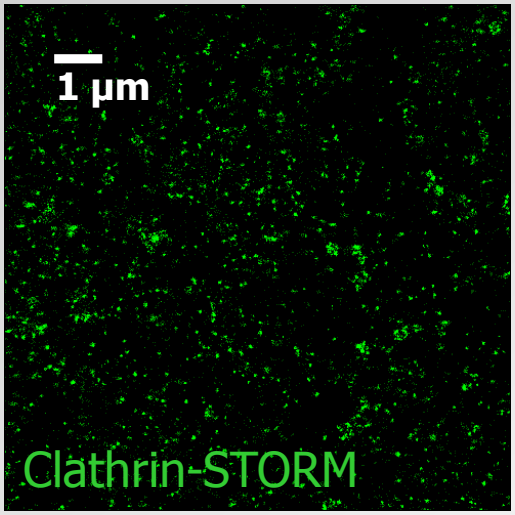
Sample Quality and Image Quality

- Bad sample = bad STORM image

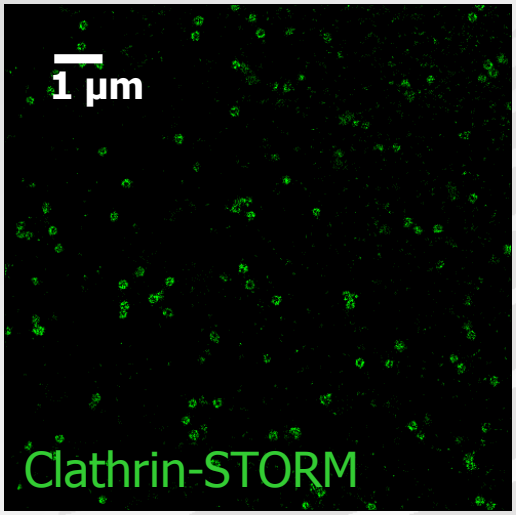
BAD

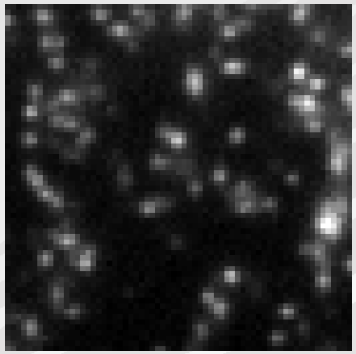


Widefield
Clathrin



Good





Widefield
Clathrin

What makes a good STORM sample?

Influences on Sample Quality

1. Probe Choice
   - Dyes that work for N-STORM
2. Labeling Strategies
   - Fixation
   - Immunostaining

1. Probe Choice

The most important considerations are….

- High photon number

$$\sigma = \sigma(\mathrm{PSF})/N^{1/2}$$

- High Localization Density

$$\text{Nyquist resolution} = \frac{2}{(\text{localization density})^{1/D}}$$

- High Photostability

Longer imaging time

- Laser Power

A Small # of Dyes Work for N-STORM (Despite what is published)

Dye	Excitation maximum (nm)[a]	Emission maximum (nm)[a]	Extinction (M^{-1} cm^{-1})[b]	Quantum yield[c]	Detected photons per switching event		Equilibrium on-off duty cycle (400–600 s)		Survival fraction after illumination for 400 s		Number of switching cycles (mean)	
					MEA	βME	MEA	βME	MEA	βME	MEA	βME
Blue-absorbing												
Atto 488	501	523	90,000	0.8	1,341	1,110	0.00065	0.0022	0.98	0.99	11	49
Alexa Fluor 488	495	519	71,000	0.92	1,193	427	0.00055	0.0017	0.94	1	16	139
Atto 520	516	538	110,000	0.9	1,231	868	0.0015	0.00061	0.92	0.86	9	17
Fluorescein	494	518	70,000	0.79	1,493	776	0.00032	0.00034	0.51	0.83	4	15
FITC	494	518	70,000	0.8	639	1,086	0.00041	0.00031	0.75	0.9	17	16
Cy2	489	506	150,000	0.12	6,241	4,583	0.00012	0.00045	0.12	0.19	0.4	0.7
Yellow-absorbing												
Cy3B	559	570	130,000	0.67	1,365	2,057	0.0003	0.0004	1	0.89	8	5
Alexa Fluor 568	578	603	91,300	0.69	2,826	1,686	0.00058	0.0027	0.58	0.99	7	52
TAMRA	546	575	90,430	0.2	4,884	2,025	0.0017	0.0049	0.85	0.99	10	59
Cy3	550	570	150,000	0.15	11,022	8,158	0.0001	0.0003	0.17	0.55	0.5	1.6
Cy3.5	581	596	150,000	0.15	4,968	8,028	0.0017	0.0005	0.89	0.61	5.7	3.3
Atto 565	563	592	120,000	0.9	19,714	13,294	0.00058	0.00037	0.17	0.26	4	5
Red-absorbing												
Alexa Fluor 647	650	665	239,000	0.33	3,823	5,202	0.0005	0.0012	0.83	0.73	14	26
Cy5	649	670	250,000	0.28	4,254	5,873	0.0004	0.0007	0.75	0.83	10	17
Atto 647	645	669	120,000	0.2	1,526	944	0.0021	0.0016	0.46	0.84	10	24
Atto 647N	644	669	150,000	0.65	3,254	4,433	0.0012	0.0035	0.24	0.65	9	39
Dyomics 654	654	675	220,000	–	3,653	3,014	0.0011	0.0018	0.79	0.64	20	19
Atto 655	663	684	125,000	0.3	1,105	657	0.0006	0.0011	0.65	0.78	17	22
Atto 680	680	700	125,000	0.3	1,656	987	0.0019	0.0024	0.65	0.91	8	27
Cy5.5	675	694	250,000	0.28	5,831	6,337	0.0069	0.0073	0.87	0.85	16	25
NIR-absorbing												
DyLight 750	752	778	220,000	–	712	749	0.0006	0.0002	0.55	0.58	5	6
Cy7	747	776	200,000	0.28	852	997	0.0003	0.0004	0.48	0.49	5	2.6
Alexa Fluor 750	749	775	240,000	0.12	437	703	0.00006	0.0001	0.36	0.68	1.5	6
Atto 740	740	764	120,000	0.1	779	463	0.00047	0.0014	0.31	0.96	3	14
Alexa Fluor 790	785	810	260,000	–	591	740	0.00049	0.0014	0.54	0.62	5	2.7
IRDye 800 CW	778	794	240,000	–	2,753	2,540	0.0018	0.038	0.6	1	3	127

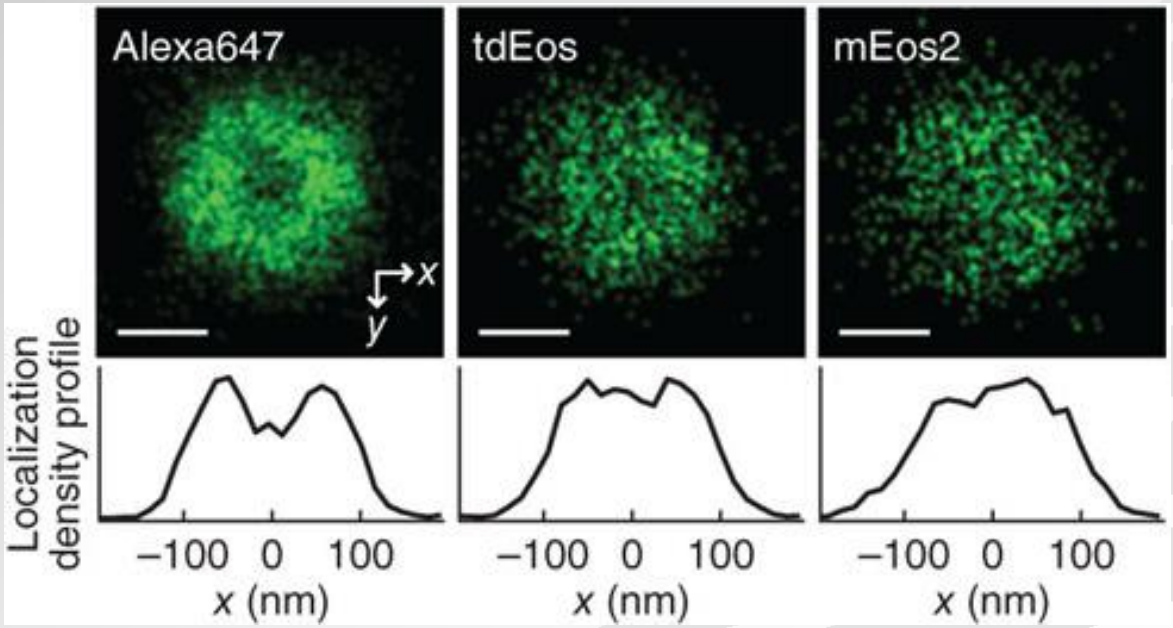
Dempsey et al., 2011

N-STORM Fluorophores

N-STORM Reporter Dyes
Alexa647
Cy5
Alexa568
Cy3B
Atto488

Fluorescent Proteins

Patterson, J. Microsc., 2011



Jones et al., 2011, Nat. Methods

Linkage Error and STORM Dyes

- Synthetic dyes are coupled to antibodies which recognize specific proteins
- The secondary antibody (conjugated to the dye) is usually used to label a primary antibody against the protein of interest

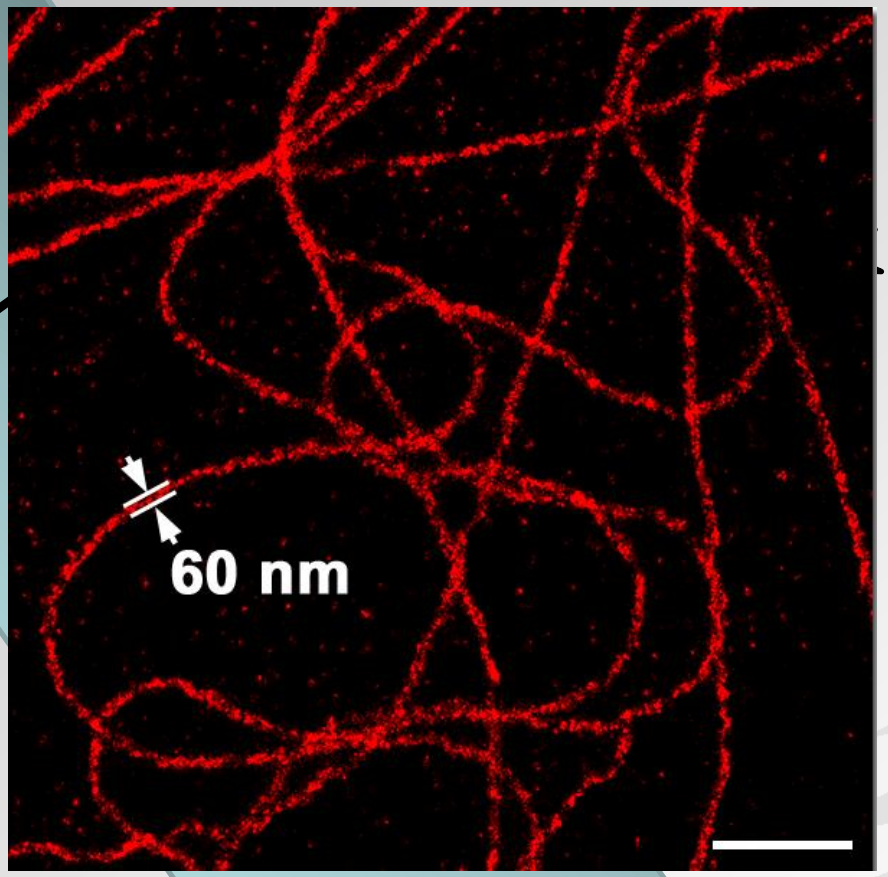
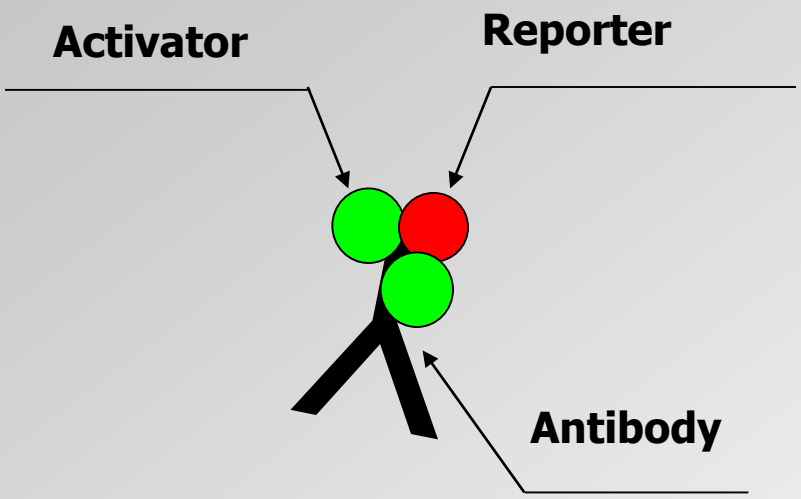


Image courtesy of Michael Davidson, FSU

- This positions dyes up to 20 nm away from the target
- Using shorter Fab secondary antibody fragments helps
- Directly conjugating dyes to proteins or primary antibodies is even better
- FP's typically positions fluorophores close to the protein of interest

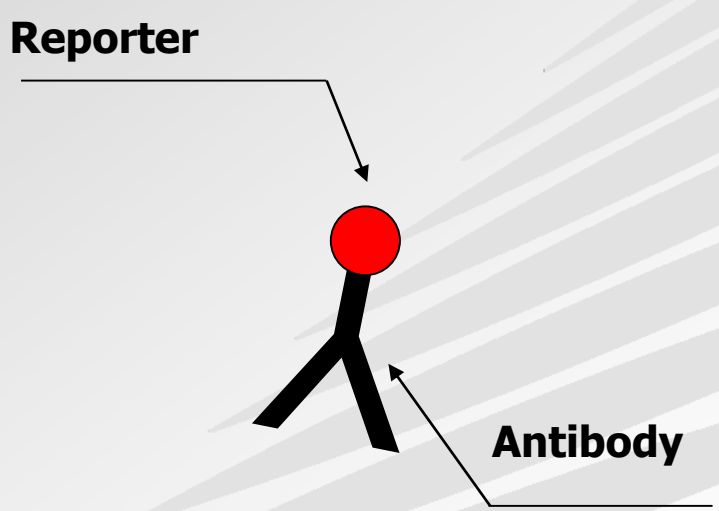
Two Methods of STORM Labeling/Imaging

Tandem Dye Pair



Alexa405-Alexa647
Cy2-Alexa647
Cy3-Alexa647

Reporter Only (cSTORM)



Alexa647
Atto488
Alexa568

Multi-channel STORM Methods: Pros and Cons

Tandem dye pair-based multicolor (Cy2-647 + Cy3-647)		**Reporter-based multicolor (Alexa 647+Atto488)**	
Pro	Con	Pro	Con
Same high photon reporter can be used for each channel (Alexa 647)			Most reporters emit fewer photons than Alexa 647
Free of chromatic aberration			Chromatic aberration
	High color crosstalk	Low crosstalk	
	Probes must be made	Commercially available probes	

Secondaries and Sources for cSTORM

cSTORM 2º Antibodies	Sources
Alexa647	Life Technologies, Jackson
Cy5	Jackson
Alexa568	Life Technologies
Cy3B	DIY (Dye from GE)
Atto488	Rockland, Sigma

Fixation/Labeling Strategies

The goals of fixation are to preserve ultrastructure and ability of antibodies to bind

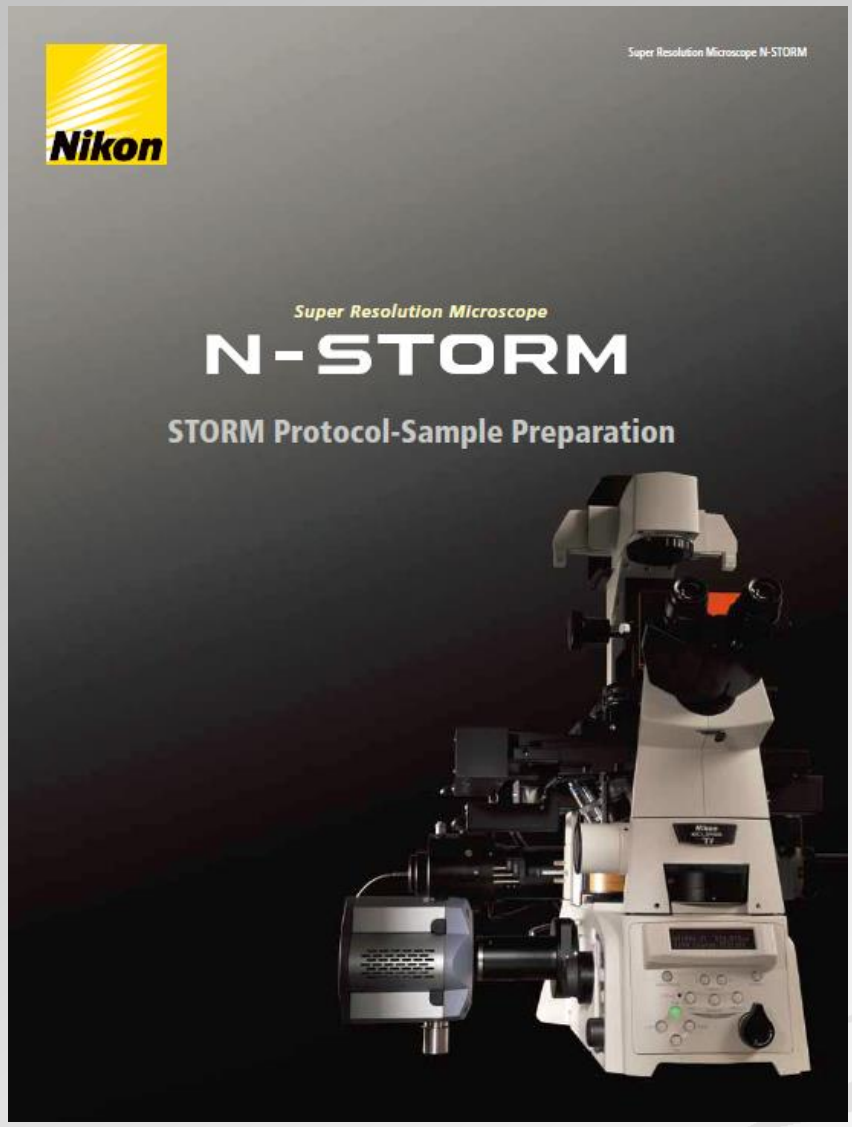
Fixatives

Methanol – solvent (lipids) and coagulant (proteins)

Aldehydes – cross-linkers that create bridges between molecules

- The best fixatives and concentrations are protein dependent
- 3% PFA and 0.1% glut is good starting point

N-STORM Protocol



These are general guidelines to be used as a **starting point**

The following steps are necessary….

Tips for STORM Sample Preparation

- Compare performance of antibodies from multiple sources.
- Optimize fixation (fixative concentration, permeabilization, etc.) to maximize structural preservation and antibody binding.
- **Minimize background** signal levels by titrating primary antibody.
- Block with heat-treated sterile filtered **blocking serum**.
- Don’t skip on the washing steps and use 1% blocking serum to remove antibodies AT EVERY STEP.
- Lock secondary antibodies in place with **post-staining fixation**.
- Remove residues with Tween 80 wash.

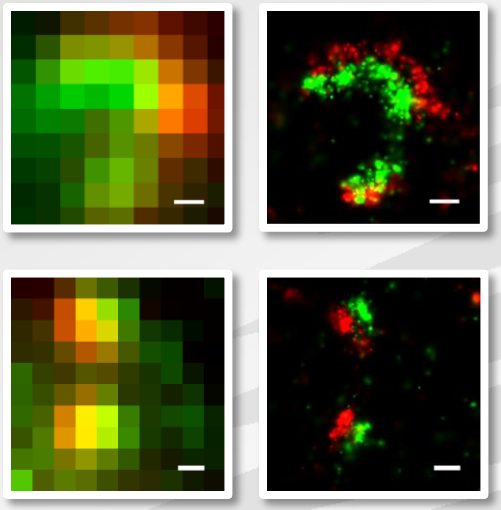
STORM and Tissue Sections

Superresolution Imaging of Chemical Synapses in the Brain

Adish Dani,[1,2,4,5] Bo Huang,[1,3,4,6] Joseph Bergan,[1,2] Catherine Dulac,[1,2,*] and Xiaowei Zhuang[1,3,*]
[1]Howard Hughes Medical Institute

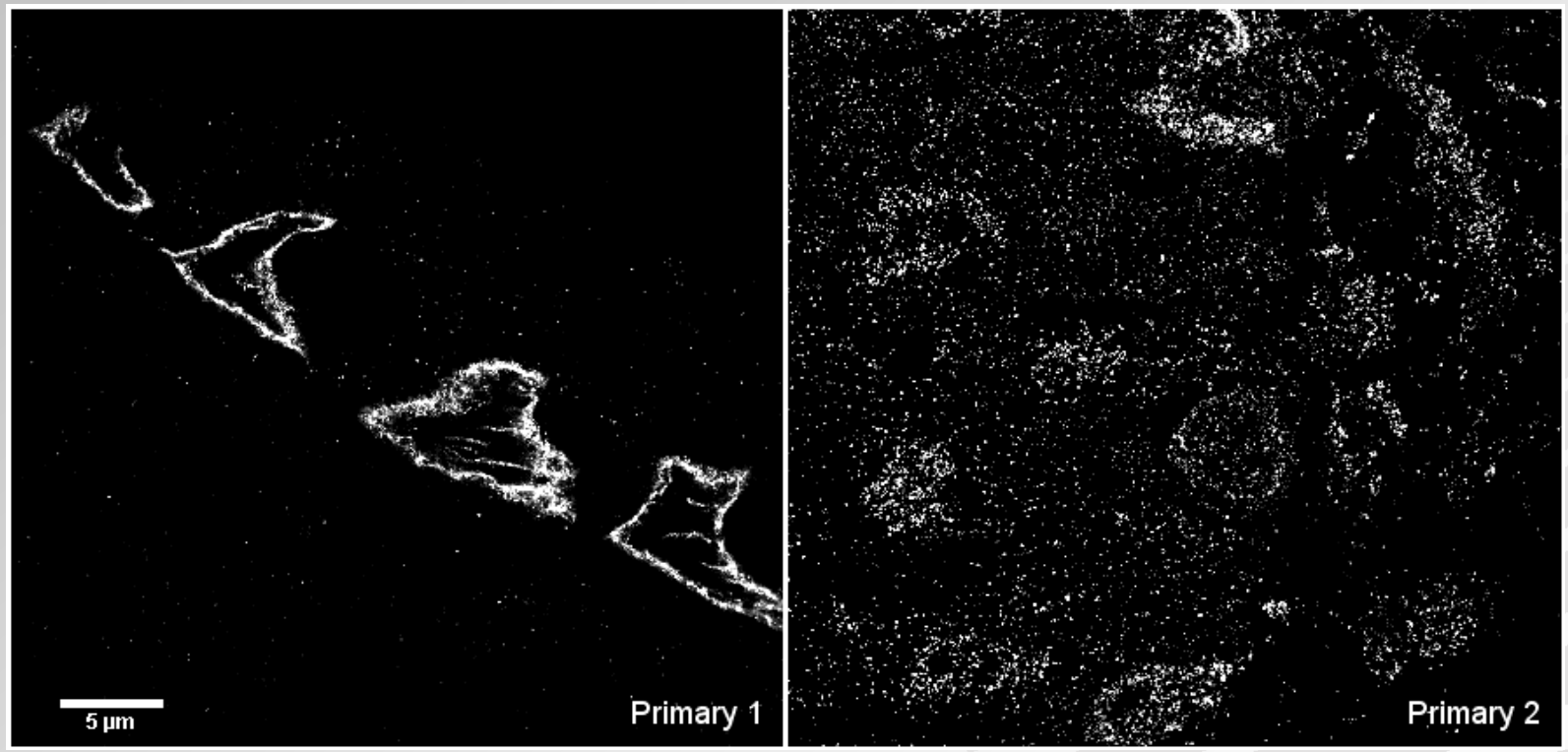
Immunohistochemistry

Two different types of mouse brain tissue sections, thick vibratome sections (~70 μm) and thin cryostat sections (~10 μm), were initially tested, yielding no appreciable difference in the observed synapse ultrastructure. For all experiments reported here, we used cryosections to provide a higher yield of sections and more consistent antibody penetration.



200 nm

N-STORM and Tissue Sections



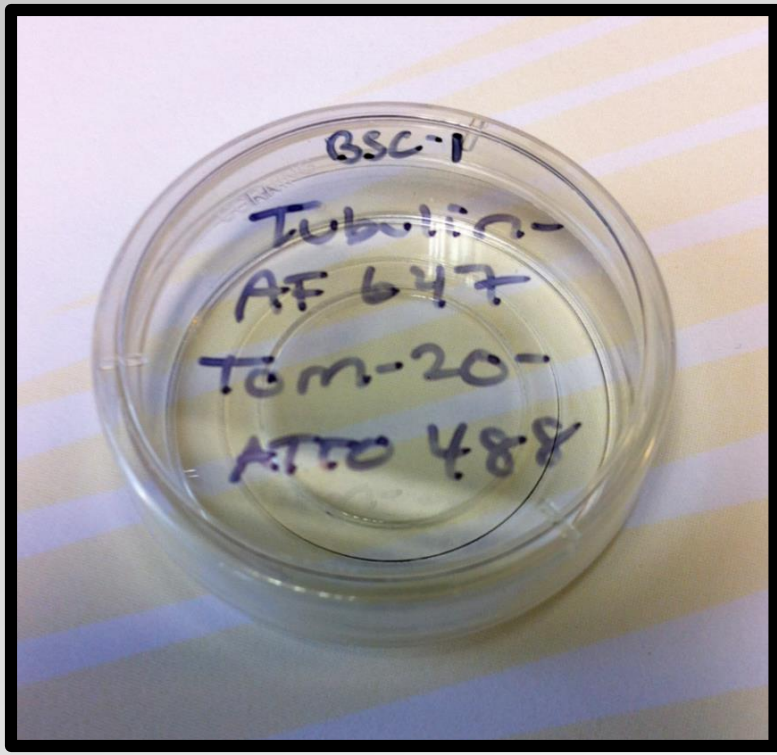
Keratin8 in 10 µm mouse skin sections

Subbing solution applied to coverslip to help sections stick

Handling of N-STORM Demo Sample

Samples prepared in the lab of Michael Davidson, Florida State University

For use with continuous activation of Alexa647 or multi-reporter imaging with Atto488 and Alexa647



- Store at room temperature (do not refrigerate)
- Protect from light and humidity
- Gently add imaging buffer
- Gently rinse 2X with distilled water, air dry, and reuse