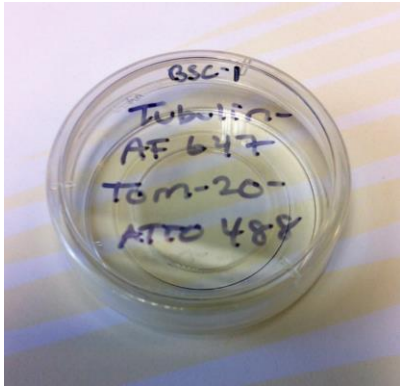
Cell Imaging Core
N-STORM
Sample Prep and Dye Tips

Determinants of Sample Quality

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



* Don't forget to use glass-bottom (#1.5) dishes to hold STORM imaging buffer!

Dyes That Work for N-STORM

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

STORM Sample Prep Summary

- Use glass-bottom (#1.5) dishes
- Label with Alexa 647 and Atto 488
- Block and wash samples thoroughly
- Use MEA-containing imaging buffer and keep it fresh
- *Optimization is usually necessary for the best results!*

1. Probe Choice

The most important considerations are….

- High photon number

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

- High Localization Density

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

- High Photostability

Longer imaging time

- Laser Power

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.

N-STORM Imaging Buffer Types

- The N-STORM protocol describes Method A and Method B
- These methods use different sources of thiol in the imaging buffer
  - MEA (Method A)
  - BME (Method B)
- MEA buffer lasts 1-2 hours during imaging
- BME buffer lasts 20-30 min during imaging
- Our demo buffer is almost always the MEA (Method A) buffer
- Adjust using the protocol recipe if you ever need to use BME

Buffer Components and Handling

- Imaging buffer is made of three components, which must be stored properly and mixed right before imaging

Buffer B:
Tris (to keep pH stable)
NaCl (to keep proteins folded properly)
Glucose (metabolite for glucose oxidase)

Store at room temperature for several weeks. Keeping it at room temp helps prevent drift

GLOX:
Glucose oxidase (to remove oxygen)
Catalase (to remove H2O2)

Store in a refrigerator to preserve activity. Good for about 2 weeks

MEA:
Cysteamine (MEA)
HCl (Solvent for MEA)

Store in a refrigerator to preserve activity. Good for up to a month. Has strong “rotten egg” smell when fresh

OR

BME (As an alternative to MEA):
BME directly from stock

Store in a refrigerator to preserve activity. Good for several months

Sample Dish Manufacturers

- MatTek
  - https://www.mattek.com/products/glass-bottom-dishes/
- Willco Wells
  - https://willcowells.com/
- Thermo
  - https://www.thermofisher.com/order/catalog/product/150680