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Supplementary information

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The DNA-PAINT palette: a comprehensive performance analysis of fluorescent dyes

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Supplementary Information

The DNA-PAINT palette: A comprehensive performance analysis of fluorescent dyes

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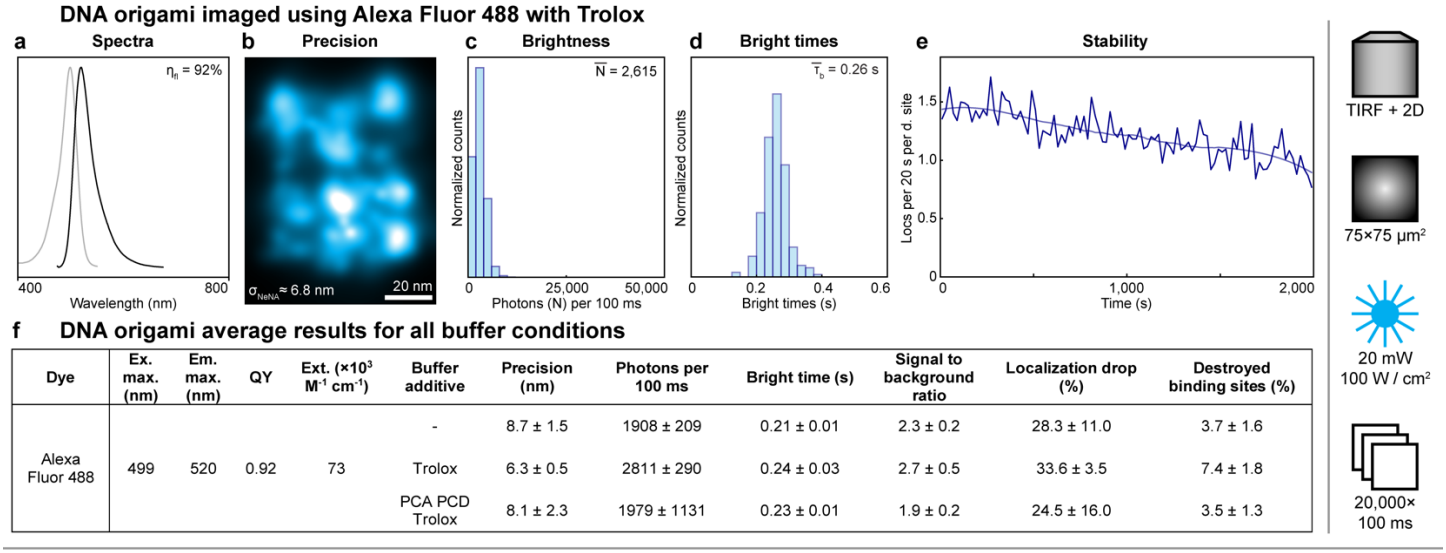
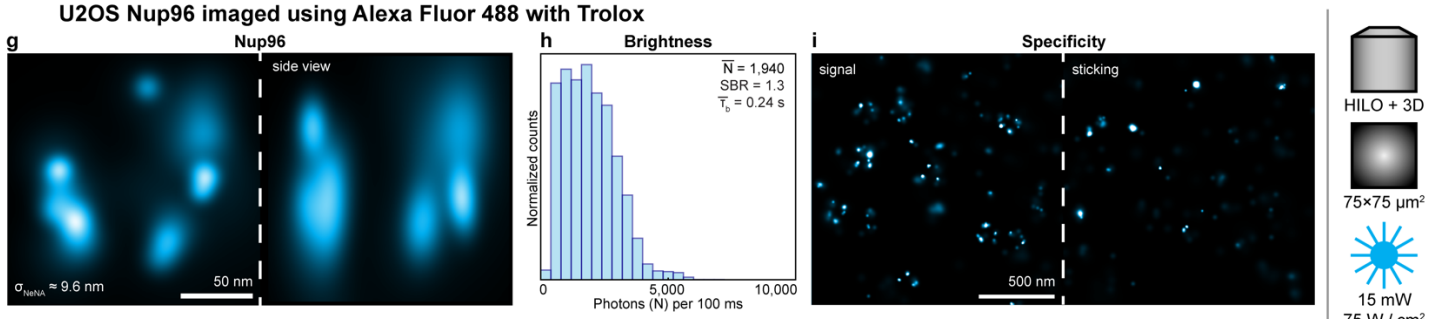
Supplementary Figs. 1-18 | Dye analysis. a, Absorption (grey) and emission (black) spectra with QY where available. **b,** Achievable precision illustrated by 20 nm DNA origami. **c,** Emission photon distribution and mean number of photons ($\overline{N}$) per 100 ms. **d,** Bright time distribution and mean bright time ($\overline{\tau_b}$). **e,** Dye stability expressed as localizations over time (per 20 seconds per docking site) over the course of the measurement. **f,** Mean DNA origami-based dye analysis results for all tested buffer conditions. Spectra, quantum yields (QY) where available and extinction coefficients from respective manufacturers. Uncertainties represent s.d. from 3 repeat measurements. **g,** Representative NPC (Nup96 labeled) illustrating achievable resolution in 3D, in cells. Left: top view, right: side view. **h,** Emission photon distribution. **i,** Comparison of representative nuclear (left) vs. cytoplasmic (right) localizations. **j,** Mean dye analysis results for cellular experiments. Uncertainties represent s.d. from 3 repeat measurements.



f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10³ M⁻¹ cm⁻¹)	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Alexa Fluor 488	499	520	0.92	73	-	8.7 ± 1.5	1908 ± 209	0.21 ± 0.01	2.3 ± 0.2	28.3 ± 11.0	3.7 ± 1.6
					Trolox	6.3 ± 0.5	2811 ± 290	0.24 ± 0.03	2.7 ± 0.5	33.6 ± 3.5	7.4 ± 1.8
					PCA PCD Trolox	8.1 ± 2.3	1979 ± 1131	0.23 ± 0.01	1.9 ± 0.2	24.5 ± 16.0	3.5 ± 1.3

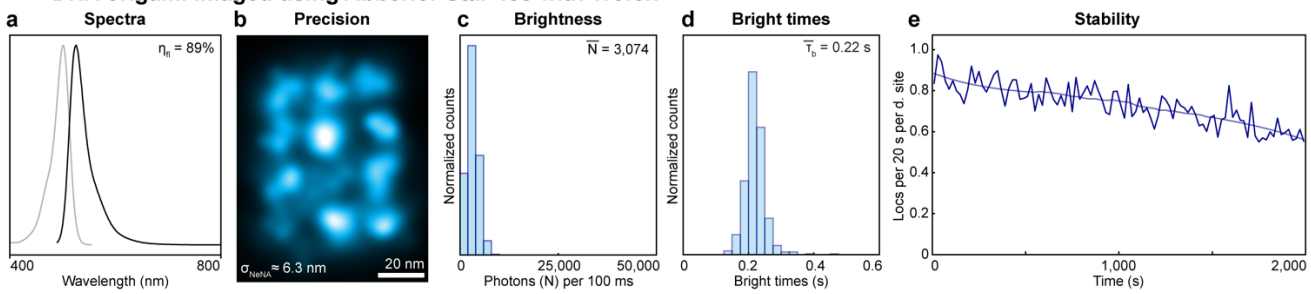


j U2OS Nup96 average results with Trolox

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Alexa Fluor 488	Trolox	9.3 ± 0.4	2191 ± 860	0.23 ± 0.01	1.3 ± 0.3	21.2 ± 7.0	0.42 ± 0.14

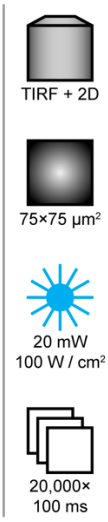
Supplementary Figure 1 | Dye analysis, Alexa Fluor 488

DNA origami imaged using Abberior Star 488 with Trolox

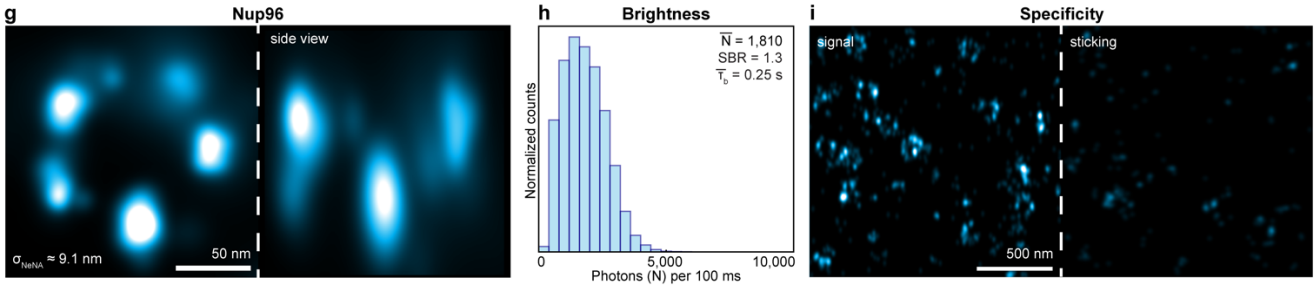


f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10^3 M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Abberior Star 488	503	524	0.89	65	-	7.8 ± 0.0	1874 ± 137	0.17 ± 0.00	2.0 ± 0.6	28.2 ± 19.1	8.7 ± 6.4
					Trolox	6.3 ± 0.6	2969 ± 365	0.21 ± 0.02	2.7 ± 0.7	27.6 ± 4.5	9.1 ± 2.7
					PCA PCD Trolox	8.9 ± 2.7	2317 ± 910	0.22 ± 0.00	1.9 ± 0.2	8.3 CI (0.0, 20.0)	3.2 ± 0.0



U2OS Nup96 imaged using Abberior Star 488 with Trolox

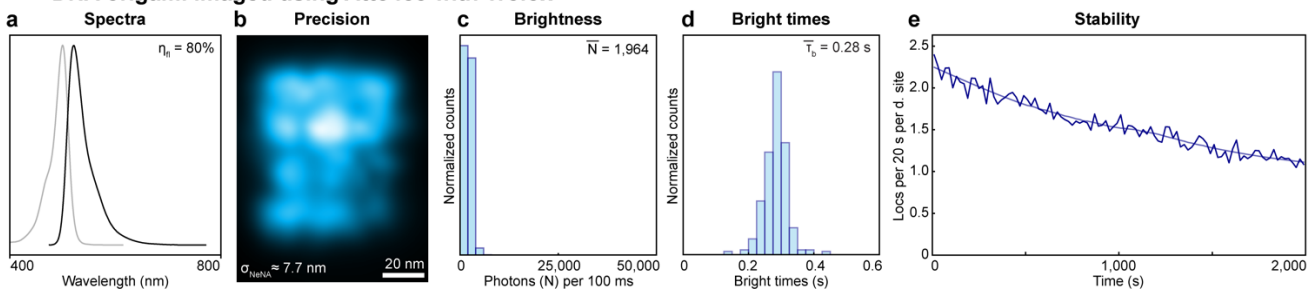


j U2OS Nup96 average results with Trolox

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Abberior Star 488	Trolox	11.5 ± 2.9	1532 ± 274	0.23 ± 0.03	1.0 ± 0.2	11.4 ± 10.5	0.55 ± 0.11

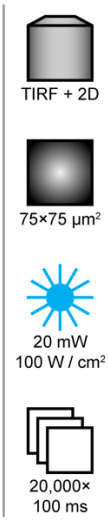
Supplementary Figure 2 | Dye analysis, Abberior Star 488

DNA origami imaged using Atto488 with Trolox

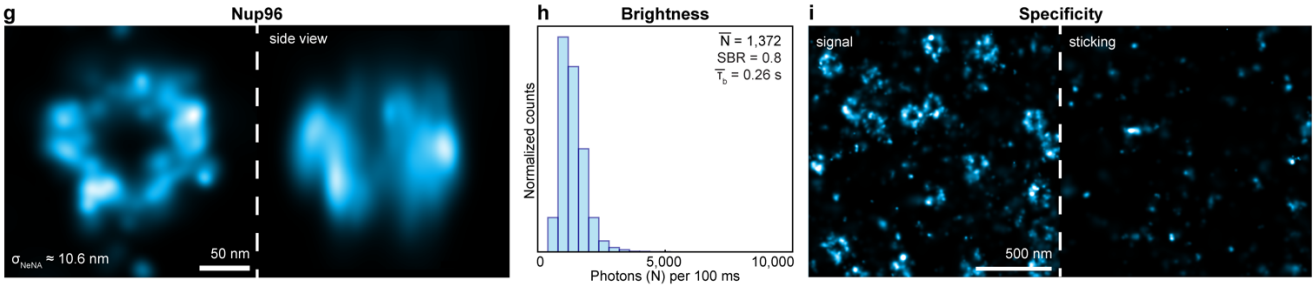


f **DNA origami average results for all buffer conditions**

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10^3 M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Atto488	500	520	0.8	90	-	8.7 ± 0.8	1849 ± 465	0.22 ± 0.01	1.4 ± 0.1	48.3 ± 8.6	19.3 ± 0.6
					Trolox	7.4 ± 0.3	2073 ± 249	0.27 ± 0.02	1.6 ± 0.2	44.3 ± 2.8	17.1 ± 3.6
					PCA PCD Trolox	8.4 ± 0.2	1804 ± 264	0.27 ± 0.00	1.4 ± 0.2	16.4 ± 8.3	9.2 ± 0.3



U2OS Nup96 imaged using Atto488 with Trolox

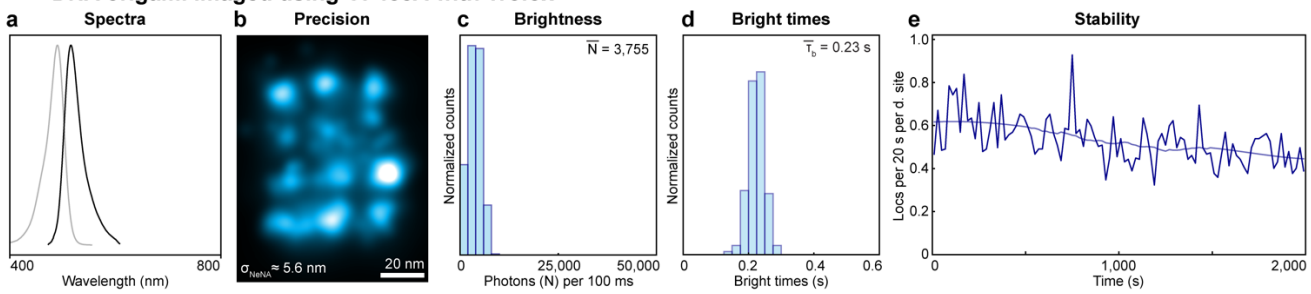


j **U2OS Nup96 average results with Trolox**

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Atto488	Trolox	11.4 ± 1.4	1388 ± 181	0.26 ± 0.00	0.8 ± 0.1	16.4 ± 3.5	0.71 ± 0.19

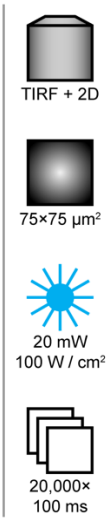
Supplementary Figure 3 | Dye analysis, Atto488

DNA origami imaged using CF488A with Trolox

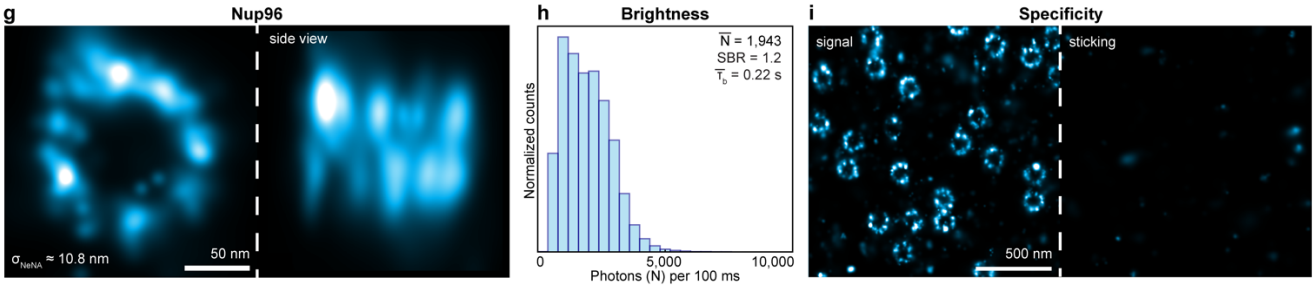


f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10³ M⁻¹ cm⁻¹)	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
CF488A	490	515	-	70	-	6.9 ± 0.8	2492 ± 578	0.16 ± 0.00	2.0 ± 0.2	32.0 ± 0.4	8.9 ± 2.8
					Trolox	5.4 ± 0.6	3879 ± 732	0.20 ± 0.03	3.2 ± 0.8	33.7 ± 10.4	8.3 ± 3.6
					PCA PCD Trolox	5.9 ± 0.8	3901 ± 412	0.22 ± 0.00	2.5 ± 0.0	11.2 ± 5.9	5.4 ± 2.1



U2OS Nup96 imaged using CF488A with Trolox

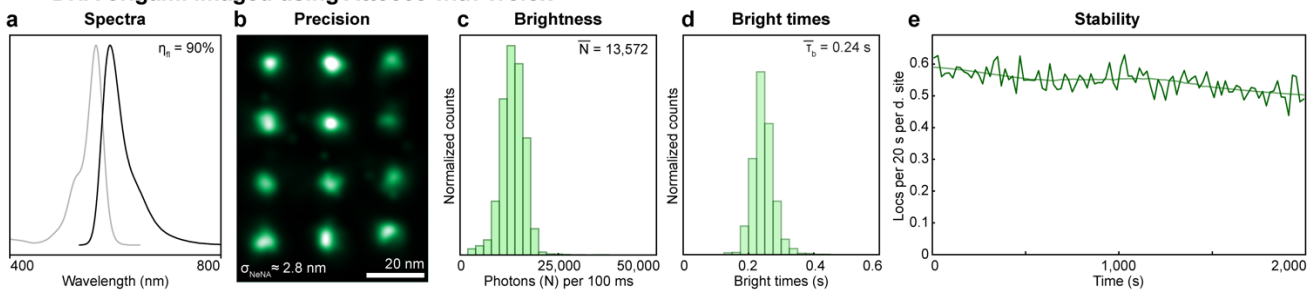


j U2OS Nup96 average results with Trolox

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
CF488A	Trolox	10.7 ± 0.1	2240 ± 305	0.21 ± 0.01	1.3 ± 0.1	15.5 ± 7.4	0.48 ± 0.07

Supplementary Figure 4 | Dye analysis, CF488A

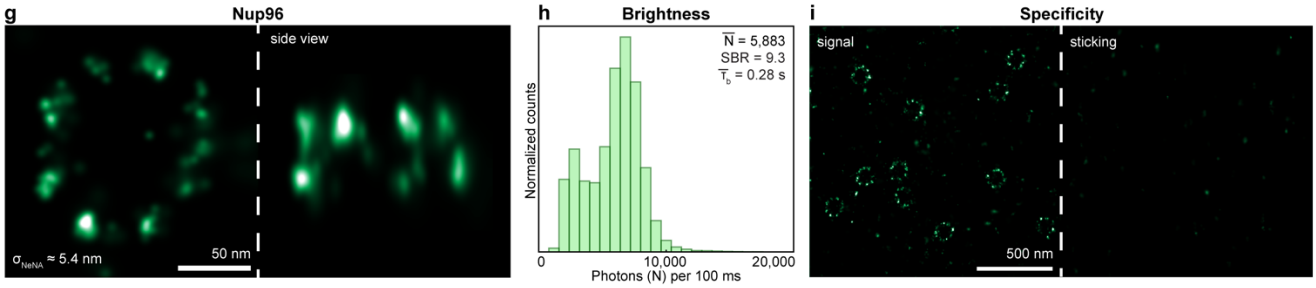
DNA origami imaged using Atto565 with Trolox



f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10^3 M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Atto565	564	590	0.9	120	-	3.2 ± 0.2	9804 ± 1907	0.24 ± 0.01	10.3 ± 0.3	18.8 ± 4.0	3.6 ± 1.6
					Trolox	2.9 ± 0.1	11600 ± 1896	0.24 ± 0.01	11.1 ± 2.8	16.4 ± 4.0	1.3 ± 0.5
					PCA PCD Trolox	3.3 ± 0.2	10295 ± 3494	0.23 ± 0.01	11.3 ± 0.3	12.1 ± 0.7	5.8 ± 4.5

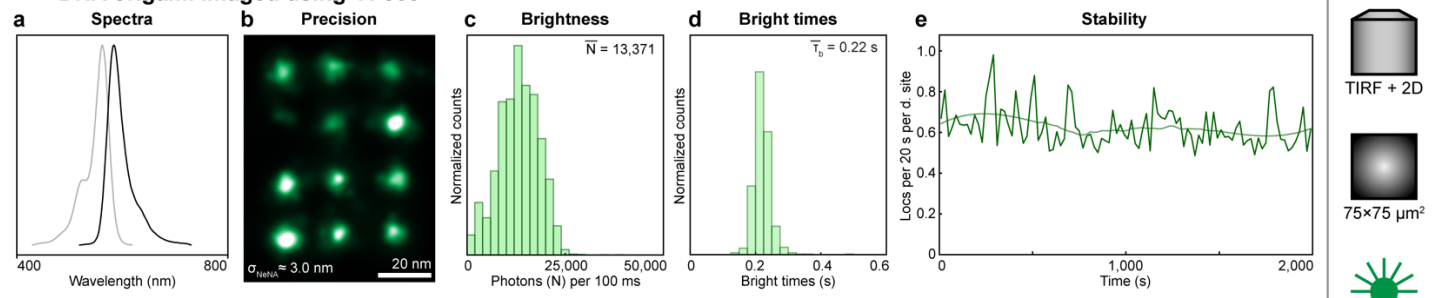
U2OS Nup96 imaged using Atto565 with Trolox



j U2OS Nup96 average results with Trolox

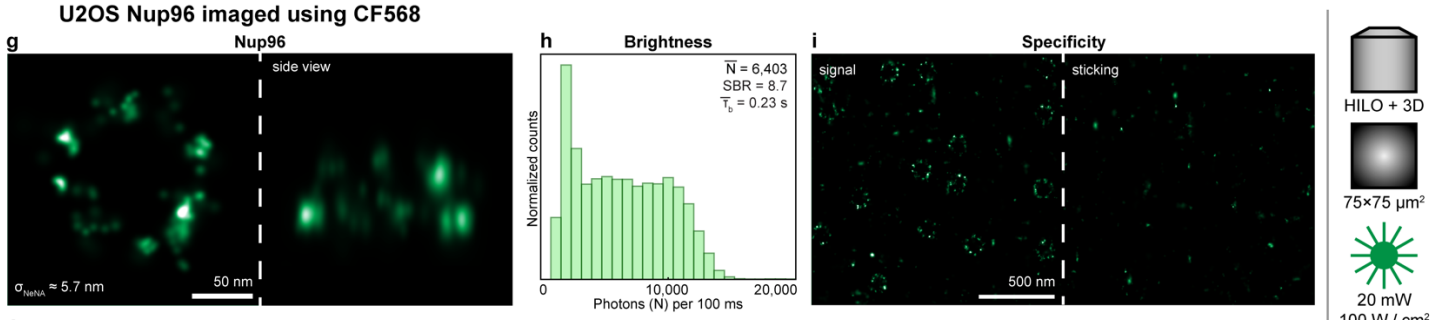
Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Atto565	Trolox	6.0 ± 0.6	6287 ± 917	0.28 ± 0.01	8.4 ± 0.9	4.2 CI (0.0, 9.3)	0.87 ± 0.39

Supplementary Figure 5 | Dye analysis, Atto565



f DNA origami average results for all buffer conditions

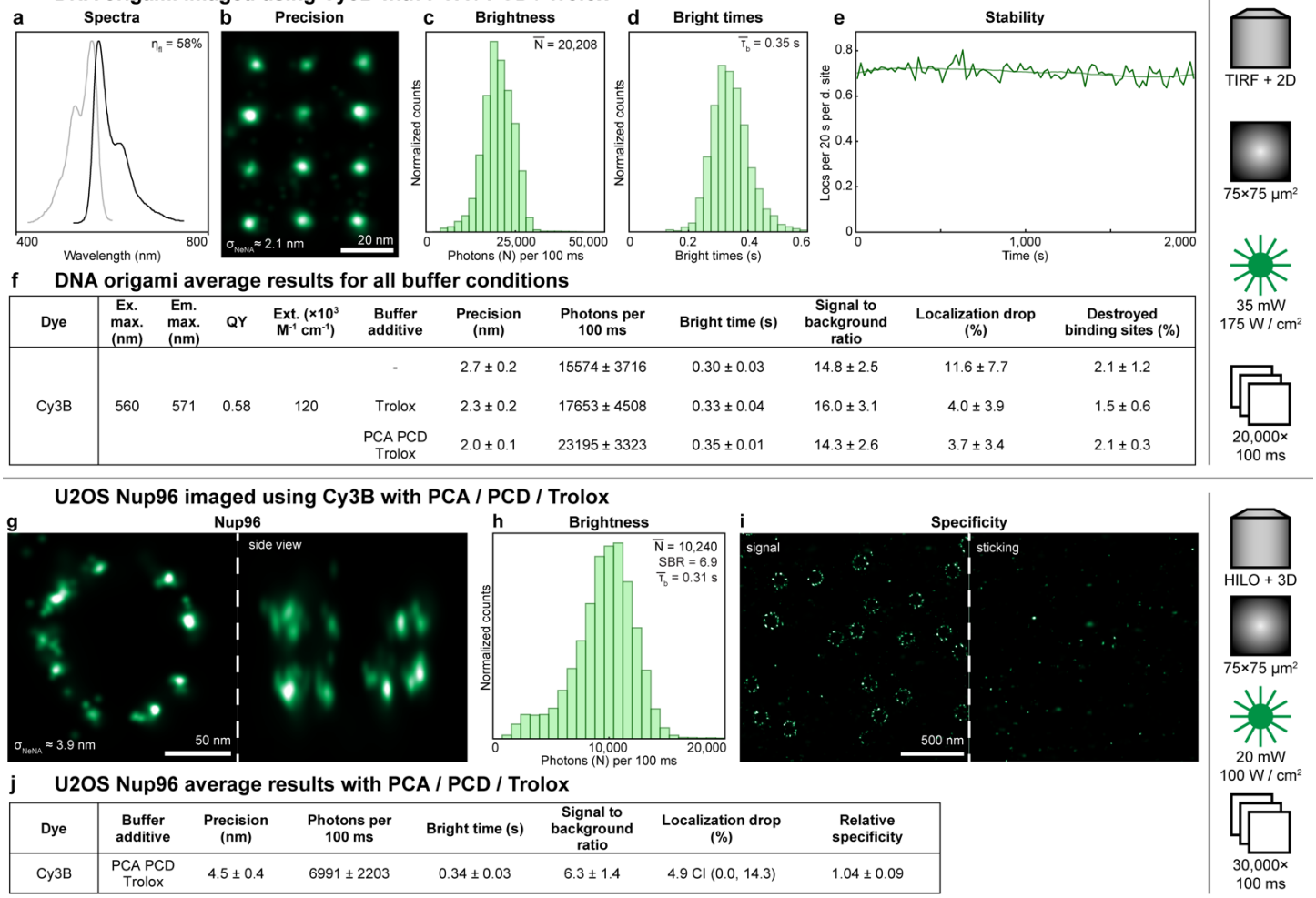
Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10³ M⁻¹ cm⁻¹)	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
CF568	562	583	-	100	-	3.0 ± 0.2	13388 ± 2033	0.21 ± 0.01	10.9 ± 2.5	11.5 ± 1.1	2.3 ± 0.2
					Trolox	3.1 ± 0.2	14729 ± 1465	0.21 ± 0.00	13.6 ± 0.7	6.3 CI (0.0, 10.0)	1.1 CI (0.0, 2.4)
					PCA PCD Trolox	3.8 ± 0.3	10456 ± 264	0.22 ± 0.01	10.8 ± 1.4	6.1 ± 1.8	1.4 ± 0.8



j U2OS Nup96 average results

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
CF568	-	6.0 ± 0.8	6332 ± 62	0.24 ± 0.02	7.1 ± 2.5	2.4 CI (0.0, 6.6)	0.51 ± 0.21

Supplementary Figure 6 | Dye analysis, CF568



f DNA origami average results for all buffer conditions

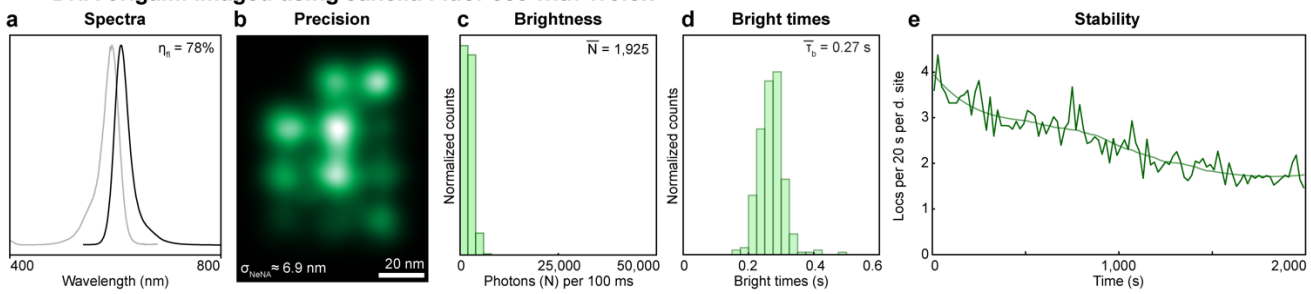
Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10³ M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Cy3B	560	571	0.58	120	-	2.7 ± 0.2	15574 ± 3716	0.30 ± 0.03	14.8 ± 2.5	11.6 ± 7.7	2.1 ± 1.2
					Trolox	2.3 ± 0.2	17653 ± 4508	0.33 ± 0.04	16.0 ± 3.1	4.0 ± 3.9	1.5 ± 0.6
					PCA PCD Trolox	2.0 ± 0.1	23195 ± 3323	0.35 ± 0.01	14.3 ± 2.6	3.7 ± 3.4	2.1 ± 0.3

j U2OS Nup96 average results with PCA / PCD / Trolox

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Cy3B	PCA PCD Trolox	4.5 ± 0.4	6991 ± 2203	0.34 ± 0.03	6.3 ± 1.4	4.9 CI (0.0, 14.3)	1.04 ± 0.09

Supplementary Figure 7 | Dye analysis, Cy3B

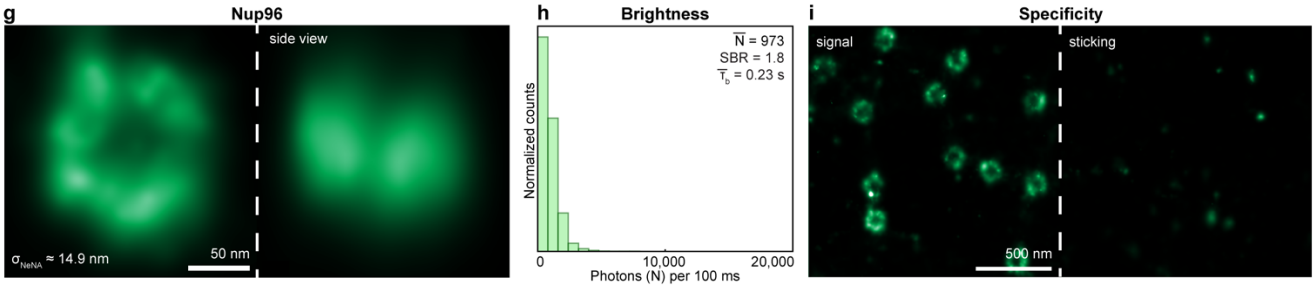
DNA origami imaged using Janelia Fluor 585 with Trolox



f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10^3 M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Janelia Fluor 585	585	609	0.78	156	-	7.6 ± 0.6	1791 ± 224	0.28 ± 0.01	3.5 ± 0.1	30.5 CI (0.0, 62.9)	20.2 CI (0.0, 45.3)
					Trolox	6.9 ± 1.1	2429 ± 1137	0.26 ± 0.02	3.6 ± 0.3	35.6 ± 28.7	17.7 ± 13.4
					PCA PCD Trolox	8.5 ± 0.6	1715 ± 214	0.26 ± 0.00	3.3 ± 0.0	10.2 CI (0.0, 24.5)	5.8 ± 4.5

U2OS Nup96 imaged using Janelia Fluor 585 with Trolox

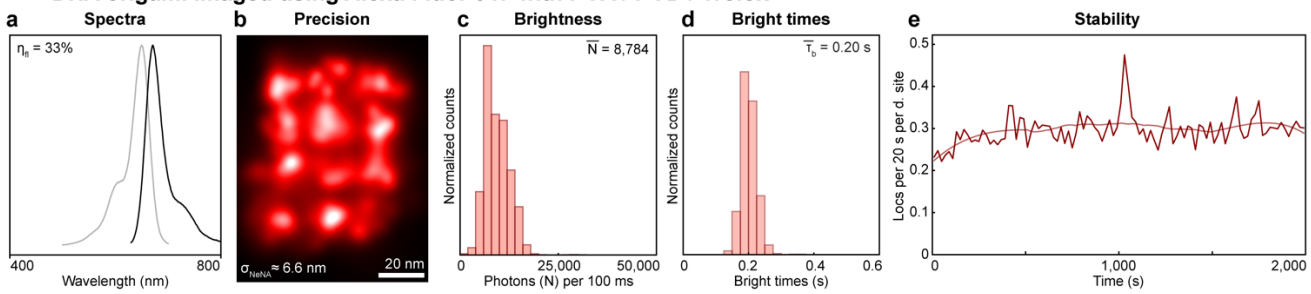


j U2OS Nup96 average results

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Janelia Fluor 585	Trolox	17.4 ± 2.5	741 ± 219	0.22 ± 0.01	1.0 ± 0.7	8.8 CI (0.0, 19.6)	1.30 ± 0.47

Supplementary Figure 8 | Dye analysis, Janelia Fluor 585

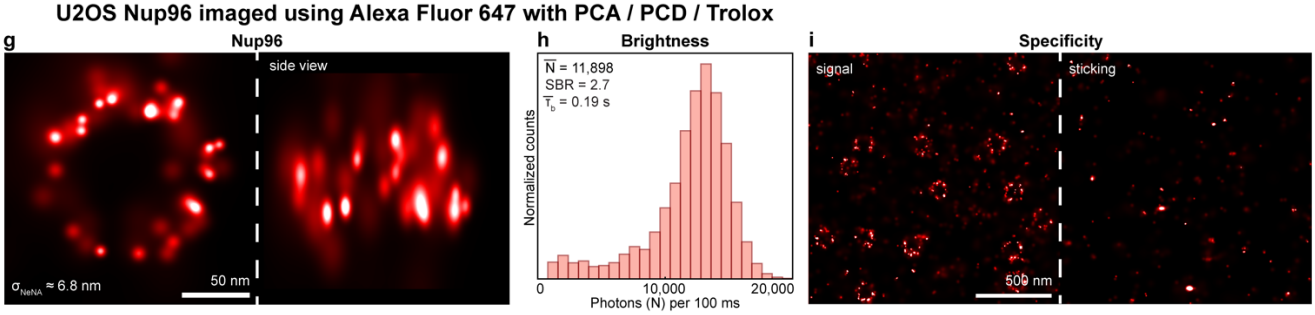
DNA origami imaged using Alexa Fluor 647 with PCA / PCD / Trolox



f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10^3 M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Alexa Fluor 647	650	671	0.33	270	-	10.0 ± 1.9	8928 ± 1015	0.18 ± 0.01	1.9 ± 0.3	43.6 ± 20.3	15.6 ± 9.8
					Trolox	9.5 ± 1.2	9293 ± 1622	0.18 ± 0.01	2.3 ± 0.0	41.6 ± 17.3	12.1 ± 8.5
					PCA PCD Trolox	6.3 ± 0.2	10348 ± 1358	0.19 ± 0.01	2.1 ± 0.2	1.4 CI (0.0, 2.9)	1.2 ± 0.1

U2OS Nup96 imaged using Alexa Fluor 647 with PCA / PCD / Trolox

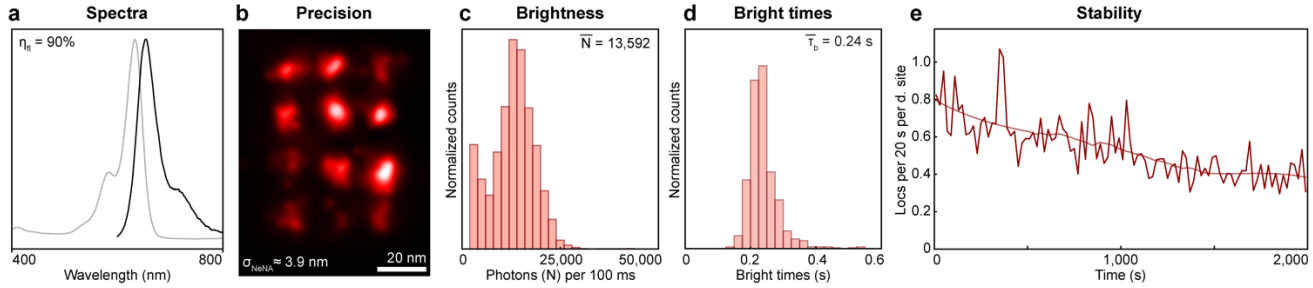


j U2OS Nup96 average results with PCA / PCD / Trolox

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Alexa Fluor 647	PCA PCD Trolox	8.9 ± 2.2	10213 ± 2592	0.19 ± 0.01	2.1 ± 0.9	51.3 ± 5.2	0.69 ± 0.41

Supplementary Figure 9 | Dye analysis, Alexa Fluor 647

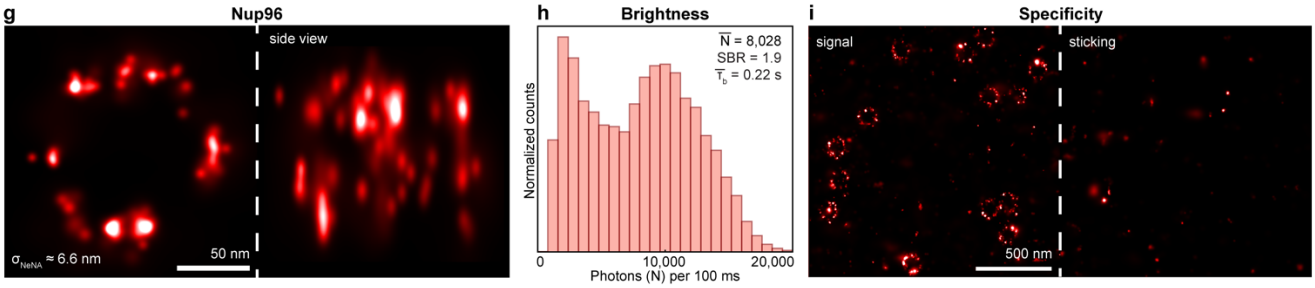
DNA origami imaged using Abberior Star 635p with Trolox



f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10^3 M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Abberior Star 635p	638	651	0.9	120	-	4.9 ± 0.5	11526 ± 825	0.24 ± 0.02	2.1 ± 0.1	45.6 ± 20.3	14.0 ± 6.9
					Trolox	4.5 ± 0.7	12731 ± 1037	0.23 ± 0.01	2.3 ± 0.3	53.3 ± 24.8	17.9 ± 11.6
					PCA PCD Trolox	7.0 ± 0.3	3813 ± 227	0.40 ± 0.00	0.9 ± 0.0	81.6 ± 2.3	68.6 ± 4.3

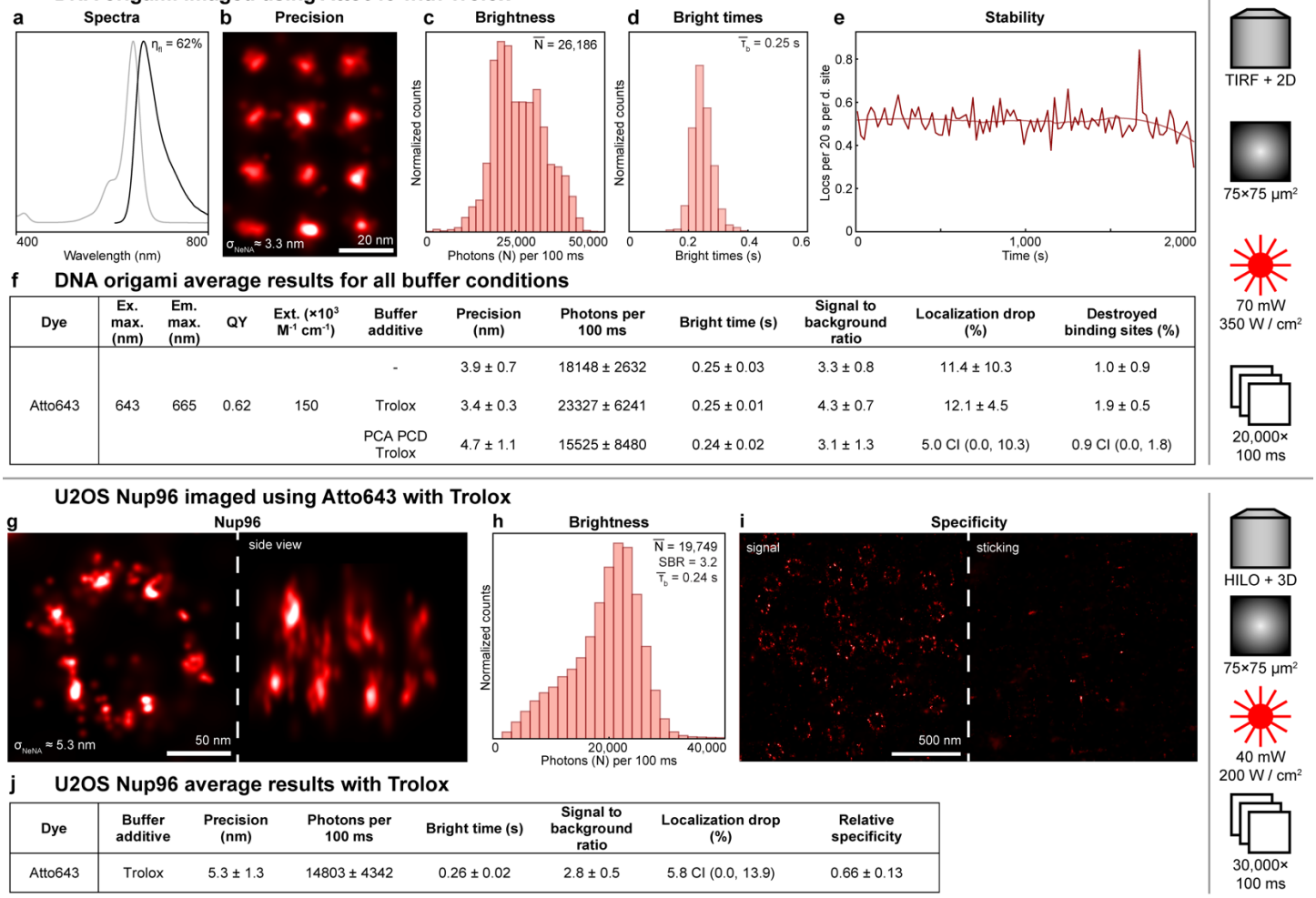
U2OS Nup96 imaged using Abberior Star 635p with Trolox



j U2OS Nup96 average results with Trolox

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Abberior Star 635p	Trolox	8.0 ± 1.3	9609 ± 2651	0.22 ± 0.01	1.7 ± 0.3	6.7 ± 6.1	0.37 ± 0.29

Supplementary Figure 10 | Dye analysis, Abberior Star 635p



f DNA origami average results for all buffer conditions

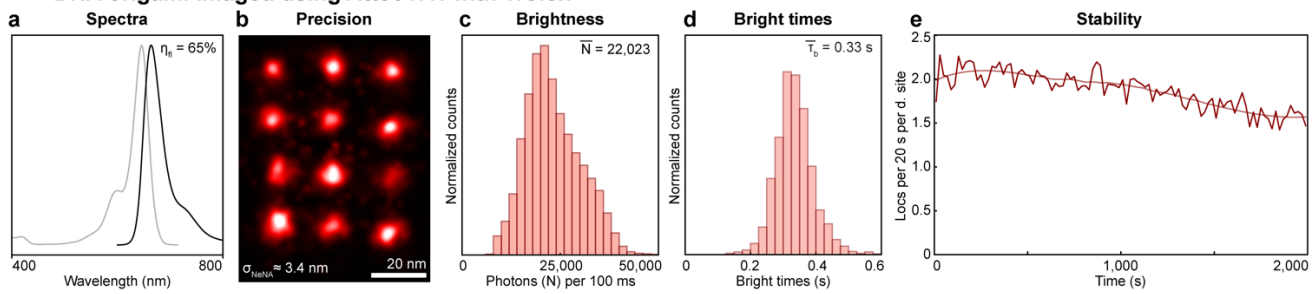
Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10³ M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Atto643	643	665	0.62	150	-	3.9 ± 0.7	18148 ± 2632	0.25 ± 0.03	3.3 ± 0.8	11.4 ± 10.3	1.0 ± 0.9
					Trolox	3.4 ± 0.3	23327 ± 6241	0.25 ± 0.01	4.3 ± 0.7	12.1 ± 4.5	1.9 ± 0.5
					PCA PCD Trolox	4.7 ± 1.1	15525 ± 8480	0.24 ± 0.02	3.1 ± 1.3	5.0 CI (0.0, 10.3)	0.9 CI (0.0, 1.8)

j U2OS Nup96 average results with Trolox

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Atto643	Trolox	5.3 ± 1.3	14803 ± 4342	0.26 ± 0.02	2.8 ± 0.5	5.8 CI (0.0, 13.9)	0.66 ± 0.13

Supplementary Figure 11 | Dye analysis, Atto643

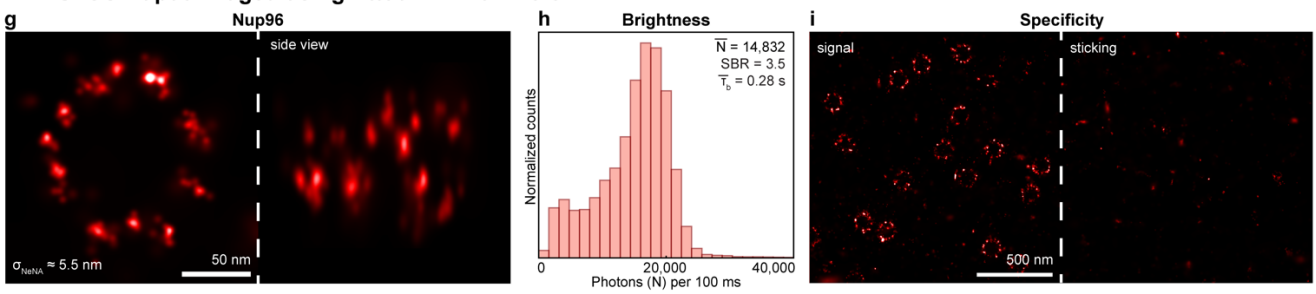
DNA origami imaged using Atto647N with Trolox



f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10^3 M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Atto647N	646	664	0.65	150	-	4.6 ± 0.4	13071 ± 2270	0.21 ± 0.01	2.9 ± 0.1	2.0 CI (0.0, 5.5)	7.0 ± 4.0
					Trolox	3.4 ± 0.1	18448 ± 3523	0.32 ± 0.01	4.0 ± 0.1	18.7 ± 9.2	4.0 ± 1.9
					PCA PCD Trolox	4.5 ± 0.5	14699 ± 5124	0.29 ± 0.04	2.9 ± 0.6	1.6 CI (0.0, 4.4)	3.2 ± 1.1

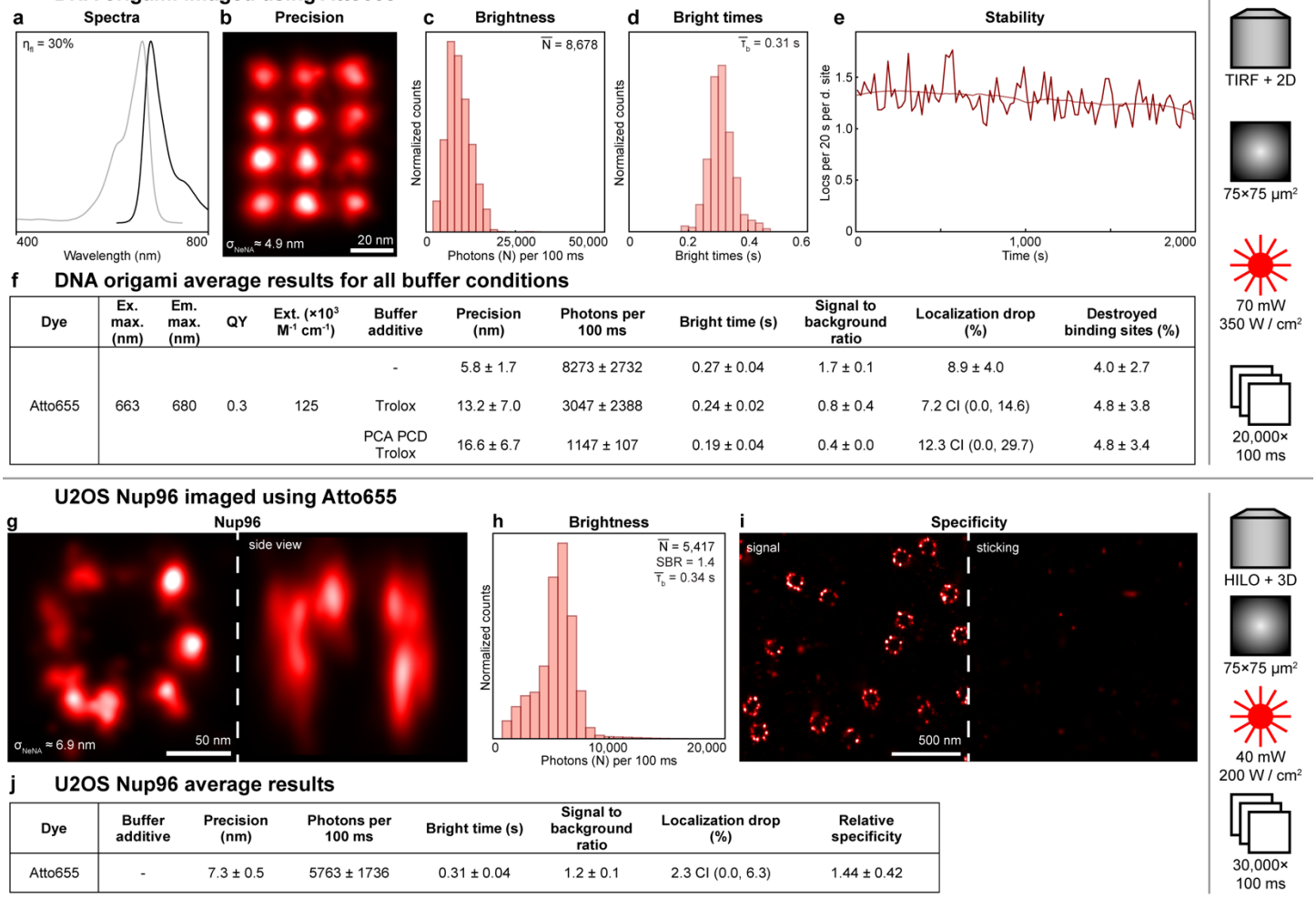
U2OS Nup96 imaged using Atto647N with Trolox



j U2OS Nup96 average results with Trolox

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Atto647N	Trolox	6.1 ± 0.8	15396 ± 1254	0.27 ± 0.01	3.2 ± 0.3	4.8 ± 2.7	0.38 ± 0.03

Supplementary Figure 12 | Dye analysis, Atto647N



f DNA origami average results for all buffer conditions

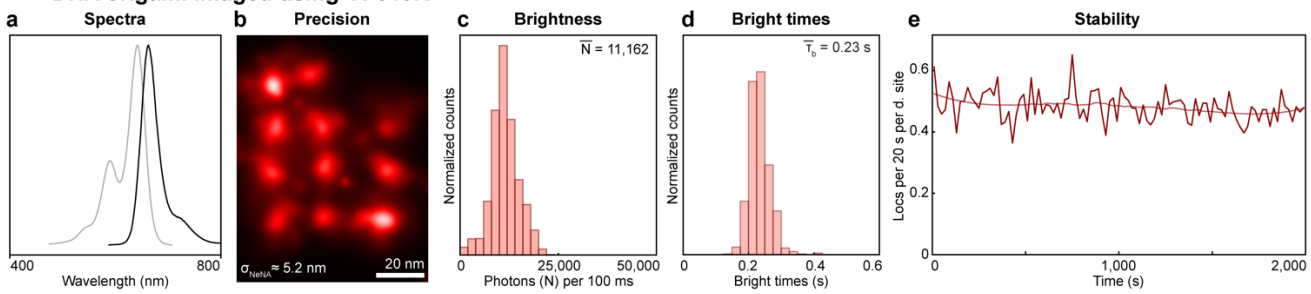
Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10³ M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Atto655	663	680	0.3	125	-	5.8 ± 1.7	8273 ± 2732	0.27 ± 0.04	1.7 ± 0.1	8.9 ± 4.0	4.0 ± 2.7
					Trolox	13.2 ± 7.0	3047 ± 2388	0.24 ± 0.02	0.8 ± 0.4	7.2 CI (0.0, 14.6)	4.8 ± 3.8
					PCA PCD Trolox	16.6 ± 6.7	1147 ± 107	0.19 ± 0.04	0.4 ± 0.0	12.3 CI (0.0, 29.7)	4.8 ± 3.4

j U2OS Nup96 average results

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Atto655	-	7.3 ± 0.5	5763 ± 1736	0.31 ± 0.04	1.2 ± 0.1	2.3 CI (0.0, 6.3)	1.44 ± 0.42

Supplementary Figure 13 | Dye analysis, Atto655

DNA origami imaged using CF640R



TIRF + 2D

75×75 µm²

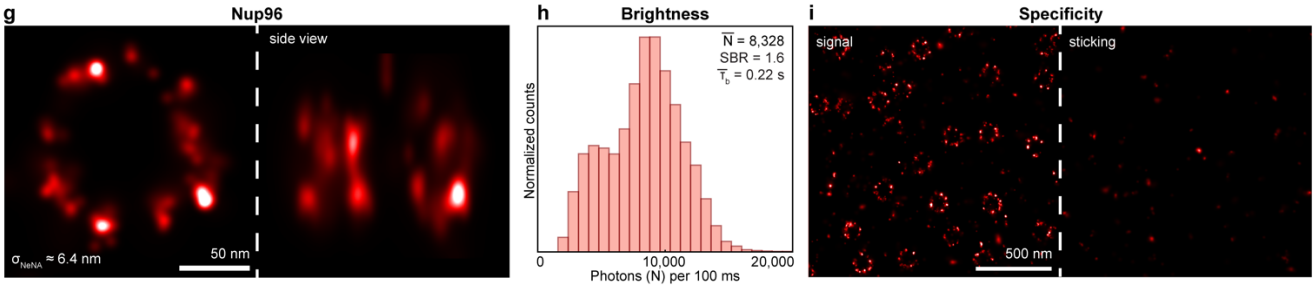
70 mW
350 W / cm²

20,000×
100 ms

f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10³ M⁻¹ cm⁻¹)	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
CF640R	642	662	-	105	-	5.5 ± 1.0	12024 ± 3240	0.22 ± 0.01	2.2 ± 0.2	7.5 ± 2.5	2.7 ± 1.2
					Trolox	6.8 ± 0.9	7951 ± 1883	0.22 ± 0.01	1.8 ± 0.1	21.8 ± 0.8	3.3 ± 1.3
					PCA PCD Trolox	10.4 ± 0.1	4176 ± 858	0.18 ± 0.01	1.1 ± 0.4	9.9 ± 0.5	3.7 ± 1.1

U2OS Nup96 imaged using CF640R



HILO + 3D

75×75 µm²

40 mW
200 W / cm²

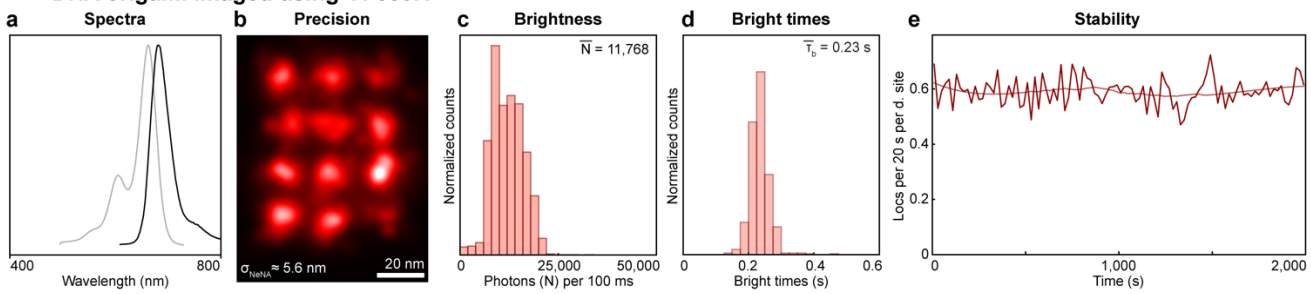
30,000×
100 ms

j U2OS Nup96 average results

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
CF640R	-	7.3 ± 0.8	7539 ± 2114	0.21 ± 0.01	1.3 ± 0.3	5.7 ± 5.0	1.15 ± 0.39

Supplementary Figure 14 | Dye analysis, CF640R

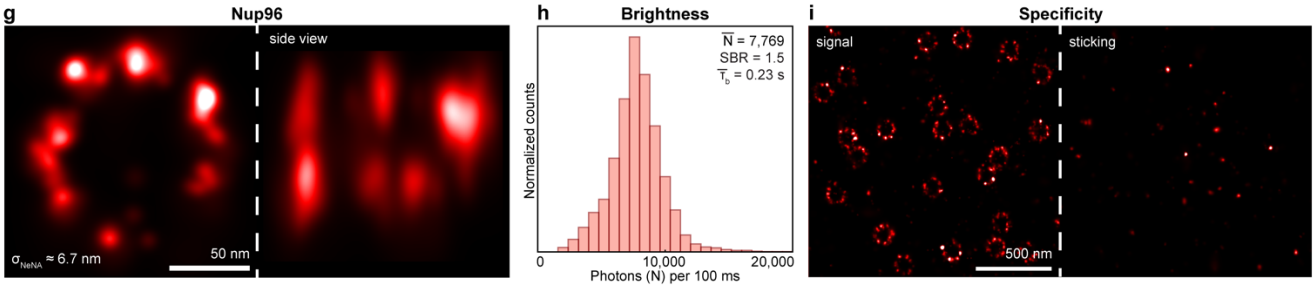
DNA origami imaged using CF660R



f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10³ M⁻¹ cm⁻¹)	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
CF660R	663	682	-	100	-	6.6 ± 0.9	10399 ± 1309	0.21 ± 0.02	2.3 ± 0.3	4.2 ± 3.8	2.8 ± 0.8
					Trolox	6.7 ± 0.8	8894 ± 1493	0.21 ± 0.02	2.2 ± 0.0	11.3 ± 2.5	1.8 ± 0.5
					PCA PCD Trolox	9.4 ± 1.3	6200 ± 468	0.18 ± 0.01	1.6 ± 0.5	7.8 ± 6.2	2.8 ± 1.3

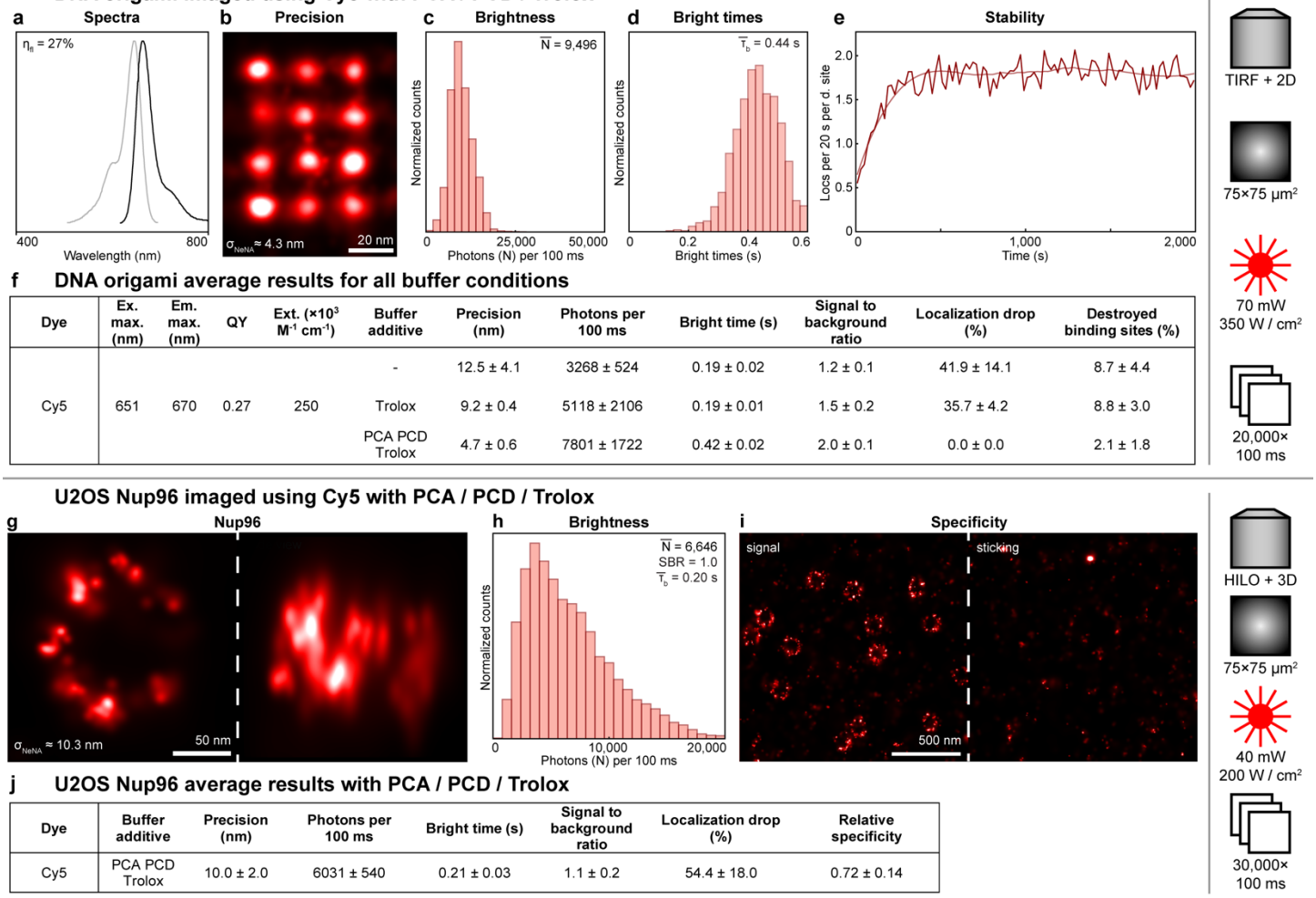
U2OS Nup96 imaged using CF660R



j U2OS Nup96 average results

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
CF660R	-	6.8 ± 0.1	7219 ± 583	0.24 ± 0.01	1.6 ± 0.1	1.8 ± 1.7	0.83 ± 0.20

Supplementary Figure 15 | Dye analysis, CF660R



f DNA origami average results for all buffer conditions

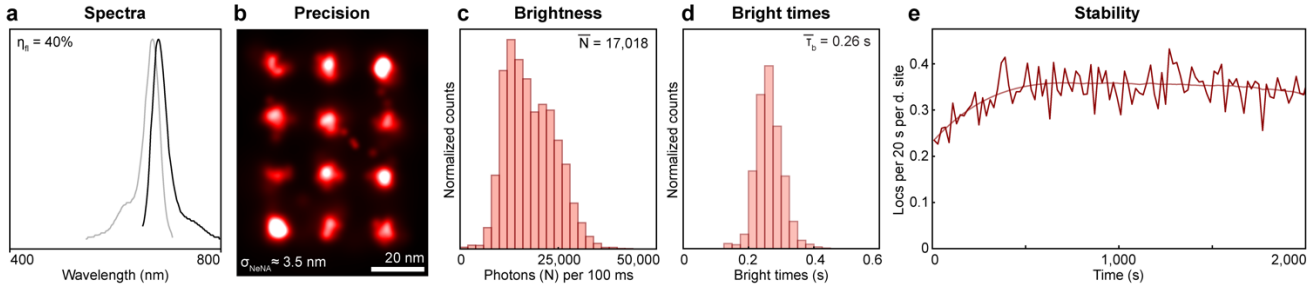
Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10³ M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Cy5	651	670	0.27	250	-	12.5 ± 4.1	3268 ± 524	0.19 ± 0.02	1.2 ± 0.1	41.9 ± 14.1	8.7 ± 4.4
					Trolox	9.2 ± 0.4	5118 ± 2106	0.19 ± 0.01	1.5 ± 0.2	35.7 ± 4.2	8.8 ± 3.0
					PCA PCD Trolox	4.7 ± 0.6	7801 ± 1722	0.42 ± 0.02	2.0 ± 0.1	0.0 ± 0.0	2.1 ± 1.8

j U2OS Nup96 average results with PCA / PCD / Trolox

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Cy5	PCA PCD Trolox	10.0 ± 2.0	6031 ± 540	0.21 ± 0.03	1.1 ± 0.2	54.4 ± 18.0	0.72 ± 0.14

Supplementary Figure 16 | Dye analysis, Cy5

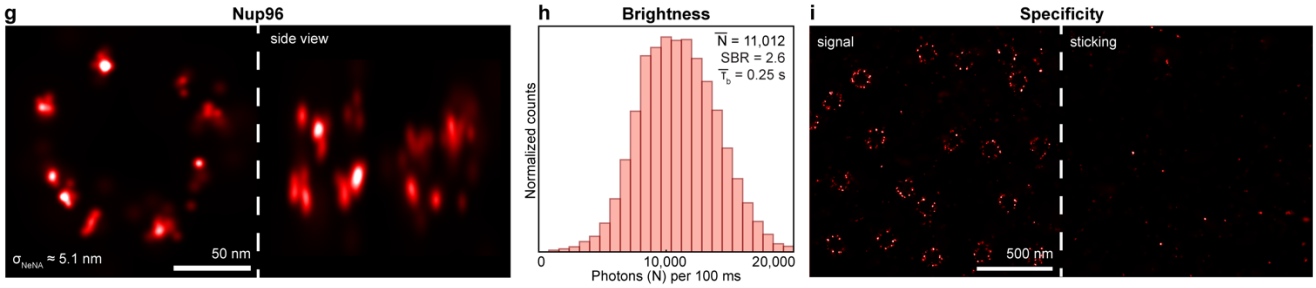
DNA origami imaged using Cy5B with PCA / PCD / Trolox



f **DNA origami average results for all buffer conditions**

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10^3 M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Cy5B	671	682	0.4	241	-	4.9 ± 0.1	16123 ± 2066	0.19 ± 0.01	3.1 ± 0.7	17.1 ± 0.6	3.3 ± 0.6
					Trolox	4.1 ± 0.4	20804 ± 4255	0.21 ± 0.02	3.7 ± 0.9	11.5 ± 6.9	2.8 ± 0.5
					PCA PCD Trolox	4.0 ± 0.4	17090 ± 2765	0.25 ± 0.02	2.9 ± 0.5	0.3 CI (0.0,0.8)	2.1 ± 0.5

U2OS Nup96 imaged using Cy5B with PCA / PCD / Trolox

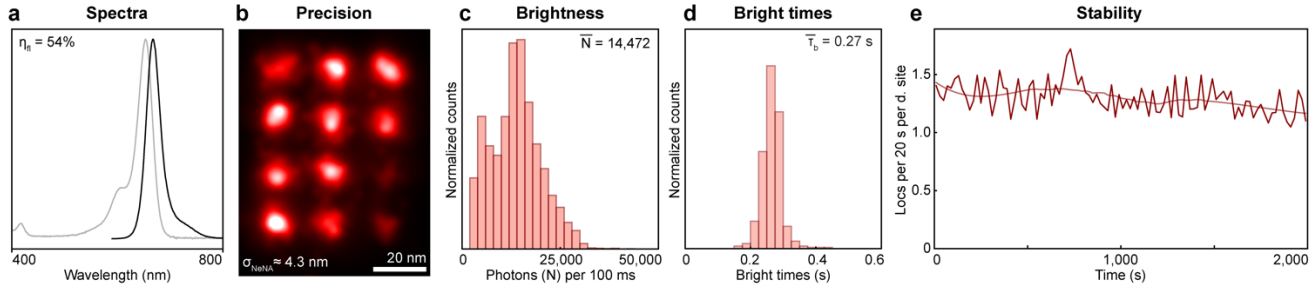


j **U2OS Nup96 average results with PCA / PCD / Trolox**

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Cy5B	PCA PCD Trolox	5.6 ± 0.5	9483 ± 2068	0.25 ± 0.01	2.2 ± 0.6	20.9 ± 18.4	0.77 ± 0.16

Supplementary Figure 17 | Dye analysis, Cy5B

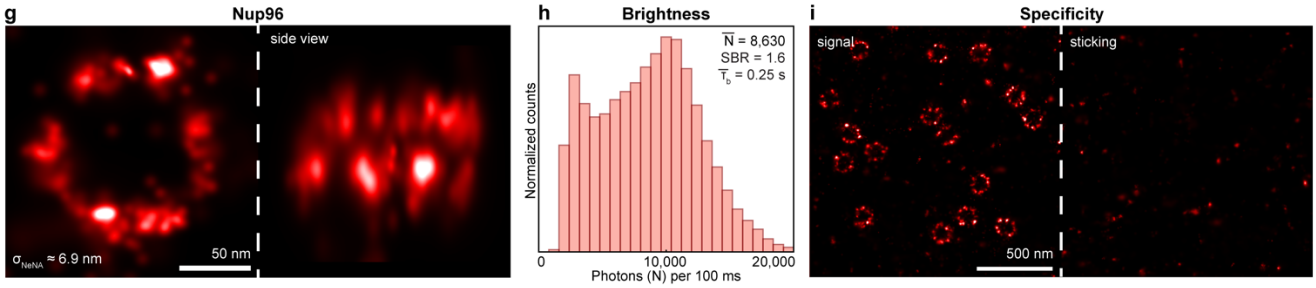
DNA origami imaged using Janelia Fluor 646



f DNA origami average results for all buffer conditions

Dye	Ex. max. (nm)	Em. max. (nm)	QY	Ext. (×10^3 M^{-1} cm^{-1})	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Destroyed binding sites (%)
Janelia Fluor 646	646	664	0.54	152	-	4.3 ± 0.1	14440 ± 3596	0.25 ± 0.03	2.6 ± 0.4	23.3 ± 9.1	5.6 ± 2.6
					Trolox	5.8 ± 2.0	12660 ± 4664	0.25 ± 0.04	2.6 ± 0.4	14.4 CI (0.0, 29.0)	2.5 CI (0.0, 6.0)
					PCA PCD Trolox	5.6 ± 0.2	10743 ± 1597	0.25 ± 0.01	2.4 ± 0.7	1.6 CI (0.0, 3.9)	4.0 ± 0.1

U2OS Nup96 imaged using Janelia Fluor 646



j U2OS Nup96 average results

Dye	Buffer additive	Precision (nm)	Photons per 100 ms	Bright time (s)	Signal to background ratio	Localization drop (%)	Relative specificity
Janelia Fluor 646	-	9.3 ± 3.0	8221 ± 1152	0.26 ± 0.01	1.7 ± 0.1	7.0 ± 6.8	0.43 ± 0.06

Supplementary Figure 18 | Dye analysis, Janelia Fluor 646

Supplementary Table 1 | Imaging parameters

Target & imaging round	Laser [nm]	Laser power at objective [mW]	Laser power density [W/cm^2]	Imager	Imager conc. [pM]	Buffer and additive	Number of frames	Imaging time [min]
DNA origami, barcode & reference	560	35	175	R1-Cy3B R3-Cy3B	500 500	B with PCA, PCD, Trolox	10,000	16.7
DNA origami, blue dyes	488	20	100	R1-Dye of interest	500	B, all conditions	20,000	33.3
DNA origami, green dyes	560	35	175	R1-Dye of interest	500	B, all conditions	20,000	33.3
DNA origami, red dyes	640	70	350	R1-Dye of interest	300 (Cy5, Atto643, Atto647N), 500 (rest)	B, all conditions	20,000	33.3
DNA origami, var. powers, CF488	488	10, 20, 40, 60, 80, 100	50, 100, 200, 300, 400, 500	R1-CF488A	500	B with Trolox	20,000	33.3
DNA origami, var. powers, Cy3B	560	35, 50, 100, 150	175, 250, 500, 750	R1-Cy3B	500	B with PCA, PCD, Trolox	20,000	33.3
DNA origami, var. powers, Atto643	640	35, 70, 100, 150, 200	175, 350, 500, 750, 1000	R1-Atto643	500	B with Trolox	20,000	33.3
U2OS NPC, reference	560	20	100	R1-Cy3B	80	C with PCA, PCD, Trolox	30,000	50
U2OS NPC, blue dyes	488	15	75	R1-Dye of interest	80 - 120	C with optimal additive	30,000	50
U2OS NPC, green dyes	560	20	100	R1-Dye of interest	80 - 100	C with optimal additive	30,000	50
U2OS NPC, red dyes	640	40	200	R1-Dye of interest	40 - 80 (Atto643), 20 - 50 (Atto647N), 80 -100 (rest)	C with optimal additive	30,000	50
U2OS Tom20, reference	560	20	100	R1-Cy3B	100	C with PCA, PCD, Trolox	30,000	50
U2OS Tom20, blue dyes	488	15	75	R1-Dye of interest	100	C with optimal additive	30,000	50
U2OS Tom20, green dyes	560	20	100	R1-Dye of interest	100	C with optimal additive	30,000	50
U2OS Tom20, red dyes	640	40	200	R1-Dye of interest	100	C with optimal additive	30,000	50
Neurons, round 1	488 560 640	15 20 40	75 100 200	R1-CF488A R4-Cy3B R6-Atto643	200 150 350	C with Trolox	30,000	50
Neurons, round 2	488 560 640	15 20 40	75 100 200	R5-CF488A R3-Cy3B R2-Atto643	150 300 100	C with Trolox	30,000	50

Imaging conditions for all measurements. The exposure time was set to 100 ms for all measurements. DNA origami "dye of interest" experiments were carried out with only buffer B, buffer B with Trolox, and buffer B with PCA, PCD, and Trolox for all dyes. U2OS NPC and Tom20 "dye of interest" experiments were carried out with the optimal buffer additive for each dye, specified in **Supplementary Figures 1-18**.

Supplementary Table 2 | Sequences

Imager name	Imager Sequence	Docking strand sequence
R1	AGGAGGA	TCCTCCTCCTCCTCCTCCT
R2	TGGTGGT	ACCACCACCACCACCACCA
R3	GAGAGAG	CTCTCTCTCTCTCTCTCTC
R4	TGTGTGT	ACACACACACACACACACA
R5	GAAGAAG	CTTCTTCTTCTTCTTCTTC
R6	TTGTTGTT	AACAACAACAACAACAACAA

Supplementary Table 3 | Antibodies

Antibody	Vendor	Information	Dilution
Mouse monoclonal (SAP7F407) anti-Bassoon	Enzo	Cat# ADI-VAM-PS003-F; RRID:AB_11181058	1 in 200
Rabbit polyclonal anti-VGAT	invitrogen	Cat# PA5-27569; RRID:AB_2545045	1 in 300
Rabbit monoclonal (EPR15581-54) anti-Tom20	Abcam	Cat# ab186735; RRID:AB_2889972	1 in 200
Mouse monoclonal (69H10) anti-Neurofilament L	Synaptic Systems	Cat# 171011; RRID:AB_2891275	1 in 200
Mouse monoclonal (42/B) anti-βII Spectrin	BD Biosciences	Cat# 612562; RRID:AB_399853	1 in 100
sdAb anti-PSD95 (1B2)	NanoTag Biotechnologies	Cat# N3705	1 in 200
sdAb anti-GFP (1H1)	NanoTag Biotechnologies	Cat# N0305	1 in 200
sdAb anti-Mouse IgG (10A4)	NanoTag Biotechnologies	Cat# N2005	1 in 300
sdAb anti-Rabbit IgG (10E10)	NanoTag Biotechnologies	Cat# N2405	1 in 300
Multiplexing Blocker Mouse	NanoTag Biotechnologies	Cat# K0102-50	1 in 200
Multiplexing Blocker Mouse	NanoTag Biotechnologies	Cat# K0202-50	1 in 200

Supplementary Table 4 | Cross-talk in 3-channel imaging

Dye added	Atto643	Cy3b	CF488
Number of locs detected in red channel	1145912	894	1720
Number of locs detected in green channel	1497	1798626	6253
Number of locs detected in blue channel	4818	14587	993884
Total number of locs	1152227	1814107	1001857
Percent of locs detected in red channel	99.45	0.05	0.17
Percent of locs detected in green channel	0.13	99.15	0.62
Percent of locs detected in blue channel	0.42	0.80	99.20
Ratio red / correct	1.000000	0.000497	0.001731
Ratio green / correct	0.001306	1.000000	0.006291
Ratio blue / correct	0.004205	0.008110	1.000000

Supplementary Table 5 | DNA origami scaffold sequence

TTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGA
TTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTG
ATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAA
CACTCAACCCTATCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGAACCACCATCAAACAGGAT
TTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAAT
CAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCG
CGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCA
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TTAACGTTTACAATTTAAATATTTGCTTATACAATCTTCCTGTTTTTGGGGCTTTTCTGATTATCAACCGGGGTACA
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TGATTTTTGATTTATGGTCATTCTCGTTTTCTGAACTGTTTAAAGCATTTGAGGGGGATTCAATGAATATTTATGA
CGATTCCGCAGTATTGGACGCTATCCAGTCTAAACATTTTACTATTACCCCCTCTGGCAAAACTTCTTTTGCAAAA
GCCTCTCGCTATTTTGGTTTTTATCGTCGTCTGGTAAACGAGGGTTATGATAGTGTTGCTCTTACTATGCCTCGTA
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TTCTTAAAATCGCATAAGGTAATTCACAATGATTAAAGTTGAAATTAAACCATCTCAAGCCCAATTTACTACTCGT
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TCTTTCAAAGTTGGTCAGTTCGGTTCCCTTATGATTGACCGTCTGCGCCTCGTTCCGGCTAAGTAACATGGAGCA
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