Supplementary Information

Circumvention of common labelling artifacts using secondary nanobodies

Shama Sograte-Idrissi[1,2,3], Thomas Schlichthaerle[4,5], Carlos J. Duque-Afonso[6,7,8,9], Mihai Alevra[1], Sebastian Strauss[4,5], Tobias Moser[6,7,8,9], Ralf Jungmann[4,5], Silvio Rizzoli[1,2,8], Felipe Opazo[1,2,10 *]

[1]Institute of Neuro- and Sensory Physiology, University Medical Center Göttingen, 37073 Göttingen, Germany

[2]Center for Biostructural Imaging of Neurodegeneration (BIN), University of Göttingen Medical Center, 37075 Göttingen, Germany

[3] International Max Planck Research School for Molecular Biology, Göttingen, Germany

[4] Faculty of Physics and Center for Nanoscience, LMU Munich, 80539, Munich, Germany

[5] Max Planck Institute of Biochemistry, 82152, Martinsried, Germany

[6] Institute for Auditory Neuroscience and InnerEarLab, University Medical Center Göttingen, 37075 Göttingen, Germany.

[7] Max Planck Institute for Experimental Medicine, 37075 Göttingen, Germany

[8] Multiscale Bioimaging Cluster of Excellence (MBExC), Göttingen, Germany

[9]University of Göttingen, 37075 Göttingen, Germany

[10] NanoTag Biotechnologies GmbH, 37079, Göttingen, Germany

Key words: DNA-PAINT, STED microscopy, immunostaining artifacts, linkage error.

* Corresponding Author: fopazo@gwdg.de

Supplementary Table 1 | Antibody used

Probe name	Company	Catalogue number	Dilution used
affibody® anti-IgM coupled to the Star635P	Abcam, Cambridge, UK	ab36088	1:25
Anti-IgM polyFab' coupled to the Star635P	Jackson ImmunoResearch, Cambridgeshire, UK	cleaved with Papain from 109-006-129	1:50
Mouse monoclonal anti IgM	Abcam, Cambridge, UK	ab193159	1:200
Secondary donkey anti rabbit-Star635P	Abberior, Goettingen, Germany	2-0012-007-2	1:200
Secondary nanobody anti rabbit-Star635P; FluoTag-X2 anti Rabbit	NanoTag Biotechnology, Goettingen, Germany	N1002-Ab635P	1:50
Monoclonal mouse Anti-GM130	BD bioscience	610822	1:62,5
Monoclonal mouse anti NPC	Abcam, Cambridge, UK	ab24609	1:200
Mouse monoclonal anti alpha Tubulin	Synaptic Systems, Goettingen, Germany	302211	1:500
Secondary nanobody anti mouse FluoTag-X2 anti Mouse kLC	NanoTag Biotechnology, Goettingen, Germany	N1202	1:100
Secondary donkey anti-mouse antibody	Jackson ImmunoResearch, Cambridgeshire, UK	715-005-151	1:100
Monoclonal mouse anti Beta actin	Sigma-Aldrich, Missouri, USA	A1978	1:100
Polyclonal rabbit anti Lamin B	Sigma-Aldrich, Missouri, USA	HPA050524)	1:100
FluoTag-X2 anti Mouse kLC LiCor800CW	NanoTag Biotechnology, Goettingen, Germany	N1202-Li800	1:500
FluoTag-X2 anti Mouse kLC LiCor680RD	NanoTag Biotechnology, Goettingen, Germany	N1202-Li680	1:500
FluoTag-X2 anti Rabbit LiCor800CW	NanoTag Biotechnology, Goettingen, Germany	N1202-Li800	1:500

Supplementary Table 2 | Handle sequences

Handle Name	Sequence	5'-mod	3'-mod	Company
P1	TTATACATCTATTTT	Azide	Atto488	Biomers.net
P3	TTTCTTCATTATTTT	Azide	Atto488	Biomers.net
P5	TTTCAATGTATTTTT	Azide	Atto488	Biomers.net

Supplementary Table 3 | Imager sequences

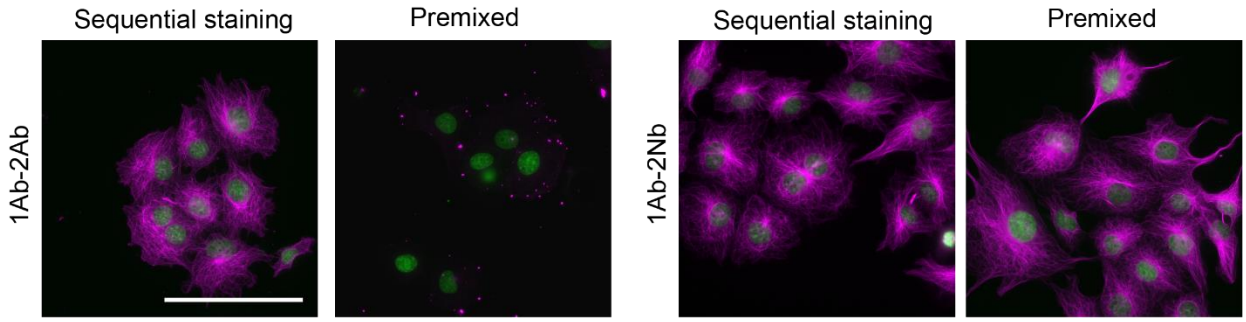
Imager name	Sequence	5'-mod	3'-mod	Company
P1*	CTAGATGTAT	None	Cy3b	Eurofins Genomics
P3*	GTAATGAAGA	None	Cy3b	Eurofins Genomics
P5*	CATACATTGA	None	Cy3b	Eurofins Genomics

Supplementary Table 4| Imaging parameters

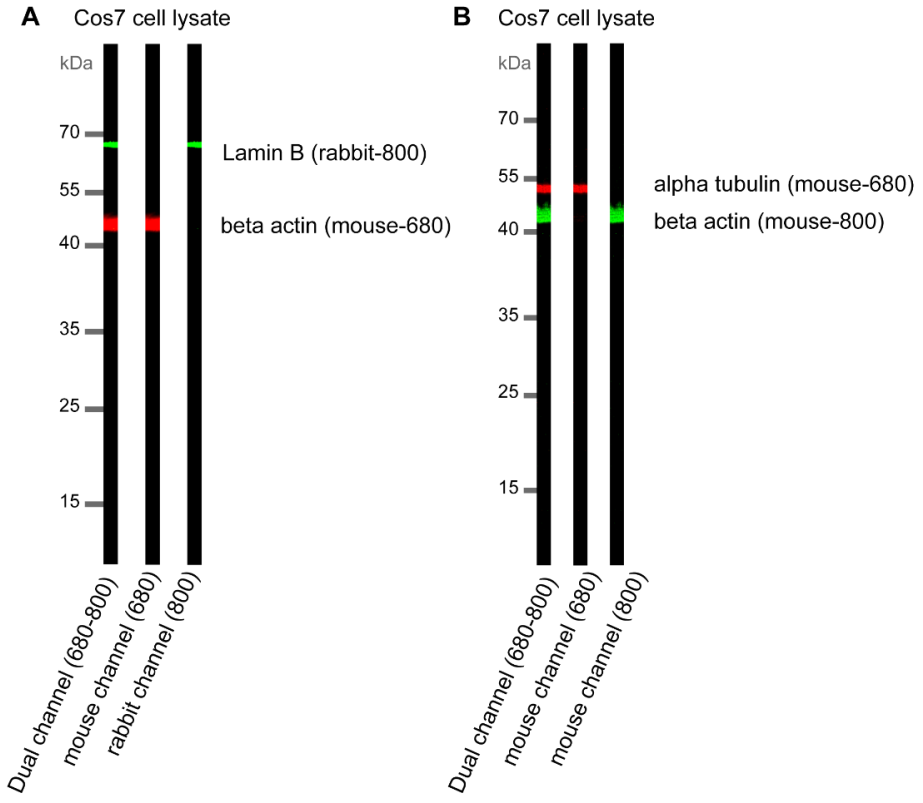
Dataset	Parameters	Power @561 nm
Figure 2A-C: DNA-PAINT Microtubule secondary nanobody	200ms, 2D, 60k Frames, 2nM. P1*	1 kW/cm^2
Figure 2D-F: DNA-PAINT Microtubule secondary antibody	200ms, 2D, 60k Frames, 2nM. P1*	1kW/cm^2
Figure 3C-E: Bassoon	150ms, 3D, 30k Frames, 3nM, P5*	1 kW/cm^2
Figure 3C-E: Homer	150ms, 3D, 30k Frames, 6 nM, P3*	1 kW/cm^2

Supplementary Table 5| Statistics on BCR autocorrelation Analysis. One-way Anova with Tukey Multiple Comparison Test. ns= non-significant, *= p ≤ 0.05, **= p ≤ 0.01, ***= p ≤ 0.001, ****= p ≤ 0.0001

	polyFab' live	**Affibody live**	**1.Ab+2.Ab live**	**1.Ab+2Nb live**
polyFab' live		ns	****	**
Affibody live			****	ns
1.Ab+2.Ab live				***
	1.Ab+2.Ab live	**1.Ab+2.Ab 10 min 4% PFA**	**1.Ab+2.Ab 30 min 4% PFA**	**1.Ab+2.Ab 30 min 4% PFA+ 0.1% GLU**
1.Ab+2.Ab live		ns	***	****
1.Ab+2.Ab 10 min 4% PFA			*	ns
1.Ab+2.Ab 30 min 4% PFA				ns
	1.Ab+2Nb live	**1.Ab+2Nb 10 min 4% PFA**	**1.Ab+2Nb 30 min 4% PFA**	**1.Ab+2Nb 30 min 4% PFA+ 0.1% GLU**
1.Ab+2Nb live		ns	ns	ns
1.Ab+2Nb 10 min 4% PFA			ns	ns
1.Ab+2Nb 30 min 4% PFA				ns
	polyFab' live	**polyFab' 10 min 4% PFA**	**polyFab' 30 min 4% PFA**	**polyFab'30 min 4% PFA+ 0.1% GLU**
polyFab' live		ns	ns	ns
polyFab' 10 min 4% PFA			ns	ns
polyFab'30 min 4% PFA				ns

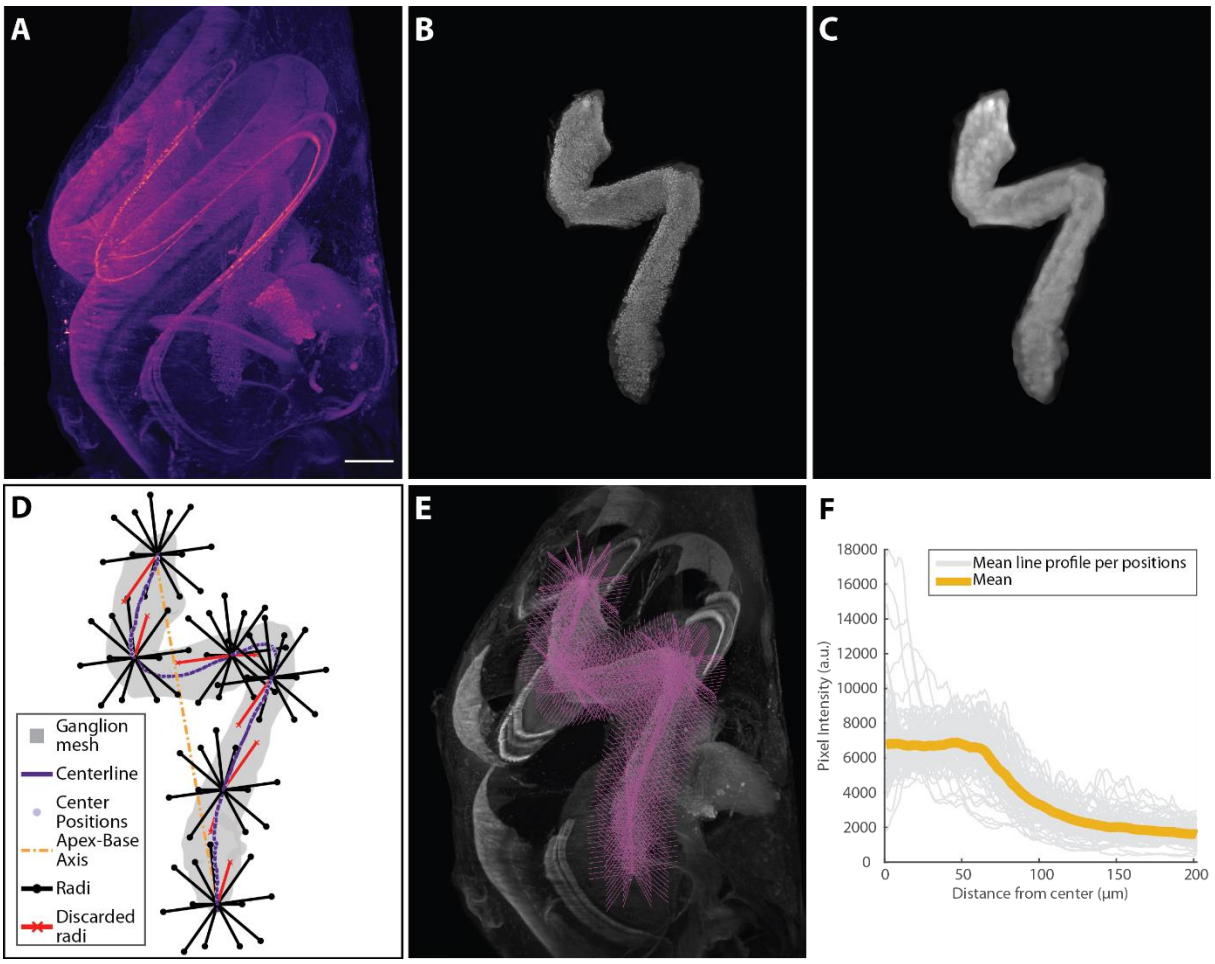


Supplementary Figure 1: Pre-mixing antibodies in a centrifuge tube prior incubation on the cell. Immunostaining is commonly done by sequential incubation of the primary probe and the secondary probe. Pre-mixing the two probe in a centrifuge tube prior incubation leads to no staining for 1.Ab-2.Ab while staining is maintained for 1.Ab-2.Nb. Hoechst staining (nucleus) in green, microtubule staining in magenta. Scale bar= 50 µm.

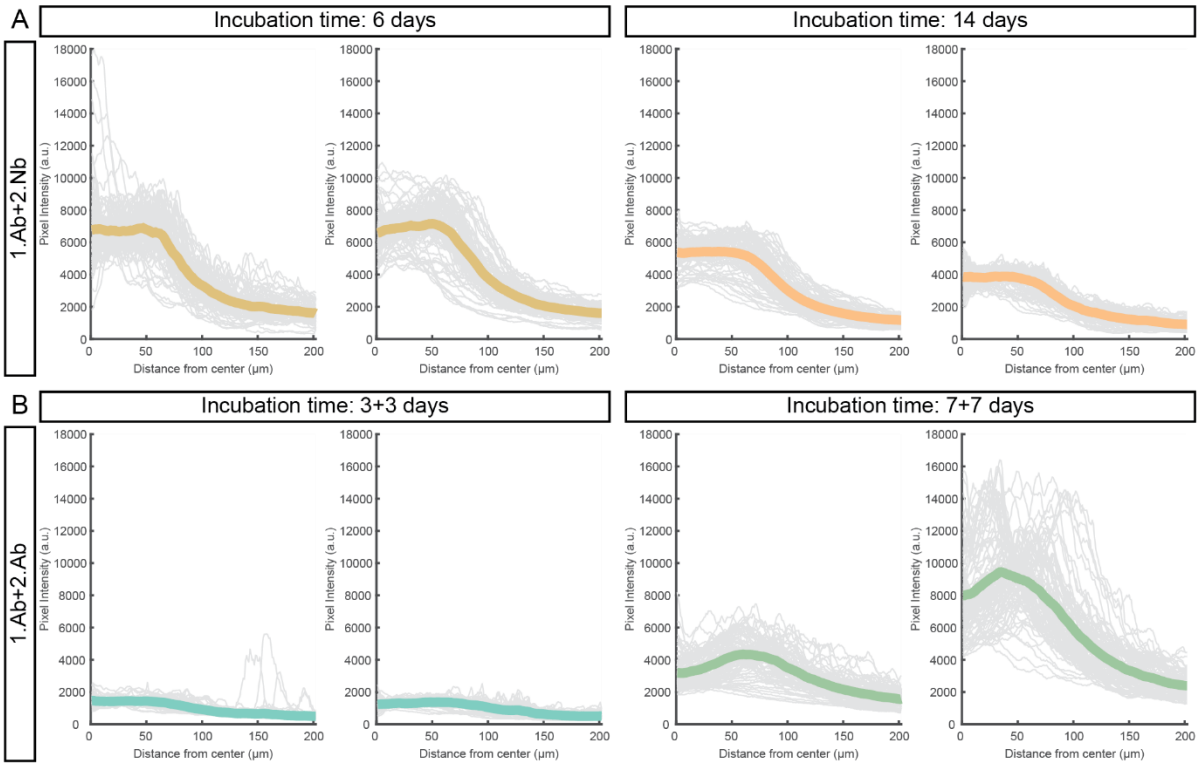


Supplementary Figure 2: Pre-mixing 1.Ab-2.Nb for Western Blot. COS-7 cell lysate blotted on nitrocellulose membrane. **A)** Pre-mixing allows shorter protocol by one single step staining. The membrane was stained with 1.Ab beta actin pre-mixed with 2.Nb anti Mouse coupled to IRDye680RD and 1.Ab anti Lamin B pre-mixed with 2.Nb anti

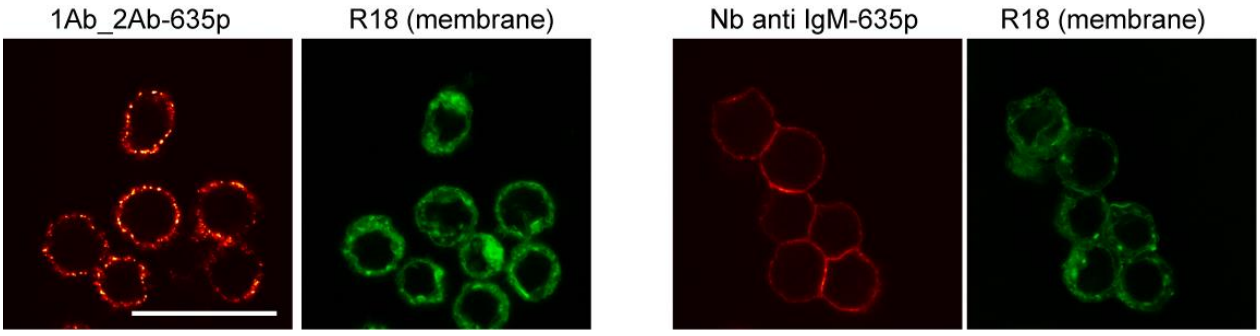
Rabbit-IRDye800CW **B)** Pre-mixing allows use of same species antibodies in the same westen blot. The membrane was stained with 1.Ab beta actin pre-mixed with 2.Nb anti Mouse-IRDye800CW and 1.Ab anti alpha tubulin pre-mixed with 2.Nb anti Mouse-IRDye800CW



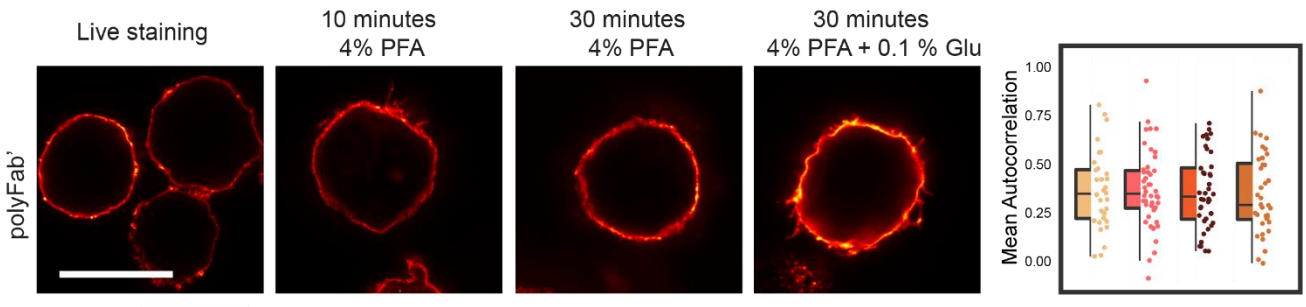
Supplemental Figure 3. Method to investigate the sample penetration of different labelling approaches in cochlear staining**. A)** Maximal intensity projection of a cleared cochlea stained with 1.Ab against parvalbumin-α premixed with 2.Nb anti-guinea pig. **B)** Coarse manual segmentation of the ganglion**. C)** Median filtered image of the ganglion (kernel: 10x10x1). **D)**. 2D projection of the mesh created from a threshold segmentation of C), its centerline, the apex-base axis, the center positions where the radii fan out and the used and discarded radii. Only 6 out of the 100 center positions and their corresponding radii used are displayed for clarity. **E)** Maximal intensity projections of a sub-stack of the slices that contains only the ganglion. In magenta, all the radii mapped back in the image space. **F)**. Mean line profile per position (n=100 positions) and mean line profile for this sample is plotted against the distance from the center position. Scalebar for A-C and E: 200 µm.



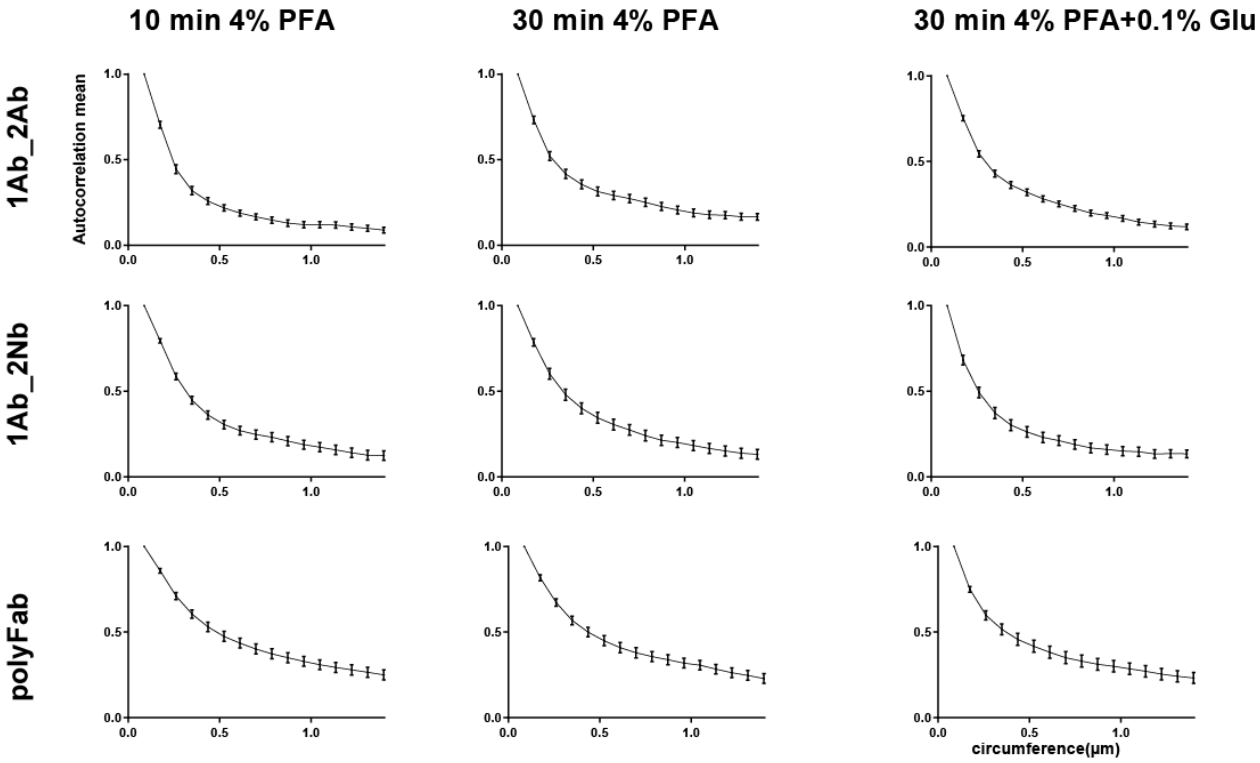
Supplemental Figure 4. Line profile from individual cochlear samples. Mean profile per position (n= 100 per sample, grey thin traces) and mean profile per sample (N=2 per staining method and incubation time, color thick traces) are displayed against distance from center position from **A)** Samples stained with a 1.Ab against parvalbumin-α premixed with 2.Nb against guinea pig, labeled with Alexa Fluor 546, and **B)** Samples stained with a 1.Ab against parvalbumin-α revealed by a 2.Ab against guinea pig, labeled with Alexa Fluor 568.



Supplementary Figure 5: Diffraction limited images (confocal microscope) of B cells stained with 1.Ab-2.Ab (left panel) or primary nanobody 1Nb (right panel) targeting the IgM of the BCR receptor. In green a membrane staining is performed (R18) to show the integrity of the membrane. z



Supplementary Figure 6: B cells fixed in different conditions and subsequently stained with polyFab'. STED images and autocorrelation analysis as explained in Fig.4.



Supplementary Figure 7: Autocorrelation curve of B cells fixed prior staining with different fixation conditions. Selected images and analyses are in Figure1 (d-e)