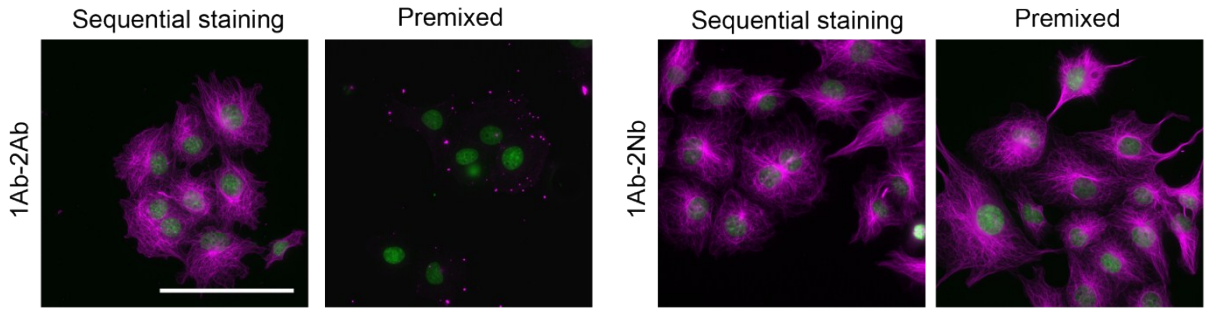
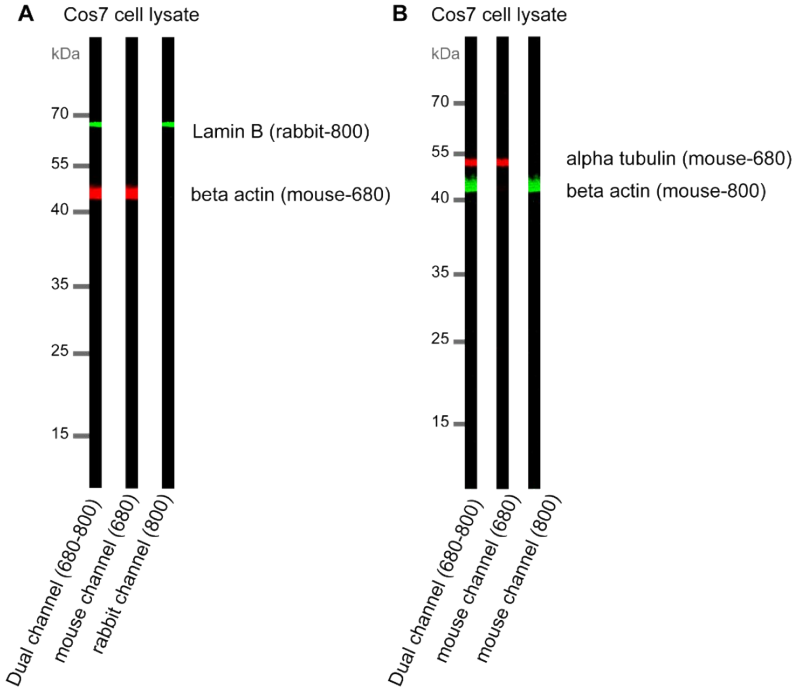
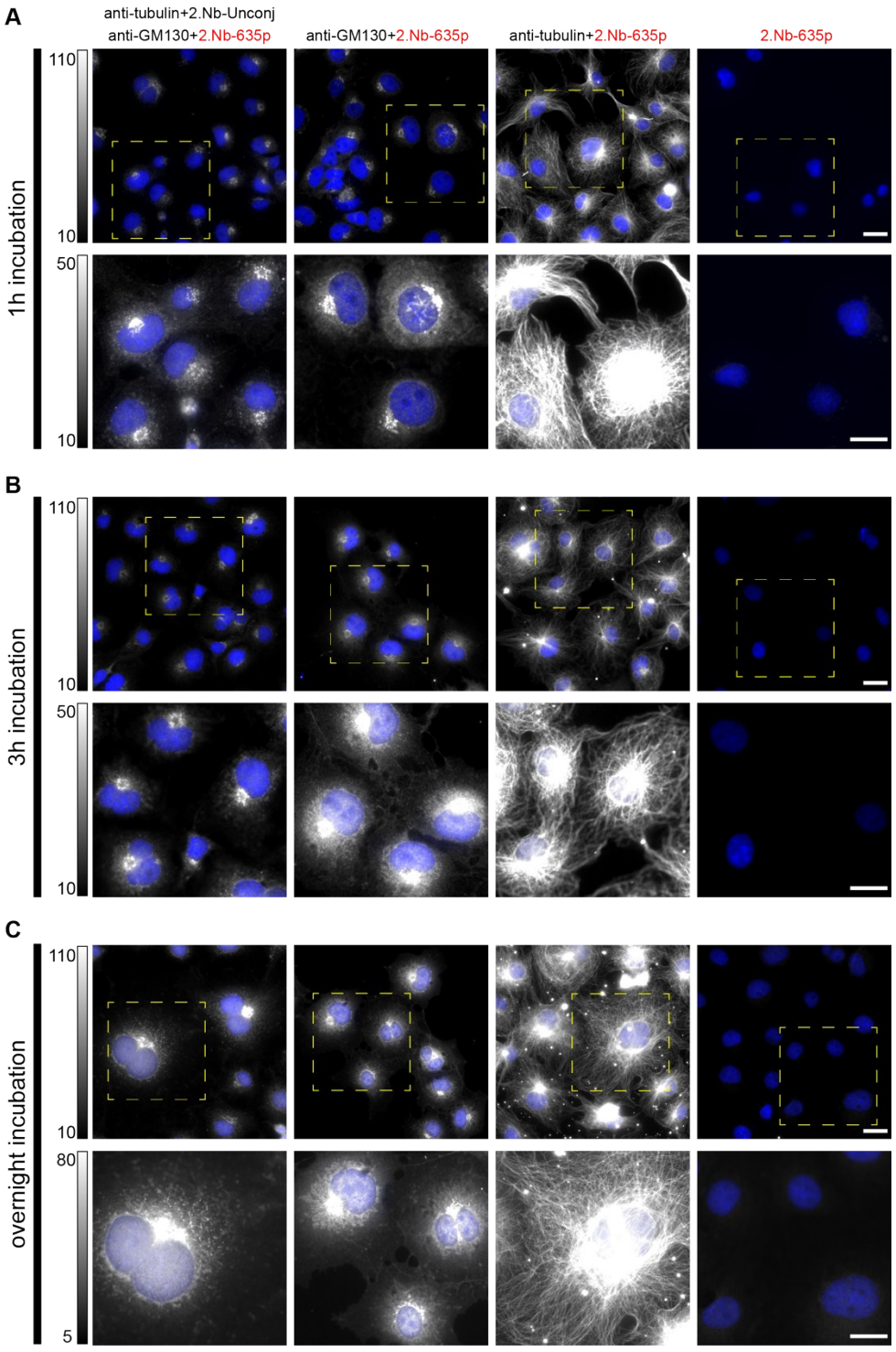
Supplementary Materials



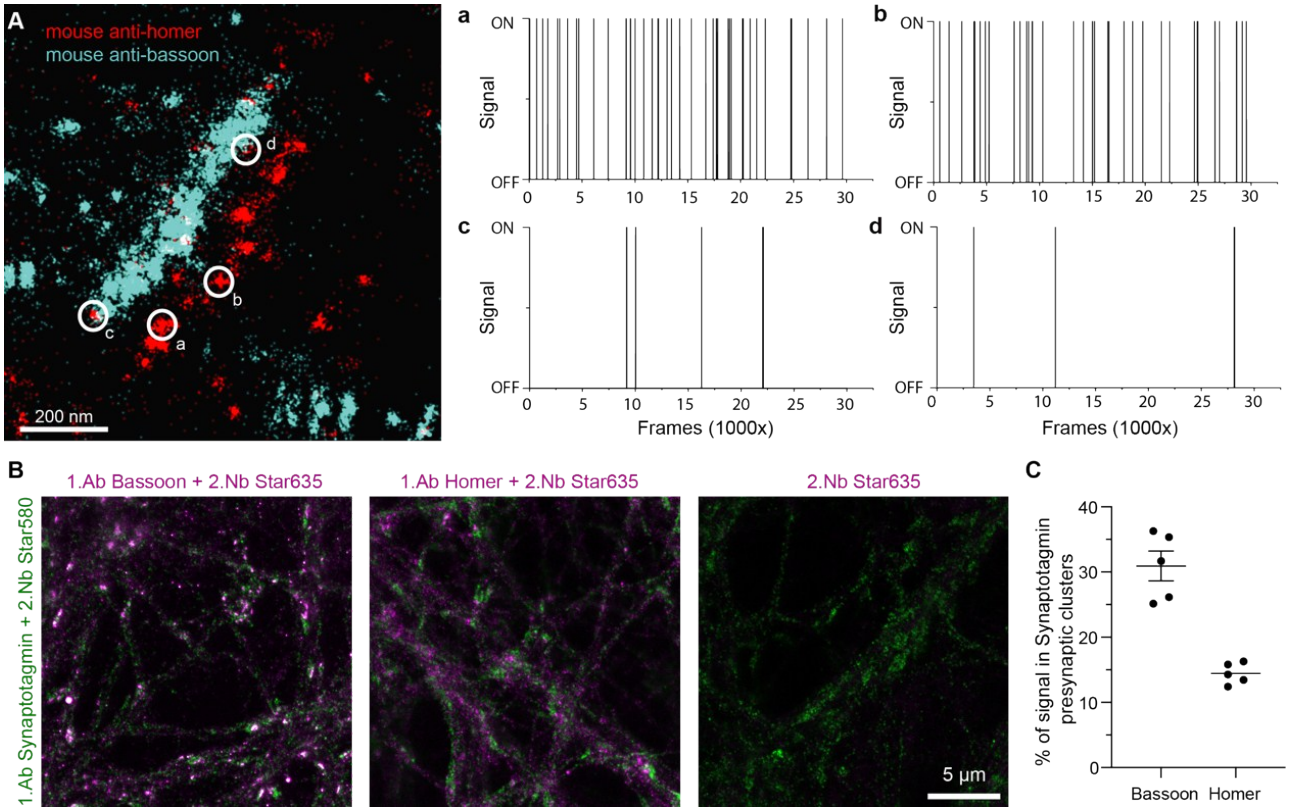
Supplementary Figure 1: Pre-mixing antibodies in a centrifuge tube prior incubation on the cell. Immunostaining is commonly done by sequential incubation of the primary probe and the secondary probe. Pre-mixing the two probe in a centrifuge tube prior incubation leads to no staining for 1.Ab-2.Ab while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



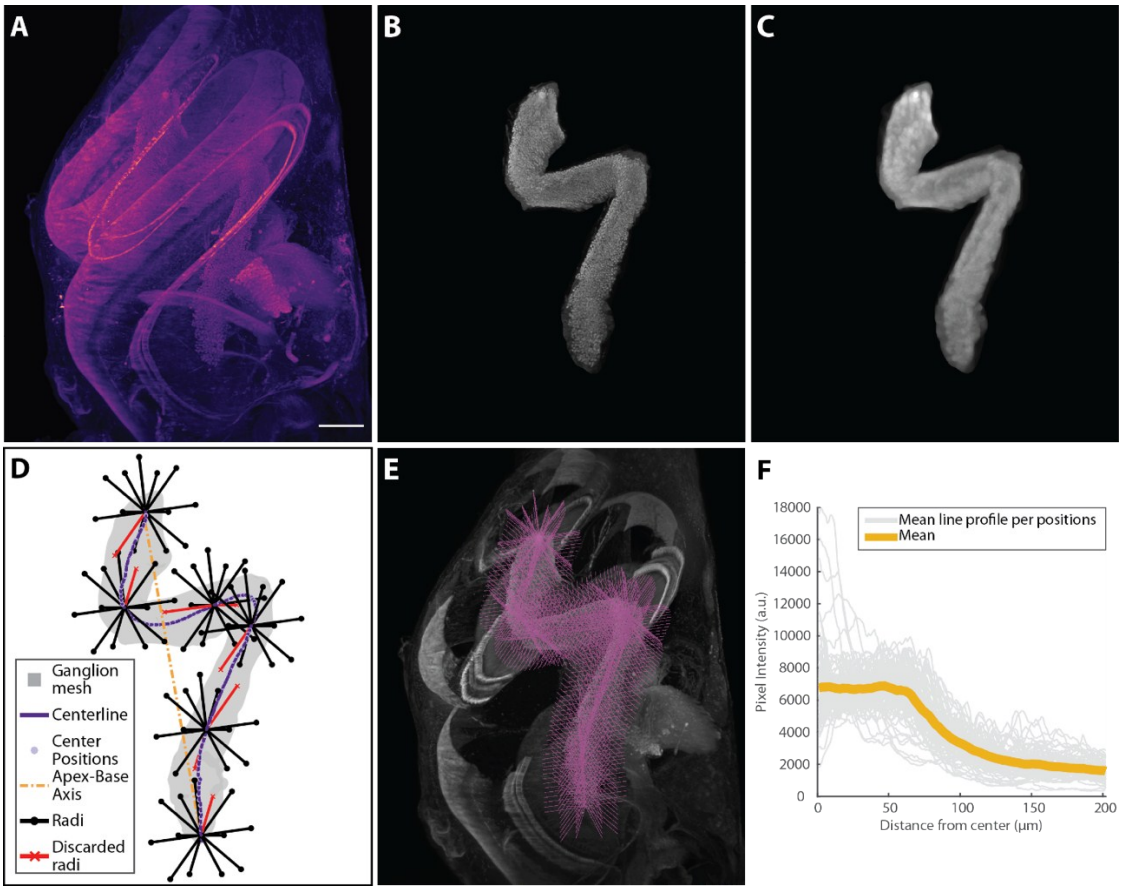
Supplementary Figure 2: Pre-mixing 1.Ab-2.Nb for Western Blot. COS-7 cell lysate blotted on nitrocellulose membrane. **A)** Pre-mixing allows shorter protocol by one single step staining. The membrane was stained with 1.Ab beta actin pre-mixed with 2.Nb anti Mouse coupled to IRDye680RD and 1.Ab anti Lamin B pre-mixed with 2.Nb anti Rabbit-IRDye800CW **B)** Pre-mixing allows use of same species antibodies in the same Western 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-IRDye680RD.



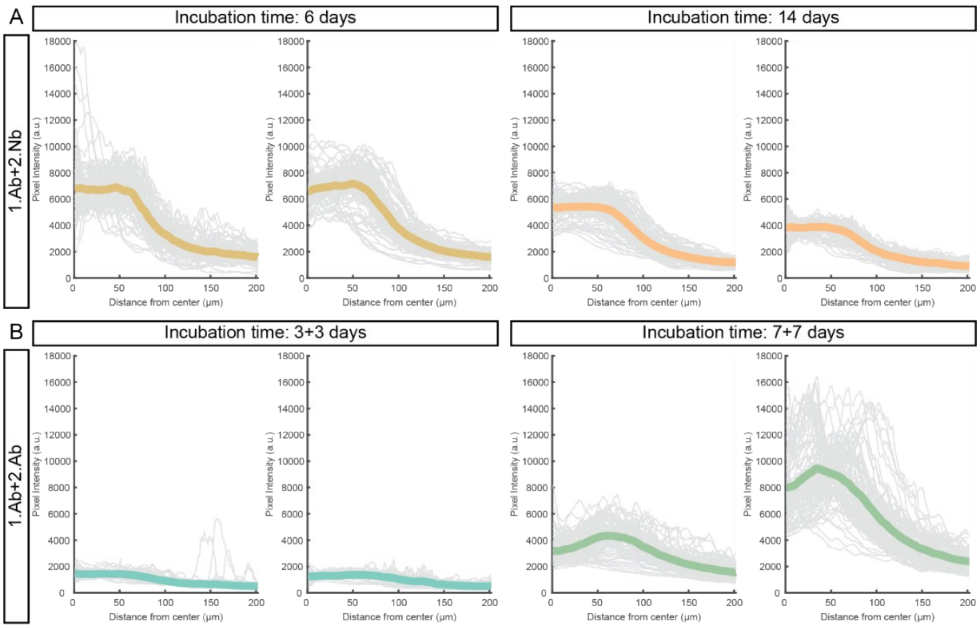
Supplementary Figure 3. Assessment of cross-contamination of 2.Nbs if pre-mixtures of same species are co-incubated on the sample simultaneously. (**A**) Top-left panel shows the epifluorescence images of COS-7 cells co-incubated for 1h at room temperature with the following pre-mixtures: anti-tubulin with unconjugated 2.Nb (2.Nb-Unconj), and anti-GM130 with 2.Nb-Star635p. The other top panels are controls. From left to right: anti-GM130 with 2.Nb-Star635p, without the anti-tubulin primary antibody; only anti-tubulin premixed with 2.Nb-Star635p; only the fluorescently labeled secondary nanobody (2.Nb-Star635p). The lower panels show the zoom areas depicted with dashed squares. Gray levels were equally set for all images (depicted with the gradient bar shown on the left). Scale bar for top and zoomed panels represents 20 µm. (**B, C**) same as A, with pre-mixtures incubated for 3h and overnight (~16h), respectively.



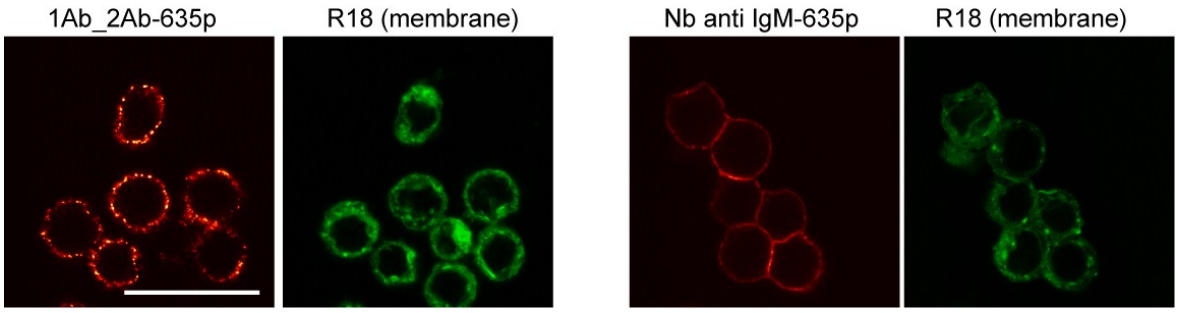
Supplementary Figure 4. The presence of homer signal in pre-synapses. (**A**) Example synapse taken from Fig. 3I. Analysis of homer DNA-PAINT localizations on the bassoon-rich localization area (pre-synapses) where the low frequency of visits by imager (shown in c & d) suggest that these "homer" localizations are non-specific events caused by the imager "stickiness". In contrast, homer localizations on the post-synaptic area can be clearly attributed to specific and repeated annealing of imager to the docking DNA strand present on the 2.Nb (a & b). (**B**) 2-Color STED microscopy images of primary hippocampal neurons stained with a guinea pig anti-Synaptotagmin1 antibody (pre-synaptic marker found in synaptic vesicles) and the same anti-homer or anti-bassoon used for the DNA-PAINT in Fig. 3H. (**C**) An analysis of B suggests that a substantial fraction of the primary antibody anti-homer (post-synaptic marker) can be found within Syt1-marked pre-synapses. An average of ~14.45±1.6% (mean±SD) of the homer signal is present there. Bassoon, which is a bona fide pre-synaptic protein, shows a stronger correlation to Syt1-marked synaptic vesicles, as expected. The graph displays the mean±sem with an N = 5.



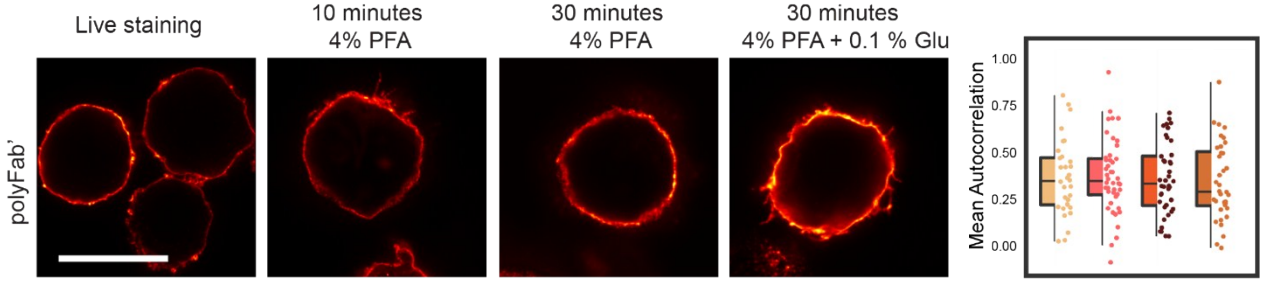
Supplemental Figure 5. Method to investigate the sample penetration of different labelling approaches in cochlear staining**. A)** Maximal intensity projection of a cleared cochlea stained with 1.Ab against parvalbumin-α premixed with 2.Nb anti-guinea pig. **B)** Coarse manual segmentation of the ganglion**. 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. Scale bars for A-C and E represent 200 µm each.



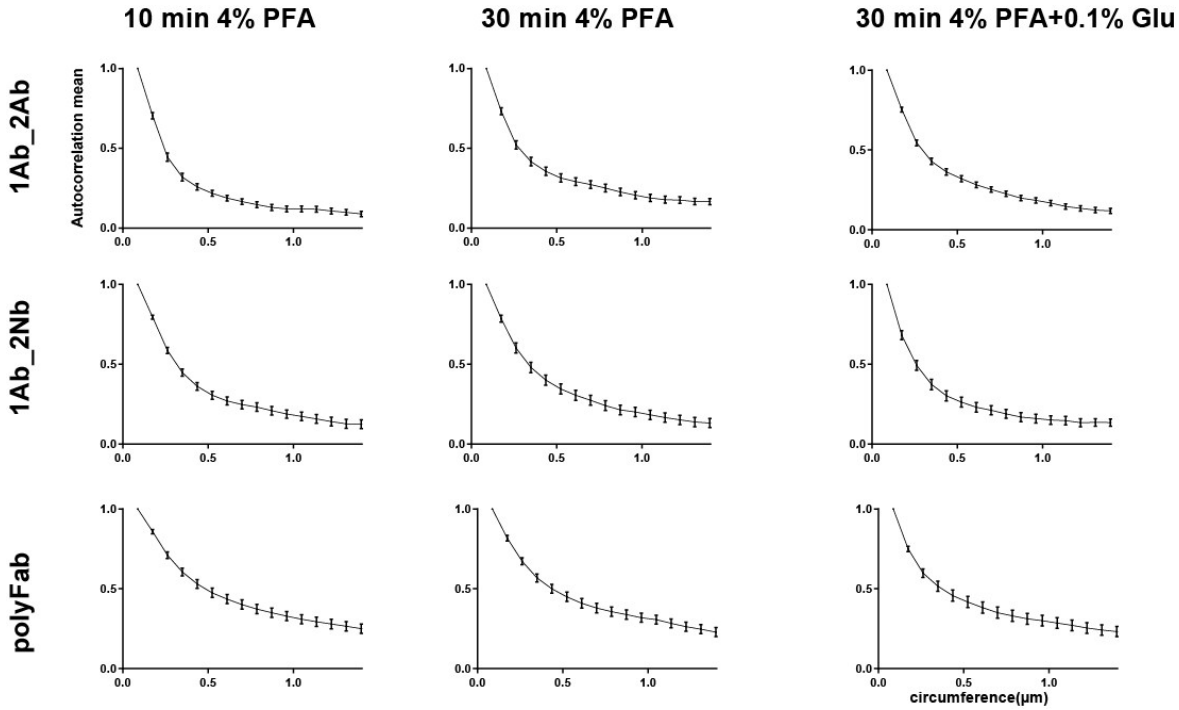
Supplemental Figure 6. Line profile from individual cochlear samples. Mean profile per position (n= 100 per sample, grey thin traces) and mean profile per sample (N=2 per staining method and incubation time, color thick traces) are displayed against distance from center position from **A)** Samples stained 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 7: Diffraction limited images (confocal microscope) of B cells stained with 1.Ab-2.Ab (left panel) or primary nanobody 1.Nb (right panel) targeting the IgM of the BCR receptor. In green a membrane staining is performed (R18) to show the integrity of the membrane. Scale bar represents 50 µm



Supplementary Figure 8: B cells fixed in different conditions and subsequently stained with monovalent polyFab`. STED images and autocorrelation analysis as explained in Fig.5.



Supplementary Figure 9: Autocorrelation curve of B cells fixed prior staining with different fixation conditions. Selected images and analyses are in Fig.6 and Supp.Fig.6.

Supplementary Table 1 | Antibodies

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
Monoclonal mouse anti-IgM	Abcam, Cambridge, UK	ab193159	1:200
Secondary donkey anti-rabbit-Star635P	Abberior, Göttingen, Germany	2-0012-007-2	1:200
FluoTag-X2 anti-rabbit Star635P	NanoTag Biotechnology, Göttingen, 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, Göttingen, Germany	302211	1:500
FluoTag-X2 anti-Mouse kLC CF633, Alexa488, Alexa546, Star635P	NanoTag Biotechnologies, Göttingen, 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 Biotechnologies, Göttingen, Germany	N1202-Li800	1:500
FluoTag-X2 anti-Mouse kLC LiCor680RD	NanoTag Biotechnologies, Göttingen, Germany	N1202-Li680	1:500
FluoTag-X2 anti-rabbit LiCor800CW	NanoTag Biotechnologies, Göttingen, Germany	N1202-Li800	1:500
Polyclonal Guinea Pig anti-Synaptotagmin1	Synaptic Systems, Göttingen, Germany	105105	1:1000

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 2: DNA-PAINT Microtubule with 2.Nbs	200ms, 2D, 60k Frames, 2nM. P1*	1 kW/cm^2
Figure 2: DNA-PAINT Microtubule with 2.Abs	200ms, 2D, 60k Frames, 2nM. P1*	1kW/cm^2
Figure 3: bassoon	150ms, 3D, 30k Frames, 3nM, P5*	1 kW/cm^2
Figure 3: homer	150ms, 3D, 30k Frames, 6nM, P3*	1 kW/cm^2

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

	polyFab live	**Affibody live**	**1.Ab+2.Ab live**	**1.Ab+2Nb live**
From Figure 5				
polyFab live		ns	****	**
Affibody live			****	ns
1.Ab+2.Ab live				***
From Figure 6a	**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
From Figure 6b	**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
From Supp. Figure 8	**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