Cell Reports Methods, Volume 2

Supplemental information

Rapid 3D-STORM imaging

of diverse molecular targets in tissue

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Figure S1. Optical Diagram of Vutara, Related to Figure 1.



Figure S1. Optical Diagram of Vutara, related to Figure 1.

Layout and optical design of the Bruker Vutara SRX352, which allows 3D STORM imaging and PSF localization via a biplane module in place of a cylindrical lens. M: Mirror, DM: Dichroic mirror, ND: Neutral density filter, L: lens, BS: Beam splitter, I: iris/aperture, OL: Objective lens, EF: Emission filter. Figure S2. RAIN-STORM resolution and localizations as a function of secondary labeling density, Related to Figure 1.



Figure S2. RAIN-STORM resolution and localization as a function of secondary labeling density, related to Figure 1.

A. Total localizations acquired when the concentration of the secondary antibody (AF647) is changed while the primary antibody concentration (Calbindin) is kept constant. Total acquired localizations decrease as secondary antibody concentration is reduced, showing 6.07x10⁶±0.490x10⁶ localizations at 5.0µg/ml, 3.73x10⁶±0.239x10⁶ at 0.5μ g/ml, and $1.19\times10^{6}\pm0.038\times10^{6}$ at 0.1μ g/ml. **B-C**. The associated XY (**B**) and XZ (**C**) resolutions as secondary antibody concentration is changed. At 5.0µg/ml, R_{xy} =63.4±3.3nm and R_{xz} =84.0±2.1nm, at 0.5µg/ml, R_{xy} =44.0±1.0nm and R_{xz}=62.6±2.8nm, while at 0.1µg/ml, R_{xy}=38.4±1.7nm and R_{xz}=56.0±4.7nm. **D**. Total localizations acquired when the concentration of the secondary antibody (CF568) is changed while the primary antibody concentration (PSD95) is kept constant. As for AF647, total acquired localizations decrease as secondary antibody concentration is reduced showing $6.30 \times 10^6 \pm 0.347 \times 10^6$ localizations at $5.0 \mu \text{g/ml}$, $3.43 \times 10^6 \pm 0.254 \times 10^6$ at 0.5μ g/ml, and $0.656 \times 10^6 \pm 0.079 \times 10^6$ at 0.1μ g/ml. E-F. The associated XY (E) and XZ (F) resolutions as secondary antibody concentration is changed. At 5.0µg/ml, $R_{xy}=29.0\pm1.1$ nm and $R_{xz}=36.6\pm2.6$ nm, at 0.5μ g/ml, $R_{xy}=35.5\pm2.0$ nm and R_{xz} =51.6±2.6nm, while at 0.1µg/ml, R_{xy} =29.8±2.2nm and R_{xz} =44.8±2.3nm. N = 3. Data are represented as the mean ± the s.e.m.

Figure S3. Protein target validation and RAIN-STORM imaging of diverse molecular targets, Related to Figure 4.

Schematic	Confocal Imaging	RAIN-STORM Imaging
	α-Tubulin	α-Tubulin
	Calbindin	Calbindin
	CAR	CAR
	CD31	CD31
	Collagen IV	Collagen IV
	Соппехіп 43	Соппехіп 43

Schematic	Confocal Imaging	RAIN-STO	RM Imaging
	CSPG4	CSPG4	
	Desmin	Desmin	
	Dystrophin	Dystrophin	
	GFAP	GFAP	
	GS	GS	
	Iba1	Iba1	10 μm

Schematic	Confocal Imaging	RAIN-STOF	RM Imaging
	Islet1	Islet1	
	ΡΚCα	ΡΚCα	
	PSD95	PSD95	
	RIBEYE	RIBEYE	
	SCGN	SCGN	
	Таи <u> 10µт</u> <u> 1µт</u>	Tau III 10µm	10 μm 0 μm 1 μm

Schematic	Confocal Imaging	RAIN-STORM Imaging
	Tomm20	Tomm20
	VGluT1	VGluT1
	VGluT3 10μm	VGluT3

Figure S3. Protein target validation and RAIN-STORM imaging of diverse molecular targets, related to Figure 4.

A representative retina schematic for each target is shown in which the region of interest is boxed (left column). Cellular structure and labeling patterns from the fluorescence confocal based images were obtained (middle columns) and used as a baseline with which to compare the reliability and robustness of RAIN-STORM imaging for various targets. Boxed regions in confocal images are magnified in images to the right. For each target, tissue was then prepared using our optimized RAIN-STORM protocol (right most columns). Representative images for each antibody are shown, followed by a magnified view of the same general structure (boxed region) shown in the fluorescence image. RAIN-STORM optimized imaging was robust across these diverse targets and antibodies, demonstrating the combability of this protocol with a wide variety of proteins. Images are representative from N = 3 animals. All confocal images are intensity-based representations of fluorescence while STORM-based images show reconstructions formed from individual localizations. STORM images are color-coded by depth (purple, to yellow, 10µm). Scale bars = 10 and 1 µm.

Figure S4. RAIN-STORM delivers robust imaging in the Nikon N-STORM system, Related to Figure 1.



2D

CF568

2D

AF647

3D

CF568

3D

AF647

10 µm

Figure S4: RAIN-STORM delivers robust imaging in the Nikon N-STORM system. Mouse retina cryosections were prepared using our optimized RAIN-STORM protocol. Horizontal cells in the outer plexiform layer (OPL) were immunolabeled with Calbindin + AF647 secondary labeling (magenta), and pre-synaptic photoreceptor terminals (spherules and pedicles) were labeled with PSD-95 + CF568 secondary labeling (cyan). STORM acquisition and analyses were performed on a Nikon N-STORM system. A. A. 2D-STORM example reconstruction is shown with an adjacent magnified example of an individual photoreceptor terminal (yellow arrow) containing distinct Calbindin+ horizontal cell processes (white arrows). Analysis settings for 2D-STORM data were based on (Robichaux et al., 2019) and were as follows: Minimum PSF height: 1,000, Maximum PSF height: 65,636, Minimum PSF Width: 200 nm, Maximum PSF Width: 400 nm, Initial Fit Width: 300 nm, Max Axial Ratio: 1.15, Max Displacement: 1 pixel. B. A 3D-STORM example reconstruction after Z-position astigmatism fitting is shown with another example magnified photoreceptor terminal (yellow arrow) encircling horizontal cell processes (white arrows). More permissive 3D-STORM analysis settings were used to enable astigmatism fitting: Minimum PSF height: 1,000, Maximum PSF height: 65,000, Minimum PSF Width: 200 nm, Maximum PSF Width: 700 nm, Initial Fit Width: 300 nm, Max Axial Ratio: 2.5, Max Displacement: 1 pixel. The z-sectioning depth of 3D-STORM reconstructions was 810 nm. All data points in the STORM reconstructions were visualized as "Gaussians" based on individual brightness and localization accuracy values. C. Total localizations and D. mean localization accuracy values are plotted as circles for both channels from 3 replicate 2D- and 3D-STORM acquisitions demonstrating reproducibility and high-quality fitting. Gray bars indicate the range, and

horizontal lines indicate the mean values. Graphs were generated using PlotsOfData (Postma and Goedhart, 2019).

Data S1. Sample and imaging conditions affect image quality and metrics, Related to Figure 4.



Staining - Blocking, Triton-X100

Unprocessed



Staining - Blocking, Saponin

Unprocessed

Saponin 0.1% Les ^{xs} =	= 43.9±0.7nm	Res _{xy} = 27.0 \pm 0.8nm	Res _{xy} = 45.0±2.3nm	Res _{xy} = 25.7±0.9nm
	= 63.6±3.6nm	Res _{xz} = 37.9 \pm 1.3nm	Res _{xz} = 62.8±8.5nm	Res _{xz} = 33.4±3.6nm
	Calbindin <u>sum</u>	PSD95	Calbindin <u>₅™</u>	PSD95
Res _{xy} =	= 48.9±2.0nm	Res _{xy} = 33.9 ± 2.3 nm	Res _{xy} = 54.8±3.1nm	Res _{xy} = 27.9 \pm 0.8nm
Res _{xz} =	= 73.3±1.2nm	Res _{xz} = 51.3 ± 4.0 nm	Res _{xz} = 81.0±6.4nm	Res _{xz} = 37.7 \pm 2.3nm
Saponin 0.3				
Res _{xy} =	64.2±5.7nm	Res _{xy} = 41.0 \pm 5.4nm	Res _{xy} = 68.7 \pm 5.6nm	$\operatorname{Res}_{xy} = 35.6 \pm 4.6 \operatorname{nm}_{xy}$ $\operatorname{Res}_{xz} = 44.5 \pm 6.2 \operatorname{nm}_{xy}$
Res _{xz} =	86.9±3.3nm	Res _{xz} = 55.9 \pm 5.7nm	Res _{xz} = 91.8 \pm 5.0nm	
Saponin 0.5				
Res _{xy} =	= 50.8±0.6nm	$Res_{xy} = 32.3 \pm 1.4nm$ $Res_{xz} = 45.4 \pm 3.4nm$	Res _{xy} = 56.0±1.9nm	$\text{Res}_{xy} = 23.3 \pm 1.0 \text{nm}.$
Res _{xz} =	= 74.5±1.5nm		Res _{xg} = 80.0±1.7nm	$\text{Res}_{xz} = 36.1 \pm 1.4 \text{nm}.$
Saponin 1.0 ⁶				
$\frac{\operatorname{Res}_{xy}}{\operatorname{Res}_{xz}} = \frac{8}{2}$: 71.1±4.2nm	Res _{xy} = 38.8±3.8nm	Res _{xy} = 79.7±3.6nm	Res _{xy} = 36.0±4.0nm
	: 100.8±8.6nm	Res _{xz} = 55.7±6.6nm	Res _{xz} = 105.9±11.0nm	Res _{xz} = 49.8±2,3nm
Saponin 2				

Figure S4. Sample and imaging conditions affect image quality and metrics, Related to Figure 4.

Staining - Blocking, NDS			
Unpro	cessed	Proce	essed
Res _{xy} = 59.1±3.5nm	Res _{xy} = 29.3±2.8nm	Res _{xy} = 67.1 ± 3.4 nm	Res _{xy} = 33.0±3.2nm
Res _{xz} = 84.1±4.5nm	Res _{xz} = 44.9±2.4nm	Res _{xz} = 95.6 ± 1.6 nm	Res _{xz} = 43.0±2.0nm
Calpingin	PSD95 Res _{xy} = 29.6±4.0nm Res _{xz} = 44.2±4.0nm	Res _{xy} = 63.7 ± 1.1 nm Res _{xz} = 86.0 ± 1.2 nm	PSD95 Res _{xy} = 34.8±3.9nm Res _{xz} = 46.3±7.5nm
Res _{xy} = 53.7±0.4nm	Res _{xy} = 30.5±2.1nm	Res _{xy} = 59.1±2.6nm	Res _{xy} = 27.5±3.8nm
Res _{xz} = 80.5±1.2nm	Res _{xz} = 42.0±3.0nm	Res _{xz} = 92.0±4.1nm	Res _{xz} = 36.0±6.8nm
Res _{xy} = 69.6±5.8nm	Res xy = 34.9±2.2nm	Res _{xy} = 69.8±1.9nm	Res _{xy} = 34.2±4.1nm
Res _{xz} = 102±12nm	Res xz = 48.5±4.8nm	Res _{xz} = 96.4±0.4nm	Res _{xz} = 48.0±7.0nm
Res _{xy} = 48.8±1.2nm	Res _{xy} = 32.4±2.1nm	Res _{xy} = 50.4±2.0nm	Res _{xy} = 31.0±3.0nm
Res _{xz} = 71.1±1.8nm	Res _{xz} = 46.5±3.6nm	Res _{xz} = 72.7±2.8nm	Res _{xz} = 43.6±1.5nm

Staining - Blocking, Triton-X100

Unprocessed



Staining - Blocking, Saponin

Unprocessed

Saponin 0.1% Les ^{xs} =	= 43.9±0.7nm	Res _{xy} = 27.0 \pm 0.8nm	Res _{xy} = 45.0±2.3nm	Res _{xy} = 25.7±0.9nm
	= 63.6±3.6nm	Res _{xz} = 37.9 \pm 1.3nm	Res _{xz} = 62.8±8.5nm	Res _{xz} = 33.4±3.6nm
	Calbindin <u>sum</u>	PSD95	Calbindin <u>₅™</u>	PSD95
Res _{xy} =	= 48.9±2.0nm	Res _{xy} = 33.9 ± 2.3 nm	Res _{xy} = 54.8±3.1nm	Res _{xy} = 27.9±0.8nm
Res _{xz} =	= 73.3±1.2nm	Res _{xz} = 51.3 ± 4.0 nm	Res _{xz} = 81.0±6.4nm	Res _{xz} = 37.7±2.3nm
Saponin 0.3				
Res _{xy} =	64.2±5.7nm	Res _{xy} = 41.0 \pm 5.4nm	Res _{xy} = 68.7 \pm 5.6nm	$\operatorname{Res}_{xy} = 35.6 \pm 4.6 \operatorname{nm}_{xy}$ $\operatorname{Res}_{xz} = 44.5 \pm 6.2 \operatorname{nm}_{xy}$
Res _{xz} =	86.9±3.3nm	Res _{xz} = 55.9 \pm 5.7nm	Res _{xz} = 91.8 \pm 5.0nm	
Saponin 0.5				
Res _{xy} =	= 50.8±0.6nm	$Res_{xy} = 32.3 \pm 1.4nm$ $Res_{xz} = 45.4 \pm 3.4nm$	Res _{xy} = 56.0±1.9nm	$\text{Res}_{xy} = 23.3 \pm 1.0 \text{nm}.$
Res _{xz} =	= 74.5±1.5nm		Res _{xg} = 80.0±1.7nm	$\text{Res}_{xz} = 36.1 \pm 1.4 \text{nm}.$
Saponin 1.0 ⁶				
$\frac{\operatorname{Res}_{xy}}{\operatorname{Res}_{xz}} = \frac{8}{2}$: 71.1±4.2nm	Res _{xy} = 38.8±3.8nm	Res _{xy} = 79.7±3.6nm	Res _{xy} = 36.0±4.0nm
	: 100.8±8.6nm	Res _{xz} = 55.7±6.6nm	Res _{xz} = 105.9±11.0nm	Res _{xz} = 49.8±2,3nm
Saponin 2				

Staining - Post-Fixation, Time & Concentration

Unprocessed



Staining - Post-Fixation, Time & Concentration

Unprocessed

Res_{xy} = 47.6 \pm 2.9nm Res_{xz} = 71.5 \pm 4.0nm IUN YH 90 Calbindin <u>sum</u> PS







S	staining - Blocking,	Time & Tempteratur	re
Res _{xy} = 41.7±0.8nm	Res _{xy} = 37.4±0.7nm	Res _{xy} = 51.7±2.1nm	Res _{xy} = 32.2±2.2nm
Res _{xz} = 63.9±3.2nm	Res _{xz} = 57.6±1.3nm	Res _{xz} = 81.8±6.3nm	Res _{x2} = 55:6±2.8nm
Res _{xy} = 44.0±2.0nm	$\text{Res}_{yy} = 42.7 \pm 3.6 \text{nm}$	Res _{xy} = 50.3±1.5nm	$\text{Res}_{xy} = 41.0 \pm 3.3 \text{ nm}$
Res _{xz} = 64.9±3.7nm	$\text{Res}_{yz} = 64.6 \pm 10.3 \text{nm}$	Res _{xz} = 78.4±5.1nm	$\text{Res}_{xz} = 63.5 \pm 6.5 \text{ nm}$
Res _{xy} = 44.0 ± 1.0 nm	Res _{xy} = 45.5±2.0nm	Res _{xy} = 55.8±4.7nm	Res _{xy} = 38.8±3.7nm
Res _{xz} = 62.6 ± 2.8 nm	Res _{xz} = 51.6±2.6nm	Res _{xz} = 78.0±1.8nm	Res _{x2} = 57.0±5.2nm
Res _{xy} = 48.1±2.2nm	Res _{xy} = 34.6±1.0nm	$Res_{xy} = 64.6 \pm 6.4 nm$	$Res_{xy} = 34.0\pm 2.9nm$
Res _{xz} = 74.6±4.4nm	Res _{xz} = 50.7±1.4nm	$Res_{xz} = 90.9 \pm 7.5 nm$	$Res_{xz} = 47.9\pm 2.5nm$

Staining - Blocking, Time & Temperature



Tissue Preparation - Primary Fixation, PFA Concentration

Res _{vv} = 55.7±2.9nm	Res, = 39.8±2.4nm	$\text{Res}_{yy} = 63.0 \pm 4.6 \text{nm}$	$\text{Res}_{yy} = 52.9 \pm 3.8 \text{nm}$
Res ^w _{xz} = 81.8±2.8nm F2 VH 90 VH %1	Res ^w _{xz} = 53.7±5.3nm	Res _{xz} ² = 94.5±2.5nm	Res ⁵ _{xz} = 69.4±12.3nm
Res _{xy} = 50.3±1.2nm Res _{xz} = 73.6±1.1nm	Res _{xy} = 34.2±0.7nm Res _{xz} = 49.0±1.3nm	$Res_{xy} = 57.6 \pm 0.8 nm$ $Res_{xz} = 84.0 \pm 0.3 nm$	Res _{xy} = 37.5±0.7nm Res _{xz} = 54.1±4.2nm
Res _{xy} = 60.2±3.9nm Res _{xz} = 82.7±3.2nm Iz Iuiuu09 Yell %	Res _{xy} = 36.6±1.8nm Res _{xz} = 53.7±0.9nm	Res _{xy} = 68.6±1.0nm Res _{xz} = 93.2±1.1nm	Res _{xy} = 33.3±2.0nm Res _{xz} = 49.8±3.4nm

Tissue Preparation - Primary Fixation, Temperature Unprocessed Processed $Res_{xy} = 36.6 \pm 1.8 nm$ $Res_{xz} = 53.7 \pm 0.9 nm$ $\text{Res}_{xy} = 68.6 \pm 1.0 \text{ nm}$ $Res_{xy} = 33.3 \pm 2.0nm$ $Res_{xz} = 49.8 \pm 3.4nm$ $\text{Res}_{xy} = 60.2 \pm 3.9 \text{nm}$ $\frac{xy}{Res} = 93.2 \pm 1.1 nm$ $\text{Res}_{xz}^{y} = 82.7 \pm 3.2 \text{nm}$ 4% PFA, 60min RT Calbindin Calbindin PSD95 PSD95 5 µm $Res_{xy} = 56.6 \pm 2.1 nm$ $Res_{xz} = 83.8 \pm 3.1 nm$ $Res_{xy} = 62.5 \pm 1.2 nm$ $Res_{xz} = 91.3 \pm 3.6 nm$ Res_{xy} = 38.5±3.3nm Res_{xz} = 62.6±3.1nm $\text{Res}_{xy} = 35.6 \pm 0.9 \text{nm}$ $\text{Res}_{xx} = 57.6 \pm 1.2 \text{nm}$ 4% PFA, 60min 4°C Tissue Prenaration - Primary Fixation Time

	ssaerreparation		
Res _{xy} = 54.3±0.6nm	Res _{xy} = 31.3±1.2nm	Res _{xy} = 61.7±2.3nm	Res _{xy} = 33.2±1.5nm
Res _{xz} = 77.4±1.5nm	Res _{xz} = 43.3±1.5nm	Res _{xz} = 90.0±5.3nm	Res _{xz} = 40.7±5.1nm
Res _{xy} = 60.2±3.9nm	$Res_{xy} = 36.6 \pm 1.8nm$	Res _{xy} = 68.6±1.0nm	Res _{xy} = 33.3±2.0nm
Res _{xz} = 82.7±3.2nm	$Res_{xz} = 53.7 \pm 0.9nm$	Res _{xz} = 93.2±1.1nm	Res _{xz} = 49.8±3.4nm
Res _{xy} = 48.5±2.0nm	Res _{xy} = 32.6±2.3nm	Res _{xy} = 58.6±2.3nm	Res _{xy} = 32.8±2.6nm
Res _{xz} = 73.6±1.9nm	Res _{xz} = 48.8±4.2nm	Res _{xz} = 83.6±3.1nm	Res _{xz} = 45.5±6.5nm

Staining - Secondary Quenching, Concentration & Reagent

Unprocessed Processed $\text{Res}_{xy} = 55.7 \pm 1.0 \text{nm}$ $\text{Res}_{xy} = 67.8 \pm 2.3 \text{nm}$ $\text{Res}_{xy} = 46.2 \pm 2.2 \text{nm}$ $\text{Res}_{xy} = 71.4 \pm 4.9 \text{nm}$ $\text{Res}_{xz}^{y} = 75.7 \pm 4.2 \text{ nm}$ $\text{Res}_{xz}^{y} = 105.5 \pm 5.4 \text{nm}$ $\text{Res}_{x_{2}}^{2} = 124.5 \pm 9.4$ nm $\text{Res}_{xz}^{y} = 92.4 \pm 1.6 \text{nm}$ 0mM NH_aCI Calbindin PSD95 Calbindin 5 µm 5 µm PSD95 $\text{Res}_{xy} = 50.5 \pm 2.1 \text{nm}$ $\operatorname{Res}_{xy} = 69.8 \pm 3.8 \operatorname{nm}$ $\text{Res}_{xy} = 43.5 \pm 2.8 \text{nm}$ $\text{Res}_{xy} = 66.4 \pm 3.5 \text{nm}$ $\text{Res}_{xz}^{y} = 79.3 \pm 2.4 \text{nm}$ $\operatorname{Res}_{xx}^{2} = 109.0 \pm 3.0 \operatorname{nm}$ $\text{Res}_{xy} = 90.1 \pm 11.9 \text{ nm}$ Res _ = 69.3±6.3nm 00mM NH_ACI $\text{Res}_{xy} = 55.4 \pm 6.2 \text{nm}$ $\text{Res}_{xy} = 65.7 \pm 12.1 \text{nm}$ $\text{Res}_{xv} = 98.9 \pm 28.2 \text{nm}$ $\text{Res}_{xy} = 69.1 \pm 11.9 \text{ nm}$ $\text{Res}_{xz} = 116.8 \pm 32.4 \text{nm}$ $\text{Res}_{xz}^{y} = 79.5 \pm 8.4 \text{nm}$ $\text{Res}_{xz}^{\gamma} = 149.8 \pm 50.2 \text{ nm}$ $\text{Res}_{xz}^{2} = 98.7 \pm 19.3 \text{ nm}$ **IOmM Glycine** $\operatorname{Res}_{xy} = 46.5 \pm 10.5 \operatorname{nm}$ $\text{Res}_{xy} = 44.4 \pm 5.4 \text{nm}$ $\operatorname{Res}_{xy} = 84.0 \pm 3.9 \operatorname{nm}$ $\operatorname{Res}_{xy} = 67.9 \pm 6.4 \operatorname{nm}$ $\text{Res}_{xz}^{'} = 69.2 \pm 8.6 \text{nm}$ $\text{Res}_{xz} = 84.7 \pm 16.9 \text{nm}$ Res _ = 139.1±5.8nm $\text{Res}_{xy} = 95.7 \pm 9.3 \text{ nm}$ mM Glycine

Imaging - Electron Sinks, Concentration & Reagent









Imaging - Electron Sinks, Concentration & Reagent Unprocessed Processed $\text{Res}_{xy} = 85.5 \pm 2.1 \text{nm}$ $\text{Res}_{xz} = 137.3 \pm 10.1 \text{nm}$ $\text{Res}_{xy} = 79.6 \pm 3.9 \text{nm}$ $\text{Res}_{xy} = 78.9 \pm 1.6 \text{nm}$ $\text{Res}_{xy} = 89.9 \pm 5.7 \text{nm}$ $\text{Res}_{y}^{xy} = \frac{125.3 \pm 9.3 \text{ nm}}{125.3 \pm 9.3 \text{ nm}}$ $\text{Res}_{x}^{2} = 114.3 \pm 4.4 \text{nm}$ $\text{Res}_{x_{7}}^{2} = 123.9 \pm 7.3 \text{ nm}$ COT Mm Calbindin Calbindin PSD95 5 µm PSD95 5 µn $\text{Res}_{xy} = 84.4 \pm 5.7 \text{nm}$ $Res_{xy} = 76.9 \pm 3.4 nm$ $Res_{xz} = 128.1 \pm 3.7 nm$ $\text{Res}_{xy} = 73.8 \pm 6.2 \text{nm}$ $\text{Res}_{xy} = 62.2 \pm 2.9 \text{nm}$ $\operatorname{Res}_{x_{7}}^{2} = 137.7 \pm 5.3 \operatorname{nm}$ $\text{Res}_{x_{z}} = 113.01 \pm 8.3 \text{ nm}$ $\text{Res}_{xz} = 99.3 \pm 5.6 \text{nm}$ $\text{Res}_{xy} = 82.3 \pm 1.1 \text{ nm}$ $\operatorname{Res}_{xv} = 76.5 \pm 1.0 \operatorname{nm}$ Res_{vv} = 79.3±2.9nm $Res_{xy} = 82.1 \pm 0.9 nm$ $\text{Res}_{xz}^{xy} = 117.9 \pm 8.4 \text{nm}$ $\text{Res}_{xz} = 106.7 \pm 6.1 \text{nm}$ $Res_{xz}^{2} = 123.8 \pm 2.5 nm$ $\text{Res}_{xz}^{y} = 123.0 \pm 4.4 \text{nm}$ <u>M</u> Trolox $\text{Res}_{xy} = 75.9 \pm 1.5 \text{nm}$ $\text{Res}_{xy} = 85.5 \pm 2.0 \text{nm}$ $\operatorname{Res}_{xy} = 88.8 \pm 9.8 \operatorname{nm}$ Res_{xy} = 79.8±1.5nm $\text{Res}_{xz} = 111.1 \pm 6.0 \text{nm}$ $\text{Res}_{x_{7}}^{*} = 139.3 \pm 26.4 \text{nm}$ $\text{Res}_{x} = 131.6 \pm 10.9 \text{ nm}$ Res_ = 117.3±7.1nm Trolox $\text{Res}_{xy} = 70.8 \pm 0.8 \text{nm}$ $\text{Res}_{xy} = 73.1 \pm 6.9 \text{ nm}$ $\text{Res}_{xy} = 70.5 \pm 5.3 \text{nm}$ $\text{Res}_{xy} = 84.3 \pm 11.6$ nm $\text{Res}_{xz}^{xy} = 113.3 \pm 5.3 \text{nm}$ $\text{Res}_{xz}^{y} = 96.1 \pm 11.9 \text{nm}$ Res _ = 129.5±16.2nm $Res_{2}^{2} = 92.4 \pm 14.2 nm$ **Tolxo**

Tissue Preparation-Primary Fixation & Quenching, Reagent & Conc.

Unprocessed



Tissue Preparation-Primary Fixation & Quenching, Reagent & Conc.

Unprocessed



Tissue Preparation-Primary Fixation & Quenching, Reagent & Conc. Unprocessed Processed $Res_{xy} = 75.8 \pm 4.6 nm$ $Res_{xz} = 100.6 \pm 6.2 nm$ $\operatorname{Res}_{xy} = 40.8 \pm 2.9 \operatorname{nm}$ Res_ = 63.2±3.6nm $\text{Res}_{xy} = 37.3 \pm 1.4 \text{nm}$ $\operatorname{Res}_{xz}^{xy} = 51.4 \pm 3.4 \operatorname{nm}$ $\text{Res}_{xz}^{xy} = 55.4 \pm 5.5 \text{ nm}$ $\text{Res}_{xz}^{2} = 88.6 \pm 6.2 \text{ nm}$ 2% PFA, 0.0% GA 100mM Glycine Calbindin 5 µm PSD95 Calbindin PSD95 5 µn $\text{Res}_{xy} = 53.6 \pm 1.3 \text{nm}$ Res_{xy} = 37.7±1.0nm Res_{xy} = 75.8±2.6nm Res_{xy} = 108.4±6.5nm Res_{xv} = 42.3±1.2nm $Res_{xx} = 57.5 \pm 2.2 nm$ $Res_{y}^{2} = 65.8 \pm 6.9 nm$ $Res^{2} = 77.1 \pm 4.1 nm$ GA 2% PFA, 0.0% 0 0.1% NaBH Res_{xy} = 36.4±3.2nm $Res_{xy} = 52.2 \pm 2.3 nm$ $Res_{xz} = 73.6 \pm 2.4 nm$ Res, = 32.6±1.3nm $Res_{xy} = 72.8 \pm 1.4 nm$ $Res_{xz} = 98.3 \pm 1.8 nm$ $\operatorname{Res}_{xz}^{xy} = 54.7 \pm 2.6 \operatorname{nm}$ $\text{Res}_{y_{z}} = 45.8 \pm 2.1 \text{ nm}$ 2% PFA, 0.0% GA 0.5% NaBH₃

Staining - Secondary, Channel & Primary Target



Tissue Preparation-Primary Fixation & Quenching, Reagent & Conc. Unprocessed Processed $Res_{xy} = 48.0 \pm 4.7 nm$ Res_{xy} = 40.3±1.0nm $\text{Res}_{xy} = 37.3 \pm 1.3 \text{nm}$ $Res_{m} = 42.0 \pm 1.2 nm$ $\text{Res}_{xz} = 56.6 \pm 0.9 \text{nm}$ $Res_{\chi} = 69.9 \pm 6.9 nm$ $\text{Res}_{xy}^{xy} = 58.1 \pm 1.7 \text{nm}$ $= 60.8 \pm 4.1$ nm Res 0% PFA, 0.3% GA 10mM NH_sCI Calbindin 5 µm PSD95 Calbindin PSD95 5 µm $Res_{xy} = 35.3 \pm 2.1 nm$ $Res_{xz} = 51.5 \pm 4.0 nm$ $\text{Res}_{yy} = 40.0 \pm 3.4 \text{nm}$ $\text{Res}_{xy} = 40.7 \pm 1.6 \text{nm}$ 37.1±6.5nm Res $Res_{xz} = 56.4 \pm 8.4 nm$ 55.9±11.5nm $Res_{xz} = 55.7 \pm 2.1 nm$ 0% PFA, 0.3% GA 100mM NH₄Cl $\text{Res}_{yy} = 37.3 \pm 2.7 \text{nm}$ $\text{Res}_{xy} = 30.3 \pm 2.1 \text{ nm}$ $Res_{v} = 35.1 \pm 0.3 nm$ Res_ = 39.6±4.3nm $\text{Res}_{xz} = 56.9 \pm 5.3 \text{nm}$ $\text{Res}_{xz} = 45.9 \pm 3.0 \text{nm}$ $\text{Res}_{xz} = 53.3 \pm 1.0 \text{nm}$ $Res_{-} = 59.6 \pm 7.6 nm$ 0% PFA, 0.3% GA 10mM Glycine Res_{xy} = 51.7±**0.**5nm $\text{Res}_{xy} = 73.4 \pm 11.0$ nm Res_{xv} = 39.3±1.4nm $Res_{w} = 37.2 \pm 1.0 nm$ $Res_{xz} = 86.0 \pm 2.4$ nm $\text{Res}_{2}^{2} = 56.0 \pm 1.8 \text{ nm}$ $Res_{xx} = 75.8 \pm 3.5 nm$ $\text{Res}_{2} = 59.3 \pm 1.5 \text{ nm}$ 0% PFA, 0.3% GA 100mM Glycine Res_{xy} = 29.3±2.5nm $Res_{m} = 38.5 \pm 2.7 nm$ $Res_{...} = 43.5 \pm 5.0 nm$ $Res_{xy} = 31.3 \pm 4.7 nm$ $\text{Res}_{xx}^{xy} = 47.0 \pm 8.1 \text{ nm}$ $Res_{x} = 58.9 \pm 6.6 nm$ Res, = 40.0±5.5nm $\text{Res}_{2} = 63.1 \pm 7.1 \text{ nm}$ 0% PFA, 0.3% GA 0.1% NaBH,



Imaging & Analysis - Imaging Buffer, Pyrannose Oxidase Buffer

Unprocessed

5 μm

Res_{xy} = 59.2±5.5nm Res_{xz} = 96.5±6.8nm

PSD95

 $Res_{xy} = 82.5 \pm 11.9 nm$ $Res_{xz} = 134.7 \pm 18.4 nm$

Calbindin

DC: 71.5 mM BME, 10U PyOx 20mM MEA, 100U Cat. 7

Res_{xy} = 98.8±21.2nm Res_{xz} = 147.7±24.6nm

Calbindin

Processed $\text{Res}_{xy} = 73.6 \pm 13.7 \text{nm}$ $\text{Res}_{xz} = 113.5 \pm 17.3 \text{nm}$ 5 μm

PSD95

Imag	ing & Analysis - Ima	ge Acquisition, Ste	o Size
Res _{xy} = 51.2 \pm 3.4nm	$Res_{yz} = 29.6 \pm 1.4 nm$	Res _{xy} = 56.4±6.2nm	Res _{xy} = 25.5±2.0nm
Res _{xz} = 70.7 \pm 3.5nm	$Res_{yz} = 42.0 \pm 3.6 nm$	Res _{xz} = 70.8±4.3nm	Res _{xz} = 38.1±3.9nm
Res $_{xy}$ = 53.3±0.8nm	$Res_{xy} = 31.5 \pm 1.0 nm$	Res _{xy} = 75.9±2.5nm	Res _{xy} = 33.2 \pm 4.4nm
Res $_{xz}$ = 77.1±2.0nm	$Res_{xz} = 43.0 \pm 3.4 nm$	Res _{xz} = 95.3±7.9nm	Res _{xz} = 45.3 \pm 6.8nm
Res _{xy} = 54.9±4.2nm	Res _{xy} = 33.2±2.0nm	Res _{xy} = 70.5±8.6nm	Res _{xy} = 34.8±3.2nm
Res _{xz} = 82.6±4.3nm	Res _{xz} = 47.5±1.4nm	Res _{xz} = 110±15.6nm	Res _{xz} = 48.5±0.3nm
Res _{xy} = 45.9±4.7nm Res _{xz} = 64.3±3.5nm ^{BZS} days lever uog	Res _{xy} = 28.8±3.8nm Res _{xz} = 42.2±5.3nm	$\text{Res}_{xy} = 47.5 \pm 6.1 \text{nm}$ $\text{Res}_{xz} = 63.8 \pm 5.4 \text{nm}$	Res _{xy} = 30.1±6.1nm Res _{xz} = 46.2±9.5nm



Imaging & Analysis - Imaging Buffer, Pyrannose Oxidase Buffer

Res xy = 63.9±6.2nm	Res _{xy} = 44.8±1.4nm	Res _{xy} = 79.2±10.0nm	Res _{xy} = 64.3±2.5nm
Res xz = 106.3±13.2nm	Res _{xz} = 69.8±3.7nm	Res _{xz} = 129.1±15.7nm	Res _{xz} = 107.2±1.5nm
Res _{xy} = 75.8±6.1nm Res _{xz} = 130.4±10.5nm ^{Of} unt Yaw WWW Yugo Toon Yang Yang Yang Yang Yang Yang Yang Yang	Res _{xy} = 55.6±1.7nm Res _{xz} = 87.5±2.5nm	Res _{xy} = 91.2±3.8nm Res _{xz} = 154.3±8.5nm	Res _{xy} = 72.1±0.8nm Res _{xz} = 117.5±3.0nm

Imaging & Analysis - Imaging Buffer, Pyrannose Oxidase Buffer

Unprocessed



Data S1. Sample and imaging conditions affect image quality and metrics,

related to Figure 4.

Representative images of conditions are shown for each channel of a given condition. Unprocessed images are shown alongside their processed counterparts, as well as the resolutions that were measured for each condition. Each row shows a different condition tested. Calbindin-labeled horizontal cells are shown in magenta, and PSD95-labeled rod terminals in cyan, unless otherwise noted. Scale bars = 5 μ m.

Supplemental Table 1: Primary antibodies used.

Antigen	Labeling specificity	Source	Dilution (confocal)	Dilution (RAIN- STORM)
Calbindin D-28K	Horizontal cells, subsets of amacrine cells, and retinal ganglion cells	Swant, Cat# CB38a, RRID: AB_10000340	1:5000	1:1000
CD31	Blood vessels, endothelial cells	Fisher, Cat# BDB5500274, RRID: AB_393571	1:200	1:50
Cone arrestin	Cone photoreceptors	Millipore, Cat# AB15282, RRID: AB_11210270	1:2000	1:1000
Collagen IV	Blood vessels	Millipore, Cat# AB769, RRID: AB_92262	1:1000	1:500
Connexin 43	Pericyte gap junctions	Sigma, Cat#C6219, RRID: AB_476857	1:1000	1:500
Desmin	Pericytes	Thermo Fisher, Cat# MA513259, RRID: AB_11000611	1:500	1:500
Dystrophin	Photoreceptor synapses	Abcam, Cat# ab15277, RRID: AB_301813	1:200	1:100
Glutamine synthetase (GS)	Muller glia	BD Biosciences, Cat# 610517, RRID: AB_397879	1:1000	1:500
GFAP	Astrocytes	Sigma, Cat# G3893, RRID: AB_477010	1:500	1:500
lba1	Microglia	Abcam, Cat# ab5076, RRID: AB_2224402	1:500	1:500
Islet1	ON bipolar cells, starburst amacrine cells, subset of retinal ganglion cells	R&D system, Cat# AF1837, RRID: AB_2126324	1:2000	1:1000
NG2	Pericytes	Abcam, Cat# ab129051, RRID: AB_2877152	1:1000	1:500
ΡΚCα	Rod bipolar cells	Abcam, Cat# ab31, RRID: AB_303507	1:500	1:500
PSD95	Photoreceptor terminals	Abcam, Cat# ab12093, RRID: AB_298846	1:500	1:500
RIBEYE	Ribbon synapses	Synaptic system, Cat#192103, RRID: AB_2086775	1:500	1:500
Secretagogin (SCGN)	Cone bipolar cells	BioVendor, Cat# RD181120100, RRID: AB_2034060	1:1000	1:500
Tau	Microtubule-associated protein	Proteintech, Cat#66499-1- Ig, RRID: AB_2881863	1:1000	1:500
α-Tubulin	α-Tubulin protein	Sigma, Cat#T51682ML, RRID: AB_477579	1:1000	1:500
Tomm20	Mitochondria	Abcam, Cat# ab78547, RRID: AB_2043078	1:1000	1:500
Tyrosine hydroxylase (TH)	Dopaminergic amacrine cell subset	EMD Millipore, Cat# AB1542, RRID: AB_90755	1:2000	1:500
Vesicular glutamate transporter 1 (VGlut1)	Photoreceptor ribbon synapses	Abcam, Cat# ab77822, RRID: AB_2187677	1:500	1:250
Vesicular glutamate transporter 3 (VGlut3)	Subset of amacrine cells	Millipore, Cat#AB5421, RRID: AB_2187832	1:1000	1:500

Supplemental Table 2: Summary of condition variations tested for RAIN-STORM, Related to Figure 1.

Stage	Parameter	Variations	Specifics 1% PFA, RT, 60min	8213357	Background Localizations 2255413	AF64/ XY Res 55.7234	39.7570
		Conc./Temp	2% PFA, RT, 60 min 4% PFA, RT, 60 min	8970285 9146043	2398174 3144002	50.3231 60.1768	34.2027 36.6071
	Primary Fixation		4% PFA, 4C, 60 min	6779812 4014695	2792175	56.5597	35.5531
		Туре	2% PFA, 0.3% GA, RT, 60 min	5030786	3181862	45.1080	29.3187
Tissue		Timing	4% PFA, RT, 30 min 4% PFA, RT, 120 min	8642310 10108757	3722096 3498380	54.3354 48.5256	31.3137 32.5775
Preparation		Conc./Type	10mM Glycine 100mM Glycine	9751623 12209377	2847049 3525533	51.7930 63.2148	31.7254 37.2768
	Primary Quenching	Conc./Type	10mM NH3Cl 100mM NH3Cl	9751623 11649151	3043674	50.2599 55.7147	39.2483 37.4105
		Conc./Type	0.1% NaBH4	10066425	2222657	53.5908	37.7183
	Embedding Method	Thickness	0.5% NaBH4 10µm	9146043	26/3369 3144002	52.2041 60.1768	32.6448 36.6071
	Embedding method	monteos	20µm 1% NDS	3775049 12554580	2494498 3953868	63.0147 59.1086	66.6873 29.2762
		Serum Permeabilizer Type	3% NDS	14772243	4423472	58.0771 53.7408	29.6001
			10% NDS	9029693	2928266	69.5874	34.9191
	Blocking Buffer		0.1% Triton	13108963 9958189	3920780 3309736	48.8157 57.0414	32.4246 25.8641
			0.3% Triton 0.5% Triton	13145415 9647404	4107653 3040177	60.7596 57.8182	35.5746 35.4601
			1.0% Triton 2.0% Triton	10495550	3467400	50.3823 50.8491	30.9056
		Permeabilizer Type	0.1% Saponin	7443945	3638982	43.9059	26.9680
			0.3% Saponin 0.5% Saponin	6084083 6343524	2769395 2916079	48.9074 64.1917	33.9253 41.0107
			1.0% Saponin	7614606	3039943	50.7969	32.2521
			2.0% Saponin No Block	6165974	2849269 2671417	41.6942	38.7984 37.3806
	Blocking Step	Temp/Time	4°C, 30 min 4°C, 120 min	5067694 5032729	2089784 2042575	45.6442 45.9341	35.7788 35.4185
		Temp/Time	RT, 30 min RT, 120 min	5802683	2586804	43.9987	42.6722
			Calbindin	3832088	2382430	40.0723	54.0511
			PKCa		NA		
			Ribeye PSD95			-	
			Desmin				
Staining			SCGN	-			
		Protein Targets	Dystrophin GS			H	
	Primary Antibody		GFAP Islet1	N/A		N/A	N/A
			Connexin43	1		A I	
			Tomm20	-			
			CD31 Collagen IV				
			VGluT1 VGlut3	-		-	
			Tau				
			AF647 1:100	6073156	1867141	65.7862	
	Secondary Antibody	Fluorophore/Conc.	AF647 1:1000 AF647 1:5000	3739319 1188205	1475533 478033	43.9605 38.4452	N/A
	Secondary Antibody	Fluorophore/Conc.	CF568 1:100 CF568 1:1000	6326942 3430488	2239627 1453105	N/A	30.8011 35.4525
			CF568 1:5000	655764	445964	46 1414	29.7523
		Conc./Time	2% PFA, 10 min	6750477	2604387	48.1055	32.2951
	Post-Fixation		4% PFA, 10 min 1% PFA, 30 min	8659132 5374777	3367457 2091335	54.9271 55.6734	36.2804 33.1415
		Conc./Time	2% PFA, 30 min 4% PFA 30 min	7270614 8267553	2700060	54.2458 47.6018	42.6215
		Conc./Type	10mM Glycine	5330971	1691966	65.6889	55.4278
	Post-Quenching	Conc./Type	10mM NH3Cl	4791254	2077605	55.6635	46.1726
			100mM NH3CI 0mM BME	5210985 13030311	<u>1932965</u> 3553994	50.52240 78.36503	43.52333 61.88567
		BME	71.5mM BME	10612112	2539832	70.96677	46.30657
			286mM BME	9332914	2006797	73.10220	72.54603
		Catalase	20U Catalase 20U Catalase	7104374 7137281	1453545 2047426	77.87803 72.29080	71.28623 76.41330
		oddadoe	100U Catalase 500U Catalase	9595769 10195843	2075109 2537068	84.14287 71.02247	65.44140 64.64657
	GLUX		0U Glu. Ox. 10U Glu. Ox	7104374	1453545	77.87803	71.28623
		Glucose Ox.	20U Glu. Ox.	9595769	2075109	84.14287	65.44140
			40U Glu. Ox. 0mM MEA	10195843 10036933	2537068 2362114	71.02247 82.48007	64.64657 67.48763
		MEA	10mM MEA 20mM MEA	11484612 8917782	2540791 2567084	74.56673 71.94410	75.99387 66.66003
			40mM MEA	9962371	2736129	73.49853	75.60517
		BME	71.5mM BME	6632177	21/2067 2110500	75.79637	44.79063 55.62603
		DIVIC	143mM BME 286mM BME	6865185 6990130	1935796 2025152	71.76753 64.24200	63.42270 49.40647
			0U PCD ~0.09U PCD	1879393	1100339	50.68107 68.00433	42.61873
		PCD	~0.17U PCD	3176465	1258718	71.34050	60.59277
Imaging	PPT		~0.35U PCD 0mM MEA	2138172 4388805	1068920 1545313	53.55533 53.90987	44.69093 54.86183
		MEA	10mM MEA 20mM MEA	4151177 3063824	1465681 1336807	73.73497 70.74163	68.12620 56.03840
			40mM MEA	2834421	1417678	58.42780	54.92740
		PCA	1mM PCA	4425433	1679832	74.27553	74.12297
	L		2mm PCA 4mM PCA	3473750 2099282	1008543	72.93673 72.42587	55.05123 63.55333
		DME	0mM BME 71.5mM BME	6344927 6632177	2172067 2110500	63.9457 75.7964	44.7966 55.6260
		BME	143mM BME 286mM BME	6865185	1935796	71.7675	63.4227 49.4065
			0U Py. Ox.	5160037	1609622	69.9593	44.6356
		Pyranose Ox. MEA	10 Pý. Ox. 5U Py. Ox.	7348113 6057925	1/53947 1699933	85.9037 86.5024	59.7095 60.1876
	POC		10U Py. Ox. 0mM MEA	7268535 8838811	2161980 2172407	82.4528 85.3317	59.1651 60.0705
			10mM MEA	6045768	1873840	77.7011	52.7179
			40mM MEA	6479168	1771027	74.9191	50.7731
		Catalase	20U Catalase	6053582 8486951	1916488 2078212	67.6008 88.8667	57.5033 63.1553
			100U Catalase 500U Catalase	7462499 8097364	2063145 2179157	83.4481 79.8785	58.2703 50.1370
	Electron Sinks	Conc /Type	1mM Trolox	10297129	2172781	76.4754	82.0630
		оонолтуре	5mM Trolox	2270131	862325	70.5364	70.7546
		Conc./Type	1mM COT 2mM COT	7249814 8668903	2213086 1677820	68.4071 79.6488	58.4930 78.9307
			5mM COT	7412183	1543031	76.8966	62.1923
C	Optimized RAIN-STO	RM		12804183.3333	7082472.0000	61.1141	31.2234
2% PFA 4C 60 mir	n, 100mM Gly 4C 60n	nin, 5% NDS, 0.5% Tr	riton, 60min RT Block, 1:500 Pri I	Dil 12hr, 1:100 Sec Dil 60min, 4% I	PFA 30min, 100mM NH4CI 30n	nin, 1U Py. Ox.	
		143mM BME, 200U	Catalase, 40mM MEA, 2mM CO1	r, 200nm Step size,250f, 3 cycle, I	0.16µm, 25p		
							6
850000						1.50E+07	localizations
850000						1.50E+07	localizations
850000						1.50E+07	localizations
850000 						1.50E+07	localizations nm
850000 20						1.50E+07	localizations
850000 20 Abbreviations RT	room temperature					90	nm