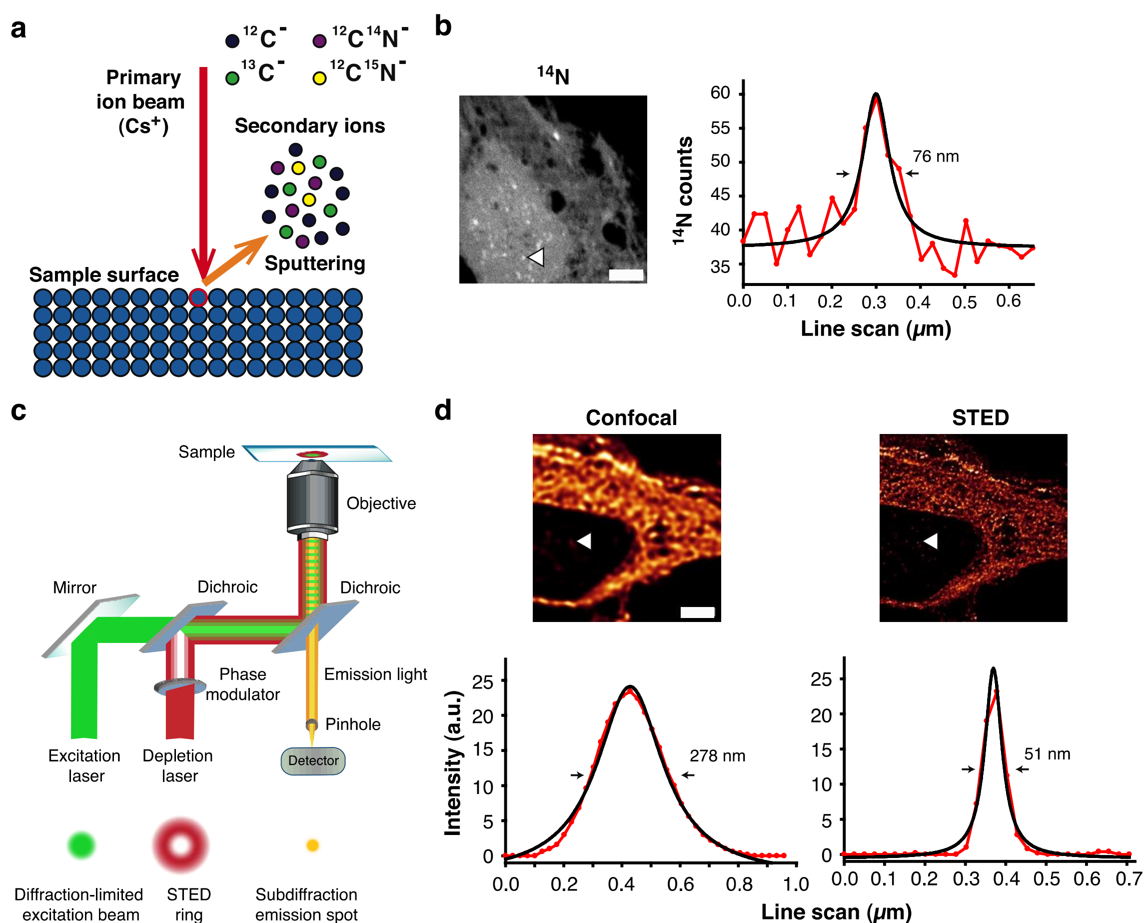
SUPPLEMENTARY INFORMATION

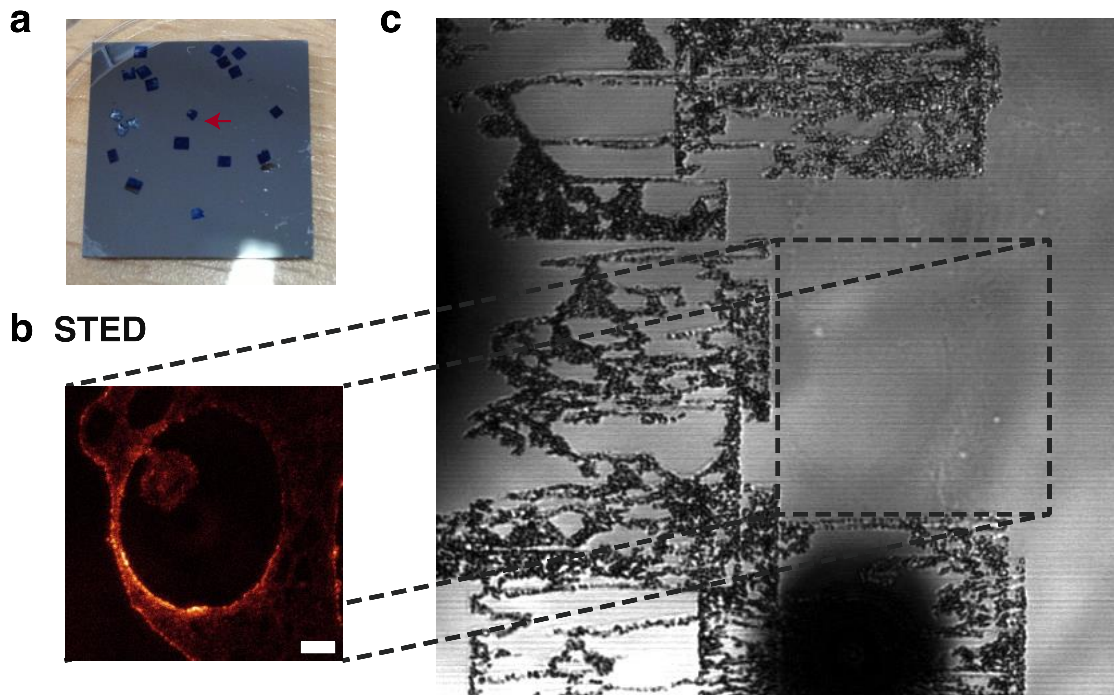
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Principles and examples for SIMS and STED imaging

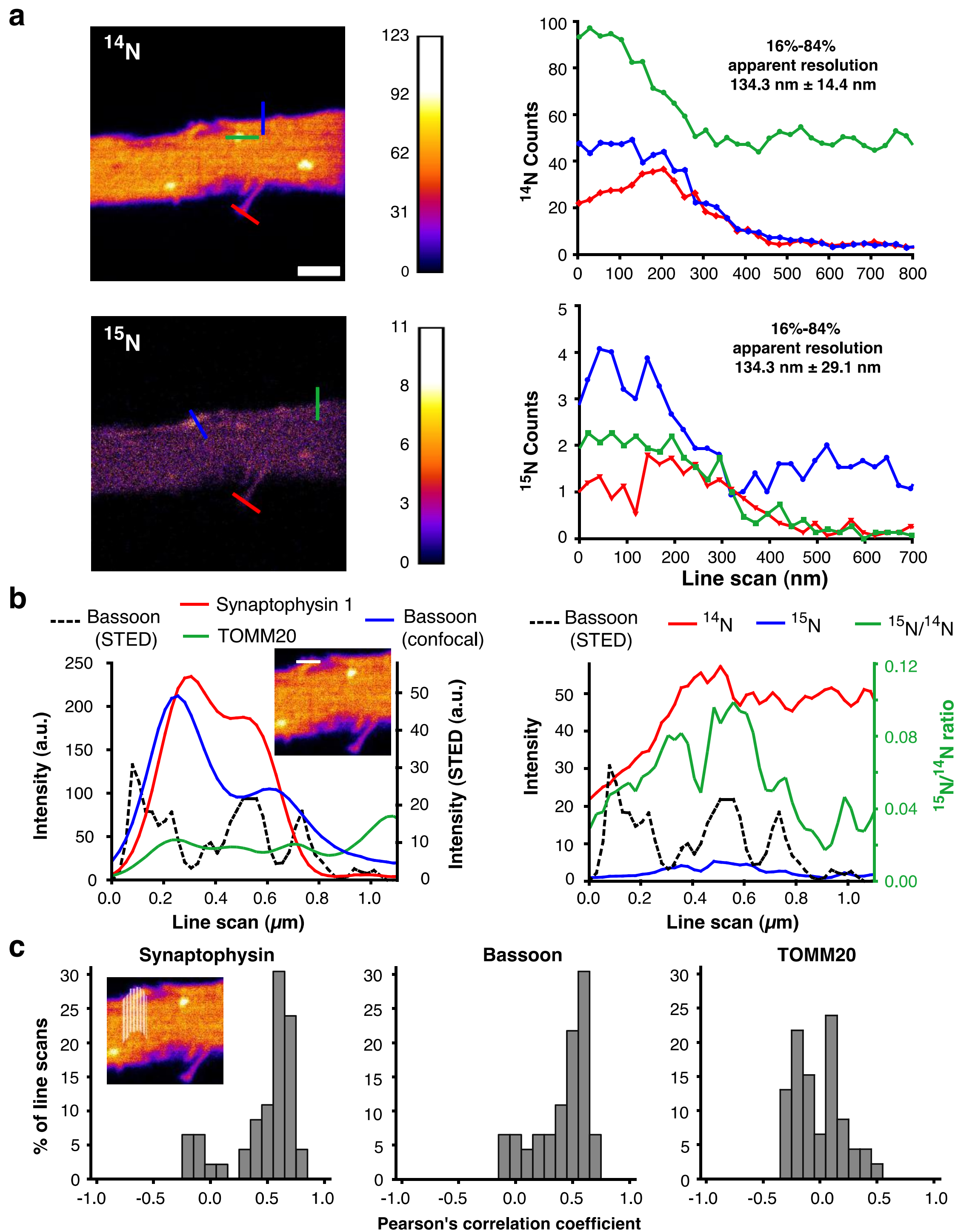
(a) SIMS imaging principle. The primary Cs^+ beam irradiates the sample, causing sputtering of secondary particles from the sample surface. These partly ionized particles (only a subset of which are depicted in the figure) are then identified by mass spectrometry. **(b)** An example SIMS image of $^{12}C^{14}N^-$ (labelled as ^{14}N) ions from a neuronal cell body section. The graph shows a line scan (red) along the spot indicated by the white arrowhead. The black curve represents a Lorentzian fit to the line scan with a full width at half maximum (FWHM) of 76 nm. Applying the 16%-84% criterion[6], instead of FWHM fitting, gives similar values. **(c)** STED imaging principle.

A toroid-shaped depletion beam (red, adjusted through a phase modulator plate) is superimposed on the excitation beam (green), resulting in stimulated emission depletion of all fluorophores except those located in the zero-intensity centre of the depletion beam. This combination of STED optics with the confocal elements (such as the detection pinhole or beam scanner) yields an effective fluorescent emission of sub-diffraction size (orange). **(d)** Examples of confocal and STED images of a neuronal cell body immunostained for calnexin, processed by deconvolution. The graphs show line scans (red) through the protein cluster indicated by the white arrowheads. Signal intensities are shown as arbitrary fluorescence units (a.u.). The FWHM obtained from Lorentzian fits (black) corresponds to 278 nm for the confocal and 51 nm for the STED images. Size bars in **b** and **d**, 2 μm.



Supplementary Figure 2. Sample preparation for correlated imaging

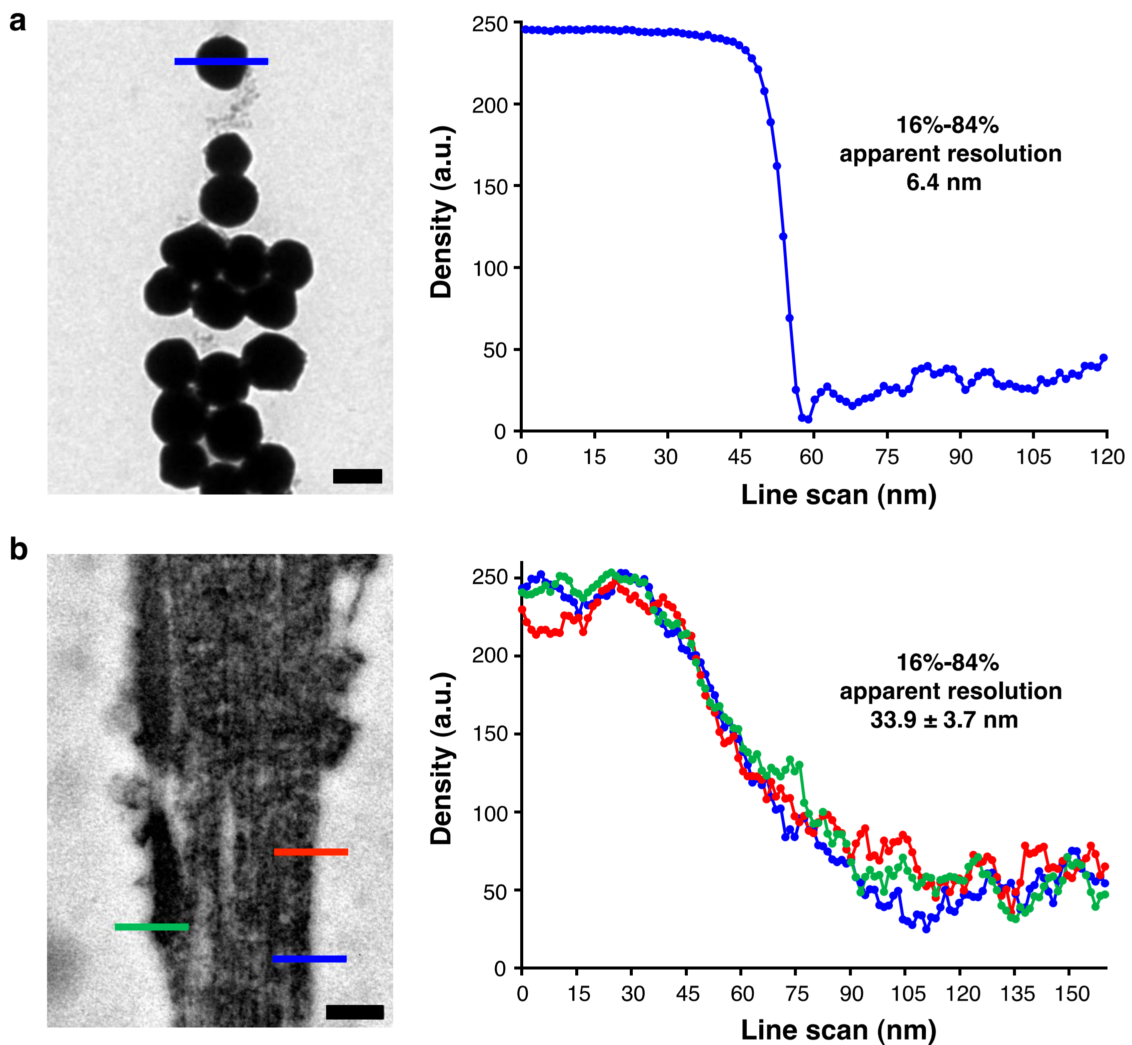
(a) The cells are embedded in LR White and 200-nm sections are cut with an ultramicrotome. The sections (blue rectangles; one indicated by the red arrow) are placed onto a silicon wafer (grey square). **(b)** The chosen cell area is imaged in the confocal or STED mode (only the STED image is shown here). Size bar, 2.5 µm. **(c)** Afterwards the multiphoton laser used for STED depletion (a Spectra-Physics Mai Tai tunable laser) is applied at maximum intensity, at 750 nm, onto the zone surrounding the imaged area, resulting in the heat-dependent deformation of the LR White resin. The deformed areas of section can be seen as black markings in a reflection image obtained from the bottom surface of the silicon wafer. The markings can later be recognized using a brightfield camera in the SIMS setup, enabling the identification of the area that needs to be imaged.



Supplementary Figure 3. Line scans performed on the correlated images from Figure 3

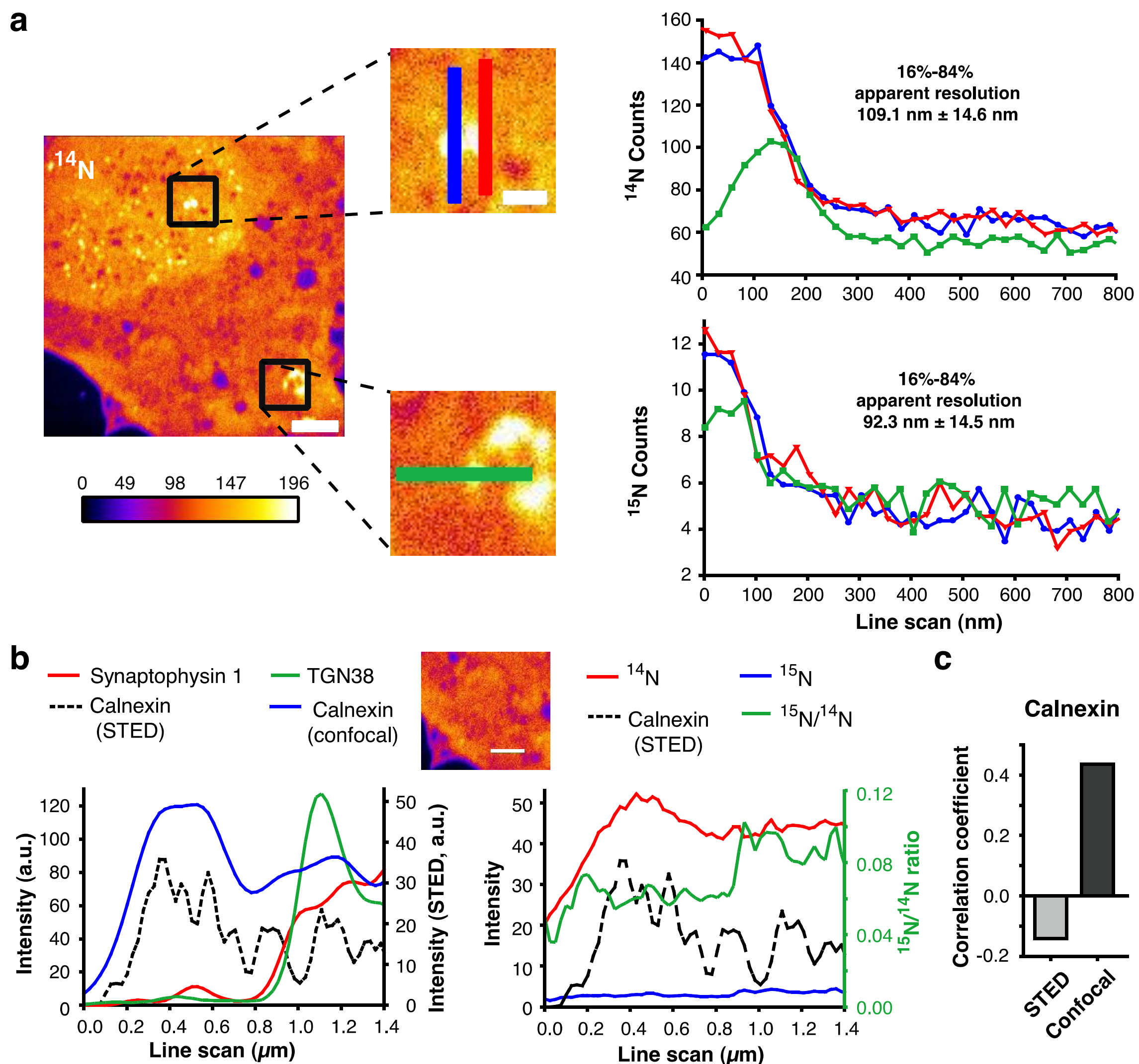
(a) To determine the lateral resolution, line scans were performed (as in **Fig. 1)** in the ^{14}N and ^{15}N images from **Fig. 3**, **(b)** Line scans were drawn through the synapse region (indicated by the white line on the inset image). The two graphs show the

different signals (fluorescence and SIMS). The fluorescence intensities are indicated in arbitrary units (a.u.). The SIMS data are indicated in counts. The SIMS data were smoothed using a moving average spanning 3 values. **(c)** We performed 46 adjacent vertical line scans over the synaptic area (marked by the white lines on the inset image), carefully avoiding the region at the edge of the axon. The Pearson's correlation coefficients of all of the line scans are now shown in the histograms. Both synaptophysin and bassoon correlate well with the $^{15}N/^{14}N$ ratio, while the mitochondria (TOMM20) signal does not. The correlation coefficients of synaptophysin and bassoon are significantly higher than that of mitochondria (one-way Anova test followed by a multiple comparison test; $p<0.05$; Matlab). Note, however, that ROI analyses, rather than line scans, are preferred in SIMS images, since the latter analysis is more prone to noise-induced errors.



Supplementary Figure 4. Analysis of the axonal edge by electron microscopy

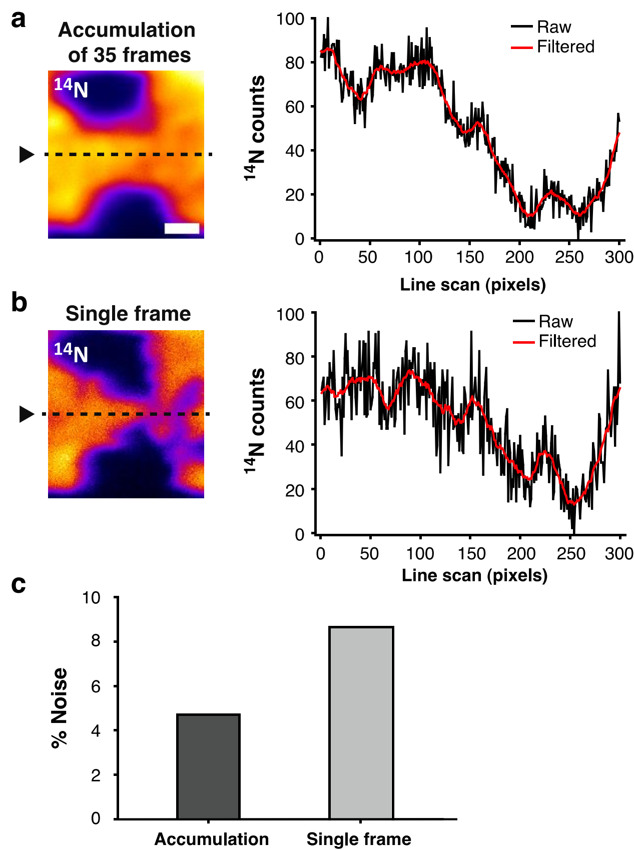
(a) 100 nm gold particles were imaged using a JEOL JEM1011 electron microscope (JEOL GmbH, Munich, Germany) and an Orius SC1000A 1 camera (Gatan Inc., Pleasanton, CA). Size bar, 100 nm. A line scan drawn over a gold particle indicates a sharp edge with an apparent resolution of 6.4 nm. **(b)** The same procedure was performed with an axonal segment. The edge of the axon is much less defined than that of the gold particle, indicating the presence of biological material at the outer boundary of the cell (such as extracellular matrix components). Size bar, 200 nm.



Supplementary Figure 5. Line scans performed on the correlated images from Figure 4

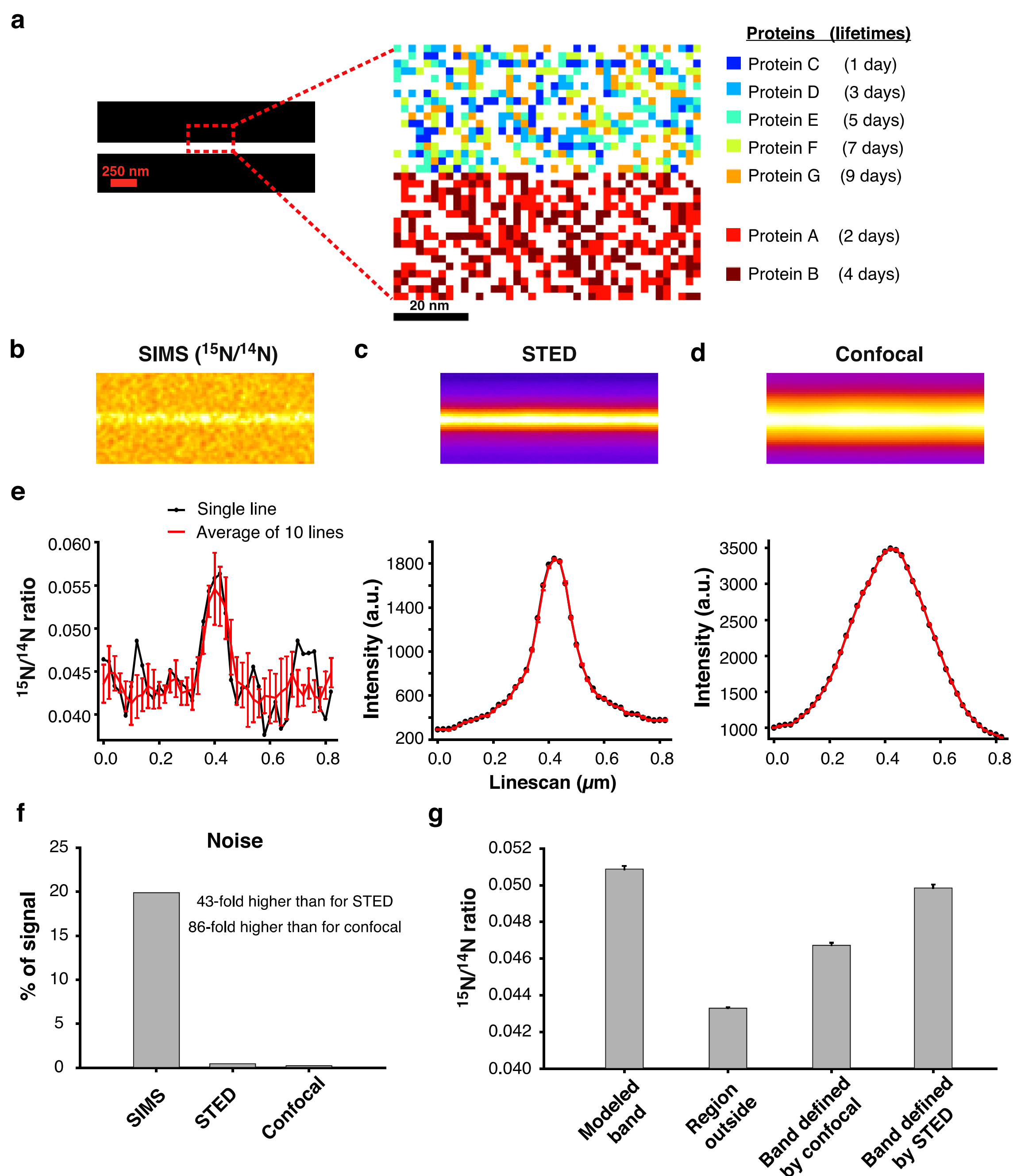
(a) Line scans were performed in the ^{14}N and ^{15}N images (from **Fig. 4**) along the marked areas, to determine the lateral resolution, as in **Fig. 1**. Size bars, 2 µm and 500 nm. **(b)** Line scans were drawn through the cell body region indicated by the white line on the inset image. The two graphs show the different signals (SIMS and fluorescence). The fluorescence intensities are indicated in arbitrary units (a.u.). The SIMS data are indicated in counts. The SIMS data were smoothed using a moving average spanning 3 values. **(c)** Pearson's correlation coefficients were calculated from the line scans in **b**, to quantify the correlation between calnexin and the $^{15}N/^{14}N$ ratio,

using the STED or confocal images of calnexin. The values were normalized to the maximum expected correlation. This example indicates that widely different conclusions can be derived from STED and confocal comparisons with the SIMS images. The noise level of the SIMS images, however, needs to be taken into account when interpreting such line scan analyses.



Supplementary Figure 6. Analysis of the effects of frame accumulation on SIMS imaging noise

(a) We accumulated 35 consecutive frames of ^{14}N images. We then performed the line scan indicated by the arrowhead. The ^{14}N counts along the scan were background-subtracted and normalized to 100%. To determine the noise levels along the scan, the values were filtered using a 15-pixel running average (red line). The noise is equivalent to the deviation from the filtered data. Size bar, 500 nm. **(b)** Same analysis, performed for a single ^{14}N frame. **(c)** The noise level, expressed as percentage, is compared for the two curves. The frame accumulation reduces noise by ~2-fold. However, this is a small gain, compared to the potential damage to the preparation caused by repeated exposure to the ion beam, and the possibility of uncontrolled drift during frame accumulation.



Supplementary Figure 7. The source of noise in metabolic protein labelling and SIMS imaging

(a) We generated an *in silico* model of a cellular membrane consisting of the following: a 100-nm wide horizontal band made of proteins A-B (white), bordered by a broader outside region, made of proteins C-G (black). We used realistic volumes for the proteins (2x2x 4-6 nm), which occupied approximately 50% of the membrane surface (in line with previous comprehensive studies of membranes[31]). The space

surrounding the individual proteins (white pixels in the zoom-in picture) is occupied by other cellular elements (lipids, sugars). We provided realistic lifetimes for the 7 proteins, as indicated by the colour code. Average lifetime of the proteins is 3 and 5 days for the white band and for the outside region, respectively. The modelled membrane was subjected to a 3-day incorporation of ^{15}N-leucine, which was taken up into each of the newly-formed proteins, according to their given lifetimes. The old proteins, which were made before the ^{15}N-leucine addition, present a $^{15}N/^{14}N$ ratio of 0.0036 (0.36%, Earth ratio). The new ones have a ratio of 0.1536 (15.36%, assuming that leucine makes for ~15% of all N atoms of the proteins). Scale bars, 250 nm for the red and 20 nm for the black (zoom-in). **(b)** We generated a SIMS image for this membrane by modelling a 50-nm broad Cs^+ beam. For that, $^{15}N/^{14}N$ ratio information was integrated using a 50-nm wide disk filter (Matlab). **(c-d)** We assumed that protein A was immunostained, and we generated STED **(c)** or confocal **(d)** images of it, integrating the protein positions with experimentally measured STED and confocal point-spread-functions (from the same microscope used in Figs. 1, 3 and 4). **(e)** Black lines indicate single vertical line scans for the SIMS, STED or confocal images. Red lines indicate averages of 10 adjacent scans (± s.d.). Note the much higher noise of the SIMS ratio image, created by the noise of ^{15}N incorporation. The noise level of the STED and confocal images is negligible. **(f)** A comparison of the noise levels, indicated as % of signal. **(g)** Despite the relatively large noise level in SIMS images, the differences in ratio between the central band and the outside region are clear: the modelled band has a $^{15}N/^{14}N$ ratio of ~0.05. The region outside has a ratio of ~0.043. We then used the confocal and STED images to identify the position of the central band, and measured the $^{15}N/^{14}N$ ratios at these positions. The confocal image provides a blurry reference region as the position of the band, which results in a ratio value that

is midway between the real values of the band and the outside region. The STED image provides a position that is almost identical to the real one, and therefore the resulting ratio value is only slightly lower than the real ratio. The bars indicate average values for the pixels in the measured area (+ s.e.m).

SUPPLEMENTARY DISCUSSION

Parameters tested to determine optimal antibody labelling and structure preservation conditions:

1. Fixation reagents.

Paraformaldehyde, in PBS or TBS buffers (concentrations of 2-4%, weight per volume). Results in good tissue preservation and allows for good epitope availability. No negative effects (such as loss of immunogenicity) were observed with the higher concentrations, and therefore 4% paraformaldehyde was used in most experiments. Substantially higher concentrations (up to 10%) did not appear to improve fixation.

Glutaraldehyde. Applied alone in PBS or TBS buffers (concentrations of 2-4%, weight per volume), it results in optimal preservation (especially clear when tested by electron microscopy). However, it removes most epitopes, and was therefore unusable for most immunofluorescence applications.

Glutaraldehyde, applied together with paraformaldehyde (0.1-0.2% glutaraldehyde, 2-4% paraformaldehyde). The combination of the fixation reagents does improve sample preservation (when compared to paraformaldehyde alone). However, even 0.1% glutaraldehyde results in a sizeable loss of epitopes. We do use this procedure for most experiments where tissue preservation needs to be optimal, but we cannot use it when all available epitopes should be labelled.

Methanol (100%, cooled to -20°C). Methanol fixation results in very poor preservation of organelle morphology, and therefore cannot be used in projects such as the present one (concerned with endosome structure). We use methanol fixation exclusively for testing antibodies that do not recognize aldehyde-fixed epitopes. In addition, as methanol results in the partial fragmentation of membranes, it would not improve staining in the present experiments.

High-pressure freezing followed by freeze-substitution and Lowacryl, LR White or LR Gold embedding. We tested these procedures for a total of 7 months, and obtained exclusively "spotty" images. As only the surface of the freeze-substituted sample sections is labelled by the antibodies, this is entirely to be expected. We also found that only ~10% of the antibodies we tested still functioned in labelling sections, and that background issues were also highly problematic.

2. Fixation time and temperature.

We experimented with fixation from ~10 minutes (for cell cultures), to 14 hours (overnight; typically for tissue samples). For cell cultures we found that fixation times of over ~20 minutes were sufficient for sample preservation; we typically use fixation times of ~45 minutes. Fixation temperature has been varied as follows: 0°C (in an ice-water bath), 4°C (refrigerator), 21-24°C (room temperature), 37°C (incubator) and short-time microwave heating (~70°C, for ~30 seconds). We found room temperature fixation to be adequate, although we do use ice-cold fixation for the first 15 minutes to inhibit any rapid cellular processes, especially in neuronal cultures (where this procedure is followed by a further 30 minutes of fixation at room temperature).

3. Sample permeabilization.

We used several different methods of sample permeabilization:

Triton X-100, applied in PBS or TBS, at concentrations ranging from 0.01 to 0.5% (volume per volume). Low concentrations (below ~0.05-0.1%) do not appear to permeabilize the samples efficiently, and accessibility of antibodies to the sample interior is therefore poor. We found the samples to lose proper morphology at concentrations substantially above 0.3%. We typically use 0.1% Triton for cell culture experiments.

Digitonin, applied at 40 μg/ml does not appear to be substantially different from Triton X-100.

Repeated freeze-thaw cycles (1 to 3 cycles). Permeabilization is obtained, and antibodies do access the sample. However, morphology does not seem to be optimal, at least for cell culture, and we also observed occasionally high background staining.

Permeabilization time is typically short – more than ~15-20 minutes are not necessary for cell cultures in our experiments.

4. Blocking.

We incubate the samples with blocking solutions, which inhibit the unspecific binding of the antibodies.

Bovine serum albumin (BSA). We tested it at ~0.1 to 10% concentrations, dissolved in PBS or TBS. It does inhibit non-specific binding of several well-described

antibodies (for example, it reduces extra-synaptic binding of antibodies directed to synaptic proteins). However, increasing BSA concentrations reduce specific binding as well, and we therefore chose ~1.5% for most experiments.

Tryptone/Peptone, alone or in combination with BSA, in concentrations of up to ~5%. This does reduce the binding of antibodies to glass coverslips, for example. High concentrations may also inhibit specific binding and therefore are not typically used in cell culture experiments.

Gelatin (up to ~2% concentrations). Blocks non-specific binding to coverslips efficiently. However, in the present project, where the emphasis has been on increasing signal (increasing antibody binding), we decided not to include this blocking reagent.

Sample blocking times have varied from ~14 hours, to no pre-blocking time (0 minutes). We chose here an incubation time of -15-20 minutes at room temperature (blocking reagents are also applied together with the antibody dilution).

5. Primary antibody labelling.

For immunostaining we apply the antibodies in blocking buffer. We start with a 1:100 dilution of antibody stocks (typically prepared at ~1 mg/ml), and we adjust the concentration according to the results – increase in the case of low signals, or decrease upon strong signals accompanied by strong background labelling. We show here that varying the antibody concentration over two orders of magnitude does not change the spotty aspect of antibody-stained preparations.

Antibody incubation time was varied between ~30 minutes and 14 hours. We found incubation times of ~1-2 hours to be sufficient for most applications, provided the antibody concentration is sufficiently high. Incubation temperature has been varied between 4°C and 37°C. Room temperature conditions (21-24°C) are sufficient for this step.

It is also possible to perform live uptake of antibodies in relatively high concentrations, in buffers lacking blocking reagents. This has been sufficient to detect short-lived events such as synaptic vesicle exocytosis[19,32]. The binding is specific; application of antibodies directed against intracellular epitopes results in negligible signal. Logically, the incubation time here is limited by the biological process to be investigated (typically several minutes up to ~1 hour).

6. Antibody washing.

We experimented with several buffers for antibody washing. Minimal buffers included PBS, TBS, low-salt PB (containing only 20 mM phosphate buffer) and high-salt PBS (containing 500 mM NaCl). Other buffers contain additionally blocking reagents such as BSA and tryptone/peptone. Washing time has been varied from 10 minutes to 14 hours. We found prolonged washing to produce little effect, especially if a second extensive washing period follows secondary antibody staining (see below). Therefore, we typically wash the primary antibody away from the preparation by 3-5 buffer changes (each for 5-10 minutes), in either PBS or a PBS-BSA buffer (1.5% BSA).

7. Secondary antibody labelling.

This procedure has been tested as above (see primary antibody labelling). We tested concentrations of secondary antibodies over three orders of magnitude, and found that for most applications good signal intensity is obtained at final concentrations of ~4-7.5 µg/ml (from stocks of Dianova antibodies, at 0.75 mg/ml in 50% glycerol in PBS). These antibody concentrations also avoid high background intensities (tested by secondary antibody application to samples not treated with primary antibodies). These conditions apply especially for Dianova or Jackson Immunoresearch antibodies (our preferred secondary antibody source). However, since this company does not commercialize Atto647N-labeled secondary antibodies, we purchase them from Sigma Aldrich. In previous years we also produced Atto647N-coupled antibodies by NHS-ester labelling of goat, sheep or donkey antibodies, either alone or with the help of the Göttingen company Synaptic Systems. For both our antibodies and the Sigma Aldrich ones, we adjust carefully the blocking buffer, so that the relatively high hydrophobicity of the Atto647N dye does not result in substantial background staining. Addition of BSA to the incubation buffer is critical. We tested extensively the concentrations/incubation times for Atto647N-labelled antibodies, and found that incubations of 0.5-1 hours and concentrations of up to ~100 µg/ml provide optimal results.

8. Final wash-off.

The preparations are now washed extensively, to remove any primary or secondary antibodies that were not specifically bound to the preparation. Several buffers were tested (see above, antibody washing). Wash-off was performed in different buffers, for ~30 minutes to several hours (especially for tissue samples). We found that after

an initial 30-45 minutes of washing, further washing steps are superfluous, especially if numerous buffer changes are performed. We currently perform several 3 to 5-minute buffer changes, first in high-salt PBS, followed by normal PBS. The addition of BSA to the first few buffer changes is useful for antibodies that give substantial background staining.

9. Sample embedding.

We tested several embedding media, such as DABCO-containing polyvinyl alcohol mounting media, Mowiöl-based media, 2,2'-thio-di-ethanol (TDE), Epon, LR White, LR Gold, or 2,4,6-Tris[bis(methoxymethyl)amino]-1,3,5-triazine (melamin). Only the latter three embedding media are suitable for Nano-SIMS procedures. LR White provided the best sample preservation, as judged by electron microscopy, and was therefore preferred in our study. Epon provides good sample preservation, but cannot be used in fluorescence microscopy, due to its high autofluorescence.

SUPPLEMENTARY REFERENCES

31. Takamori, S. *et al.* Molecular Anatomy of a Trafficking Organelle. *Cell* **127,** 831–846 (2006).

32. Opazo, F. *et al.* Limited Intermixing of Synaptic Vesicle Components upon Vesicle Recycling. *Traffic* **11,** 800–812 (2010).