The same synaptic vesicles drive active and spontaneous release

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Brief communication:

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Synaptic vesicles release neurotransmitter both actively (upon stimulation) and spontaneously (at rest). It has been long assumed that identical vesicles use both modes of release; however, recent evidence has challenged this view. Using several assays (FM dye imaging, pHluorin imaging and antibody-labeling of synaptotagmin), in neuromuscular preparations from *Drosophila*, frog and mouse as well as rat cultured neurons, we suggest that the same vesicles participate in active and spontaneous release.

Synaptic vesicles fuse with the neuronal plasma membrane to release their neurotransmitter contents both upon the arrival of action potentials (active release) and at rest (spontaneous release). It has been assumed for almost six decades that identical vesicles exocytose in both cases, an assumption which has been used to generate the quantal (vesicular) release theory[1]. This assumption is in agreement with the fact that prolonged active[2] or spontaneous release[3] can both release essentially all vesicles (see also[4,5]). The issue of whether the same vesicles can be released both spontaneously and actively has been tested recently by investigating the loading and unloading of styryl (FM) dyes during vesicle recycling[6]. In cultured hippocampal neurons, the vesicles loaded with FM dyes during active or spontaneous recycling were unloaded efficiently only by the same release paradigm. Also, inhibiting synaptic vesicle re-acidification (and therefore refilling with neurotransmitter) by use of folimycin during spontaneous release specifically depleted the spontaneous pool[6]. Surprisingly, both of these findings have been later contested in similar experiments – FM dyes taken up either at rest or during stimulation could be released with identical kinetics, and the inhibition of re-acidification during depolarization led to a cessation of spontaneous release[7] (see also[5]). However, later studies have again found different FM dye release kinetics when using different loading paradigms[8, 9], and labeling experiments using the expression of a biotinylated variant of the synaptic vesicle marker VAMP2 (synaptobrevin) also supported the hypothesis that spontaneously and actively recycling vesicles are different[10].

We tested here this controversial issue by combining several fluorescence imaging assays. We sought to reproduce the FM dye loading/unloading paradigms used in the past. A number of FM dyes have been used, including FM 1-43 and FM 2-10[6–9]; both dyes have provided evidence for a difference between the spontaneously and actively recycling vesicles[8,9]. It has been recently suggested that FM 2-10 reports this difference better than FM 1-43[9]. However, as FM 2-10 is less bright when compared to FM 1-43, it is typically used at very high concentrations (400 µM; ~4-fold higher than its membrane dissociation constant[11]). Since the FM dyes have a detergent-like structure, they inhibit vesicle recycling at high concentrations[11]. We therefore decided to employ FM 1-43 in our work (the concentration typically used, ~10 µM, is 3-fold lower than the dissociation constant[11]).

The systematic investigation of all possible loading/unloading paradigms (active-active, active-spontaneous, spontaneous-active and spontaneous-spontaneous) had never been applied in past studies addressing the issue[6–10]. We tested four different synaptic preparations: the neuromuscular junctions (NMJs) of mouse, frog and *Drosophila* third instar larvae, cultured hippocampal neurons. The preparations were incubated with 10 µM FM 1-43 during electrical stimulation (5–10 sec at 10–30 Hz for NMJs, 30sec at 20 Hz for the cultured neurons), and the dye was then unloaded either by electrical stimulation or at rest (spontaneously). Conversely, FM 1-43 was loaded at rest (incubation times of 15 minutes for hippocampal cultures, and 30–60 minutes for the NMJs, see Supplementary Materials and Methods) and was then unloaded again either actively or spontaneously (Fig. 1a). All procedures (loading/unloading) are well within the dynamic range (see Suppl. Fig. 1) of the preparations, i.e. they do not saturate the vesicle pools involved (none of the paradigms completely release the pools involved). All loading/unloading combinations resulted in substantial FM 1-43 release (Fig. 1b and Suppl. Fig. 2; note that as none of our unloading paradigms are saturating, full unloading cannot take place). Both actively and spontaneously loaded preparations lost similar amounts of fluorescence, either at rest or under stimulation (Fig. 1c). Finally, note that photobleaching and loss of dye through leakage were negligible (Supplementary Figure 3).

This result is in agreement with a model in which the same vesicles recycle both spontaneously and actively. However, as most of the previous work[6–10] dealt with hippocampal cultures, we employed several additional experimental approaches in this model system. We first designed an assay in which the synaptic vesicle marker synaptotagmin was identified by biotinylated antibodies during one round of loading (actively for 30 seconds at 20 Hz, or spontaneously for 15 minutes). After removal of the antibody, the vesicles were subjected to a second round of loading (again actively or spontaneously) in presence of fluorescently labeled streptavidin (Fig. 2a). This paradigm ensures selective labeling of vesicles which recycled during both loading rounds (Fig. 2b). All active/spontaneous labeling combinations provided the same amount of fluorescence (Fig. 2c).

To directly visualize the vesicles, we modified this assay by employing two different synaptotagmin antibodies, applied during the two loading rounds (Fig. 2d), and imaged the vesicles using a newly developed improvement of stimulated emission depletion microscopy, isoSTED[12], which allows a three-dimensional resolution on the size of single synaptic vesicles (~40–50 nm). Many small vesicle-sized spots could be identified (Fig. 2e). Their colocalization in the two color channels was analyzed by drawing line-scans through the individual spots (Fig. 2f) and calculating the corresponding Pearson's correlation coefficients; similar distributions were obtained for all active/spontaneous labeling combinations (Fig. 2g). About 30–40% of all red vesicles were also green labeled (Supp. Fig. 5), for all conditions.

Finally, to test whether overexpressed synaptic proteins would behave differently (see[10]), we used the well-described synaptopHluorin[13] (spH) construct, in which a pH-sensitive GFP moiety is attached to the intravesicular domain of VAMP2 (synaptobrevin-2). The low pH within the synaptic vesicle quenches synaptopHluorin, with fluorescence increasing strongly upon the pH shift associated with exocytosis[13]. We visualized vesicle populations using a quenching approach: spH-expressing neurons were stimulated in presence of folimycin, to inhibit re-acidification, which allowed us to measure the size of the vesicle pool released actively. After allowing for complete compensatory endocytosis (90 s) we applied bromophenol blue (BPB, 2mM) to quench the remaining surface spH fluorescence[14]. The non-acidified vesicles that had been endocytosed remained fluorescent. We then either applied a second round of stimulation or allowed the preparations to release spontaneously for 15 minutes (both still in presence of BPB). Under these conditions, the exocytosis of non-acidified vesicles quenches their fluorescence, and thus provides a measurement for the re-use of these vesicles (protocol described in Fig. 3a). Both active and spontaneous recycling in presence of BPB resulted in quenching (Fig. 3b), to similar levels (Fig. 3c), consistent with our other findings.

Our results indicate that the same lipid membranes, the same synaptotagmin molecules and the same synaptopHluorin-labeled vesicles are used both spontaneously and actively. While there still could be conditions that favor (in a limited way) some bias toward how synaptic vesicles form following one type of stimulation, and how they are later released, our results indicate that there is no strict segregation between vesicles used for evoked and spontaneous release. This conclusion disagrees with some of the previously published works[6, 8–10]. The source of this discrepancy is not completely clear, although pitfalls of the data analysis methods used in some of the previous studies have already been pointed to[7]. Also, recent work[10] has employed multiple rounds of high potassium depolarization for testing vesicle pool identity – a non-physiological treatment which saturates release, and may have damaging effects. Finally, many of the previous studies[6–10] failed to address all combinations of sequential active and spontaneous release – which in our view is essential to obtaining reliable conclusions.

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Author contributions: BGW, TWG and SOR designed and performed experiments. BGW and TWG evaluated the manuscript. SOR supervised the project and wrote the manuscript.

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Figure Legends

Figure 1. Synaptic vesicle recycling visualized with FM 1-43.
(a) Experimental procedure: Preparations were loaded with FM 1-43 either actively (electrical stimulation) or spontaneously. After a brief wash (10 minutes, room temperature) the preparations were imaged, followed by unloading either spontaneously or actively.
(b) Typical images of mouse NMJ preparations (see Suppl. Fig. 1 for the other preparations). Scale bar: 10 µm. All image pairs are scaled identically.
(c) Fraction of fluorescence decrease after unloading, for the four experimental loading/unloading combinations (A-A, active loading-active unloading; A-S, active loading-spontaneous unloading; S-S, spontaneous loading-spontaneous unloading; S-A, spontaneous loading-active unloading). Bars show average ±SEM of 4 to 10 independent experiments (on average, 15 synapses were analyzed in each experiment). The exact loading/unloading protocols are presented under Supplementary Methods. No statistically significant differences could be found ($p>0.05$, one-way ANOVA tests). As the dye washing time for hippocampal cultures (10 minutes) was close to the spontaneous release time (15 minutes), we reproduced these experiments with a shorter washing time (2 minutes, Supplementary Figure 4).

Figure 2. Repeated recycling visualized with synaptotagmin antibodies.
(a)-(c) Single-label marking of synaptic vesicles.
(a) The preparation is incubated with biotinylated antibodies recognizing the lumenal domain of synaptotagmin. This antibody is internalized upon active or spontaneous compensatory endocytosis. Antibodies bound to surface synaptotagmin molecules which were not endocytosed were then blocked with unlabeled streptavidin (gray). In a subsequent period of active or spontaneous exocytosis Cy3-conjugated streptavidin was applied, thus labeling vesicles which had recycled during both periods of release.
(b) Typical images. Scale bar: 40 µm.
(c) Average intensity ±SEM for 4 independent experiments. No significant differences could be found ($p>0.7$, one-way ANOVA test).
(d)-(g) Double-label marking of synaptic vesicles.
(d) The preparation is incubated with unlabeled rabbit synaptotagmin antibodies (green), which label the vesicles recycling in a first round of active or spontaneous release. Unlabeled mouse synaptotagmin antibodies (black, thick drawing) are applied to block surface epitopes. During a second round of release (active or spontaneous) fluorescently coupled mouse synaptotagmin antibodies (Atto647N, magenta, thick drawing) are applied. After fixation and permeabilization the rabbit antibody is detected by conventional immunostaining (anti-rabbit secondary antibodies fluorescently labeled with Dyomics 480XL, green). Vesicle membranes containing both antibodies (both colors) must have recycled during both release rounds.
(e) Typical isoSTED images. Scale bar: 0.5 µm. Arrowheads indicate double-labeled vesicles.
(f) Horizontal line scans through the vesicles indicated in (e). The full width at half maximum (FWHM), obtained from fitting Gaussian curves to the data, is indicated as a measure of the spot size.
(g) Correlation between Syt1 (green) and Atto647N (magenta) positive vesicles (cumulative histograms). 128–174 punctae were analyzed for each condition, from two independent experiments. Inset shows the average Pearson's correlation coefficient (±SEM). No significant differences between the A-A /A-S, or S-A/S-S labeling conditions could be found ($p>0.15$, t-tests).

Figure 3. Synaptic vesicle recycling visualized with synaptopHluorin.
(a) Experimental paradigm. SynaptopHluorin-expressing neurons are stimulated in presence of folimycin, to inhibit re-acidification of newly endocytosed vesicles, leading to a fluorescence increase (dF1). Application of bromophenol blue (BPB) quenches surface fluorescence, while the newly endocytosed, non-acidified vesicles remain fluorescent. Following a 1-minute break (to allow for thorough penetration of the BPB into the cultures) a second electrical stimulation is performed (left), or the preparation is incubated for 15 minutes at rest (right). SynaptopHluorin fluorescence of non-acidified vesicles is quenched when they are exocytosed in presence of BPB (dF2). Numbers indicate points of image acquisition.
(b) Typical images of hippocampal cultures stimulated as described in (a), and then releasing in BPB either actively (top) or spontaneously (bottom). The arrowheads point to representative synapses. Image numbers refer to different experimental steps as indicated in (a). Scale bar: 10 µm.
(c) Comparison between the fluorescence increase (amount of endocytosed vesicles) under stimulation in presence of folimycin (dF1), and the fluorescence loss by exocytosis from this pool in BPB (dF2) either actively (open symbol) or spontaneously (closed symbol). Inset indicates the fraction of the active pool which was re-released in BPB (dF2/dF1; average ±SEM); no significant differences

were found ($p>0.7$, t-test). 263 and 164 (A-S) boutons have been analyzed, from 2 independent experiments.

SI Guide

Supplementary Item & Number	Title or Caption
Supplementary Figure 1	FM 1-43 loading in the different preparations.
Supplementary Figure 2	Synaptic vesicle recycling visualized with FM 1-43 at frog and *Drosophila* NMJs and hippocampal cultures.
Supplementary Figure 3	Loss of FM dye through photobleaching or leakage is negligible.
Supplementary Figure 4	FM1-43 loading/unloading in hippocampal cultures with shorter washing times.
Supplementary Figure 5	Percentage of double-labeled vesicles in the different loading combinations in isoSTED imaging.
Supplementary Materials and Methods	

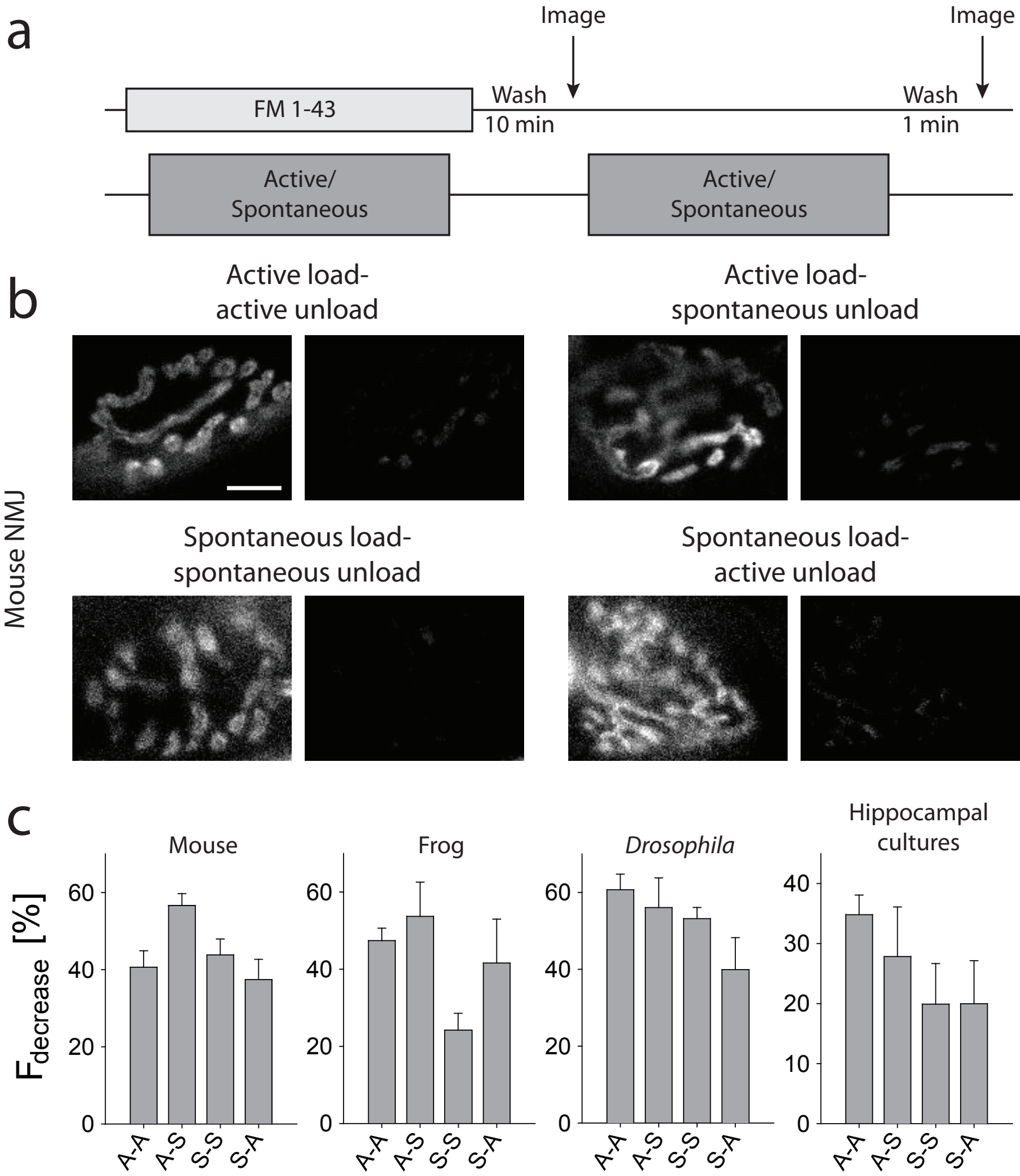
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Image
Image
FM 1-43
Wash
10 min
Wash
1 min
Active/
Spontaneous
Active/
Spontaneous
b
Active load-
active unload
Active load-
spontaneous unload
Spontaneous load-
spontaneous unload
Spontaneous load-
active unload
Mouse NMJ
c
Mouse
Frog
Drosophila
Hippocampal
cultures
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40
20
0
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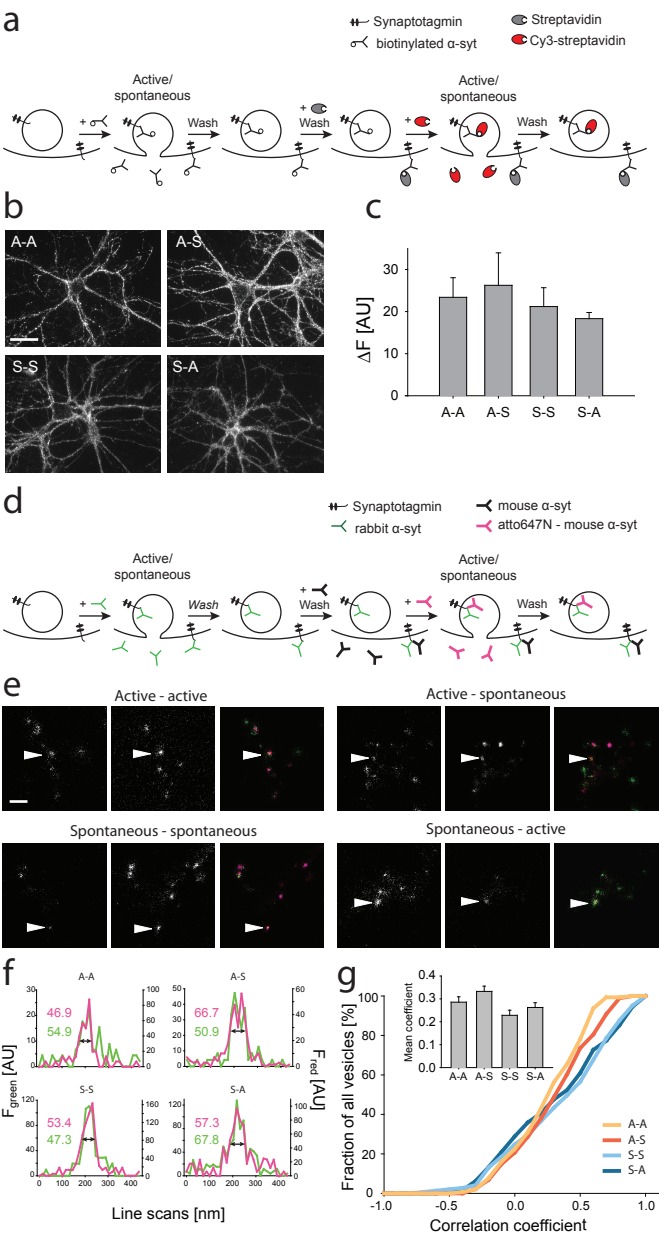
30
20
10
0
A-A
A-S
S-S
S-A


a
Synaptotagmin
biotinylated α-syt
Streptavidin
Cy3-streptavidin
Active/
spontaneous
Wash
Wash
Active/
spontaneous
Wash
b
A-A
A-S
S-S
S-A
c
ΔF [AU]
30
20
10
0
A-A
A-S
S-S
S-A
d
Synaptotagmin
rabbit α-syt
mouse α-syt
atto647N - mouse α-syt
Active/
spontaneous
Wash
Wash
Active/
spontaneous
Wash
e
Active - active
Active - spontaneous
Spontaneous - spontaneous
Spontaneous - active
f
A-A
46.9
54.9
A-S
66.7
50.9
S-S
53.4
47.3
S-A
57.3
67.8
F green [AU]
F red [AU]
Line scans [nm]
g
Mean coefficient
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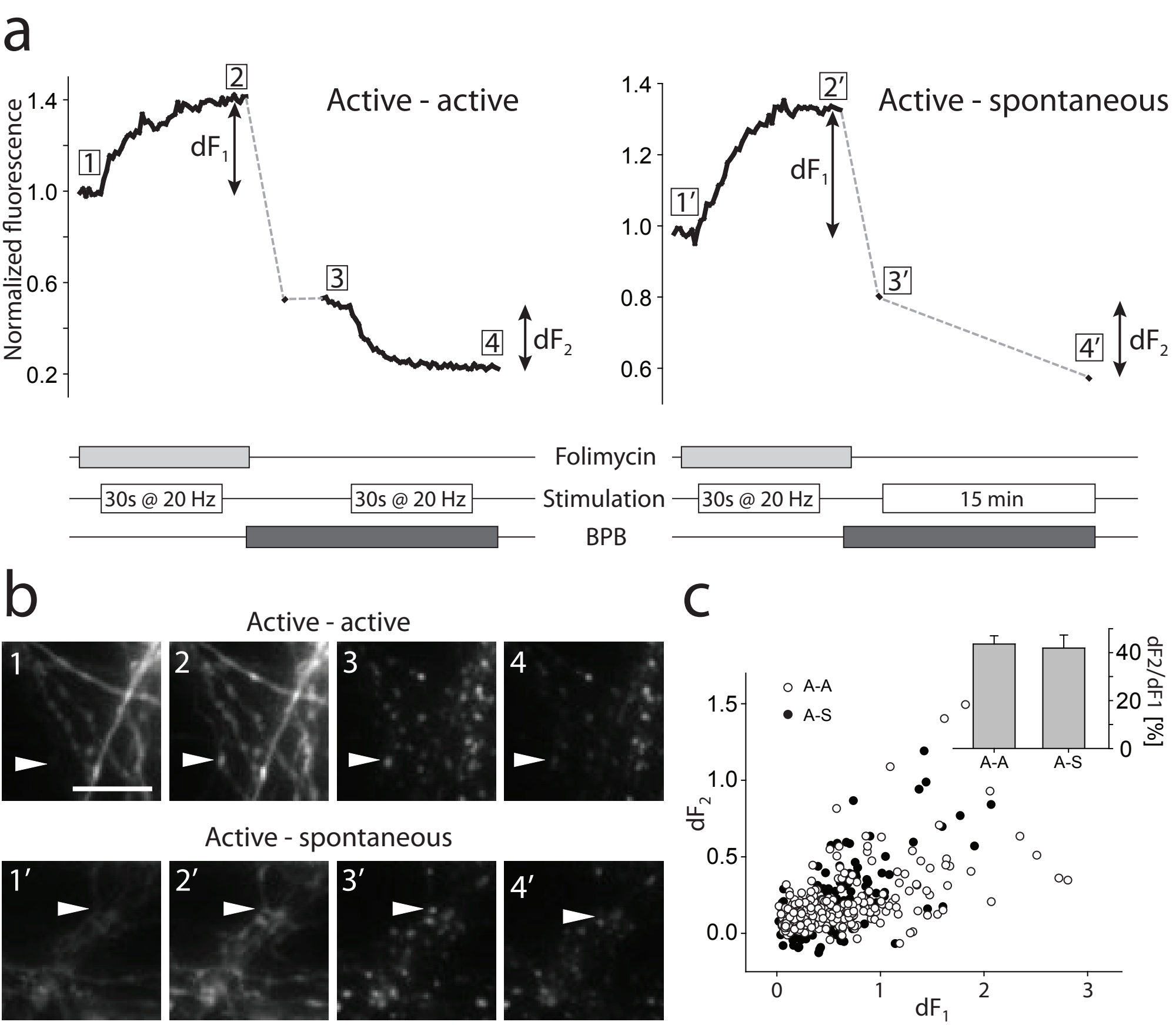
0.3
0.2
0.1
0.0
A-A
A-S
S-S
S-A
Fraction of all vesicles [%]
100
80
60
40
20
0
-1.0
-0.5
0.0
0.5
1.0
Correlation coefficient
A-A
A-S
S-S
S-A


a
Active - active
Active - spontaneous
Normalized fluorescence
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1.0
0.6
0.2
1.2
0.8
1
2
3
4
1′
2′
3′
4′
dF1
dF2
Folimycin
Stimulation
BPB
30s @ 20 Hz
30s @ 20 Hz
30s @ 20 Hz
15 min
b
Active - active
Active - spontaneous
c
A-A
A-S
dF2/dF1 [%]
40
20
0
dF2
dF1
1.5
1.0
0.5
0.0
0
1
2
3