Comment on "The Dynamic Control of Kiss-and-Run and Vesicular Reuse Probed with Single Nanoparticles"

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Zhang *et al.* (Research Articles, 13 March 2009, p. 1448) reported that synaptic vesicles usually release neurotransmitter through a kiss-and-run mechanism occurring within 1 second but that full collapse of the vesicles becomes more prevalent with repeated stimuli. We report that the kinetics of vesicle retrieval do not change during a stimulus train, with endocytosis occurring in 10 to 15 seconds.

The process by which synaptic vesicles interact with the surface membrane to release neurotransmitter has been debated since Katz described his quantal theory of neurotransmission (*1*). Do vesicles collapse into the surface membrane? Or can neurotransmitter release occur through a short-lived fusion pore by a process of kiss-and-run? This fundamental issue has been difficult to resolve because the techniques used to investigate it provide measurements open to different interpretations (*2*). One of the most direct approaches for assaying vesicle fusion and retrieval is expression of pH-sensitive green fluorescent protein molecules (pHluorins) in the lumen of synaptic vesicles by fusion to membrane proteins (*3*, *4*). When exocytosis is triggered, the loss of protons from the vesicle interior unquenches the pHluorin to generate a fluorescence increase, and this signal decays when the vesicle is reacidified after retrieval. Recent studies using pHluorins report only full collapse of synaptic vesicles, with retrieval occurring in 10 to 15 s through a clathrin-dependent mechanism (*5*–*7*). In contrast, Zhang *et al.* (*8*) reported the dominance of kiss-and-run under certain conditions by using quantum dots to assay fusion.

The fluorescence of quantum dots is also pH-dependent and increased by 15% on vesicle fusion. In Zhang *et al.* (*8*), recovery of the signal followed two patterns: A rapid return to baseline was interpreted as retention of the particle by kiss-and-run, while a much larger fall below baseline was interpreted as escape of the particle from the synaptic cleft after vesicle collapse. On this basis, the authors concluded that kiss-and-run (0.5 to 1 s) occurs with a probability of ~60% if the synapse is activated after a period of rest, falling to ~10% after the sixth stimulus delivered at 0.1 Hz. The observation that transient signals becomes less prevalent at 0.1 Hz might lead one to expect that their steady-state probability will be even lower when the frequency of stimulation is increased further, but the opposite was observed: a 3- to 4-fold increase in the probability of transient signals at 10 Hz [figure 4C in (*8*)]. Zhang *et al.* suggest that this biphasic modulation of the fusion process by stimulation frequency makes the protocol we used to investigate endocytosis inappropriate for detecting kiss-and-run using synaptophysin-pHluorin (sypHy) (*5*). In order to measure the pHluorin signal generated by a single action potential (AP) while avoiding bias introduced by selection of events from a noisy background, we delivered single APs at very low frequency (0.02 Hz) and then simply averaged traces to obtain the behavior of a population of vesicles. Using simulations, Zhang *et al.* (*8*) concluded that this approach will not have the sensitivity to detect fast kiss-and-run unless the response to the first AP after a period of rest is separated from responses to later stimuli. We have examined this prediction experimentally (*9*, *10*) and find that it does not hold.

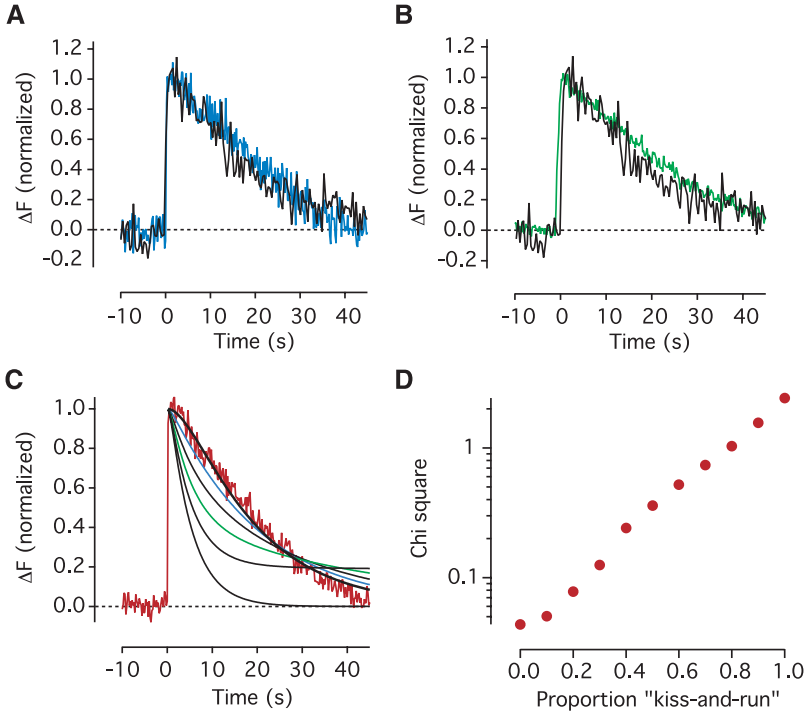

Fig. 1. Analysis of endocytosis using synaptophysin-pHluorin (sypHy). (**A**) Averaged sypHy response to the first AP in a train (black trace, $n = 139$) compared to the 8th to 10th APs (blue trace, $n = 443$) when APs are repeated at 0.02 Hz. The average for the first AP has been decimated to a frequency of 2.5 Hz from an original acquisition rate of 5 Hz. (**B**) Averaged sypHy response to the first AP in a train (black trace, $n = 139$) compared to a burst of 20 APs at 20 Hz (green trace, $n = 79$). There was no obvious change in the sypHy signal under these different conditions. (**C**) The decay of sypHy fluorescence modeled with different proportions of fast and slow endocytosis ($\tau_{KR} = 0.95$ s; τ_{FF} = open parameter) followed by reacidification ($\tau_r = 5$ s). The model predictions were fit with a least sum of squares method to the average fluorescence trace from 1480 single APs (red). The model with 100% slow endocytosis was most similar to the data (heavy black line). Colored lines are models with 20% (blue) and 60% (green) kiss-and-run, and both clearly deviate from measurements. The expected time course of decline in the sypHy signal is similar for any value of $\tau_{KR} < 2$ s. The experimental trace was collected at a sampling frequency of 5 Hz, which might not be sufficient for precisely quantifying the speed of any fast component of endocytosis occurring in a fraction of a second but is quite sufficient for the detection of any significant amount of endocytosis occurring with a time constant of 2 s or less. (**D**) Graph relating chi-square values of the fit to the model (log scale) to the proportion of kiss-and-run. Assuming just 10 to 20% kiss-and-run caused an increase in chi-square value (i.e., a deterioration in the quality of the fit).

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First, we averaged responses to the first AP delivered after ~5 min rest, when Zhang *et al*. (*8*) predict ~60% of fusion events to occur by kiss-and-run (Fig. 1A, black trace). We then made a separate average of the responses to the 8th to 10th AP during stimulation at 0.02 Hz, when ~20% of events are predicted to be kiss-and-run (Fig. 1A, blue trace). However, the time-course of recovery was indistinguishable in the two cases, indicating that the history of stimulation did not alter the kinetics of endocytosis at this low frequency. Recovery was best accounted for by a single mode of endocytosis with a time constant of 14 s (Fig. 1C). As a further test, we averaged the sypHy signal observed in response to a burst of 20 APs at 20 Hz, when more vesicles are released (Fig. 1B, green trace). The prediction of Zhang *et al*. is that ~80% of events should be kiss-and-run (*8*), but again, a fast mode of endocytosis could not be detected.

Zhang *et al*. (*8*) also suggested that pHluorins may not be suited to detection of fast endocytosis because the fall in fluorescence lags after endocytosis by the time required to reacidify the vesicle—about 5 s at room temperature. However, reacidification occurs in ~1 s at 35°C, when it is still not possible to detect a fast mode of endocytosis (*11*). We also analyzed pHluorin responses using a model in which there are two competing modes of endocytosis, as suggested by Zhang *et al*. (*8*): fast (τ_{KR} = 0.95 s) and slow (τ_{FF} = open parameter). The equation describing fluorescence recovery is

$$F(t) = \frac{KR}{k_r - k_{KR}}\left[k_r\left(1 - e^{-k_{KR}t}\right) - k_{KR}\left(1 - e^{-k_r t}\right)\right] +$$

$$\frac{1 - KR}{k_r - k_{FF}}\left[k_r\left(1 - e^{-k_{FF}t}\right) - k_{FF}\left(1 - e^{-k_r t}\right)\right]$$

where KR is the proportion of vesicles undergoing kiss-and-run and k_r is the rate-constant of acidification (0.2 s^{-1} at room temperature). Figure 1C shows the least-squares fit of the model to the average response to a single AP for different KR. The lowest chi-square value was obtained assuming that there is no kiss-and-run, whereas assuming 10 to 20% kiss-and-run caused a significant deterioration in the quality of the fit (Fig. 1D). A very large deviation from the experimental trace and increase in the chi-square value was observed assuming 60% kiss-and-run (Fig. 1C, green trace), as estimated by Zhang *et al*. (*8*). The reacidification time of 5 s would not prevent the detection of fast endocytosis using pHluorin signals acquired at 5 Hz. Measurements made with sypHy therefore argue against a major component of kiss-and-run at hippocampal synapses, even under the very specific conditions proposed in (*8*). We conclude that the dominant mechanism of endocytosis in hippocampal synapses occurs with τ = 14 s at room temperature, a conclusion also reached by Balaji and Ryan (*6*) using VGlut1-pHluorin.

It will be important to understand why the interpretations of measurements made using pHluorins and quantum dots do not coincide. We suspect that this will become clearer with further characterization of the two techniques. The advantages of pHluorin reporters is that they are targeted to all synaptic vesicles by normal cellular processes, and they remain associated with vesicle membrane through multiple rounds of exocytosis and endocytosis. In comparison, quantum dots are large (~15 nm) relative to synaptic vesicles (~24 nm diameter lumen) and the synaptic cleft (width ~20 nm) and must be loaded by a period of concerted stimulation. It is also unknown whether quantum dots hinder collapse of the vesicle membrane after fusion, when the dense particle is sandwiched between pre- and postsynaptic membranes. In particular, we question the assumption that a transient fluorescent signal with retention of the particle always equates with kiss-and-run. If exogenously applied quantum dots are taken up into the synapse during stimulation, then why should not one that has just been released directly into the synaptic cleft?

References and Notes

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9. Dissociated neurons from hippocampi dissected from E18 rats were cocultured with glia and transfected to express a plasmid coding for sypHy using Lipofectamine2000 after 8 days in vitro (DIV). Live fluorescent imaging was carried out at 14 to 21 DIV using an inverted microscope equipped with a Photometrics Cascade 512 B camera, frame rate 5 Hz. Action potentials were evoked by field stimulation. For details, see (*10*).
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Response to Comment on "The Dynamic Control of Kiss-and-Run and Vesicular Reuse Probed with Single Nanoparticles"

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Granseth *et al*. argue that vesicle retrieval at hippocampal synapses is fully accounted for by a single mode of endocytosis. However, their assay focused on readily releasable pool vesicles (RRP), not RRP + reserve pool vesicles in tandem, and therefore cannot detect pool-dependent changes in vesicle-retrieval kinetics during a stimulus train. Using a probe similar to theirs, we observed rapid vesicle retrieval consistent with kiss-and-run fusion.

Vesicle recycling is critical for continued synaptic transmission at nerve terminals. We used single quantum dots (Qdots) to investigate synaptic vesicle behavior and found that kiss-and-run (K&R) vesicle fusion dominates at the beginning of stimulus trains and then gradually gives way to classical full-collapse fusion (FCF) (*1*). Based on previously reported data (*2*), Granseth *et al*. (*3*) argue that the pattern of vesicle recycling using pooled signals from a pH-sensitive green fluorescent protein (GFP) probe (synaptophysin-pHluorin or sypHy) is best explained by FCF only. They averaged the sypHy signals to the first and the 8th to 10th stimulus pulses during the 0.02-Hz stimulation train and observed no detectable difference between the two averages. We welcome this opportunity to clarify an advantage of the Qdot method: the ability to monitor single-vesicle behavior across the total recycling vesicle pool [readily releasable pool (RRP) + reserve pool]. In our study (*1*), changes in the dynamics of fusion/retrieval

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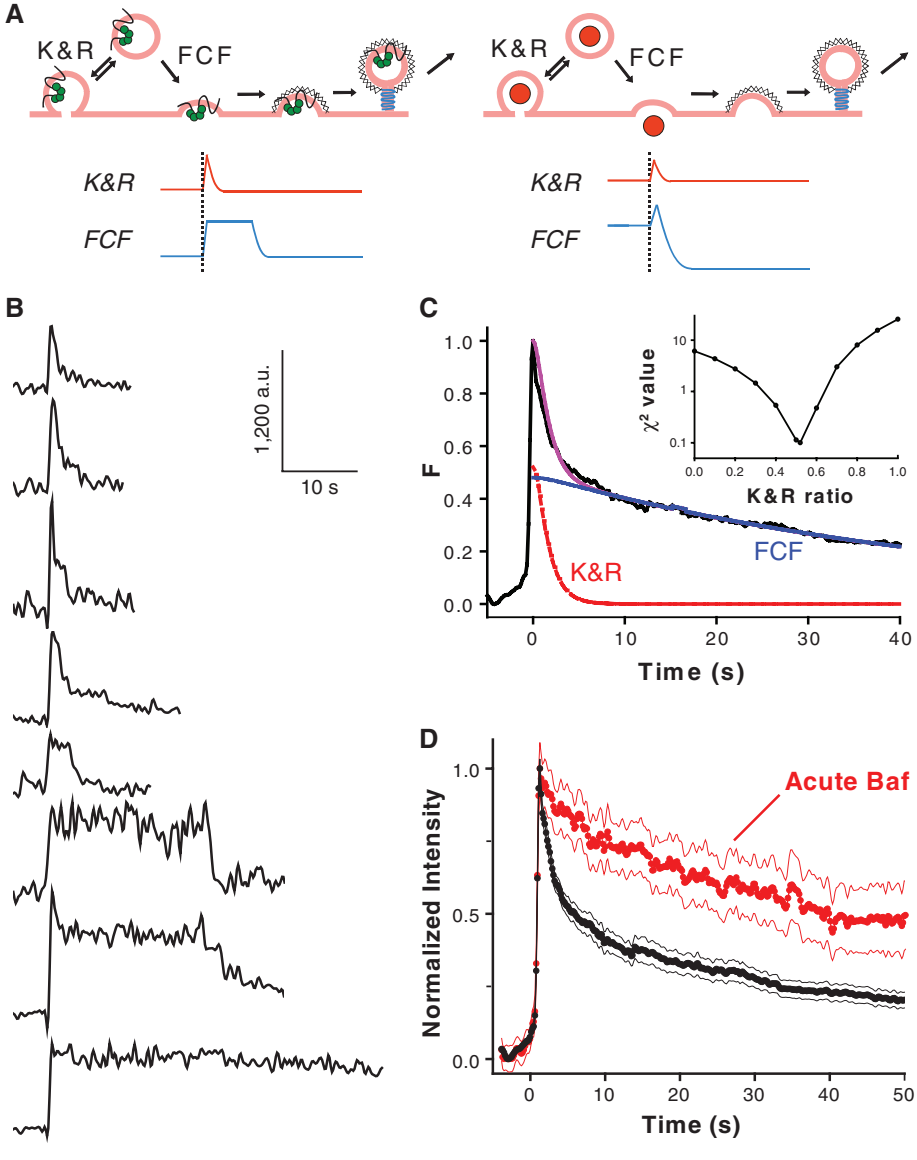

Fig. 1. Single-vesicle fusion events probed with SypH4X. (**A**) Cartoon and hypothetical traces for SypH4X- and Qdot-based measurements. (**B**) Representative traces of SypH4X fluorescence signals reporting single fusion events (0.3 Hz stimulation). (**C**) Ensemble average (black trace) and the best fit with the same equation used by Granseth *et al*. (*2*) (solid purple line), consisting of a fast K&R component (red dashed line, 52%, τ_{KR} = 0.53 s) and a slow FCF component (blue dashed line, 48%, τ_{FCF} = 49 s). We fixed the following parameters according to our experiments: $k_r = 1/\tau_r = 1/1.45s = 0.70s^{-1}$, $k_{KR} = 1/\tau_{KR} = 1/0.53\ s = 1.89s^{-1}$, and allowed $\tau_{FF} = 1/k_{FF}$ to vary between 10 and 60 s. The values of τ_{KR} and τ_r are faster than those estimated in (*4*) (both 2.6 s), though the interpretations are otherwise compatible. (Inset) The χ^2 value falls to a minimum at 52% K&R. (**D**) Same ensemble average (black circles and line: mean ± SEM), compared with pooled SypH4X signals in 1μM bafilomycin A1 (applied 30 s before the first stimulus pulse). The remaining slow decay is likely due to the post-FCF lateral diffusion of SypH4X out of the region of interest. Cultured rat hippocampal CA3-CA1 neurons (*1*) transfected with SypH4X (*4*) at 8 to 11 days in vitro (DIV) and studied at 14 to 20 DIV. Neurons stimulated at 0.3 Hz for 2 min and imaged at 3 Hz with a Cascade 512B camera, standard GFP filter cube (Chroma), and a 40×, 1.3 NA objective with a 4× projection lens. Positive events were identified in kymographs with a moveable region of interest (*12*) to track an individual SypH4X cluster over time while minimizing the effect of its relocation. The spatial distribution of fluorescence was fitted to a two-dimensional Gaussian to define the center of a 2 μm-diameter circle for integrating total fluorescence.

that originate from differences in behavior of RRP and reserve pool vesicles were evident during stimulus trains as the minority Qdot-loaded RRP vesicles were quickly depleted, giving way to the majority Qdot-loaded reserve pool vesicles, which are less likely to support K&R. In contrast, the sypHy signals of Granseth *et al*. (*3*) would arise almost entirely from RRP vesicles. At the low-frequency stimulation they used (0.02 Hz), each and every stimulus recruits vesicles with high vesicle release probability $P_{r,v}$—those residing in the RRP. Because all vesicles were sypHy-labeled, the RRP was continually replenished with labeled vesicles. Thus, the lack of deviation between their first and 8th to 10th responses is entirely to be expected. Our own experiments with a pHluorin-based probe confirm this expectation.

To explore whether signals corresponding to K&R can be obtained with a proteinaceous probe, we used synaptophysin tagged with four copies of pHluorin (SypH4X) (*4*). We were able to detect single-vesicle fusion events reliably, using 0.3 Hz stimulation to increase the yield of unitary signals (examples in Fig. 1B). We observed at least three distinct patterns of decay of the SypHy4X signal (Fig. 1B): (i) fast, complete decay (*4*, *5*); (ii) staggered decay, with an early partial downstep followed by a later recovery (*4*); and (iii) a simple, late recovery (*2*, *6*). The overall time course (averaged from 244 records) was not consistent with a single slow mode of retrieval but was well-fitted by a combination of fast and slow modes, using the same equation as Granseth *et al*. (*2*) (Fig. 1C). The best fit by χ^2-test (Fig. 1C, inset) was obtained with a 52% contribution of K&R events. A similar biphasic time course was seen with unitary transients at the beginning of the stimulus train, after a long quiescence. Acute exposure to a vesicular H^+-pump blocker, bafilomycin, abolished the rapid phase (Fig. 1D), indicating that it corresponds to internalization of probe into a reacidifying compartment, the lumen of retrieved vesicles. The reacidification time constant for brief events, 1.45 ± 0.18 s, was not significantly different from that obtained with Qdots 0.95 ± 0.18 s ($P > 0.05$, unpaired *t* test), which also agrees with the results from (*5*) (Fig. 2A).

There are many potential explanations for the discrepancy between Granseth *et al*. (*2*, *3*) and data obtained with Qdots (*1*) and with SypH4X (*4*). One may consider differences in cultures, experimental conditions, cytosolic calcium concentration, or the degree of G-protein activation, known to affect the balance between K&R and FCF (*7*). A broadly accommodating scenario is that while the Qdot (15 nm in diameter) remained trapped inside the vesicle and reported a K&R event, the fusion pore could dilate to a lesser degree (0.5 to 14 nm), perhaps even allowing escape of a subset of vesicular protein markers by lateral diffusion within the membrane. Thus, pHluorin-labeled vesicle proteins might escape during an event identified as K&R by Qdot analysis, akin to "cavicapture" (*8*).

Regarding the concern that Qdots induced fast vesicle retrieval, our results indicated that even maximal loading of the total recycling pool with Qdots does not affect vesicle trafficking as monitored with the styryl reporter dye FM 4-64 (*9*), electrophysiology (*9*), and a pHluorin-based probe (*1*). Granseth *et al*. (*3*) question whether the uptick equates with K&R rather than full fusion followed by retrieval of a Qdot. We can exclude the latter on two grounds. Co-occurring uptake of a fresh Qdot can be firmly excluded; there were no Qdots on the surface during destaining (removed by the 20-min wash), and only a single, vesicle-enclosed Qdot remained visible in the region of interest. The alternative scenario, immediate recapture of the exocytosed Qdot by an adjacent clathrin-coated, endocytosis-ready vesicle, was disfavored by the limited movement of Qdots during the deacidification period, which was mostly less than the minimal center-to-center distance (100 nm) between a fused vesicle and a putative endocytosis-ready vesicle next to it (Fig. 2B). Finally, we considered whether Qdots might be recaptured by endocytosis of the original vesicle itself (Fig. 2C). This scenario predicts that Qdot photoluminescence signal would generate an "upstep," reflecting Qdot externalization after FCF and brightening before re-internalization. Evidence on clathrin-mediated endocytosis predicts an upstep with a lifetime of many seconds [$\tau = 14$ s, according to (*3*)], but nothing like this was ever observed (Fig. 2C). Instead, the plateau of the uptick lasted on average ~0.5 to 1 s (*1*), consistent with capacitance measurements (*10*) and much too fast for conventional clathrin coat formation (*11*). Taken together, our results preclude the idea that the same Qdot was recaptured, either by an adjacent endocytotic vesicle (Fig. 2B) or by endocytosis of the original vesicle (Fig. 2C).

A final scenario, inconsistent with the proposal of Granseth *et al*. (*3*) but worth considering, is that K&R occurs but is supported by a hypothetical clathrin precoat rather than de novo clathrin assembly. Clathrin-coated vesicles have rarely been seen at the active zone of quiescent nerve terminals, nor is it clear how a clathrin precoat could coexist with the SNARE proteins of the fusion machinery. Nonetheless, if K&R arises because of mechanical constraints on what would otherwise be FCF, clathrin precoating is a possible candidate (*2*) that would be consistent with Zhu *et al*. (*4*).

Each of the approaches used to study vesicle recycling has its own virtues and drawbacks. In addition to their favorable optical properties, Qdots can selectively label one pool of vesicles (*1*), whereas pHluorin-based indicators cannot. Single vesicles take up only one Qdot at most (*1*, *9*), allowing unambiguous tracking of the vesicle that harbors it, even if the vesicle were to exchange some proteins or lipids with the plasma membrane. However, Qdots do not yet track vesicle proteins or lipids. Ultimately, and perhaps already (Fig. 2A), Qdot- and pHluorin-based measurements will provide complementary insights into intriguing aspects of vesicle fusion, retrieval, and reuse.

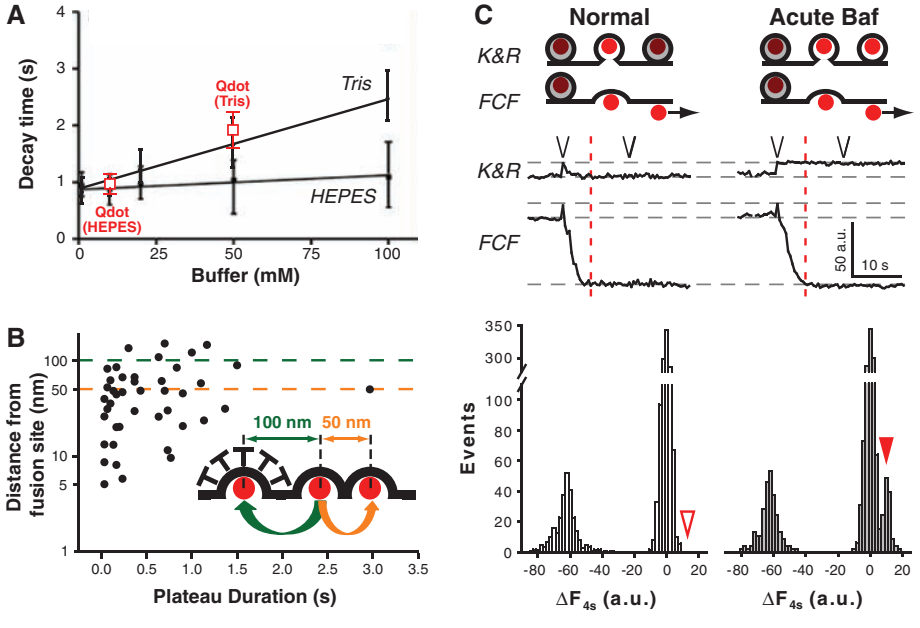

Fig. 2. Interpretation of Qdot signals from vesicle fusion experiments. (**A**) Time constants of reacidification measured by Qdots (red symbols) are consistent with pHluorin-based data of (*5*) (black line and symbols). (**B**) Movements of single Qdots during fusion pore open time are usually < 50 nm, and almost always < 100 nm. (Inset) Scenarios of hypothetical Qdot reuptake. (**C**) Contradicting Qdot reuptake scenario, lack of upstep, documented with analysis 4 s (dashed line) after stimulation under normal conditions. Complete absence of upstep (open arrowhead), contrasts with clear upstep in acute bafilomycin (filled arrowhead).

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