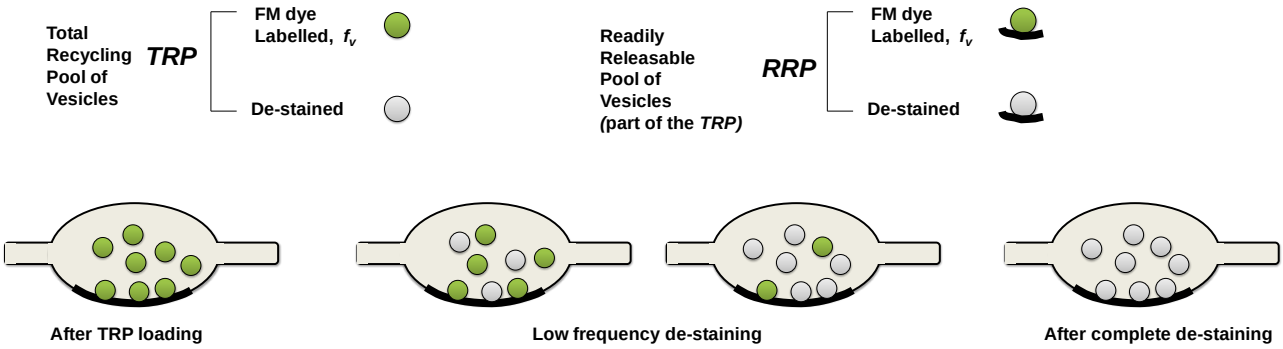
**Text S1. Quantification of FM dye (SRC1) de-staining during low frequency stimulation.**

In this section we consider a quantitative model which allows estimating basic presynaptic parameters using SRC1 de-staining profiles recorded in individual synaptic boutons.




Notation:

$f_v$ specific fluorescence of a single SRC1 labeled vesicle

$RRP$ number of readily-releasable vesicles

$TRP$ total number of recycling vesicles

$p_v$ average release probability of individual vesicles within RRP

$F_{FM}(t)$ specific vesicular FM dye fluorescence intensity in individual synaptic boutons

Model assumptions:

In general, FM dye de-staining profiles have complex shapes that depend on the type of FM dye used and on loading/stimulation protocols [1–5]. It has however been shown that vesicles within RRP and TRP co-exist in a dynamic equilibrium [6,7]. Therefore we hypothesized that, during low frequency (0.5 Hz) stimulation, there is enough time for

newly endocytosed fluorescence-free vesicles to re-equilibrate between the TRP and RRP between successive APs. To test this hypothesis we compared SRC1 de-staining at 0.5 Hz and 0.25 Hz. If SRC1 de-staining was significantly affected by depletion of RRP vesicles or by preferential recycling of RPP vesicles (for example as has been reported after bursts of high frequency stimulation [7]), then the SRC1 de-staining per AP should be higher at 0.25 Hz than at 0.5 Hz. However, no such difference between SRC1 de-staining rates at these two frequencies was found when $k_{AP}$ was normalized by the number of APs (Figure S1 G - I). We therefore conclude that, during low frequency stimulation, fluorescence-labeled and fluorescence-free vesicles were equally distributed between RRP and TRP.

We then obtain:

$$\frac{d_{FM}F(t)}{dt} = -\nu \cdot N_{AP} \cdot f_v \cdot \frac{F_{FM}(t)}{\Delta F_{FMtotal}} \qquad (1.1)$$

where $\Delta F_{FMtotal} = TRP \cdot f_v$ is the specific fluorescence of FM dye labeled vesicles corresponding to completely labeled TRP, and $N_{AP}$ is the average number of vesicles released during a single AP. Under the binomial model [8–10], the average number of vesicles released during a single AP (i.e. vesicular release rate) follows the relationship: $N_{AP} = RRP \cdot p_v$. This yields:

$$\frac{dF_{FM}(t)}{dt} = -\nu \cdot [RRP/TRP] \cdot p_v \cdot F_{FM}(t)$$

and

$$F_{FM}(t) = \Delta F_{FMtotal} \cdot \exp(-\nu \cdot [RRP/TRP] \cdot p_v \cdot t)$$

Thus the experimentally determined AP-specific SRC1 de-staining rate can be expressed as:

$$k_{AP} = \nu \cdot \left[RRP/TRP\right] p_v \qquad (1.2)$$

Consequently, another experimentally determined parameter – the vesicular release rate ( $R_{rel} = \frac{\Delta F_{FMtotal} k_{AP}}{\nu}$ ) – is proportional to the average number of vesicles released during a single AP - $R_{rel} \propto N_{AP}$ :

$$R_{rel} = \frac{\Delta F_{FMtotal} k_{AP}}{\nu} = TRP \cdot f_v \cdot [RRP/TRP] \cdot p_v = f_v \cdot RRP \cdot p_v = f_v \cdot N_{AP}$$

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