

The pH Probe CypHerTM5E is Effectively Quenched by FM Dyes

Oliver Welzel · Kristina Loy · Carsten H. Tischbirek · Alina Tabor · Peter Gmeiner · Johannes Kornhuber · Teja W. Groemer

Received: 4 July 2012 / Accepted: 31 January 2013 / Published online: 10 February 2013
© Springer Science+Business Media New York 2013

Abstract Concurrent imaging of spectrally distinct fluorescence probes has become an important method for live-cell microscopy experiments in many biological disciplines. The technique enables the identification of a multitude of causal relationships. However, interactions between fluorescent dyes beyond an obvious overlap of their fluorescent spectra are often neglected. Here we present the effects of the well-established fluorescent dyes FM[®]2-10 or FM[®]1-43 on the recently introduced pH-dependent probe CypHerTM5E. Spectrophotometry as well as live-cell fluorescence microscopy revealed that both FM dyes are effective quenchers of CypHerTM5E. Control experiments indicated that this effect is reversible and not due to bleaching. We conclude that, in general, parallel measurements of both dyes are possible, with low FM dye concentrations. Nevertheless, our results implicate that special care has to be taken in such dual colour experiments especially when analysing dynamic CypHerTM5E signals in live-cell microscopy.

Electronic supplementary material The online version of this article (doi:10.1007/s10895-013-1164-3) contains supplementary material, which is available to authorized users.

O. Welzel (✉) · K. Loy · C. H. Tischbirek · J. Kornhuber · T. W. Groemer
Department of Psychiatry and Psychotherapy,
Friedrich-Alexander-University of Erlangen-Nuremberg,
Schwabachanlage 6,
91054 Erlangen, Germany
e-mail: oliver.welzel@uk-erlangen.de

A. Tabor · P. Gmeiner
Department of Chemistry and Pharmacy, Emil Fischer Center,
Friedrich-Alexander-University of Erlangen-Nuremberg,
Schuhstrasse 19,
91052 Erlangen, Germany

Keywords FM dye · CypHerTM5E · Fluorescence microscopy · Synaptic vesicles · Hippocampal neurons

Abbreviations

FM [®] 2-10	N-(3-Triethylammoniumpropyl)-4-(4-(Diethylamino) Styryl) Pyridinium Dibromide
FM [®] 1-43	N-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino) Styryl) Pyridinium Dibromide
GFP	Green fluorescent protein
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMC	Critical micelle concentration

Introduction

The parallel monitoring of multiple fluorescence probes has become increasingly important for the microscopy of cellular processes in many biological disciplines. E.g. the use of spectrally distinct styryl FM dyes revealed information about endocytosis kinetics [1] as well as spontaneous recycling synaptic vesicles [2]. However, interactions between fluorescent dyes beyond an obvious overlap of their fluorescent spectra might confound experimental results and have to be carefully evaluated.

In this study, we focused on the spectrally distinct fluorescent dyes FM[®]2-10 or FM[®]1-43 and CypHerTM5E. Individually, these dyes are extensively used for the analysis of synaptic transmission. Their combined use might present an interesting tool to investigate the dynamics of synaptic vesicle recycling as these dyes have individual advantages and could in principle easily be combined.

FM dyes are particularly useful for the analysis of lipid membrane trafficking. Due to their about two orders of

magnitude higher fluorescence in membranes compared to polar solvents such as water [3] they offer the advantage to determine the brightness of single synaptic vesicles [4–7]. In neuronal cells these dyes offer the possibility to stain different pools of recycling synaptic vesicles dependent on the electrical stimulus [8, 9]. Thus, FM dyes allow the direct measurement of exocytosis of previously stained vesicles [10], but only an indirect measurement of endocytosis and related kinetics [10, 11]. Additionally, repetitive stimulation experiments are complex due multiple rounds of staining, washing extracellular dye away and subsequent destaining [12, 13]. Several different FM dyes with different spectra and distinct membrane partitioning characteristics have been designed [3, 14]. E.g. FM[®]2-10 and FM[®]1-43, in which FM[®]2-10 partitions faster corresponding to better dye unloading from synaptic vesicles [3]. Differences between FM dyes were used to analyze several aspects of synaptic vesicle recycling [1, 2].

Many of these disadvantages of FM dyes have been overcome with the introduction of the pH-dependent CypHer[™]5E [15–17]. CypHer[™]5E is a variant of the dye Cy5[™], that only fluoresces in acidic conditions ($pK_a=6.1$) [15, 16]. Due to its pH-dependency this probe reports changes of the vesicular pH-gradient during the different phases of vesicle recycling when coupled to an antibody against the luminal domain of a synaptic vesicle protein. Recently, many questions regarding synaptic vesicle recycling have been answered with this probe [6, 18–21]. Compared to homologs of the pH-dependent GFP variant pHluorin [22–24], antibodies labelled with CypHer[™]5E have the advantage that they can be flexibly applied to native tissue. As pHluorins have to be genetically encoded in transgenic animal species or used in overexpression systems, sensitive physiological measurements might be obscured due to the artificial expression of the protein.

The combination of the pH-dependent probe CypHer[™]5E coupled to a vesicular antibody with either one of the FM dyes FM[®]2-10 or FM[®]1-43 would offer various advantages with respect to synaptic vesicle recycling. As especially FM[®]1-43 is widely used in single vesicle analysis [4–7], a combination with CypHer[™]5E give the opportunity to calibrate the CypHer[™]5E fluorescence to the quantal nature of synaptic transmission [25]. Especially experiments using repetitive stimulation or focusing on endocytosis would benefit from such a quantal analysis of synaptic vesicle recycling, e.g. study of drug effects [12, 26]. Furthermore as FM dyes as well as CypHer[™]5E coupled to an antibody are able to label individual vesicle pools a combined use could solve questions regarding the intermixing, origin and reuse of different synaptic vesicle pools [2, 9, 27]. In contrast to spectrally different FM dyes like FM[®]1-43 and FM[®]5-95 [2], which allow only one measurement of exocytosis, the use of CypHer[™]5E offers the possibility for multiple measurements due to the permanent binding of the antibody.

Here, we analysed the potential combination of the FM dyes FM[®]2-10 or FM[®]1-43 with CypHer[™]5E for parallel imaging experiments. We report the concentration-dependent quenching of CypHer[™]5E fluorescence by the FM dyes. Furthermore, we show how this effect influences the dynamic CypHer[™]5E signal monitored in *in vitro* experiments with primary rat hippocampal neurons. While we have found that a parallel measurement with both dyes using low FM dye concentrations is generally possible, our results might warrant a caveat for experimenters conducting similar imaging protocols.

Experimental

Cell Culture

Hippocampal neuronal cultures were prepared from 1 to 3 days old Wistar rats (Charles River, USA) as described [21]. Newborn rats were sacrificed by decapitation in accordance with the guidelines of the State of Bavaria. Hippocampi were removed from the brain and transferred into ice cold Hank's salt solution, and the dentate gyrus was cut away. After digestion with trypsin (5 mg ml^{-1}) cells were triturated mechanically and plated in MEM medium, supplemented with 10 % fetal calf serum and 2 % B27 Supplement (all from Invitrogen, Taufkirchen). Experiments were performed between 25 and 30 days *in vitro*.

Fluorescent Labeling

For the exemplary FM[®]2-10 experiment (Fig. 1c, e) recycled synaptic vesicles were labeled with $100 \mu\text{M}$ FM[®]2-10 (Invitrogen, Karlsruhe). To stain the total recycling pool nerve terminals were loaded with 1,200 pulses at 40 Hz [28]. Dye was allowed to remain on the cells for 60 s after the end of the stimulation to permit complete endocytosis, and was subsequently removed by a 7 min wash-out.

For the αSyt1 -CypHer[™]5E experiments, cultured hippocampal neurons were incubated for 20 min with $0.6 \mu\text{g}$ of CypHer[™]5E labeled anti-Synaptotagmin1 antibody (αSyt1 -CypHer[™]5E; Synaptic Systems, Goettingen) in extracellular medium containing 40 mM K^+ to stain the total synaptic vesicle recycling pool.

Imaging

Experiments were conducted at room temperature on a Nikon TI-Eclipse inverted microscope equipped with a $60\times$, 1.2 NA water immersion objective and a Perfect Focus System[™]. Fluorescent dyes were excited by a Nikon Intensilight C-HGFI through excitation filters centered at 482 nm and 640 nm using dichroic longpass

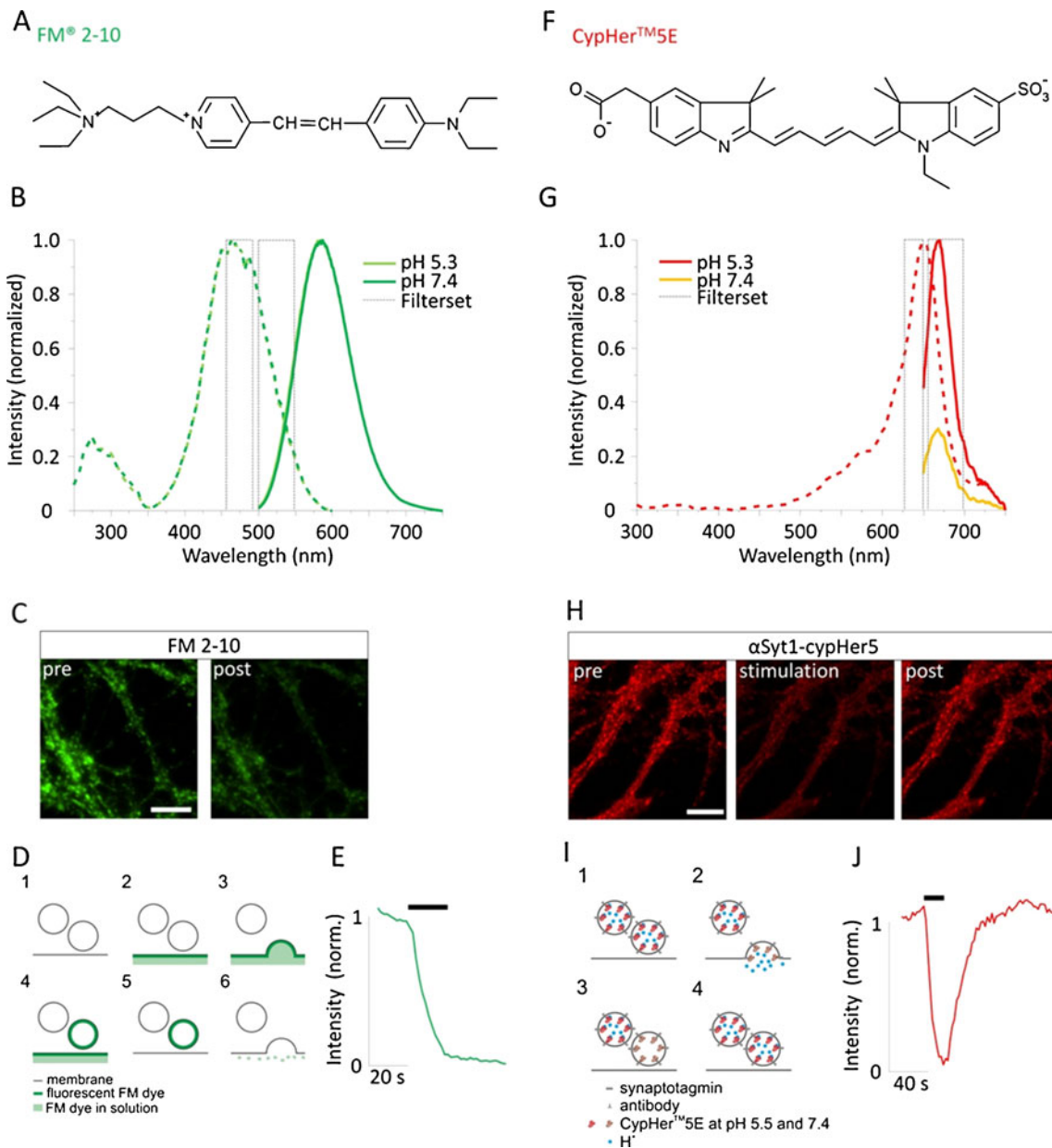


Fig. 1 Dye structure and corresponding spectra of the styryl dye FM[®]2-10 and the pH-dependent Cy5TM variant CypHerTM5E. **a** Dye structure of FM[®]2-10 [14]. **b** Excitation (*dashed*) and emission spectra of the dye FM[®]2-10 for two different pH values. Typical excitation and emission filters are depicted as *grey rectangles* which were chosen to avoid any bleed-through of the two fluorophors in dual colour experiments. **c** Exemplary fluorescence images of hippocampal neurons stained with FM[®]2-10 following the wash-out of the external dye before and after electrical stimulation with 200 pulses at 10 Hz. **d** Schematic of membrane staining with FM dyes. After adding FM dye to the extra cellular medium, the dye incorporates preferentially into the membrane of hippocampal neurons (2). Dye incorporated into lipid membranes exhibits a higher fluorescence compared to dye in solution. The membrane of synaptic vesicles, which fuses with the plasma membrane upon an electrical stimulus, is stained with the dye (3). After compensatory endocytosis stained synaptic vesicles are inside the synapse (4). After washing the extracellular dye away, only fluorescent synaptic vesicles remained (5). Upon an electrical stimulus these vesicles again fuse with the membrane and lose their fluorescent dye due to the concentration gradient (6), which

enables the measurement of exocytosis. An exemplary fluorescence profile of the synapses shown in (c) upon a stimulation with 200 pulses at 10 Hz is shown in (e). **f** Dye structure of CypHerTM5E [16]. **g** Excitation (*dashed*) and emission spectra of the dye CypHerTM5E for two different pH values. Typical excitation and emission filters are depicted. **h** Exemplary fluorescence images of hippocampal neurons labelled with α Syt1-CypHerTM5E before and after electrical stimulation with 200 pulses at 10 Hz and after recovery. **i** Illustration of α Syt1-CypHerTM5E labelling and its fluorescence properties. Antibodies labelled with CypHerTM5E against the synaptic vesicle protein synaptotagmin1 are inside the vesicle (1). CypHerTM5E is fluorescent in the acidic lumen of the synaptic vesicle. Upon an electrical stimulus vesicles fuse with the plasma membrane and the intravesicular protons are lost. CypHerTM5E is less fluorescent (2). After compensatory endocytosis, (3) synaptic vesicles are reacidified and the fluorescence of CypHerTM5E increases (4). Thus, α Syt1-CypHerTM5E enables the measurement of exocytosis and endocytosis/reacidification of synaptic vesicles. An exemplary fluorescence profile of the synapses shown in (h) upon a stimulation with 200 pulses at 10 Hz is shown in (j)

mirrors (cut-off wavelength 500 nm and 660 nm), respectively. The emitted light passed emission band-pass filters ranging from 500 nm–550 nm to 660 nm–700 nm (Semrock, Rochester) and was projected onto a cooled EM-CCD camera (iXon^{EM} DU-885, Andor, Belfast). The filter sets were chosen to avoid any spectral overlap or bleed-through of the respective fluorophores. Cover slips were placed into a perfusion chamber (volume=500 μ l) containing extracellular medium consisting of (in mM): 144 NaCl, 2.5 KCl, 2.5 CaCl₂, 2.5 MgCl₂, 10 Glucose, 10 HEPES, pH=7.4.

In FM experiments images were recorded with 200 ms exposure time and in α Syt1-CypHerTM5E experiments with 900 ms exposure time at 0.5 Hz frame rate each.

Synaptic boutons were stimulated by electric field stimulation (platinum electrodes, 10 mm spacing, 1 ms pulses of 50 mA and alternating polarity) with 200 pulses at 10 Hz; 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris Bioscience, Bristol) and 50 μ M D-amino-5-phosphonovaleric acid (D,L-AP5, Tocris Bioscience, Bristol) were added to prevent recurrent activity.

Data Analysis

All image analysis was performed using custom-written routines in MATLAB (The MathWorks, Natick, MA). Recorded image stacks were used to automatically define peak regions of interest (ROI) of synaptic bouton size [29], where a stimulation-evoked fluorescence decrease occurred in difference images. The average of each ROI was calculated for each image to obtain fluorescence intensity profiles for each bouton. Graphs were generated using MATLAB (The MathWorks Inc., Natick), Microsoft[®] Excel 2010 (Microsoft[®] Corporation, Redmond) or Origin[®] 8.5.1 (OriginLab Corporation, Northampton, Massachusetts).

Fluorescence Spectrophotometry

All fluorometric measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies Deutschland GmbH, Boeblingen).

All solutions were measured in quartz cuvettes (sample volume=400 μ l) at an excitation wavelength of 473 nm or 640 nm with excitation and emission slits set at 5 nm. FM[®]2-10, FM[®]1-43, CypHerTM5E and α Syt1-CypHerTM5E were solved in respective concentrations in extracellular medium with pH=7.4 or pH=5.3.

For the determination of FM dye excitation and emission spectra, samples, including calibration samples, were mixed with 20 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) [30, 31], which is a zwitterionic detergent used to increase the absolute fluorescence of FM dyes (FlukaTM, Buchs) [31].

Results and Discussion

Properties of FM Dyes and α Syt1-CypHerTM5E

Styryl dyes like FM[®]2-10 and FM[®]1-43 are widely used to analyse synaptic vesicle recycling [1, 2, 6, 10, 21]. The dye molecules (Fig. 1a) consist of a lipophilic tail region, which causes the dye to partition into lipids such as cellular membranes, a fluorescent middle region with two aromatic rings and a positively charged head, which prevents the dye from flipping across the membrane [3]. The fluorescence of these dyes shows a marked increase of the fluorophore core that lies in the middle of the FM dye structure, when they are inserted into lipid membranes [14]. Thus almost the entire signal is emitted by dye molecules in membranes in contrast to dye dissolved in polar solutions such as water [3, 31]. In our measurements FM dyes exhibited a 100-fold increase in fluorescence when CHAPS was added to the extracellular medium. Above a critical concentration this soluble amphiphile form stable aggregates called micelles [32]. FM dye molecules partition into these CHAPS micelles [31]. Hereby the lower dielectric constant of the lipid solvent (compared with non CHAPS) is responsible for the increase in fluorescence [31, 33]. The critical micelle concentration (CMC) of CHAPS is 6.50 mM [34] and is slightly reduced in our system due to the used extracellular medium [35]. Numerous similar dyes with different properties such as shifted fluorescence spectra or modified tails have been generated [3, 14]. The spectra of FM dyes are pH independent (Fig. 1b and Fig. S1B), which is crucial for the analysis of synaptic vesicle recycling as the intravesicular lumen is acidic with a pH of about 5 compared to neutral pH of the cytoplasm and extracellular space. This is due to the activity of the vacuolar H⁺ ATPase and is required for neurotransmitter accumulation [24, 36, 37]. Specific staining and destaining protocols of the dyes have been used in the past two decades to visualize many aspects of synaptic vesicles recycling [1, 2, 6, 7, 38]. An exemplary fluorescence image of hippocampal neurons stained with FM[®]2-10 is shown in Fig. 1c and the corresponding typical staining scheme with exocytosis kinetics is shown in Fig. 1d, e.

Recently, the pH-dependent Cy5TM variant CypHerTM5E (Fig. 1f) has been introduced in the analysis of synaptic vesicle recycling [6, 21]. CypHerTM5E fluoresces in the acidic milieu of synaptic vesicles (pH=5.3), but not if it is exposed to the extracellular pH (pH=7.4; Fig. 1g). In accordance with previous studies we found a reduction of the CypHerTM5E fluorescence at pH=7.4 to 30.19 % compared to pH=5.3 [16, 18]. Coupled to a monoclonal antibody directed against the intravesicular domain of synaptotagmin1 (α Syt1-CypHerTM5E), it enables the selective labelling of functional terminals by antibody uptake and visualization of synaptic exo- and endocytosis cycles according to the stimulation-dependent change in fluorescence (Fig. 1h–j).

Effect of FM Dyes on CypHerTM5E Fluorescence–Spectrophotometry

First, we measured the influence of different concentrations of FM[®]2-10 on the emission spectrum of α Syt1-CypHerTM5E. We used typical concentrations of FM[®]2-10, as they are used in live-cell experiments ranging from a relatively low concentration of 25 μ M to the highest of 400 μ M [1, 3, 27, 39]. With increasing concentrations of FM[®]2-10 the emission intensity of α Syt1-CypHerTM5E decreased (Fig. 2a; Pearson's $r=-0.90$, $p<0.01$). Additionally, we analyzed the effect of 200 μ M FM[®]2-10 on the emission maximum of pure CypHerTM5E and measured a reduction of $49.46\% \pm 0.79\%$ in accordance with the reduction of 46.36% for α Syt1-CypHerTM5E (see Fig. 2a, c). We also analyzed the α Syt1-CypHerTM5E emission spectra as a function of typical FM[®]1-43 concentrations ranging from 2.5 μ M to 100 μ M (Fig. 2b; Pearson's $r=-0.97$, $p<0.01$) [1, 2, 6, 30, 31]. At the highest concentrations of FM[®]2-10 and FM[®]1-43, emission spectra with CHAPS [30, 31] are depicted, which lead to a small shift in the emission spectrum, but not to a further reduction indicating that FM dyes do not directly quench α Syt1-CypHerTM5E (Fig. 2a, b). We used a concentration of 325.27 mM CHAPS which is high above the CMC and thus not only micelles are formed also at such high concentrations surfactants could form worm-like structures and bilayers, which encapsulate extracellular medium.

With increasing concentrations of FM dyes the emission intensity of α Syt1-CypHerTM5E decreased (Fig. 2c). This reduction is not a bleaching effect of the fluorescent dye CypHerTM5E, as multiple measurements revealed no change in amplitude of the emission spectra (Fig. S2; Pearson's $r=-0.25$, $p=0.44$). Furthermore a dimming of α Syt1-CypHerTM5E fluorescence by interception of the excitation and emission light was excluded. Therefore the absorptions at the excitation (640 nm) and emission (664 nm) wavelengths of CypHerTM5E were measured in the presence of the lowest and highest concentration of the respective FM dyes [40]. For the absorption path length of 5 mm the intensities after passing through the FM dyes containing solution were in the range of 93.39% to 99.34%, indicating a minimal effect in our system.

Effect of FM Dyes on CypHerTM5E Fluorescence–Fluorescence Microscopy

To analyse the effects of FM dyes on α Syt1-CypHerTM5E measurements under more physiological conditions, we performed live-cell experiments. Primary rat hippocampal neurons were labelled with α Syt1-CypHerTM5E in a 40 mM K⁺ solution to ensure the fluorescent labelling of the entire vesicle recycling pool. The cells were then stimulated at least two consecutive times with 200 pulses at 10 Hz (Fig. 3a). The first stimulation showed the expected fluorescence time course of

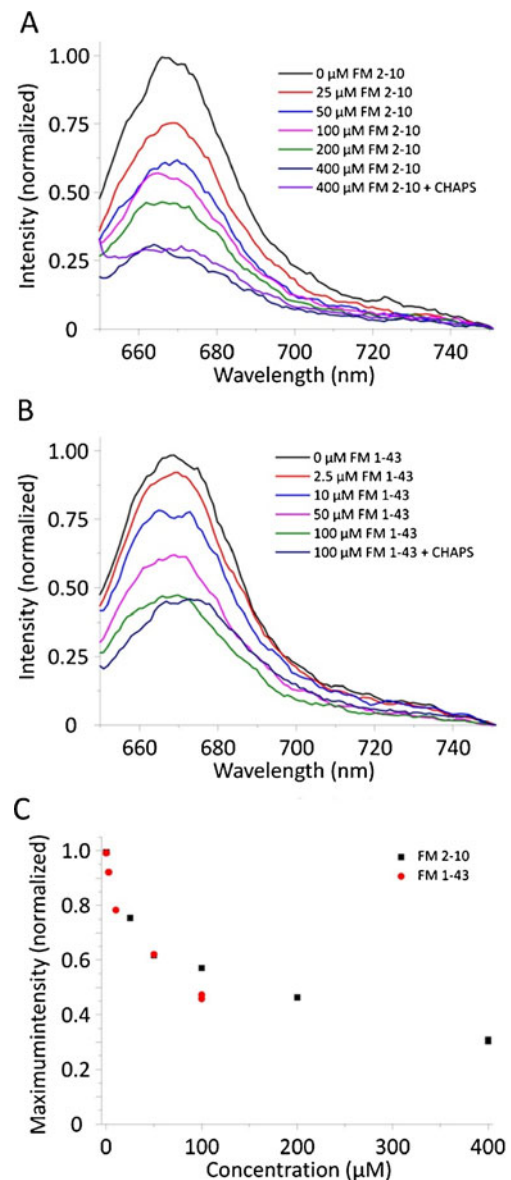


Fig. 2 Fluorescence characteristics of the probe CypHerTM5E as a function of FM dye concentration. Emission spectra of CypHerTM5E at pH=5.3 for different concentrations of the styryl dyes FM[®]2-10 (a) and FM[®]1-43 (b). At the highest FM dye concentrations CHAPS was added (molar ratios, CHAPS/FM[®]2-10=813.25 and CHAPS/FM[®]1-43=3,253). c Maximum emission intensities normalized to controls as a function of FM[®]2-10 (black squares) and FM[®]1-43 (red circles) concentration

cells labelled with α Syt1-CypHerTM5E (Fig. 3b, c) [18, 21]. The second stimulation was in the presence of FM[®]2-10. Depending on the concentration of the FM dye, the baseline fluorescence, which is the constant fluorescence level before the onset of the electrical stimulation, of the previously detected spots was lowered. As the protein Synaptotagmin1, similar like many other vesicular proteins, is located on the surface of the synapse [41, 42], the CypHerTM5E dye is accessible for the FM dyes in solution.

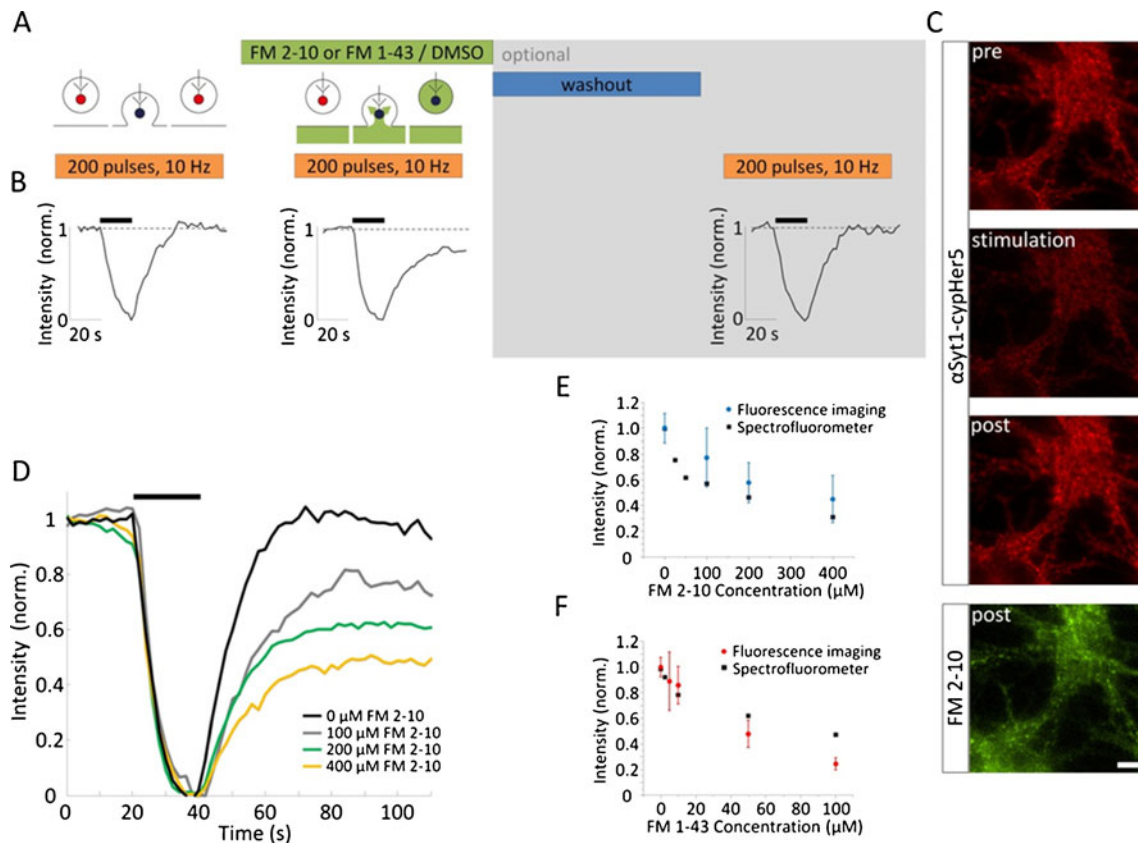


Fig. 3 Live cell experiments of hippocampal neurons labelled with α Syt1-CypHerTM5E and subsequently stained with FM[®]2-10 or FM[®]1-43. **a** Experimental procedure. Functional synapses of rat hippocampal neurons were labelled with α Syt1-CypHerTM5E, which fluoresces due to the intravesicular acidic pH. A control stimulus of 200 pulses at 10 Hz was delivered before adding various concentrations of FM[®]2-10 or FM[®]1-43 or DMSO as control. The cells were stimulated a second time with 200 pulses at 10 Hz to analyse the influence of FM dyes on CypHerTM5E intensity. Optionally, after washing away the extracellular FM dye, neurons were stimulated with 200 pulses at 10 Hz. **b** Exemplary average fluorescence profiles of α Syt1-

CypHerTM5E without FM dye, with 100 μ M FM[®]2-10 and after washout. **c** Exemplary fluorescence images of hippocampal neurons labelled with α Syt1-CypHerTM5E before and after electrical stimulation with 200 pulses at 10 Hz and after recovery. Same region at the end of the protocol stained with FM[®]2-10. Scale bar, 10 μ m. **d** Average fluorescence profiles of α Syt1-CypHerTM5E for different concentrations of FM[®]2-10 (≥ 3 experiments with >400 boutons for each). All profiles are normalized to respective controls. Relative quenching as a function of FM[®]2-10 (**e**) and FM[®]1-43 (**f**) concentration for live cells experiments (blue and red circles) and spectrofluorometer data (black squares)

Thus the baseline fluorescence is quenched. Additionally, compared to control experiments the fluorescence recovery after the end of the stimulation was significantly reduced (Fig. 3d; Kruskal–Wallis test: $\chi^2_{3,15}=8.19$, $p<0.05$) as FM[®]2-10 enters synaptic vesicles and quenches the intravesicular CypHerTM5E (Fig. 3a). This quenching would probably take place between the FM dye in lipid phase of the vesicle and the CypHerTM5E dye inside the synaptic vesicle (Fig. 1d, i). After a thorough washout of the external FM dye, an optional third stimulation revealed a recovery of the α Syt1-CypHerTM5E fluorescence signal. The amount of quenching in the live-cell experiments exhibits a similar characteristic as the data obtained from spectrofluorometry (Fig. 3e; Pearson's $r=0.97$, $p<0.05$). Furthermore, the effect of FM[®]1-43 was tested. Increasing concentrations of the dye reduced the fluorescence signal of

α Syt1-CypHerTM5E and was again comparable to the spectrofluorometer data (Fig. 3f; Pearson's $r=0.97$, $p<0.05$).

In accordance with a previous study, we observed that high concentrations of FM dyes resulted in a lower fusion probability (10 μ M FM[®]1-43: 16.73 % \pm 7.43 % compared 10 % to 17 % reduction estimated by Zhu. Y. and Stevens C.F.) [43]. However, this effect did not tamper our quenching analysis as the fluorescence profiles were normalized to their respective minimum as well as to the baseline before stimulation (Fig. 3d).

Conclusion

In this study, we report the quenching effect of the FM dyes FM[®]2-10 and FM[®]1-43 on CypHerTM5E fluorescence.

Besides the reduction of the emission spectrum of CypHerTM5E by FM dyes in spectrophotometric measurements, we showed that this effect is also apparent in in vitro experiments with primary rat hippocampal neurons labelled with these two dyes. The spectrophotometric data revealed that this reduction in fluorescence is due to the quenching effect of FM dyes on CypHerTM5E fluorescence rather than an inhibition of endocytosis as could be supposed to fluorescence microscopy data alone. The vesicle-like complexes built by the used high concentrations of the detergent CHAPS in the spectroscopy study are similar to synaptic vesicles in the in vitro experiments arguing for the comparable results obtained by these experiments.

We showed that the decrease of CypHerTM5E fluorescence by FM dyes is a process of fluorescence quenching as we were able to exclude dimming by interception of excitation and emission light [33, 40]. Analysis of the Stern–Volmer relationship [44] revealed a linear relation between the concentration of the quencher (FM dyes) and the ratio of fluorescence of CypHerTM5E with and without the quencher, indicating a dynamic or a static quenching mechanism (Fig. S3A, B). The reduced fluorescence recovery after the end of the stimulation indicated that the quenching process requires close proximity to the fluorophore and cannot occur when FM dyes and CypHerTM5E are separated by cell and vesicle membranes [40]. Furthermore the recovery of the α Syt1-CypHerTM5E fluorescence signal due to washout of FM dyes almost excluded a static quenching mechanism as possible complexes formed by FM dyes and CypHerTM5E are unlikely to be reformed. Any form of resonance energy transfer and dark quenching could additionally be excluded as the emission spectra of CypHerTM5E do not overlap with the absorption spectra of the investigated FM dyes. However, the increase of the light scattering background of the CypHerTM5E emission spectrum (Fig. 2a) together with the more effective quenching of FM[®]1-43 compared to FM[®]2-10 (Fig. 2c) give a hint to a dynamic quenching via photoinduced electron transfer upon van der Waals contact [45, 46]. Our data suggest that a parallel measurement with both dyes using low FM dye concentrations is generally possible, even though a small amount of quenching of CypHerTM5E by FM dyes have to be accepted. Special care has to be taken regarding kinetic measurements when using both dyes.

Acknowledgments We thank Katrin Ebert for excellent technical assistance.

References

- Klingauf J, Kavalali ET, Tsien RW (1998) Kinetics and regulation of fast endocytosis at hippocampal synapses. *Nature* 394(6693):581–585. doi:10.1038/29079
- Groemer TW, Klingauf J (2007) Synaptic vesicles recycling spontaneously and during activity belong to the same vesicle pool. *Nat Neurosci* 10(2):145–147. doi:10.1038/nn1831
- Gaffield MA, Betz WJ (2006) Imaging synaptic vesicle exocytosis and endocytosis with FM dyes. *Nat Protoc* 1(6):2916–2921. doi:10.1038/nprot.2006.476
- Chen X, Barg S, Almers W (2008) Release of the styryl dyes from single synaptic vesicles in hippocampal neurons. *J Neurosci* 28(8):1894–1903. doi:10.1523/JNEUROSCI.4518-07.2008
- Ryan TA, Reuter H, Smith SJ (1997) Optical detection of a quantal presynaptic membrane turnover. *Nature* 388(6641):478–482. doi:10.1038/41335
- Welzel O, Henkel AW, Stroebel AM, Jung J, Tischbirek CH, Ebert K, Kornhuber J, Rizzoli SO, Groemer TW (2011) Systematic heterogeneity of fractional vesicle pool sizes and release rates of hippocampal synapses. *Biophys J* 100(3):593–601. doi:10.1016/j.bpj.2010.12.3706
- Welzel O, Tischbirek CH, Kornhuber J, Groemer TW (2012) Pool-independent labelling of synaptic vesicle exocytosis with single vesicle resolution in rat hippocampal neurons. *J Neurosci Methods* 205(2):258–264. doi:10.1016/j.jneumeth.2012.01.011
- Murthy VN, Stevens CF (1999) Reversal of synaptic vesicle docking at central synapses. *Nat Neurosci* 2(6):503–507. doi:10.1038/9149
- Rizzoli SO, Betz WJ (2005) Synaptic vesicle pools. *Nat Rev Neurosci* 6(1):57–69. doi:10.1038/nrn1583
- Ryan TA, Reuter H, Wendland B, Schweizer FE, Tsien RW, Smith SJ (1993) The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. *Neuron* 11(4):713–724
- Ryan TA, Smith SJ, Reuter H (1996) The timing of synaptic vesicle endocytosis. *Proc Natl Acad Sci U S A* 93(11):5567–5571
- Henkel AW, Welzel O, Groemer TW, Tripal P, Rotter A, Kornhuber J (2010) Fluoxetine prevents stimulation-dependent fatigue of synaptic vesicle exocytosis in hippocampal neurons. *J Neurochem* 114(3):697–705. doi:10.1111/j.1471-4159.2010.06795.x
- Micheva KD, Taylor CP, Smith SJ (2006) Pregabalin reduces the release of synaptic vesicles from cultured hippocampal neurons. *Mol Pharmacol* 70(2):467–476. doi:10.1124/mol.106.023309
- Wu Y, Yeh FL, Mao F, Chapman ER (2009) Biophysical characterization of styryl dye-membrane interactions. *Biophys J* 97(1):101–109. doi:10.1016/j.bpj.2009.04.028
- Adie EJ, Kalinka S, Smith L, Francis MJ, Marengi A, Cooper ME, Briggs M, Michael NP, Milligan G, Game S (2002) A pH-sensitive fluor, CypHer 5, used to monitor agonist-induced G protein-coupled receptor internalization in live cells. *BioTechniques* 33(5):1152–1154, 1156–1157
- Briggs MS, Burns DD, Cooper ME, Gregory SJ (2000) A pH sensitive fluorescent cyanine dye for biological applications. *Chem Commun* 23:2323–2324
- Martens H, Weston MC, Boulland JL, Gronborg M, Grosche J, Kacza J, Hoffmann A, Matteoli M, Takamori S, Harkany T, Chaudhry FA, Rosenmund C, Erck C, Jahn R, Hartig W (2008) Unique luminal localization of VGAT-C terminus allows for selective labeling of active cortical GABAergic synapses. *J Neurosci* 28(49):13125–13131. doi:10.1523/JNEUROSCI.3887-08.2008
- Hua Y, Sinha R, Martineau M, Kahms M, Klingauf J (2010) A common origin of synaptic vesicles undergoing evoked and spontaneous fusion. *Nat Neurosci* 13(12):1451–1453. doi:10.1038/nn.2695
- Hua Y, Sinha R, Thiel CS, Schmidt R, Huve J, Martens H, Hell SW, Egnér A, Klingauf J (2011) A readily retrievable pool of synaptic vesicles. *Nat Neurosci* 14(7):833–839. doi:10.1038/nn.2838
- Ratnayaka A, Marra V, Branco T, Staras K (2011) Extrasynaptic vesicle recycling in mature hippocampal neurons. *Nat Commun* 2:531. doi:10.1038/ncomms1534
- Welzel O, Tischbirek CH, Jung J, Kohler EM, Svetlitchny A, Henkel AW, Kornhuber J, Groemer TW (2010) Synapse clusters are preferentially formed by synapses with large recycling pool sizes. *PLoS One* 5(10):e13514. doi:10.1371/journal.pone.0013514

22. Li H, Foss SM, Dobry YL, Park CK, Hires SA, Shaner NC, Tsien RY, Osborne LC, Voglmaier SM (2011) Concurrent imaging of synaptic vesicle recycling and calcium dynamics. *Front Mol Neurosci* 4:34. doi:[10.3389/fnmol.2011.00034](https://doi.org/10.3389/fnmol.2011.00034)
23. Miesenbock G, De Angelis DA, Rothman JE (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394(6689):192–195. doi:[10.1038/28190](https://doi.org/10.1038/28190)
24. Sankaranarayanan S, De Angelis D, Rothman JE, Ryan TA (2000) The use of pHluorins for optical measurements of presynaptic activity. *Biophys J* 79(4):2199–2208. doi:[10.1016/S0006-3495\(00\)76468-X](https://doi.org/10.1016/S0006-3495(00)76468-X)
25. Del Castillo J, Katz B (1954) Quantal components of the end-plate potential. *J Physiol* 124(3):560–573
26. Balaji J, Ryan TA (2007) Single-vesicle imaging reveals that synaptic vesicle exocytosis and endocytosis are coupled by a single stochastic mode. *Proc Natl Acad Sci U S A* 104(51):20576–20581. doi:[10.1073/pnas.0707574105](https://doi.org/10.1073/pnas.0707574105)
27. Pyle JL, Kavalali ET, Piedras-Renteria ES, Tsien RW (2000) Rapid reuse of readily releasable pool vesicles at hippocampal synapses. *Neuron* 28(1):221–231
28. Ryan TA, Smith SJ (1995) Vesicle pool mobilization during action potential firing at hippocampal synapses. *Neuron* 14(5):983–989
29. Sbalzarini IF, Koumoutsakos P (2005) Feature point tracking and trajectory analysis for video imaging in cell biology. *J Struct Biol* 151(2):182–195. doi:[10.1016/j.jsb.2005.06.002](https://doi.org/10.1016/j.jsb.2005.06.002)
30. Denker A, Bethani I, Krohnert K, Korber C, Horstmann H, Wilhelm BG, Barysch SV, Kuner T, Neher E, Rizzoli SO (2011) A small pool of vesicles maintains synaptic activity in vivo. *Proc Natl Acad Sci U S A* 108(41):17177–17182. doi:[10.1073/pnas.1112688108](https://doi.org/10.1073/pnas.1112688108)
31. Henkel AW, Lubke J, Betz WJ (1996) FM1-43 dye ultrastructural localization in and release from frog motor nerve terminals. *Proc Natl Acad Sci U S A* 93(5):1918–1923
32. Tanford C (1978) The hydrophobic effect and the organization of living matter. *Science* 200(4345):1012–1018
33. Lakowicz JR (1999) Principles of fluorescence spectroscopy, 2nd edn. Springer Science and Business Media
34. Neugebauer JM (1990) Detergents: an overview. *Methods Enzymol* 182:239–253
35. Chattopadhyay A, Harikumar KG (1996) Dependence of critical micelle concentration of a zwitterionic detergent on ionic strength: implications in receptor solubilization. *FEBS Lett* 391(1–2):199–202
36. Betz WJ, Mao F, Smith CB (1996) Imaging exocytosis and endocytosis. *Curr Opin Neurobiol* 6(3):365–371
37. Nelson N (1992) Structure and function of V-ATPases in endocytic and secretory organelles. *J Exp Biol* 172:149–153
38. Darcy KJ, Staras K, Collinson LM, Goda Y (2006) Constitutive sharing of recycling synaptic vesicles between presynaptic boutons. *Nat Neurosci* 9(3):315–321. doi:[10.1038/nn1640](https://doi.org/10.1038/nn1640)
39. Smillie KJ, Cousin MA (2012) Akt/PKB controls the activity-dependent bulk endocytosis of synaptic vesicles. *Traffic*. doi:[10.1111/j.1600-0854.2012.01365.x](https://doi.org/10.1111/j.1600-0854.2012.01365.x)
40. Harata NC, Choi S, Pyle JL, Aravanis AM, Tsien RW (2006) Frequency-dependent kinetics and prevalence of kiss-and-run and reuse at hippocampal synapses studied with novel quenching methods. *Neuron* 49(2):243–256. doi:[10.1016/j.neuron.2005.12.018](https://doi.org/10.1016/j.neuron.2005.12.018)
41. Opazo F, Punge A, Buckers J, Hoopmann P, Kastrop L, Hell SW, Rizzoli SO (2010) Limited intermixing of synaptic vesicle components upon vesicle recycling. *Traffic* 11(6):800–812. doi:[10.1111/j.1600-0854.2010.01058.x](https://doi.org/10.1111/j.1600-0854.2010.01058.x)
42. Wienisch M, Klingauf J (2006) Vesicular proteins exocytosed and subsequently retrieved by compensatory endocytosis are nonidentical. *Nat Neurosci* 9(8):1019–1027. doi:[10.1038/nn1739](https://doi.org/10.1038/nn1739)
43. Zhu Y, Stevens CF (2008) Probing synaptic vesicle fusion by altering mechanical properties of the neuronal surface membrane. *Proc Natl Acad Sci U S A* 105(46):18018–18022. doi:[10.1073/pnas.0809714105](https://doi.org/10.1073/pnas.0809714105)
44. Stern O, Volmer M (1919) Über die abklingungszeit der fluoreszenz. *Phys Z* 20:183–188
45. Doose S, Neuweiler H, Sauer M (2005) A close look at fluorescence quenching of organic dyes by tryptophan. *ChemPhysChem* 6(11):2277–2285. doi:[10.1002/cphc.200500191](https://doi.org/10.1002/cphc.200500191)
46. Doose S, Neuweiler H, Sauer M (2009) Fluorescence quenching by photoinduced electron transfer: a reporter for conformational dynamics of macromolecules. *ChemPhysChem* 10(9–10):1389–1398. doi:[10.1002/cphc.200900238](https://doi.org/10.1002/cphc.200900238)