Vesicular Imaging

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Acidic-pH-Activatable Fluorescence Probes for Visualizing Exocytosis Dynamics**

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Abstract: *Live imaging of exocytosis dynamics is crucial for a precise spatiotemporal understanding of secretion phenomena, but current approaches have serious limitations. We designed and synthesized small-molecular fluorescent probes that were chemically optimized for sensing acidic intravesicular pH values, and established that they can be used to sensitively and reliably visualize vesicular dynamics following stimulation. This straightforward technique for the visualization of exocytosis as well as endocytosis/reacidification processes with high spatiotemporal precision is expected to be a powerful tool for investigating dynamic cellular phenomena involving changes in the pH value.*

Exocytosis plays fundamental roles in critical cellular events, such as inflammatory response, neuronal transmission, and the release of hormones.[1] The molecular mechanisms of exocytosis have been intensively studied with biochemical and electrophysiological methods, and are well understood at the level of the whole cell.[1] However, precise regulatory mechanisms, including the spatiotemporal dynamics of individual secretory vesicles, remain unclear owing to the lack of suitable tools for their investigation. For example, the use of a vesicle-resident protein-tagged green fluorescent protein (GFP)[2] or a fluorescent styryl dye[3] in combination with total internal reflection fluorescence microscopy made it possible to monitor exocytosis phenomena in terms of diffusely diminished fluorescence; however, the practical utility of this approach is limited by the noisy background due to fluorophores in the nonvesicular region. In another study, a pH-sensitive GFP analogue, ecliptic synapto-pHluorin, was used to detect intravesicular neutralization upon exocytosis;[4] however, the pre-exocytic vesicular distribution and dynamics could not be tracked well, since synapto-pHluorin is nonfluorescent at the acidic intravesicular pH value (generally pH ≈ 5[4]). Therefore, for the sensitive visualization of vesicular dynamics, we focused on selective sensing of the characteristic acidic pH value inside the secretory vesicles by fluorescent probes. Such probes can be used to monitor intracellular vesicular dynamics, and their signals are turned off upon exocytosis (Figure 1). Several fluorescent probes activated at acidic pH values have been developed and applied to biological systems,[5–7] but these probes are not suitable for selective vesicular imaging from the viewpoints of signal activatability and photostability. For example, commercially available CypHer5E[5] and pHrodo[6] have high backgrounds at the physiological pH value of 7.4 (see Figure S1 in the Supporting Information), whereas our previously developed DiEtNBDP[7] proved to be insufficiently photostable (see Figure S2). To sensitively visualize vesicular dynamics, we need new fluorescent probes with both high signal activatability at the acidic intravesicular pH value of 5.0 versus pH 7.4, and high photostability.

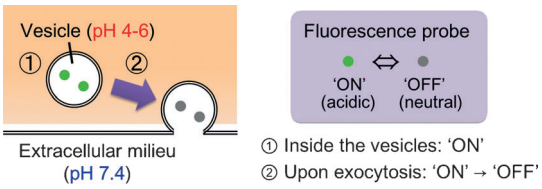

Figure 1. Visualization of vesicular dynamics with probes activated under acidic conditions.

Our strategy for the development of fluorescent probes that can be activated at acidic pH values, RhPs, was based on the selection of rhodamine as a bright and photostable fluorophore and piperazines as pH-sensitive switches that operate by a mechanism of photoinduced electron transfer (Figure 2a).[7,8] To examine the feasibility of this approach, we synthesized RhP-H as a pilot compound. Although Wu and

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Supporting information for this article, including detailed experimental procedures for the synthesis of the probes, the photochemical measurements, and the imaging experiments, is available on the WWW under http://dx.doi.org/10.1002/anie.201402030.

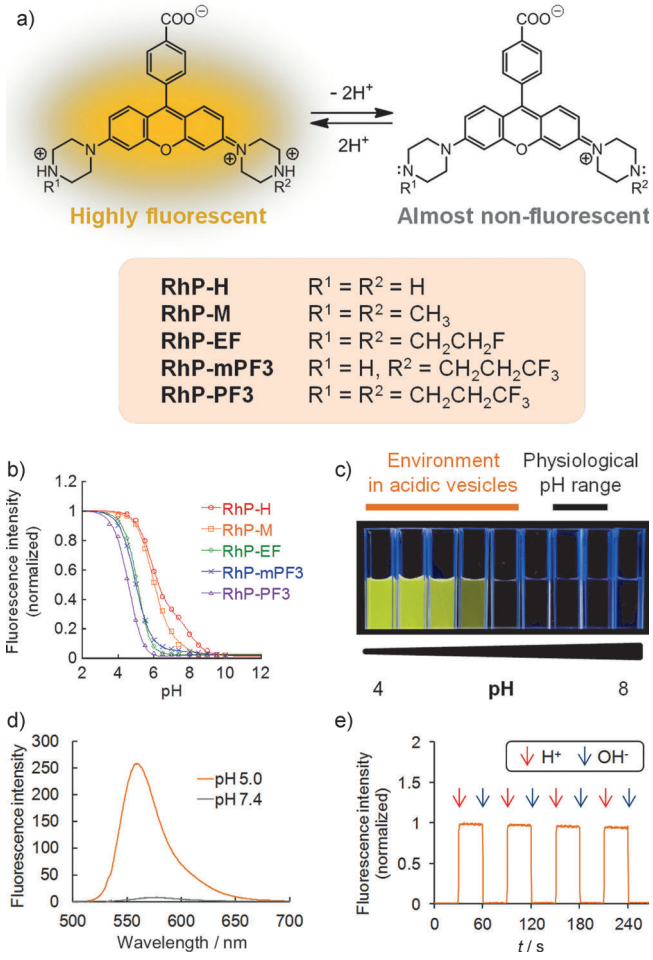

Figure 2. Design and properties of fluorescent probes activated under acidic conditions, RhPs. a) Acid–base equilibrium and b) pH profiles of RhPs. c) Photograph of cuvettes containing RhP-EF excited at 365 nm at pH values from 4 (left) to 8 (right) in increments of 0.5 pH units. d) Emission spectra of RhP-EF excited at 534 nm at pH 5.0 and 7.4. e) Fluorescence reversibility of RhP-EF during pH cycles between pH 7.2 and 3.1.

Table 1: Properties of fluorescent probes activated under acidic conditions.

	Activation[a] [-fold]	Photostability[b] [%]
RhP-EF	58	98 ± 1.9
pHrodo	1.1	98 ± 0.3
CypHer5E	4.6	74 ± 1.6
DiEtNBDP	12	8.8 ± 1.4

[a] Activation was calculated by dividing the fluorescence intensity of probes at pH 5.0 by that at pH 7.4. [b] Photostability was evaluated as the percentage fluorescence intensity of probes after irradiation at 50 J cm^{-2} as compared with that before irradiation (taken as 100%). Values are mean ± standard deviation ($n = 3$).

Burgess reported a similar molecule with pH-dependent properties, including fluorescence off–on reversibility,[9] its detailed properties, such as its pK_a values, were not evaluated. We found that the background fluorescence of RhP-H at pH 7.4 was unacceptably high, reaching approximately 30% of the maximum fluorescence (Figure 2b). Thus, RhP-H is unsuitable for the selective detection of the acidic vesicular environment. Next, to chemically optimize the pH sensitivity, we prepared a series of derivatives of RhP-H by introducing N-substituents (Figure 2a). Dependently upon the electron-withdrawing properties of the N-substituents, these RhPs showed acidic shifts of the fluorescence curves (Figure 2b; for details, see Table S1 and Figure S3 in the Supporting Information). From these derivatives, we selected RhP-EF as a fluorescent scaffold that could distinguish intravesicular pH values of 4–6 from the physiological pH value of 7.4 (Figure 2c) owing to its potent fluorescence activation upon putative acidification in the vesicles (58-fold; Figure 2d; see also Table S1). Unlike previously reported probes, RhP-EF offers both high activatability at acidic intravesicular pH values and excellent photostability (Table 1). Also, RhP-EF was extremely bright under acidic conditions ($\Phi_{fl} > 0.6$; see Figure S3), and its fluorescence was not influenced by biologically relevant substances, including other cations and glutathione (see Figure S10). Further investigation revealed rapid and complete reversibility of the response to environmental changes in the pH value (Figure 2e). We therefore concluded that RhP-EF should enable real-time monitoring of intravesicular pH-value changes, including multiple cycles. In short, these in vitro results demonstrated that RhP-EF is a promising scaffold for the selective sensing of acidic intravesicular pH values, with superior photostability and excellent reversibility.

For the visualization of exocytosis dynamics, an acidotropic fluorescent probe, named aRhP-EF (Figure 3), was developed by introducing an *N*,*N*-dimethylamino (dMA) group into RhP-EF to promote localization to the acidic vesicles, in line with pH-partitioning theory.[10] Unlike RhP-EF, aRhP-EF sensitively stained granules in RBL-2H3 cells, which are a well-studied mast-cell model[11] (see Figure S4). Moreover, we demonstrated that only aRhP-EF enabled selective granular staining with sufficiently high photostability for continuous observation; it also showed superior activation at acidic pH values (95-fold at pH 5.0 versus pH 7.4) as compared to other granule-targeted pH-activatable probes (Cy-pHer5E-dMA, pHrodo-dMA, and DiEtNBDP-dMA, all prepared in-house, and commercially available LysoSensor dye; see Table S2 and Figures S5 and S6). As a proof of concept (Figure 1), we performed time-lapse confocal imaging of degranulation induced by stimulating aRhP-EF-stained cells with the Ca^{2+} ionophore ionomycin in the presence of an extracellular fluid-phase marker

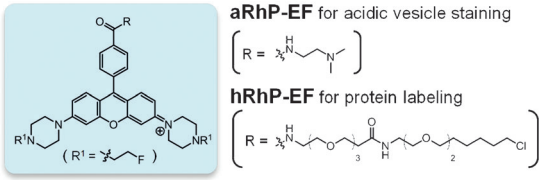

Figure 3. Chemical structures of fluorescent probes for exocytosis imaging.

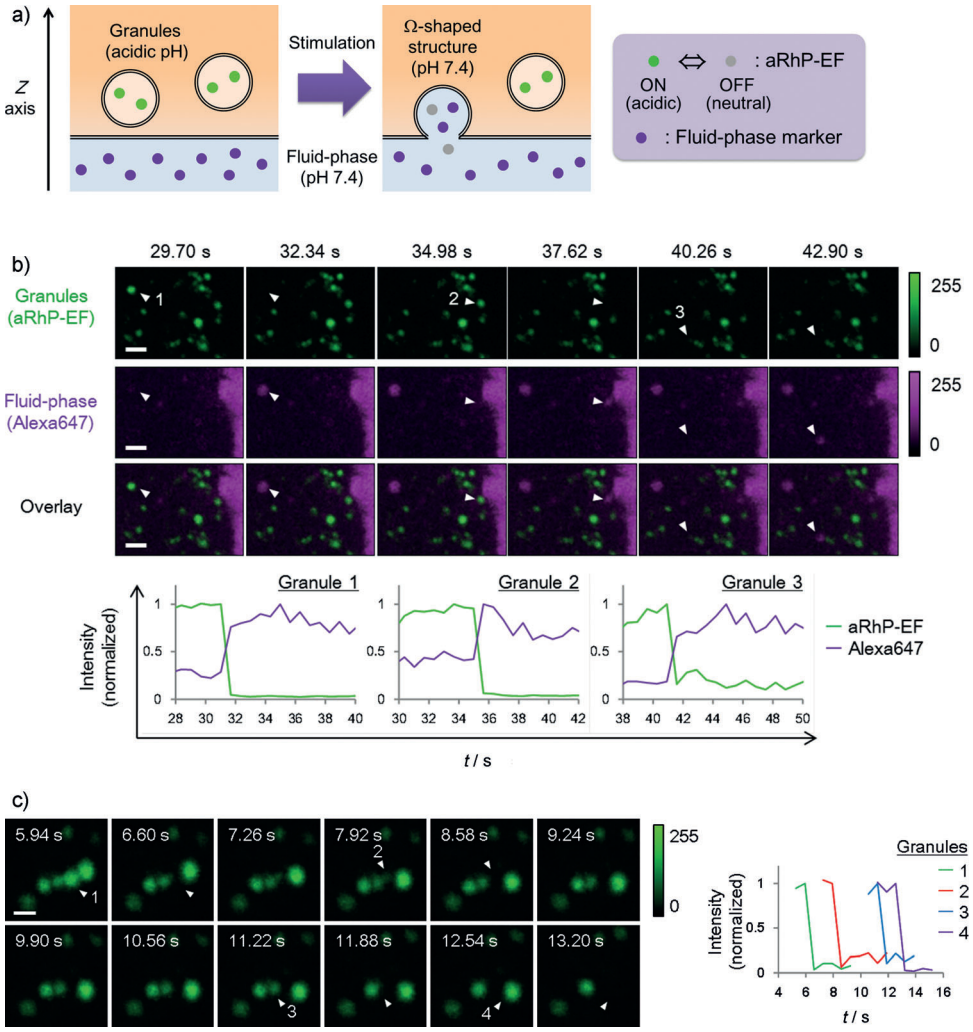

Figure 4. Exocytosis dynamics in RBL-2H3 cells visualized with aRhP-EF. a) Schematic representation of the imaging of exocytosis by our proposed method.b,c) Degranulation in ionomycin-stimulated RBL-2H3 cells. A fluid-phase marker was used in (b). Scale bars: 1 μm. The time course of normalized fluorescence intensity at numbered granules is shown.

(Figure 4a). Intriguingly, the fluorescence of some granules suddenly disappeared at different time points (Figure 4b, "Granules"; see also Movie S1 in the Supporting Information), and we observed synchronized influx of the fluid-phase marker into Ω-shaped membrane structures formed upon degranulation (Figure 4b, "Fluid phase"; see also Movies S2 and S3). These results indicate that the disappearance of the granular fluorescence corresponds to exocytosis events. To our knowledge, the dynamics of individual exocytosis events in RBL-2H3 cells has not been visualized previously without the need to artificially enlarge the granules with serotonin.[12] We also succeeded in visualizing an intriguing process of "sequential exocytosis", in which successive vesicular releases occurred around an initial event (Figure 4c; see also Movie S4), thereby providing direct evidence for a focal release mechanism that was originally proposed on the basis of the electron-microscopic observation of hormone-treated pancreatic acinar cells.[13] Thus, our technique was confirmed to enable the sensitive and reliable visualization of exocytosis dynamics at the level of individual vesicles. Furthermore, the simple technique of staining vesicles by incubating cells with the probe should enable this method to be readily applied to other cell types, including nontransfectable cells.

In secretory cells, exocytosis and compensatory endocytosis are tightly coupled, so that the releasing functionality is restored.[1] To further address the visualization of exo- and endocytosis processes, we developed hRhP-EF by coupling a HaloTag ligand to RhP-EF (Figure 3). Our imaging strategy was based on the covalent tethering of RhP-EF to granule-localizing vesicle-associated membrane protein 2 (VAMP2)[14] by using the HaloTag labeling technology[15] (Figure 5a). To test the performance of hRhP-EF, we incubated it with RBL-2H3 cell lines expressing or not expressing VAMP2-HaloTag protein. We confirmed that granular labeling occurred selectively in RBL-2H3/VAMP2-HaloTag cells, whereas only minimal nonspecific staining was observed in the original RBL-2H3 cells, presumably because of the chemically introduced poly(ethylene glycol) (PEG) spacer in hRhP-EF (see Figure S7). We also confirmed that the granular fluorescence reversibly responded to the environmental pH cycle (see Figure S8). Moreover, we found that only hRhP-EF enabled selective granular labeling of VAMP2-HaloTag-expressing cells and continuous observation, in contrast to the other HaloTag-ligand-coupled pH-activatable probes prepared in-house, CypHer5E-halo and pHrodo-halo (see Table S3 and Figures S7 and S9). Next, hRhP-EF-labeled cells were sensitized with anti-dinitrophenyl (DNP) IgE and stimulated with DNP-conjugated bovine serum albumin (DNP-BSA) to induce degranulation. Intriguingly, some granules showed cyclic "off" and "on" fluorescence changes (Figure 5b; see also Movie S5). By using a fluid-phase marker, we also revealed that this type of off–on phenomenon occurs at the identical vesicle (see Figure S11). This result is direct evidence of "kiss-and-run" events that consist of granular exocytosis in the "off" phase and endocytosis followed by intragranular reacidification in the "on" phase. Fernandez et al. reported the existence of

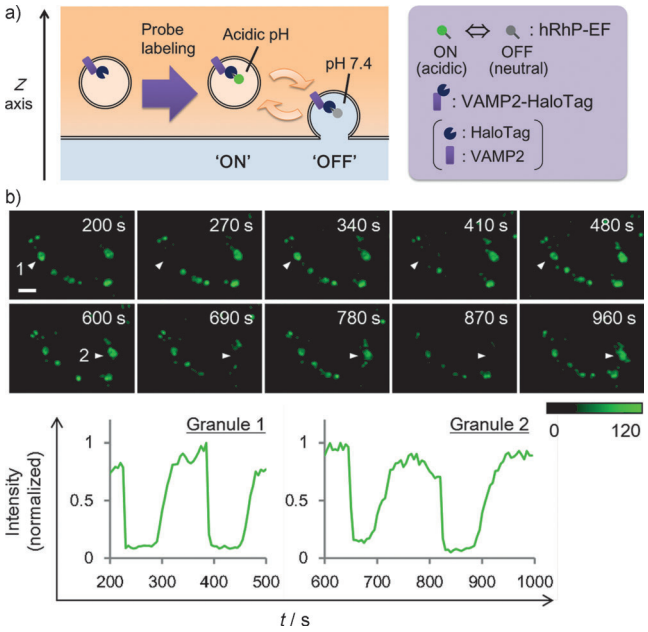

Figure 5. Exocytosis and endocytosis/reacidification dynamics in RBL-2H3 cells visualized with hRhP-EF. a) Schematic representation of the visualization of vesicular dynamics. b) Representative vesicular dynamics in anti-DNP IgE/DNP-BSA-stimulated RBL-2H3/VAMP2-HaloTag cells. Scale bar: 5 μm. The time course of normalized fluorescence intensity at numbered granules is shown.

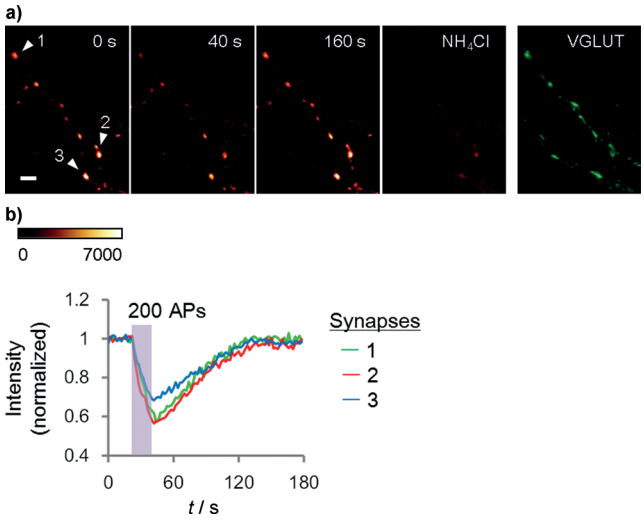

Figure 6. Exocytosis and endocytosis/reacidification dynamics in cultured neurons visualized with hRhP-EF. a) Synaptic-vesicle dynamics in cultured neurons: 200 action potentials (APs) at 10 Hz were applied at 20–40 s; intravesicular neutralization (NH_4Cl) and immunostaining (VGLUT) were also performed. Scale bar: 5 μm. b) Time course of normalized fluorescence intensity at synapses numbered in (a).

exo- and endocytosis events at whole-cell resolution by measuring the membrane capacitance of RBL-2H3 cells.[16] In comparison with these capacitance measurements, our technique represents a major technical advance that permits a fine spatiotemporal understanding of individual vesicular events, including exocytosis and endocytosis/reacidification processes, at single-granule resolution.

To further confirm the applicability of our technique, we applied it to neurons to visualize the exo- and endocytosis dynamics of synaptic vesicles during neuronal transmission. Cultured rat hippocampal neurons expressing VAMP2-HaloTag protein were labeled with hRhP-EF and electrically stimulated to release synaptic vesicles. The intensity of punctate fluorescence decreased by 30–40% during the electrical stimulation and subsequently recovered to the initial level (Figure 6; see also Movie S6). These changes in fluorescence probably correspond to synaptic-vesicle exocytosis and subsequent endocytosis/reacidification. We also confirmed that synaptic labeling was successfully performed with hRhP-EF on the basis of colocalization with a synaptic marker of vesicular glutamate transporter 1 (VGLUT1; Figure 6a "0 s" versus "VGLUT"), and we further confirmed that the synaptic fluorescence diminished in a pH-dependent manner by intravesicular neutralization with NH_4Cl (Figure 6a, "NH_4Cl"). Thus, our technique is applicable to neuronal studies. It may be primarily useful for exocytosis studies to reveal vesicular dynamics with high spatiotemporal precision. However, it would also be suitable for endocytosis studies, such as receptor internalization,[7] because proteins of interest (e.g. receptors) can be readily tracked with the probe by the use of protein-labeling technology.

In conclusion, we have developed fluorescent probes that are chemically optimized for sensing the acidic intravesicular environment. When conjugated with an acidotropic moiety or used in combination with protein-labeling technology, our chemical probes, aRhP-EF and hRhP-EF, enabled the visualization of vesicular dynamics in living cells with high intravesicular acidic activation of fluorescence, high staining specificity, and good photostability; they were greatly superior to other existing probes in these respects. Our results demonstrate that the dynamic fluorescence changes reflect exocytosis as well as endocytosis/reacidification processes. Because the background is minimal with these probes, vesicular imaging can be performed simply with confocal or conventional fluorescence microscopy. This study has provided a versatile and powerful tool for understanding vesicular events as spatiotemporally dynamic cellular phenomena, with substantial advantages over electron microscopy and electrophysiology. Furthermore, the technique has potential application in high-throughput screening systems. Secretion phenomena, such as degranulation during allergic response and synaptic transmission during neuronal function, certainly involve potential drug targets, and cell-based screening systems for compound libraries are under construction with our probes.

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Communications

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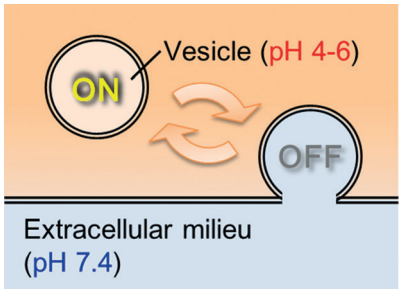
Acidic-pH-Activatable Fluorescence Probes for Visualizing Exocytosis Dynamics



Going live: Live imaging of the dynamics of exocytosis is crucial for a precise spatiotemporal understanding of secretion phenomena. Small-molecular fluorescent probes designed to be activated under acidic intravesicular conditions enabled the sensitive and reliable visualization of vesicular dynamics with high spatiotemporal precision (see picture).