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ORIGINAL PAPER

Lateral drift correction in time-laps images by the particle-tracking algorithm

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Abstract In long-term time-laps imaging of living cells, a significant lateral drift of the fluorescently labeled structures is often observed due to many reasons including superfusion of solution, temperature gradients, bolus addition of pharmacological agents and cell motility. We have detected lateral drift in long-term time-laps confocal imaging by tracking fluorescent puncta, which represent single exocytotic vesicles expressing synaptopHluorin (spH), a pH sensitive green fluorescence protein. Following the initial increase in fluorescence intensity due to alkalinization of vesicle lumen, the spH fluorescent puncta dimmed, which may be attributed to the resealing of the fusion pore and subsequent slow reacidification of the vesicle, or alternatively the dimming may be due to a significant lateral drift of the vesicle out of the region of interest (ROI). We identified and compensated the lateral drift by tracking particles present in the confocal images, without any additional mechanical and/or optical hardware components. The peak of the Gaussian two-dimensional (2D) curve fitted to the fluorescent particle intensity profile was recorded as the X and Y coordinates of the vesicle in each frame. The resulting coordinates of vesicle positions were averaged and rounded to the nearest pixel value, which was used to correct the drift in the time-laps images. In drift corrected time-laps images, the vesicle remained enclosed by the ROI, and the time dependent changes of spH fluorescence intensity averaged from the ROI remained at a constant level, revealing that endocytosis with subsequent slow reacidification of vesicles was an unlikely event.

Keywords Neuroendocrine cells · Lactotrophs · Synaptophluorin · Confocal microscopy imaging · Lateral drift

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Introduction

Time-laps imaging of living cells or tissue samples is an important technique in cell physiology. In long-term imaging, where recording takes several minutes or tens of minutes, significant lateral drift in imaged fluorescent structures may be observed. Ghosh and Webb (1994) tracked each individual fluorescently labeled low-density lipoprotein receptor molecule on the surface of human skin fibroblasts. The limitations on tracking precision were found to depend on the signal-to-noise ratio of the tracked particle and mechanical drift of the microscope system. By recording a sequence of time-lapse images and tracking the immobilized particles on glass surface, they found that the microscope system has an apparent slow drift during 2 min of recording, which resulted in a net displacement of 140 nm from the starting position. The diffusional path of single molecules in a phospholipid membrane may be imaged using phospholipids carrying one rhodamine dye molecule (Schmidt et al. 1996). A test of the mechanical stability of the imaging system was carried out by taking consecutive images of fluorescent latex spheres immobilized on a coverslip and showed that lateral positions have stability with a standard deviation of 12 nm. This analysis gave no indication

for resolvable mechanical drifts on a time scale of seconds (Schmidt et al. 1996). Bloess et al. (2002) reported optical imaging of single molecules with a reproducibility of the lateral position of 3.4 nm. This was achieved by the correction of the mechanical drift by monitoring the position of a spatial reference in the sample. A drift occurring on the time scale of tens of minutes reflects a mechanical drift of the microscope set-up, which shifts the whole image of the sample with respect to the CCD camera (Bloess et al. 2002). Moreover, Carter et al. (2007) further improved the resolution of single-molecule experiments by compensating the drift and achieved stabilization of an optical microscope by measuring the position of a fiducial mark coupled to the microscope coverslip and correcting for the drift using a piezoelectric stage. With these improvements, they achieved stabilities of 0.17 nm for tens of seconds (Carter et al. 2007).

Development of green fluorescent proteins (GFPs) has allowed the visualization of dynamic processes in living cells. The release of neurotransmitters and hormones from secretory vesicles of neurons and neuroendocrine cells is mediated by exocytosis, consisting of the fusion of vesicle and plasma membranes, leading to the formation of a fusion pore between the vesicle and the plasma membrane. Fusion pore dynamics of vesicles undergoing exocytosis has been studied using different electrophysiological, electrochemical and fluorescence techniques (Lindau et al. 2003; Stenovec et al. 2004; Vardjan et al. 2007). In contrast to single molecule imaging, in optical imaging of live cells larger image drifts are often observed, which may result from the liquid flow driven by superfusion systems, bolus addition of pharmacological agents or from cell motility. To compensate the lateral drift in long-term time-laps confocal imaging, we used an approach where single fluorescent vesicles were tracked to obtain coordinates of the image drift. To test our approach, we used confocal images of living rat lactotrophs, which express synaptopHluorin (spH), a pH sensitive GFP fused to the luminal side of the vesicle-membrane protein synaptobrevin-2 (Miesenböck et al. 1998). Rat pituitary lactotrophs spontaneously and under stimulation secrete prolactin (Walker and Farquhar 1980) and are a convenient preparation for studying vesicle exocytosis at the elementary level using real time fluorescence microscopy (Angleson et al. 1999; Cochilla et al. 2000; Stenovec et al. 2004; Vardjan et al. 2007). When expressed in exocytotic vesicles, spH enables optical discrimination between the unfused (faint or no fluorescence signal) and fused vesicles (greatly increased green fluorescence signal) (Miesenböck et al. 1998; Sankaranarayanan and Ryan 2000; Gandhi and Stevens 2003; Tsuboi and Rutter 2003; Fernandez-Alfonso and Ryan 2004).

The purpose of this study was to compensate the lateral drift in long-term time-laps confocal imaging in living cells, by tracking single fluorescent vesicles. We show here, that our approach provides an appropriate method to correct lateral drift and enables consistent analysis of fluorescence intensity changes in single exocytotic vesicles.

Materials and methods

Cell preparation and transfection

Lactotroph-enriched cell cultures were prepared from adult male Wistar rat anterior pituitaries as described earlier (Ben-Tabou et al. 1994). Cells were plated on poly-L-lysin-coated glass coverslips and maintained in DMEM high glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 10% newborn calf serum and L-glutamine in an atmosphere of humidified air (92%) and CO_2 (8%). Liposome mediated gene transfer (Lipofectamin Plus™, Invitrogen, Carlsbad, CA, USA) was used to transfect 1-day-old cultures with spH in a pCI neo vector (Promega, Madison, WI, USA) (Miesenböck et el. 1998). The spH plasmid construct was a gift of G. Miesenböck, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, NY, USA. All the experiments were carried out 2–4 days after transfection at room temperature. Standard saline solution was prepared consisting of 10 mM HEPES/NaOH (pH 7.2), 10 mM D-glucose, 130 mM NaCl, 8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM KCl. All chemicals were obtained from Sigma–Aldrich Inc. (St Louis, MO, USA).

Time-laps confocal microscopy

Laser-scanning fluorescence images were acquired using inverted confocal microscope (Zeiss LSM 510, Jena, Germany). Coverslips with transfected cells were mounted on a perfusion-stimulation chamber on the stage of the confocal microscope and supplied with a standard saline solution. spH fluorescence was excited with a 488 nm Argon Ion laser line. Fluorescence emission was collected through a plan apochromatic objective (63×, 1.4 NA) with a 505–530 nm band-pass emission filter. Time-lapse fluorescence images of lactotrophs were obtained every ~0.5 s for up to 2 min (~240 frames). The thickness of the optical section was ~2 μm.

Results

spH is expressed in prolactin-containing vesicles of lactotrophs

To measure single vesicle fusion events in real-time, lactotrophs were transfected with spH, a pH sensory protein,

consisting of the vesicle membrane targeted protein VAMP2 (synaptobrevin-2) and the pH-sensitive enhanced GFP (superecliptic pHluorin), fused to the luminal side of VAMP2 (Miesenböck et al. 1998). In transfected lactotrophs, a punctuate appearance of the green fluorescence was observed (Fig. 1), which represents prolactin-containing vesicles, as reported earlier (Vardjan et al. 2007). The fluorescence signal of spH is completely quenched at the acidic pH in resting vesicles, resulting from the activity of the vacuolar H^+-ATPase. Upon vesicle fusion with the plasma membrane, which occurs following stimulation by a depolarizing solution (Fig. 1), the acidified vesicle interior becomes accessible to more alkaline extracellular environment, allowing protons to escape, which relieves the proton-dependent quenching of spH, thus the number of fluorescent puncta per cell is increasing (Fig. 1a middle and right panels), consistent with previous reports (Stenovec et al. 2004). To monitor fluorescence intensity changes of spH over time of a single vesicle, a squared region of interest (ROI, 9 × 9 pixels) was centered on the spot of fusion event. The spH fluorescence intensity increases swiftly (Fig. 1c) and remains elevated until vesicle retrieval and/or reacidification occurs (Miesenböck et al. 1998). The rapid increase in spH fluorescence intensity was observed after cell exposure to the depolarizing medium (Fig. 1a, b) indicating that in these vesicles, fusion of vesicle membrane with the plasma membrane resulted in the exposure of luminal spH to the external alkaline medium via the fusion pore. Analysis of time-dependent spH fluorescence changes measured in the ROI revealed a rapid increase in fluorescence intensity that indicated an efflux of protons through a newly formed fusion pore. Vardjan et al. (2007) reported that following the peak of fluorescence intensity in spontaneously fusing vesicles, the green fluorescent puncta either dimmed, implying the resealing of the fusion pore due to the reacidification of the vesicle ("transient" fusion pore), or remained visible throughout the experiment, indicating that the vesicular lumen remained exposed to the extracellular space through an open fusion pore ("permanent" fusion pore). However, in some of the latter vesicles a notable lateral image drift was observed as shown on Fig. 1b.

Lateral drift correction

We have monitored single stationary vesicles for several minutes by measuring the time-dependent fluorescence intensity changes in the spH labeled vesicles. In some recordings, lateral drift in the vesicle images (Fig. 1a, b) introduced an artifact in the subsequent image analysis of time-dependent fluorescence intensity changes (Fig. 1c). In the magnified view, the drift of the selected vesicle is clearly observed as vesicle displacement from the center of ROI, marked by white edges (Fig. 1b). Shortly after the cell was exposed to the depolarizing solution (at time 0), the spH fluorescence averaged from the ROI steeply increased and subsequently slowly decreased, most likely due to the lateral drift of the imaged vesicle. If the drift is not taken into account, the decrease in spH fluorescence may be erroneously interpreted as the reacidification of the vesicle lumen, which also implies the resealing of the fusion pore (as in "transient" fusion pore). To automatically correct the lateral drift in time-laps images, we developed the software tool using Matlab (Fig. 2; Mathworks, Natick, MA, USA). Matlab is a high-level language for technical computing, where an array is a basic data element and is therefore especially well suited for image analysis. The image processing was performed on a PC with Microsoft Windows XP operating system.

Our approach to correct lateral drifts is based on tracking of imaged particles (ParticleTR, Celica, Ljubljana, Slovenia; Potokar et al. 2005, 2007), and does not need any additional mechanical and/or optical hardware components. We first exported confocal images as 8-bit tag image file format (TIFF) files. The software automatically imported the image time series and converted them into arrays. All image stacks were sequentially analyzed (Fig. 2). To track single particles, we first selected these objects; each represented a single fluorescently labeled vesicle. The software directly fitted two-dimensional (2D) Gaussian curves to the vesicle intensity profile (Anderson et al. 1992; Schutz et al. 2004; Potokar et al. 2005, 2007), using a simplex algorithm (Lagarias et al. 1998) with a least-squares estimator. The peak of the Gaussian 2D curve was recorded as the coordinates of the vesicle in each frame. Before data was fitted, the ROI was filtered using a kernel with Gaussian distribution and with a standard deviation of one pixel. The dimensions of the 2D Gaussian curve were 10 × 10 pixels. The tracking of vesicles and analysis was fully automatic (Fig. 3a). Trajectories of the movements of the vesicles, which are due to the Brownian motion of vesicles and to lateral drift in time-laps recordings, show a decline (see Fig. 3a). The resulting coordinates of vesicle positions, recorded as a function of time were averaged and rounded to the nearest pixel value (Fig. 3b). The rounded mean coordinate was used to correct individual time-laps image for the drift in the image sequence. The software shifted each image in the time stack according to the corresponding drift values for *X* and *Y*. To avoid interpolation and recalculation of pixel values, the drift correction was performed to the nearest full pixel value. We found that 75 s following stimulation (see Figs. 1, 4), the time-laps images were drifted for four pixels in both X and Y directions, which corresponds to 5.7 pixels or 283 nm in diagonal direction. In drift corrected time-laps images, the single vesicle remained within the ROI, marked by white edges (Fig. 4b), and the time course of spH fluorescence intensity averaged

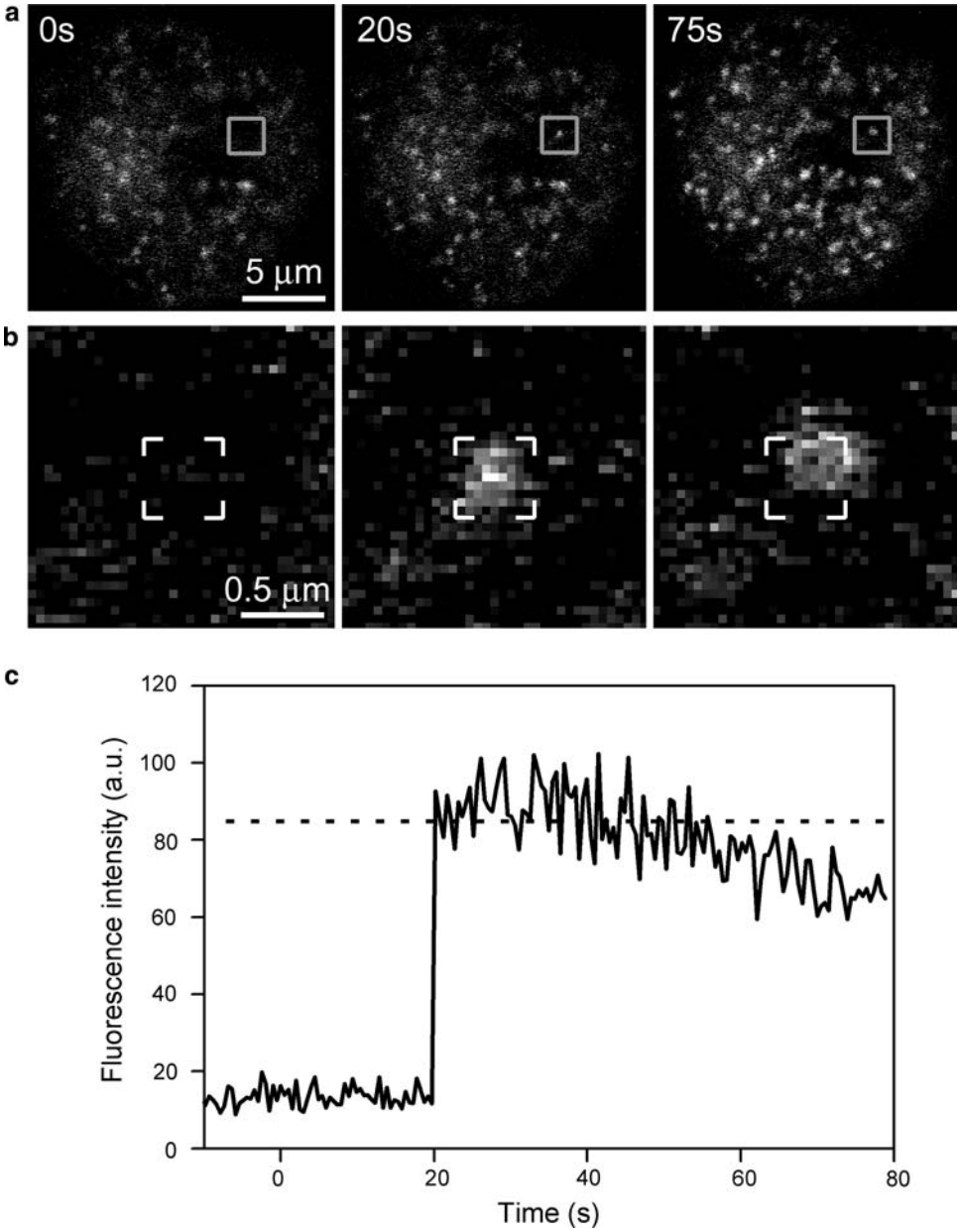

Fig. 1 The time-laps sequence of confocal images showing time-dependent brightening of spH expressing vesicles after application of depolarising KCl solution. **a** The time-laps images of the confocal section through the lactotroph, transfected by the vesicle membrane targeted spH, which is expressed in the luminal side of the vesicle membrane. *Bright fluorescent spots* represent single vesicles. Exocytosis of a prolactin vesicle resulted in a rapid increase in the spH fluorescence signal, for this more spots are visible at times beyond 20s. Time labels indicate the times of image acquisition. **b** Enlarged view on a selected vesicle labeled by the *gray* frames in **a**. Note that the image of the framed single vesicle is significantly displaced from the ROI, marked by *white* edges. **c** Time dependent changes of spH fluorescence intensity averaged from the ROI. Following a delay of around 20 s after the depolarizing solution was applied (at time 0 s), a steep increase in fluorescence intensity was recorded, after which a fluorescence decline was observed, most likely due to the drift of the imaged vesicle from the ROI (see *panel b*). The *dashed line* indicates the maximum elevation of spH fluorescence intensity

from the ROI remained at a constant level, after a steep increase. This implies that the vesicle analyzed displayed a "persistent" fusion event.

We conclude that lateral drift correction software based on the particles tracking provides appropriate means for analysis of fluorescence changes obtained in single vesicles during long-term time-laps imaging in living cells.

Discussion

Time-laps fluorescence imaging in living cells is an important technique, which allows visualization of subcellular processes. In short-term records, fluorescence changes obtained in cells are usually devoid of lateral or vertical drifts. However, if cells are monitored for several minutes or longer, or a perfusion solution is applied to the cells, or if the temperature gradients are introduced into the system, the lateral and vertical drifts may introduce serious artifacts in the subsequent image analysis. Vertical drift (focus-drift) is due to the slippage in the microscope focus mechanism and/or the thermal gradients in the microscope. To correct for the focal-drift, z-stacks of images may be taken periodically, as described earlier (Kreft et al. 2005). The software calculates the Pearson's correlation coefficient between each image in the z-stack and the reference image in the stack. The maximal correlation coefficient of pixel intensities is taken to identify the image, which corresponded to the focal plane of the reference image (Kreft et al. 2005). Here we describe an approach to correct the lateral drift in

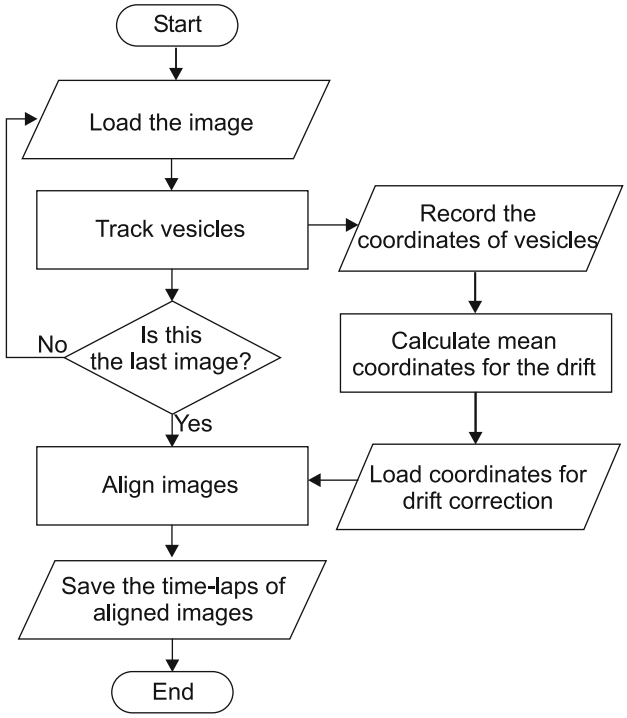

Fig. 2 Flowchart displaying the drift detection and correction processes

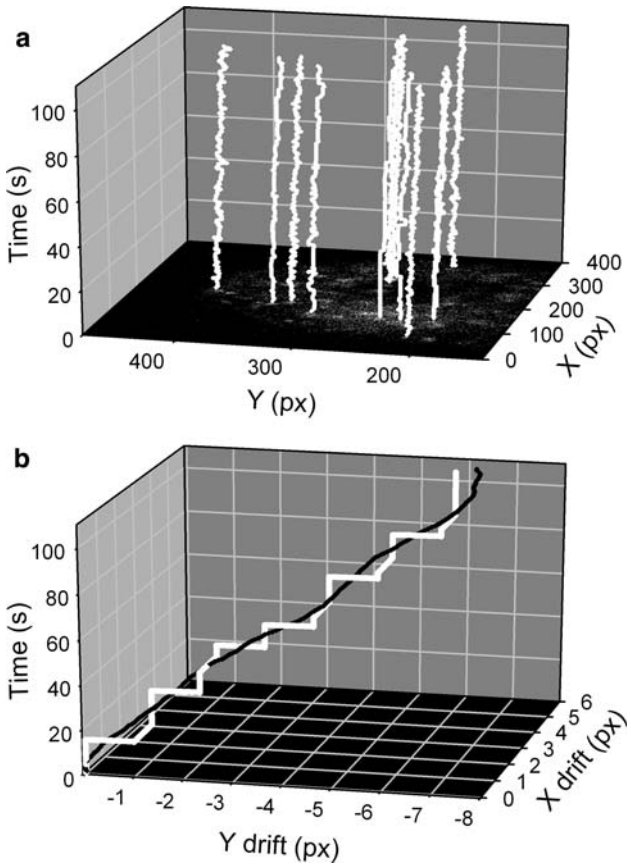

Fig. 3 Detection and measurement of lateral drift in images of fluorescently labeled vesicles. **a** Trajectories (*white*) of vesicle movements obtained in a cell, which represent the lateral drift in time-laps recordings. Trajectories are superimposed to the fluorescent image of the cell (bottom plane). In addition to Brownian motion of vesicles, a decline to the right is observed. **b** Averaged trajectories (*black*) and trajectories rounded to the nearest pixel (*px*) value (*white*). The later serves as a measure for correcting drift over time

prolonged imagining of fluorescently labeled secretory granules (diameter of around 200 nm) in live pituitary cells by tracking imaged particles.

Vesicles undergoing exocytosis have been studied using different electrophysiological, electrochemical and fluorescence techniques, including visualization of GFPs (Lindau et al. 2003; Stenovec et al. 2004; Vardjan et al. 2007). In contrast to single molecule imaging, where the reported mechanical drift is smaller than the resolution of the microscope (Ghosh and Webb 1994; Schmidt et al. 1996), in imaging of live cells several orders of magnitude larger drifts are often observed. This is due to mechanical distortions introduced by the superfusion system, temperature gradients, bolus addition of pharmacological agents or due to cell motility. In single molecule imaging, the limitation is set by the signal-to-noise ratio and by the mechanical drift of the microscope system. By tracking the immobilized particles, the drift was measured to be tens of nm per minute (Ghosh and Webb 1994). Using a spatial reference in the sample, a drift may be compensated off-line (Bloess et al. 2002), or on-line with the imaging using the piezoelectric stage (Carter et al. 2007).

We developed an approach to compensate the lateral drift in long-term time-laps confocal imaging by tracking fluorescent spH labeled exocytotic vesicle (Vardjan et al. 2007). These vesicles fluoresce due to the expression of spH, a pH sensitive GFP (Miesenböck et al. 1998), which is used to measure pH in single unfused (faint or no fluorescence signal) and fused vesicles (greatly increased green fluorescence signal) (Miesenböck et al. 1998; Sankaranarayanan and Ryan 2000; Gandhi and Stevens 2003; Tsuboi and Rutter 2003; Fernandez-Alfonso and Ryan 2004).

On monitoring time-dependent spH fluorescence intensity changes, a rapid increase in fluorescence was observed after the exposure of cells to the depolarizing medium indicating that in these vesicles fusion with the plasma membrane resulted in vesicle lumenal spH to be exposed to the extracellular, relatively more alkaline medium via the fusion pore. As also reported by Vardjan et al. (2007), following the peak of fluorescence intensity signal the fluorescent puncta dimmed, which may be attributed to the resealing of the fusion pore and subsequent reacidification of the vesicle lumen. However, in some recordings, the lateral drift caused shifts of the vesicle positions out of

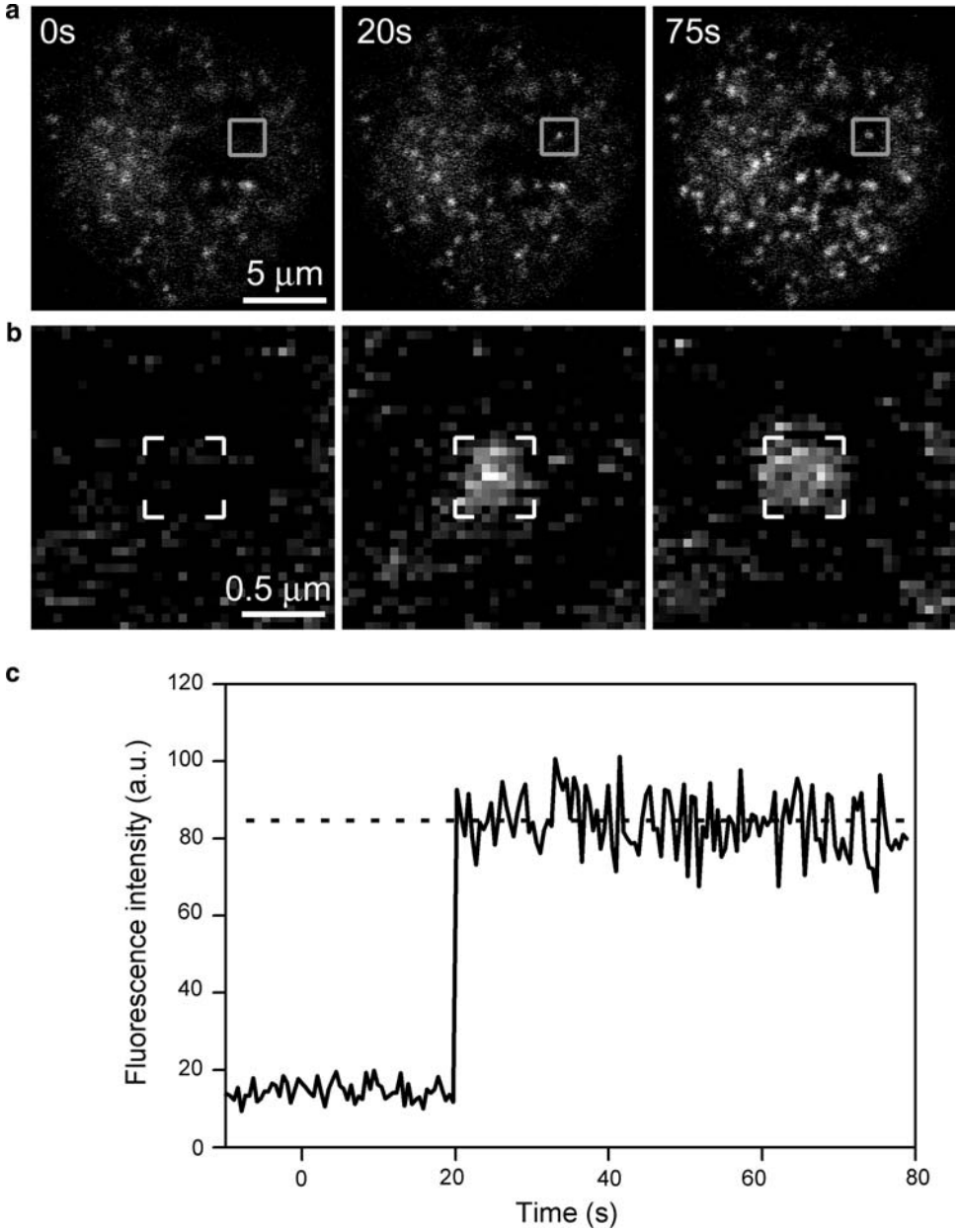

Fig. 4 The drift corrected time-laps sequence of confocal images of the same cell as in Fig. 1. **a** The drift corrected time-laps images of the confocal section in a lactotroph. Time labels indicate the times of image acquisition. **b** Enlarged view on a selected vesicle labeled by the *gray* frames in **a**. Note that the single vesicle remains within the ROI, marked by *white* edges. **c** Time dependent changes of spH fluorescence intensity averaged from the ROI. After a steep increase, the fluorescence intensity remains at a constant level. The *dashed line* indicates the maximum elevation of spH fluorescence intensity

confided ROIs, which resulted in artifacts when the time-course of vesicle fluorescence intensity was analyzed. Such artifact may be interpreted as transient event in time-dependent spH fluorescence intensity monitoring. Moreover, in any measurements of time-dependent fluorescence intensity changes, lateral drift may severely decrease the amount of data that could be further analyzed, if the lateral drift is not corrected.

Our approach to correct lateral drift is based on tracking of imaged particles (Potokar et al. 2005, 2007), and does not need any additional mechanical and/or optical hardware components. The peak of the Gaussian 2D curve fitted to the fluorescent particle intensity levels, was recorded as the coordinates of the vesicle in each frame. The resulting coordinates of vesicle position were averaged and rounded to the nearest pixel values and were used to correct the drift in time-laps images. In drift corrected time-laps images, the single vesicle remained within the ROI, and the time dependent changes of spH fluorescence intensity averaged from the ROI remained at a constant level after initial increase. This implies, that the vesicle analyzed underwent a "persistent" fusion event and not a "transient" event as appeared before lateral drift correction. The drift correction embedded in our software allows us to appropriately analyze and interpret fluorescence intensity changes in single exocytotic vesicles obtained in long term time-laps imaging in living cells.

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