METHODS AND APPROACHES

Quantitative determination of cellular [Na+] by fluorescence lifetime imaging with CoroNaGreen

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Fluorescence lifetime imaging microscopy (FLIM) with fluorescent ion sensors enables the measurement of ion concentrations based on the detection of photon emission events after brief excitation with a pulsed laser source. In contrast to intensity-based imaging, it is independent of dye concentration, photobleaching, or focus drift and has thus been successfully employed for quantitative analysis of, e.g., calcium levels in different cell types and cellular microdomains. Here, we tested the suitability of CoroNaGreen for FLIM-based determination of sodium concentration ([Na+]) inside cells. In vitro measurements confirmed that fluorescence lifetimes of CoroNaGreen (CoroNaFL) increased with increasing [Na+]. Moreover, CoroNaFL was largely independent of changes in potassium concentration or viscosity. Changes in pH slightly affected FL in the dynamic, FLIM-based determination of intracellular [Na+]. This approach thus represents a valuable tool for quantitative determination of cellular [Na+] and changes thereof in different subcellular compartments.

Introduction

A fundamental property of animal cells is that they maintain a low sodium concentration ([Na+]) in their cytosol. This is accomplished by the ubiquitous Na+/K+-ATPase (NKA), which converts ATP into energy stored in an inwardly directed Na+ gradient across the plasma membrane (Sweadner, 1989; Kaplan, 2002). The Na+ gradient is used to drive secondary-active transporters including ion transporters such as the Na+/Ca2+ exchangers (Verkhratsky et al., 2018) or transporters for metabolites (Wright and Turk, 2004). Increases in the cytosolic [Na+], e.g., following channel-mediated Na+ influx (Rose et al., 1999), may result in a reduction or even reversal of Na+-dependent transporters, a phenomenon described for Na+/Ca2+ exchange in the heart muscle or in the brain (Kirischuk et al., 2012; Aronsen et al., 2013; Gerkau et al., 2018). Na+-dependent transporters are expressed not only at the plasma membrane but also at the membranes of cellular organelles. Mitochondria, for example, express Na+/Ca2+ exchangers that shape mitochondrial calcium signaling in a Na+-dependent manner, thereby regulating mitochondrial as well as cellular functions (Maack et al., 2006; Murphy and Eisner, 2009; Parnis et al., 2013; Nita et al., 2014; Ben-Kasus Nissim et al., 2017).

The foregoing studies thus indicate that there is a tight interrelationship between regulation and signaling of Na+ and other ions, which not only includes the cytosol but also comprises intracellular organelles. Detailed understanding of these processes requires knowledge on driving forces, which means knowledge on basal [Na+] as well as changes thereof during signaling in the various subcellular compartments. Determination of [Na+] has, however, always been hampered by the limited availability of experimental tools. In essence, only a handful of chemical fluorescent indicators suitable for dynamic Na+ imaging inside cells exist and all suffer from well-known pitfalls such as interaction with proteins and...
other cellular constituents or low quantum efficacy and bleaching (Schreiner and Rose, 2012; Biskup and Gensch, 2014). The latter is especially critical when measurement of [Na+] is based on the analysis of the emission intensity of the dyes.

In addition to exhibiting a change in fluorescence intensity with ion binding, many ion-selective fluorescent dyes also show a change in their fluorescence lifetime (FL), a parameter that is independent from the number of dye molecules (Bastiaens and Squire, 1999). In FL imaging microscopy (FLIM; Suhling et al., 2005; Becker, 2012), a pulsed laser source can be employed in combination with time-correlated single-photon counting (TCSPC). The latter records the exact time at which individual photons hit the detector after the excitation event. The temporal incidence of photon emission decays exponentially, and repeating this measurement many times enables the construction of a curve from which the average FL is deducted. As mentioned above, the FL is generally independent from the absolute number of dye molecules and from dye concentration.

Since its first implementation for intensity-independent, quantitative analysis of calcium concentrations in 1992 (Lakowicz et al., 1992), this technique has been widely employed for determination of intracellular calcium in different tissues (Wilms et al., 2006; Wilms and Eilers, 2007). Among others, Ca2+-FLIM revealed globular intracellular calcium in different tissues (Wilms et al., 2006; 1992), this technique has been widely employed for determination of Na+ using FLIM with CoroNaGreen https://doi.org/10.1085/jgp.201912404

To determine the properties of CoroNaGreen for FLIM-based detection of somatic [Na+] increases in neurons in mouse brain (Untiet et al., 2017), we tested if CoroNaGreen FLIM can be employed to evaluate the dye’s properties, such as its sensitivity to potassium or pH, or a calibration to reveal its apparent Kd in situ, were performed. The goal of the present work was therefore to analyze the suitability of lifetime measurements of CoroNaGreen for quantitative determination of intracellular [Na+] in more depth. In addition, we tested if CoroNaGreen FLIM can be employed to monitor changes in [Na+] induced by metabolic inhibition or inhibition of the cellular NKA. To this end, we employed two different strategies for data analysis: TCSPC as well as a photon-count ratio method (“Ratio FLIM”), with the latter increasing the temporal resolution several-fold as compared with the former.

Materials and methods
FL determination of CoroNaGreen in vitro
To determine the properties of CoroNaGreen (Life Technologies, Thermo Fisher Scientific; C36675) in vitro, the dye was dissolved at 5 µM in 1 ml saline composed of 10 mM HEPES, 26 mM K+-glucuronate, and different [Na+] (0–150 mM); [K+] was adjusted to maintain iso-osmolality (NaCl + KCl = 150 mM), and pH was titrated to 7.3 with KOH. To evaluate the K+-sensitivity of CoroNaGreen, [Na+] was set to 15 mM and [K+] was varied between 30 and 150 mM, while NMDG was added accordingly to maintain iso-osmolality. To analyze its dependence on pH, salines with 20 mM [Na+] were used in which pH was adjusted between 5.0 and 8.0. The influence of different viscosity was tested using a saline with 20 mM Na+, to which 0, 1, 3, or 5% (g/ml) of an 80 kD dextran (Sigma-Aldrich Chemie; 00892) was added.

The time-resolved detection of CoroNaGreen’s fluorescence decay in vitro was performed with a FluoTime100 fluorescence spectrophotometer based on a picoHarp300 unit (PicoQuant). As an excitation source, a pulsed diode laser (LDH-440; center wavelength, 440 nm; pulse width, 54 ps; repetition frequency, 20 MHz; PicoQuant) was used. Fluorescence decay curves were measured by a photomultiplier, where photons were spectrally selected by a monochromator (530 nm, CoroNaGreen; or 440 nm, Instrument Response Function [IRF]) and acquired in TCSPC mode. Fig. 1, A, C, E, and G, depict the nonconvolved, as-measured fluorescence decay curves. For further processing, the fluorescence decay curves were analyzed by iterative deconvolution of a bi-exponential model function and the measured IRF using the software package FluoFit 4.4 (PicoQuant). Fig. 1, A, D, E, and G, depict the average FL calculated from the respective bi-exponential model function (see also below).

Cell culture and dye loading
HEK293T cells (“HEK cells”; human embryonic kidney, 96121229, SV40 transformed) were obtained from the European Collection of Cell Cultures (Sigma-Aldrich Chemie). Cells were grown using Dulbecco’s modified Eagle’s medium, 10% FBS, supplied with 1% penicillin streptomycin (10,000 units/ml penicillin stock, 10,000 µg/ml streptomycin stock, GIBCO, Thermo Fisher Scientific) and 2 mM glutamine (Thermo Fisher Scientific). The cells were trypsinized and replated twice a week when they reached 70–80% confluency.

For experiments, coverslips were transferred to an experimental bath and incubated in artificial cerebrospinal fluid (ACSF), composed of 125 mM NaCl, 3 mM KCl, 25 mM HEPES, 2 mM MgSO4, 2 mM CaCl2, 1.25 mM NaH2PO4, and 5 mM glucose, titrated to pH 7. For dye-loading, cells were incubated with the membrane-permeable form of CoroNaGreen (CoroNaGreen acetoxymethyl [AM] ester; C36676; Thermo Fisher Scientific) dissolved (15 µM) in ACSF for 15 min at room temperature. After dyeloading, HEK cells were washed with ACSF and placed in an imaging chamber of an upright scanning fluorescence microscope (Air MP; Nikon Instruments Europe). In some experiments, cells were additionally stained with 15 nM Mitotracker Red CMXRos (“Mitotracker”) for 15 min at 36°C (M7512; Thermo Fisher Scientific) before loading the cells with CoroNaGreen-AM at room temperature as described above.

FL imaging and calibration of CoroNaGreen in situ
Dye-loaded HEK cells were excited by 100 fs pulses at 880 nm, generated at 80 MHz by a mode-locked Titan Sapphire laser
Figure 1. Ion dependence of CoroNaGreen FL in vitro. Traces shown on the left (A, C, E, and G) represent temporal photon distributions emitted by CoroNaGreen (normalized photon count) after pulsed excitation in different salines as indicated; the insets show an enlargement of the fluorescence intensity decays as a function of time between 0.5–1.2 ns. Graphs on the right (B, D, F, and H) illustrate mean values of τ_{AVG} ± SD obtained from three experiments each. 

(A and B) Photon count and τ_{AVG} of CoroNaGreen in salines with different [Na⁺], ranging from 0–150 mM. Data in B were fit with a sigmoidal function, revealing a K_d of 42.7 mM. In A, the IRF is shown in black. 

(B) Also depicted is the structural formula of CoroNaGreen as given by the Meyers et al. Journal of General Physiology 2019.

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Photon count and \( \tau_{\text{AVG}} \) of CoroNaGreen in salines with a fixed \([\text{Na}^+]\) of 15 mM and potassium concentrations altered between 30 and 150 mM (plotted are 30, 80, 100, 120, and 150 mM). The inset on the left illustrates the nearly complete overlap obtained at 80 and 120 mM \([\text{K}^+]\).

(P and F) pH sensitivity of photon count and \( \tau_{\text{AVG}} \) of CoroNaGreen at a given \([\text{Na}^+]\) of 20 mM. Plotted are traces obtained at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. (G and H) Photon count and \( \tau_{\text{AVG}} \) of CoroNaGreen at a \([\text{Na}^+]\) of 20 mM and after addition of dextran at 1, 3, and 5%. Note that addition of dextran did not result in a detectable change in fluorescence intensity decays.
fluorescence decay of CoroNaGreen in cuvette calibrations (n = 3 experiments). As reported before (Naumann et al., 2018), the FL of CoroNaGreen increased with increasing [Na+] (Fig. 1 A). Fitting the data obtained using a sigmoidal plot revealed a Kᵩ of 42.7 ± 18.2 mM (Fig. 1 B). Because baseline [Na⁺] inside cells is typically in the range of 10–20 mM, this indicates that CoroNaGreen might be suitable for FLIM-based analysis of [Na⁺] inside cells.

We next went on to test its sensitivity to other ions and to changes in viscosity. Vertebrate cells typically maintain a baseline intracellular [K⁺] concentration ([K⁺]) of 110–130 mM (Ballanyi et al., 1983; Kofuji and Newman, 2004), which, e.g., in neurons may be transiently reduced due to K⁺ loss in response to opening of K⁺-permeable ion channels. To test the sensitivity of CoroNaGreen to K⁺, we exemplarily compared its FL in salines with a fixed [Na⁺] of 15 mM, to reflect baseline conditions, and different [K⁺], ranging from 30 to 150 mM (n = 3 experiments). Fig. 1, C and D, illustrate that CoroNaGreen exhibited only small changes in its FL upon varying [K⁺]; decay curves were essentially indistinguishable between 120 and 80 mM K⁺. Given that [K⁺] decreases inside cells are expected to largely mirror concomitant increases in [Na⁺] and anticipated changes in [Na⁺] are <50 mM, the K⁺-sensitivity of CoroNaGreen thus seems negligible for measurements under our experimental conditions in situ.

To probe for the pH sensitivity of CoroNaGreen FL, salines with a fixed [Na⁺] of 20 mM and different pH, ranging from 5 to 8, were prepared (n = 3 experiments). As illustrated in Fig. 1, E and F, the FL of CoroNaGreen was essentially independent on pH in the range between 6.0 and 8.0, whereas at pH ≤5.5, FL increased slightly. Finally, we evaluated the effect of different viscosity by adding 80 kD dextran at 0, 1, 3, or 5% to saline with a viscosity that was increased slightly. Because baseline [Na⁺] inside cells is typically in the range of 10–20 mM, this indicates that CoroNaGreen might be suitable for FLIM-based analysis of [Na⁺] inside cells.

To address the identity of the high FL/intensity compartments, cells were additionally stained with Mitotracker, which selectively labels mitochondria (Fig. 2 C). Merging the different channels revealed that compartments exhibiting a distinctly increased FL and intensity showed a remarkable spatial overlap with the Mitotracker fluorescence (Fig. 2, D and E), indicating that they contain a high number and density of mitochondria. This colocalization was especially obvious in the peri-nuclear region (see white areas in the merged images in Fig. 2). Notably, this does not exclude the copresence of other organelles like lysosomes in the region of the high coincident presence of both fluorescent signals.

To calibrate FL of CoroNaGreen in situ, cells were first exposed to ACSF followed by different calibration salines, containing a defined [Na⁺] ranging from 0 to 150 mM, as well as ouabain (1 mM), granamicin (3 μM), and monensin (10 μM; Fig. 3 A; n ≥ 25). Importantly, the latter ionophores preferentially insert into the plasma membrane and not into organelles (Haroootianian et al., 1989), allowing rapid equilibration between the [Na⁺] of the cytosol with that of the external saline (Rose and Ransom, 1996a; Langer and Rose, 2009). Under these conditions, the difference in FL between the perinuclear region and the remainder of the cell was largely maintained, and FL was consistently longer for [Na⁺] ≤50 mM (Fig. 3 A). At higher [Na⁺], however, compartments could no longer be clearly distinguished based on their FL (Fig. 3 A).

To analyze the behavior of the different cellular regions separately, ROIs were manually drawn based on differences in the intensity of CoroNaGreen to delineate the nucleus, perinuclear regions, and cytosol, respectively (see Fig. 3 D). Plotting the FL of the different compartments against the [Na⁺] confirmed their apparent divergence at low [Na⁺]: while mean FL of nuclei and cytosol were not significantly different for [Na⁺] ≤50 mM, FL of perinuclear regions was consistently higher than that of both nuclei and cytosol. This consistent difference was lost for mean values of the FL of the three compartments at [Na⁺] ≥70 mM (Fig. 3 B). These results suggested that at [Na⁺] relevant for the present study (0–50 mM), [Na⁺] was apparently fully equilibrated between cytosol and nucleus. In contrast to this, full equilibration was apparently not obtained for the perinuclear region. Therefore, these ROIs were excluded for the determination of the [Na⁺] sensitivity of CoroNaGreen FL in situ. Instead, only ROIs positioned over cytosolic and nuclear regions were included to construct a calibration curve. Plotting the average FL from these ROIs against the [Na⁺] revealed an increase from ~483 ps at 0 mM Na⁺ to ~1,032 ps at 150 mM (n ≥ 25; Fig. 3 C). Changes in FL followed a sigmoidal function, revealing an apparent Kᵩ of 79.9 ± 24.5 mM (Fig. 3 C).

Using these calibration parameters to the entire cell (ROIs positioned to include all cellular compartments) resulted in an average intracellular [Na⁺] ([Na⁺])$_i$ of 19.0 ± 4.0 mM in ACSF (n = 58). Based on the observation that FL and intensity differ between cellular regions (see Fig. 2 B), we then determined the FL from three distinct cellular ROIs of each cell separately, comprising (1) the nucleus, (2) the perinuclear region, and (3) the remaining cytosol. This analysis revealed an average [Na⁺] in the cytosol ([Na⁺]$_c$) of 17.6 ± 4.5. The nuclear [Na⁺] ([Na⁺]$_n$) was...
significantly lower, amounting to 13.0 ± 4.2 mM. Perinuclear regions, in contrast, exhibited a significantly higher [Na\(^+\)]\(_{\text{PN}}\) of 26.5 ± 4.8 mM (n = 58; Fig. 3 D).

[Na\(^+\)] changes revealed by CoroNaGreen FLIM

The experiments described above show that FL of CoroNaGreen allows quantitative determination of baseline intracellular [Na\(^+\)]. In a next step, we tested if this technique also enables detection of changes in [Na\(^+\)] in the different subcellular compartments upon different experimental manipulations.

First, we analyzed the effect of an inhibition of cellular energy metabolism by perfusing cells with glucose-free saline containing sodium azide (NaN\(_3\); 5 mM), a blocker of mitochondrial ATP production, and 2-desoxyglucose (1 mM), a blocker of glycolysis, for a period of 2 min (n = 20). This is expected to result in a decrease in cellular ATP and thereby in an inhibition of the NKA (Gerkauf et al., 2018; Lerchundi et al., 2018). Because the generation of one image required collection of photons for a period of 80 s, one image under baseline conditions (ASCF) was taken first (“T80”), before exposing the cells to the metabolic inhibitors and generating three more images (“T160, T240, T320”; Fig. 4 A). At T320, [Na\(^+\)] in all three compartments had increased significantly as compared with baseline conditions (T80): in the cytosol, [Na\(^+\)] rose by 7.4 ± 1.8 mM, in the nucleus by 6.7 ± 2.2 mM, and in the perinuclear region by 6.2 ± 2.4 mM (Fig. 4 A). While these three compartments exhibited different baseline [Na\(^+\)] (Fig. 4 A) as described before (see Fig. 3 D), the relative changes in [Na\(^+\)] induced by metabolic inhibition as well as the general time course detected by this method were similar.

In addition, we analyzed the effect of a perfusion with nominally K\(^+\)-free saline for 1 min, which causes an immediate inhibition of the NKA (e.g., Rose and Ransom, 1996a; Kelly and Rose, 2010). Analysis of the FL revealed a significant increase in [Na\(^+\)] of all subcompartments in response to switching to K\(^+\)-free saline (n = 24; Fig. 4 B). At T320, [Na\(^+\)] had increased by 15.8 ± 5.7 mM in the cytosol, by 15.8 ± 3.8 mM in the nucleus, and by 12.9 ± 4.7 mM in the perinuclear region. While the relative changes compared with baseline [Na\(^+\)] were highly significant for each compartment, the overall responses of the different compartments were again similar. As observed with metabolic inhibition (see Fig. 4 A), [Na\(^+\)] did not recover to baseline (Fig. 4 B), suggesting that NKA was still not fully operational within the given observation window.

Taken together, these experiments show that FLIM of CoroNaGreen can be employed to determine absolute changes in [Na\(^+\)] in cellular compartments in response to different manipulations. Inherent to the long acquisition times, necessary to construct reliable fits of fluorescence decay times, however, the temporal resolution is rather low.

[Na\(^+\)] changes revealed by “Ratio FLIM”

Conventional, time-domain TCSPC-based FLIM relies on the determination of \(\tau_{AVG}\) by fitting a bi-exponential decay function to the fluorescence intensity time course. As stressed above, relatively large photon numbers (2,000 or more under our experimental conditions; Rahmati, S., T. Gensch, and F.Z. Jülich, personal communication) are needed in every pixel to obtain a reliable fit of the bi-exponential function to the pixel’s fluorescence intensity decay as a function of time causing relatively long acquisition times of ~80 s.

To increase the temporal resolution of TCSPC-based FLIM, an alternative approach based on splitting the fluorescence intensity decay curve into early and late segments and estimating the number of photons in the two time periods was introduced earlier (Zheng et al., 2015, 2018; Minge et al., 2017). If the fluorescence decay of the measured compound (here CoroNaGreen) will change due to Na\(^+\) binding to the dye, the relative amount of photons in the early and late time period will change. The optimal value for splitting the decay curve cannot be calculated in general but needs to be determined for a given setup and fluorescent molecule.

We tested different splitting values and determined segment 1 from 1.12 to 1.37 ns and segment 2 from 1.37 to 11.52 ns as the best suited for our measurements. By calculating the ratio of the photon numbers of the two segments (Fig. 5 A), one obtains a parameter that reflects changes in the fluorescence decay profile.
similar to those reported by $\tau_{AVG}$. The calculated ratio of the slow to fast photon numbers ("R") is dependent on the change of the fluorophores’ fluorescence decay property, which in the case of CoroNaGreen is a measure of Na⁺ binding and hence [Na⁺]. Then the data derived from in situ calibrations of CoroNaGreen (0–150 mM Na⁺, 14 conditions, $n \geq 25$; see Fig. 3 B) were reanalyzed. When plotting the resulting ratio versus the [Na⁺] (Fig. 5 B), data could be fitted by a sigmoidal function. The apparent $K_d$ of 88.6 mM was close to that determined based on fitting decay curves (79.9; see Fig. 3 B).

Subsequently, we employed Ratio FLIM in a new set of experiments to study the response of HEK cells to removal of extracellular K⁺ for 1 min ($n = 21$). Analysis was performed on images generated within 8 s (collecting photons for 4 s followed
by 4 s of time needed for saving the image), which is 10 times faster than required for TCSPC-based FLIM with pixel fluorescence decay analysis by fitting a sum of exponential decay functions. As observed before, all three cellular compartments exhibited a significant rise in $[\text{Na}^+]$ upon perfusion with K$^+$-free saline (Fig. 5 C). After $\sim$3 min, $[\text{Na}^+]$ had increased by $\sim$15 mM. This increase was similar in amplitude to the values obtained using conventional FLIM at this time point (see Fig. 4 B). $[\text{Na}^+]$ continued to rise in all compartments for five to seven more minutes to a new plateau, 30–40 mM over the initial baseline. Again, no recovery was observed, suggesting a long-lasting inhibition of the NKA in response to removal of extracellular K$^+$.

To determine the temporal onset of the $[\text{Na}^+]$ increase, the SD of the FL obtained under baseline conditions was calculated and the first time point at which the FL exceeded $2 \times$ SD identified. This analysis revealed an increase in $[\text{Na}^+]$ about $\sim$72 s (71.9 ± 28.6 s) after K$^+$ removal within the cytosolic compartment. Nucleus and perinuclear regions showed delayed responses of $\sim$92 s (92.3 ± 31.7 s) and $\sim$118 s (117.6 ± 42.1 s), respectively. Ratio FLIM thus uncovered a clear temporal sequence in the responses.

Figure 4. $[\text{Na}^+]$ changes detected by FLIM of CoroNaGreen: (A) Top: FL images of CoroNaGreen-AM-loaded HEK cells under control conditions (ACSF) and in response to perfusion with glucose-free saline containing sodium azide (Na$_3$N$_2$; 5 mM) and 2-desoxyglucose (2-DG; 1 mM) for 2 min. Each image was collected for a period of 80 s. The first image was taken in ASCF (‘T80”), before exposing the cells to the metabolic inhibitors (red bars) and generating three more images (‘T160, T240, T320”). Bottom: $[\text{Na}^+]$ at each time point (mean ± SEM; n = 24) in the three compartments. Dotted line represents baseline $[\text{Na}^+]$ in the respective compartment. (B) Same experimental setup and illustration as in A, showing the effect of perfusion with nominally K$^+$-free saline for 1 min (indicated by green bar; n = 17). (A and B) Scale bars, 20 µm. *, P < 0.05, **, P < 0.01, and ***, P ≤ 0.001.
of the different subcellular compartments, with the cytosol leading the [Na+] increase, while the [Na+] increase occurred with a delay of another 20 s in the nucleus and 46 s in the perinuclear region as compared with the cytosol (Fig. 5 C).

In summary, these experiments show that ratiometric analysis of the FL of CoroNaGreen is suitable for the quantitative detection of [Na+] changes in subcellular compartments. As compared with conventional FLIM, the temporal resolution increases significantly by reducing the time required for image generation from 80 to 8 s.

Discussion
Suitability of CoroNaGreen FL for quantitative determination of [Na+] in situ
Our calibration of CoroNaGreen showed that it exhibits distinct changes in FL when binding Na⁺ in the range between 0 and 150 mM both in vitro and in situ. As reported before (Rungta et al., 2015), the normalized decay curves were best fit with a bi-exponential function. In contrast to the former work, the average lifetime \( \tau_{AVG} \) instead of \( \tau_1 \) was used, as it proved a more stable fit parameter at our experimental setup. In HEK cells, when \( \tau_{AVG} \) (obtained from ROIs positioned over nuclear and cytosolic regions) was plotted against [Na⁺], the data followed a sigmoidal curve revealing an apparent \( K_d \) of 79.9 mM. Earlier calibrations of CoroNaGreen’s sensitivity for Na⁺ performed in hippocampal neurons only covered the lower concentration range, still suggesting that CoroNaGreen’s \( K_d \) for Na⁺ is >60 mM (Meier et al., 2006). The data provided by Rungta et al. (2015), performing lifetime measurements with CoroNaGreen in hippocampal neurons, is also in line with this observation. Notably, the apparent \( K_d \) was significantly lower under in vitro conditions as compared with the calibrations performed in HEK cells, a phenomenon already observed for different other dyes including the sodium indicators Sodium Green (Szmacinski and Lakowicz, 1997; Despa et al., 2000b) and AsanteNatriumGreen 2 (Lamy and Chatton, 2011; Roder and Hille, 2014; Naumann et al., 2018).

CoroNaGreen’s average FL (\( \tau_{AVG} \)) in HEK cells increased from ~480 ps at 0 mM Na⁺ to ~1,030 ps at 150 mM. This change in lifetime with binding of Na⁺ is rather moderate as compared to, e.g., the calcium indicators OGB-1 and OGB-2 (Oregon-Green-BAPTA-1/2),...
which undergo a more than fivefold increase in their FL upon binding of calcium (Wilms and Eilers, 2007; Zheng et al., 2018). Other commercially available Na⁺ sensors tested so far either are not suited for FL imaging or only show moderate changes in lifetime as well.

The UV-excitable, ratiometric Na⁺ indicator sodium-binding benzofuran isophthalate (Minta and Tsien, 1989) has been widely used for intensity-based quantitative imaging with wide-field microscopy (Schreiner and Rose, 2012) as well as with two-photon microscopy (Rose et al., 1999), but cannot be employed for FL imaging (Despa et al., 2000a; Naumann et al., 2018). Sodium Green, which exhibits an absorption maximum at 507 nm, was characterized using the frequency domain (Szmacinski and Lakowicz, 1997; Despa et al., 2000b), as well as time-correlated lifetime measurements (Biskup and Gensch, 2014; Naumann et al., 2018), the former revealing an increase in the lifetime of about twofold with binding of Na⁺. Sodium Green, however, is difficult to reliably employ in situ, since it interacts strongly with cytosolic constituents leading to similar FL changes as caused by Na⁺ binding (Biskup and Gensch, 2014). Finally, AsanteNatriumGreen 2 (now distributed by abcam as ”ION NaTRIUM Green”), shows the most prominent changes in FL upon Na⁺ binding compared with the aforementioned Na⁺ indicators, but exhibits a notable sensitivity to K⁺ as well as protons (pH; Roder and Hille, 2014; Naumann et al., 2018).

As mentioned above, CoroNaGreen has been employed for Na⁺ lifetime imaging in situ before (Rungta et al., 2015). Because in the former work, the dye’s cross-selectivity to other ions was not tested, we also analyzed the K⁺- and pH-sensitivity of CoroNaGreen in the present study. With [Na⁺] kept constant, our data demonstrate no sensitivity of CoroNaGreen FL to a reduction of K⁺ from 120 to 80 mM, which is within a concentration range expected to occur during the manipulations performed in the present study (removal of extracellular K⁺ and metabolic inhibition). Moreover, no dependence on pH at values between pH 6.0 and 8.0 was detected, while FL slightly increased at pH ≤5.5. We also found that CoroNaGreen’s lifetime was independent from the presence of dextran up to a concentration of 5% (corresponding to a viscosity of roughly 2.5 mPa s; Carrasco et al., 1989). Altogether, we thus conclude CoroNaGreen is generally suited for FL imaging of Na⁺ inside cells.

Notably, possible differences in ionic concentrations or viscosity between different cellular subcompartments are not expected to strongly influence the FL properties of the dye. This is especially relevant considering the deviation of FL in perinuclear regions as compared with the nucleus and cytosol at [Na⁺] ≤50 mM (see Fig. 3B). The perinuclear regions exhibiting a high FL/intensity strongly colocalized with Mitotracker fluorescence, indicating that they contain a high number of mitochondria. Moreover, perinuclear regions also host lysosomes, which have a pH of ~5. The distinct difference in FL found in the perinuclear could thus at least partly be due to a different behavior of the dye inside these two organelles (e.g., because of a different ionic or protein environment). The overlapping of the FL response of the three compartments at [Na⁺] >70 mM, however, argues against this notion.

Baseline [Na⁺] in cellular subcompartments
Our calibrations indicated that under baseline conditions (in physiological ACSF), mean [Na⁺] in the cytosol was at 17.6 mM, which is in the range of [Na⁺], determined in other cell types, including HeLa cells (12–14 mM; Despa et al., 2000b), vertebrate neurons (15 mM; Kelly and Rose, 2010) or astrocytes (17 mM; Unichenko et al., 2012). Nuclear regions exhibited a significantly smaller [Na⁺] (~13 mM) than the cytosol. Earlier work using intensity-based imaging with Na⁺ indicator dyes in different cell types concluded that cytosolic and nuclear [Na⁺] are similar (e.g., smooth muscle cells; Borin et al., 1993), and that nuclear [Na⁺] is lower than cytosolic [Na⁺] (e.g., ventricular myocytes [Kondratev and Gallitelli, 2003] and hepatocytes [Garner, 2002]), or the other way around (e.g., heart myocytes; Bkaily et al., 1997). Notably, while the nuclear pore complexes were long thought to represent the only pathway for ions, recent work has provided strong evidence for the presence of ion channels, transporters, and receptors in the nuclear membrane. These include the sodium/calcium exchanger (Secondo et al., 2018), sodium/proton exchanger (Bkaily et al., 2012), and NKA (Garner, 2002), all of which might be involved in setting nuclear [Na⁺]. Still, while our data imply that the mean [Na⁺] in the nucleus is significantly lower than that of the cytosol, the biological relevance of this difference in [Na⁺] remains unclear at present.

In ACSF, the perinuclear areas of the cells exhibited an increased average FL as compared with the nucleus-far cytosolic areas and the nucleus itself, suggesting that [Na⁺] might differ between these compartments. Notably, our data suggest that the calibration procedure using ionophores that are preferentially inserted into the plasma membrane did not enable a reliable permeabilization of perinuclear organelles for [Na⁺] in the concentration range relevant for this study (0–50 mM). Therefore, we did not construct a separate calibration curve from this compartment. While different cellular compartments and organelles certainly differ in their chemical, ionic, and/or protein environment, our data also show that CoroNaGreen FL is rather insensitive to differences in K⁺, pH, or viscosity. As argued above, we conclude from these results that the observed differences in FL were not related to a different behavior of the dye in the different compartments.

Our data suggest differences in the [Na⁺] between subcellular compartments, with perinuclear regions exhibiting an average [Na⁺] of ~27 mM, a level significantly higher than the [Na⁺] of cytosolic as well as of nuclear regions. The perinuclear region is characterized by the presence of lysosomes and contains a high density of mitochondria as demonstrated by our Mitotracker stainings. Regions of high FL/intensity showed a remarkable overlap with Mitotracker fluorescence, suggesting that the high [Na⁺] in this region mainly reflects the [Na⁺] of mitochondria. In addition, it may, at least partly, report [Na⁺] of other organelles present, including endoplasmatic reticulum or lysosomes.

A difference between cytosolic and mitochondrial [Na⁺] was also found in an earlier study (Bernardinelli et al., 2006) using intensity-based fluorescence imaging with the red-shifted variant CoroNaRed, which preferentially enters mitochondria (see also Ben-Kasus Nissim et al., 2017).
Combining this with sodium-binding benzofuran isophthalate to monitor cytosolic [Na⁺], they reported an elevated mitochondrial [Na⁺] as compared with the cytosol (19 mM versus 13 mM; Bernardinelli et al., 2006). In contrast to this, other work indicated that mitochondrial [Na⁺] might be several millimoles lower than cytosolic [Na⁺] (e.g., Donoso et al., 1992; Murphy and Eisner, 2009). Na⁺ uptake into mitochondria is mediated by the mitochondrial Na⁺/Ca²⁺ exchanger (Palty et al., 2010; Nita et al., 2014; Ben-Kasus Nissim et al., 2017), which is antagonized by sodium efflux through a Na⁺/H⁺ exchanger (Bernardinelli et al., 2006; Nita et al., 2015). Na⁺/Ca²⁺ exchanger activity is modulated by the cytoplasmic [Na⁺] (Maack et al., 2006; Kim and Matsuoka, 2008), and depending on the driving forces, this transporter can switch between forward (Na⁺ influx) and reverse (Na⁺ efflux) mode (Samanta et al., 2018). Mitochondrial [Na⁺] is thus likely to be set in a dynamic equilibrium, determined by ion concentrations and dynamics (Na⁺, Ca²⁺, pH) and the relative strength of Na⁺ influx versus Na⁺ efflux.

Detection of changes in [Na⁺] using FLIM

To evaluate if FLIM of CoroNaGreen can be employed to reveal changes in [Na⁺], we performed two experimental approaches expected to result in an increase in cellular [Na⁺]. Metabolic inhibition by addition of blockers of glycolysis and mitochondrial respiration instead caused significant changes in the FLs of CoroNaGreen in all three cellular compartments, indicative of an increase in their [Na⁺] by 6–7 mM. Earlier work performed, e.g., in brain tissue, has shown that this induces a decrease in cellular ATP (Lerchundi et al., 2018), resulting in an inhibition of the NKA and an increase in cellular [Na⁺] (Gerkauf et al., 2018).

Similarly, removal of extracellular K⁺ for a period of 1 min resulted in increased FL in HEK cells, with an increase in [Na⁺] by 13–16 mM as detected after 3 min in the cytosol, nucleus, and perinuclear region. The [Na⁺] increase induced by removal of extracellular K⁺ was thus larger than upon addition of metabolic blockers, most likely because the former induces an immediate and complete inhibition of the NKA. As a consequence, removal of K⁺ causes a strong increase in the cellular [Na⁺] of many cell types, including neurons and astrocytes (e.g., Rose and Ransom, 1996b; Kelly and Rose, 2010).

Conventional FLIM inherits a rather low temporal resolution, and photons have to be collected over a period of 80 s for reliable fits of the pixel fluorescence decays. To increase the temporal resolution of our measurements, we introduced a modified data analysis, termed “ratiometric” photon counting (Minge et al., 2017; Zheng et al., 2018). With this approach, the photon distribution is empirically separated into a “fast” and a “slow” segment, and the quotient of both (the photon count ratio) is calculated. This procedure resulted in a strong reduction in photon collection periods and a 10-fold increase in temporal resolution to 0.125 Hz.

Ratio FLIM thereby enabled a better visualization of the dynamic onset and time course of the increase in [Na⁺] induced by removal of K⁺ in the three cellular compartments. Indeed, while it showed similar amplitudes as compared with conventional FLIM, it revealed a temporal sequence in the response time: cytosolic [Na⁺] began to rise within ~72 s upon the removal of potassium, whereas the rise in nuclear [Na⁺] was delayed by an additional 20 s. The increase in the perinuclear area [Na⁺] even lagged an additional 26 s behind. This sequence of events suggests that removal of extracellular K⁺ first results in an inhibition of the plasma membrane NKA and in an increase in cytosolic [Na⁺]. The disturbance of cytosolic [Na⁺] then evokes a secondary increase in nuclear [Na⁺] and, finally, in organelles present in the perinuclear region. Altogether, we conclude from these data that ratio FLIM is well suited to probe for dynamic changes in [Na⁺] in different cellular compartments that occur within this time range.

Acknowledgments

Sharona E. Gordon served as editor.

We thank Simone Durry and Claudia Roderigo (Institute of Neurobiology, Heinrich Heine University, Düsseldorf, Germany) for excellent technical assistance, as well as Bettina Mertens, Andrea Jansen, and Hua Tan (Forschungszentrum Jülich, ICS-4, Germany) for excellent support with cell culture. We also thank Dr. Christian Henneberger (Bonn University) for helpful comments and advice on data analysis.

J. Meyer was supported by the International Helmholtz Research School of Biophysics and Soft Matter, Forschungszentrum Jülich, Germany. This work was supported by the Deutsche Forschungsgemeinschaft (Ro2327/8-2 and 13-1).

The authors declare no competing financial interests.

Author contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. J. Meyer, V. Untiet, C. Fahlke, T. Gensch, C.R. Rose: design and conceptualization of research, data analysis; J. Meyer, V. Untiet, T. Gensch: experiments and data analysis; J. Meyer, T. Gensch, C.R. Rose: writing original draft; J. Meyer, V. Untiet, C. Fahlke, T. Gensch, C.R. Rose: review and editing of the manuscript; C. Fahlke, C.R. Rose: funding acquisition.

Submitted: 6 June 2019
Accepted: 11 September 2019

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