

Video Article

Synthesis, Cellular Delivery and *In vivo* Application of Dendrimer-based pH Sensors

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Abstract

The development of fluorescent indicators represented a revolution for life sciences. Genetically encoded and synthetic fluorophores with sensing abilities allowed the visualization of biologically relevant species with high spatial and temporal resolution. Synthetic dyes are of particular interest thanks to their high tunability and the wide range of measurable analytes. However, these molecules suffer several limitations related to small molecule behavior (poor solubility, difficulties in targeting, often no ratiometric imaging allowed). In this work we introduce the development of dendrimer-based sensors and present a procedure for pH measurement *in vitro*, in living cells and *in vivo*. We choose dendrimers as ideal platform for our sensors for their many desirable properties (monodispersity, tunable properties, multivalency) that made them a widely used scaffold for several biomedical devices. The conjugation of fluorescent pH indicators to the dendrimer scaffold led to an enhancement of their sensing performances. In particular dendrimers exhibit reduced cell leakage, improved intracellular targeting and allow ratiometric measurements. These novel sensors were successfully employed to measure pH in living HeLa cells and *in vivo* in mouse brain.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50545/>

Introduction

The use of fluorescent molecules to label specific biologically-relevant molecules has completely changed the way we study biological systems. Widefield and confocal microscopy allowed for a real-time high-resolution visualization of biological processes and nowadays are among the most popular techniques to study biological events *in vitro*, in cells and *in vivo*.¹ A relevant improvement was represented by the development of fluorescence indicators, *i.e.* dyes whose fluorescence is dependent on the concentration of a specific molecular entity. pH and calcium indicators in particular had a dramatic impact on the study of cell physiology due to the enormous relevance of H⁺ and Ca²⁺ ions in biology.^{2,3}

However, most of the sensing dyes present several intrinsic limitations related to their small molecule behavior such as: i) difficulties in subcellular targeting; ii) poor solubility in water and consequently poor biocompatibility; and iii) cell leakage and thus lack of long time-lapse imaging ability.⁴ Moreover, the signal of many probes cannot be corrected for the dependence on the dye concentration (non-ratiometric imaging) and therefore, an absolute measurement in cells or *in vivo* is not possible.

We recently described a simple and effective methodology to overcome these limitations, based on the conjugation of sensing dyes on a dendrimer scaffold.⁵ Dendrimers are monodisperse hyperbranched polymers with very appealing properties for biological applications.⁶ In particular several dendritic architectures have been developed and used for drug⁷ and gene delivery.⁸ Only very recently several groups started to explore the potential of these molecules as scaffold for sensing devices.^{9,10,11}

We previously described an easy synthetic route towards the functionalization of different polyamidoamine (PAMAM) scaffolds based on NHS-activated esters.¹² Conjugates can be obtained in a single step by means of dialysis as only purification. Interestingly this approach can easily be applied to a variety of dendritic or polymeric scaffolds.^{13,14}

To achieve ratiometric imaging dendrimers were double-labeled with two sets of dyes: i) a pH indicator (*i.e.* fluorescein) and ii) a pH-independent fluorescent moiety (*i.e.* rhodamine). This allowed us to perform accurate pH imaging as the ratio between fluorescein and rhodamine is only dependent on the pH and no more on the concentration of the probe. Another interesting approach to this issue is represented by the use of lifetime-based probes.¹⁵ As the lifetime does not depend on probe concentration these measurements do not need a ratiometric correction.

However, lifetime measurements require a more complicated instrumental setup and their temporal resolution is sub-optimal for fast physiological processes, thus limiting their potential applications.

In order to perform intracellular imaging, the probe needs to be delivered across the plasma membrane into the cytosol. As the dendrimers are not membrane permeable due to their size and hydrophilicity, intracellular delivery could be achieved through electroporation. By means of this technique, widely used in biology for transfection, labeled macromolecules can be effectively delivered into cells to perform high quality imaging. Moreover, with electroporation the complications related to dendrimer endocytosis can be avoided as the macromolecules are directly delivered to the cytoplasm. Interestingly after electroporation different dendrimers shows distinct localizations inside the cells even in absence of any specific targeting sequence.⁵ This passive targeting, only due to the physicochemical properties of the dendrimer, can be exploited to achieve organelle-specific pH imaging.

Ratiometric imaging can be performed using confocal microscopy. Fluorescein and rhodamine, covalently conjugated to the dendritic scaffold, were separately imaged and a pixel-by-pixel ratio map was created. Several procedures to control intracellular pH in living cells by means of ionophores were reported. Ionophores are small hydrophobic molecules able to transport ions across the plasma membrane; ionophores for H⁺ ion, such as nigericin, are available and can be used to calibrate dendrimer-based sensors.¹⁶ These measurements revealed a linear response to pH similarly to what observed *in vitro*. On the basis of the calibration intracellular pH could be accurately measured. These measurements demonstrated that dendrimer-based sensor can be a valuable tool in study H⁺ homeostasis in living cells and pathological processes that involve pH regulation malfunctions.

We recently demonstrated that dendrimer-based pH sensors can also be applied *in vivo*, performing pH imaging in the brain of anesthetized mice.¹⁷ Due to the complex environment of living tissues a high quality *in vivo* sensing is technically challenging. Here we show a detailed description of the experimental procedure for *in vivo* pH imaging with emphasis of the crucial issues to be addressed to perform an accurate pH imaging in the brain. Two-photon microscopy has been employed for two main reasons: i) the use of infrared light allows to overcome the lack of tissue penetration of standard confocal microscopy; ii) the broad two-photon absorption of fluorescein and rhodamine allow their simultaneous excitation avoiding the complications related to the use of two wavelengths for excitation. pH measurements in mouse brain were successfully carried out; sensors readily respond to hypoxia induce change of pH in the brain extracellular space. These measurements demonstrate that dendrimer-based indicators can be successfully used to highlight physiological and pathological change of pH *in vivo* in an animal model.

Protocol

1. Synthesis of the Sensors

1. In the following section we provide a procedure for the conjugation of pH indicators to PAMAM dendrimers. The same protocol can be applied with minimal modification to alternative amine-bearing dendrimers.^{5,17,13,14} Commercially available dendrimers and dyes can be used without further purifications.
2. Dissolve the dendrimer in anhydrous DMSO (50 μ M final concentration). Prepare 10mM stock solutions of fluorescein-NHS and tetramethyl-rhodamine-NHS (TMR) in anhydrous DMSO.
3. Add to the dendrimer solution the desired amount of fluorescein and TMR. The molar ratio in the mixture will reflect the amount of dyes loaded on the dendrimer. Typically 1 ml of G4 PAMAM dendrimer solution in a microcentrifuge tube is reacted with 8 eq (40 μ l) of fluorescein and 8 eq (40 μ l) of TMR. Stir the solution at room temperature for 12 hr.
4. Dilute 1:10 with deionized water and load the reaction mixture in a dialysis bag (MWCO=10 kDa). Dialyze for 24 hr against deionized water replacing frequently the water in the reservoir.
5. Transfer the solution to a vial and freeze-dry for 24 hr. A purple powder should be obtained. Weight the obtained solid and dissolve it in milliQ water at a final concentration of 10 μ M. Aliquot the solution and store at -20 °C.

2. In Vitro pH Measurements

1. For *in vitro* calibration prepare a solution 500nM of dendrimer in PBS (2 mM phosphate) in a quartz cuvette. The use of a very dilute PBS buffer (2 mM) to avoid abrupt changes of pH during the titration is recommended.
2. Measure the emission spectra of fluorescein (exc 488 nm) and TMR (exc 550 nm) and optimize the optical settings of the fluorimeter to achieve a good signal-to-noise ratio.
3. Perform a pH titration by adding small volumes of NaOH 0.1 N and HCl 0.1 N. After every addition shake the cuvette for mixing, wait 1 min for equilibration and measure the pH by means of a pH microelectrode. Emission spectra of fluorescein and TMR should be recorded for every step without any change in the optical settings.
4. Plot the fluorescence intensity vs pH for the titration. The rhodamine signal should be unaffected by pH (<10%). The fluorescein signal should be a sigmoidal curve and should be fitted with a single-binding model with pK = 6.4.

3. Cell Culture and Electroporation

1. Cultivate HeLa cells in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). Keep cell culture at 37 °C in a humidified 5% CO₂ atmosphere.
2. For dendrimer electroporation, when cells are confluent, remove the media and wash cells using DPBS (Dulbecco's Phosphate-Buffered Saline). Remove the DPBS and add trypsin-EDTA. Neutralize the trypsin by adding medium containing serum but not antibiotics. Centrifuge at 900-1,200 rpm for 2 min at room temperature. Remove the media and rinse the pellets using DPBS.
3. Count the cells and take 4*10⁶ cells. Centrifuge at 1,200-1,500 rpm for 2 min at room temperature.
4. Resuspend the cell pellet in 200 μ l of microporation buffer (provided by microporator manufacturer) and transfer cells to a 1.5 ml microcentrifuge tube.

5. Add dendrimer aqueous solution into resuspended cells. The amount of dendrimer required per sample is dependent on the PAMAM type (typically 250 nM for cationic and 2 μ M for neutral dendrimers).
6. Add electroporation buffer (provided by microporator manufacturer) in a microporation tube. Pipette the cells and dendrimers mixtures with electroporation tip of 100 μ l volume-sized. Insert the microporator pipette into the pipette station. Set the pulse condition for microporation: pulse voltage = 1,005 V; pulse width = 35 msec; pulse number = 2.
7. After the pulse transfer cells to a 1.5 ml microcentrifuge tube and centrifuge cells for 5 min at 1,200 rpm to remove the excess of dendrimer in the medium. Plate 10 μ l of electroporated cells onto 35 mm glass-bottom dishes (WillCo-dish GWSt-3522) with fresh medium w/o antibiotics.

4. pH Sensing in Living HeLa Cells

1. Image cells with a confocal microscope 12 hr after electroporation.
2. Standard filter set for fluorescein and rhodamine can be used. If tunable filters are available set a green channel from 500 - 550 nm and a red channel from 580 nm to 650 nm. Excitation at 488nm is optimal for fluorescein while rhodamine could be imaged either with the 543nm or the 561 laser line.
3. Focus on the specimen and adjust lasers power and detectors gain to maximize the signal-to-noise ratio. If the electroporation was successful cells should be brightly fluorescent in both channels. The localization depends on the size and charge of the dendrimer used. Often some lysosomal localization (small perinuclear vesicles) is present due to endocytosis or compartmentalization. If the lysosomal localization is predominant, *i.e.* most of the fluorescence is localized inside vesicles and poor signal is observed in the cytosol, this signifies toxicity and measurement should be discarded. Acquire sequentially the two channels, if needed acquire several images and average the images to improve image quality.
4. For calibration clamp cell pH using buffers with ionophores at different pH and acquire at least 20 cells per pH as described above. For a detailed description of the procedure and the composition of the buffers please refer to Bizzarri and Coworkers.¹⁶ We suggest to measure at least 5 points from pH = 5.5 to pH = 7.5. pH below 6 are toxic to cells but tolerated for short amount of time, we suggest to acquire the images as quick as possible. If cells demonstrate signs of apoptosis, discard the cells and restart.
5. Use ImageJ or analogous software for data analysis. Import the images of the green and red channel, subtract background and create a pixel by pixel ratio imaging with the tool "Image calculator".
6. Draw a region of interest (ROI) selecting the desired cell and measure the intracellular green-to-red ratio. Analyze all the images acquired and then plot the ratio versus the pH. In the range from 5.5 to 7.5 the trend should be linear. The linear fit of the points obtained will give the calibration curve that will be used to convert green-to-red ratio to pH.
7. As further control acquire several untreated cells (no ionophores) and try to calculate pH with the obtained calibration curve. A value between 7.2 and 7.4 should be obtained.

5. In Vivo Sample Preparation

1. Experiments were performed on C57Bl/6J (males and females) between postnatal day 28 and 70. Anesthetized the mouse with an intraperitoneal injection of Urethane (*i.e.* ethyl carbamate) (20% w/v in physiological saline, 20 mg/Kg urethane). Animals were sacrificed after experiment with an overdose of urethane followed by an intracardiac injection of the same anesthetic.
2. Perform an intramuscular injection of dexamethasone sodium phosphate (2 mg/kg body weight) to reduce the cortical stress response and cerebral edema during the surgery.
3. Shave the animal's head and apply 2.5% lidocaine gel to the scalp.
4. Use scissors to cut the flap of skin covering the skull of both hemispheres
5. Wash the exposed bone with saline and gently remove the periosteum using forceps. This will provide a better base for glue and dental cement to adhere with.
6. Apply a custom-made steel head post with a central imaging chamber and glue it with cyanoacrylate in a plane approximately parallel with the skull over the cortical region of interest and fix it in place with white dental cement (Paladur).
7. Fix the head of the mouse in order to perform a craniotomy of 2-3 mm in diameter drilled over the region of interest.
8. Try to minimize heating of the cortex during surgery, dural tears, or bleeding.
9. Keep the cortex superfused with sterile ACSF (126 mM NaCl, 3 mM KCl, 1.2 mM KH_2PO_4 , 1.3 mM MgSO_4 , 26 mM NaHCO_3 , 2.4 mM CaCl_2 , 15 mM glucose, 1.2 mM HEPES in distilled H_2O , pH = 7.4).

6. pH imaging in Mouse Brain

1. During the experiment aid animal respiration providing O_2 -enriched air. Oxygen is enriched up to 80%. O_2 partial pressure and flow is frequently adjusted to obtain a proper respiration aid. Keep body temperature constant at 37 °C with a feedback controlled heating blanket.
2. Fix the animal through the steel post under the objective of a two-photon imaging setup.
3. In order to inject the sensor in the brain cortex load a glass pipette containing an AgCl electrode (tip diameter 4 mm) with the dendrimer solution (1 μ M). The electrode will allow to record extracellular field potentials.
4. With a microinjection setup insert the pipette in the cortex at approximately 150 μ m depth. Inject for 1-2 min at a pressure of 0.5 psi.
5. Optimize the optical setup for imaging. Laser power should be adjusted to minimize photobleaching and photodamage. Typically laser power employed is around 20 mW and PMT gain was kept constant at 667 V since previous calibrations showed that this voltage gives the best S/N ratio.
6. For imaging excite the sensor at 820 nm and detect simultaneously fluorescein and rhodamine fluorescence through standard FITC and TRITC filters.
7. For time resolved measurements acquire time lapse series at 2 Hz.
8. For background correction acquire a dark frame with the laser shutter closed to measure the mean thermal noise arising in the PMTs and the pedestal usually added by the electronics.
9. For data analysis follow the same procedure reported in section 4.

Representative Results

Figure 1 shows a schematic representation of the conjugation of sensing dyes to different dendritic scaffolds. The resulting indicators can be obtained in one easy synthetic step from commercially available products. Amine-bearing dendrimers are reacted with NHS-activated dyes in DMSO and purified by dialysis. This general procedure has already been successfully used for labeling of several dendrimers: i) PAMAM dendrimer generation 2, 4 and 6;¹² pegylated PAMAM dendrimers¹⁷ and PEG-dendritic hybrids¹⁸. As distinct dendrimers show different interactions with cells and tissue (localization, toxicity, permeability, diffusion) the selection of the dendritic structure is strongly related to the desired application and to the specimen of interest.

The sensors comprise two sets of fluorescent dyes: i) pH indicators and ii) pH-insensitive moieties acting as internal references. Although a large variety of indicators and references are available, the best results have been obtained with fluorescein and tetramethylrhodamine. The ratio of the two dyes can be tuned simply using different molar equivalents in the reaction. **Figure 2** shows a typical *in vitro* titration of a pH sensor. Fluorescein and rhodamine can be separately excited at 488nm and 550nm, respectively, allowing minimum cross-talk between the two channels. As can clearly be seen, fluorescein (**Figure 2A**) shows a pH-dependent behavior while rhodamine (**Figure 2A**) signal does not change significantly in the physiological pH range. Therefore, the ratio of these two signals does not depend on sensor concentration but only on pH. This concentration independence will be of great importance for the biological measurements as intracellular probe concentration cannot be controlled.

For live cells measurements the intracellular delivery of the dendritic sensors is achieved *via* electroporation. This technique is widely used in biology for DNA transfection and can be applied with minimal variations on the manufacturer protocol. Through electroporation, the macromolecules can be delivered directly to the cytoplasm, avoiding any complication related to the vesicular system of endocytosis. **Figure 3a** shows confocal images of HeLa cells electroporated with the dendrimer sensors. A strong signal in both fluorescein (green, left) and rhodamine (red, middle) is observed. The two signals perfectly colocalize demonstrating the integrity of the sensor structure. A ratiometric map is reconstructed by dividing the two images pixel-by-pixel and represented with a pseudocolor scale (**Figure 3a**, right). pH-clamping with ionophores was performed, in order to calibrate the sensor response inside cells. Calibration with ionophores is a well-established protocol, several protocols have been extensively described and can be used without modifications.¹⁹ Indicators readily respond to pH with a change of the green-to-red ratio as shown in **Figure 3b**. This allowed us to obtain a calibration curve (**Figure 3c**) for accurate pH measurements. The linear trend in the calibration demonstrates the ability of the sensor to respond to pH without any perturbation from the cellular environment. The calibration curve has been used to determine the pH value of living HeLa cells to be 7.4 and 4.8 for cytoplasm and lysosomes, respectively. These results are in good agreement with the literature.

Finally, we showed how dendrimer-based sensors can be employed for *in vivo* imaging. Dendrimers can be injected easily through the tissue with a procedure very similar to the widely used calcium indicators.²⁰ Once in the tissue, the indicators diffuse extremely slowly, allowing long term imaging before complete tissue drainage. Typical results are shown in **Figure 4**. Fluorescein (green) and rhodamine (red) signals have been simultaneously obtained with 820 nm excitation and the ratio map was built on a pixel-by-pixel basis, similar to live cells measurements. Notably, the indicators localize in the extracellular space; the non-fluorescent areas in the image identify cellular bodies or small blood vessels. In order to verify sensor response to pH, we proposed the use of hypercapnia. Indeed, carbon dioxide is known to alter the equilibria of the carbonate buffers in blood and tissues, resulting in an acidification of the tissues.²¹ As shown in **Figure 4b**, the inhalation of 30% CO₂ is enough to induce a strong response of the sensor that is completely reversible upon re-ventilation of the mouse. These results demonstrate the potential of dendrimer-based sensors to highlight physiological and pathological changes of pH in living cells and *in vivo*.

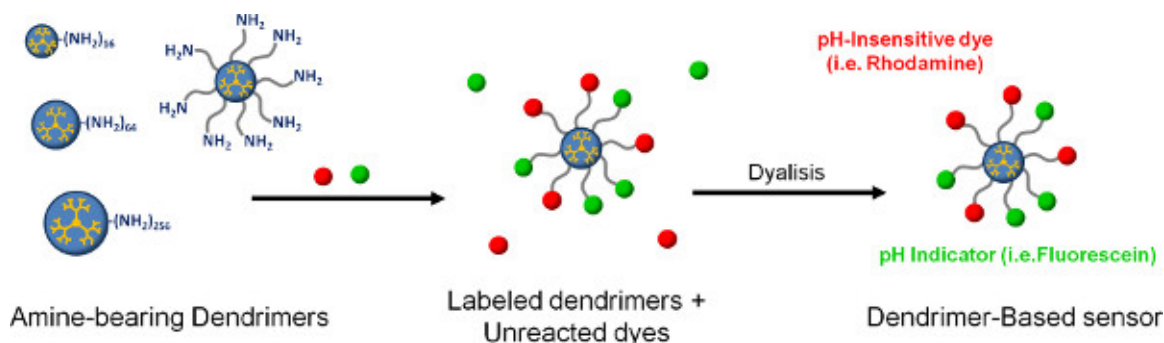


Figure 1. Synthesis of dendrimer-based sensors Schematic representation of dendrimer-based pH sensor. The same procedure can be applied to virtually every amine-bearing dendrimer (left). Product was obtained through a single conjugation reaction followed by dialysis. [Click here to view larger figure.](#)

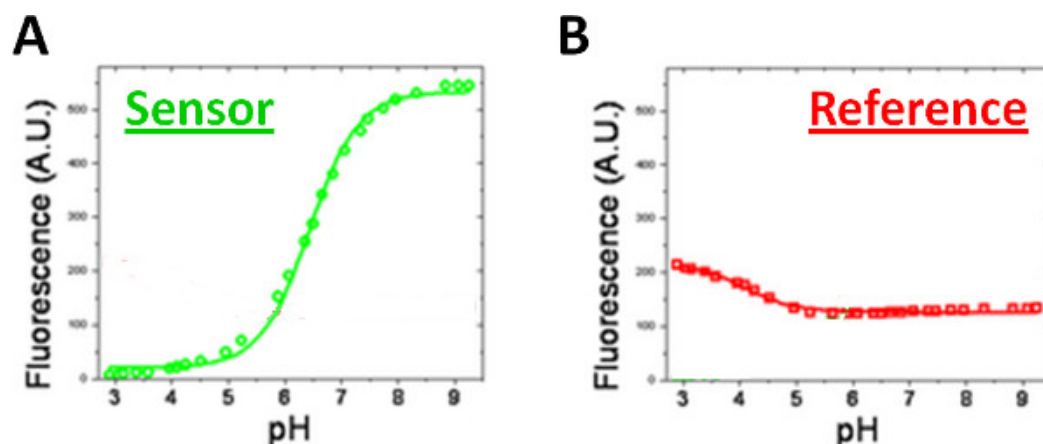


Figure 2. *In vitro* pH titration for a dendrimer-based sensor. a) Response of the pH indicator fluorescein and b) the internal reference, rhodamine, showing no change over the physiological pH range.

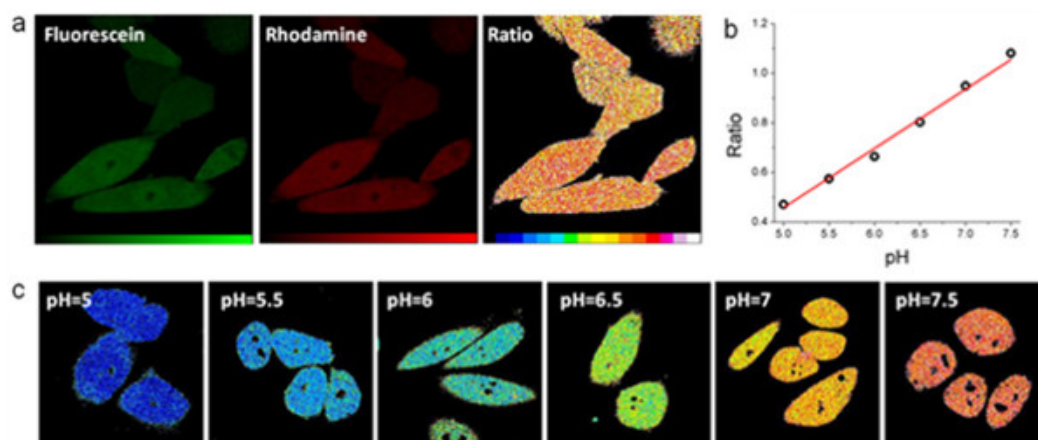


Figure 3. Representative results of pH imaging in living HeLa cells. a) Confocal imaging of fluorescein channel (left), rhodamine channel (middle) and pH ratiometric map (right). b) pH calibration with ionophores. c) representative ratiometric maps of cells clamped at different pH. Reproduced from Albertazzi L, Brondi M, Pavan GM, Sato SS, *et al.* (2011) Dendrimer-Based Fluorescent Indicators: *In Vitro* and *In Vivo* Applications. *PLoS ONE*. 6(12), e28450. doi:10.1371/journal.pone.0028450

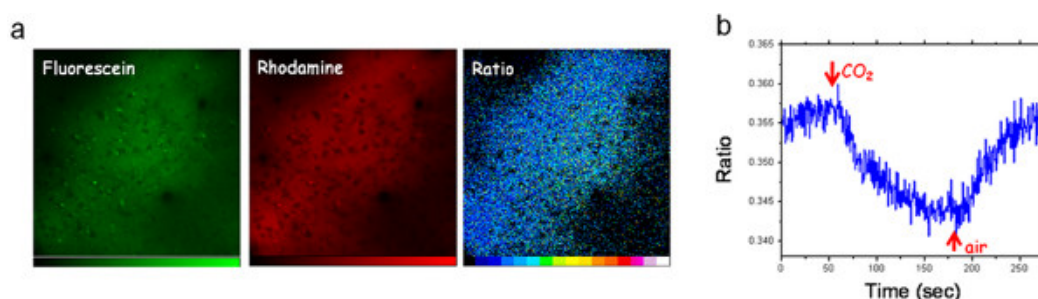


Figure 4. pH imaging in the brain of anesthetized mouse. a) green and red channel (simultaneously excited at 820 nm) and pH ratiometric map. b) typical response of the sensor to hypercapnia (30% CO₂). Adapted from: Reproduced from Albertazzi L, Brondi M, Pavan GM, Sato SS, *et al.* (2011) Dendrimer-Based Fluorescent Indicators: *In Vitro* and *In Vivo* Applications. *PLoS ONE*. 6(12), e28450. doi:10.1371/journal.pone.0028450. [Click here to view larger figure.](#)

Discussion

The critical steps for successful pH imaging with dendrimer-based sensors are: i) the selection of the correct dendritic scaffold and the number of indicators conjugated to it and ii) the optimization of sensor delivery protocol in cells or *in vivo*.

The synthetic procedure is fairly easy and can be applied virtually to every amine-bearing hyperbranched polymer. The sensors can be obtained from commercially available dendrimers and NHS-activated dyes in one single step. We believe that this modular and straightforward procedure will be beneficial for the further application of such sensors for a variety of biological questions. Purification can be effectively achieved by dialysis to remove the unconjugated dyes. The selection of the dendritic scaffold is crucial and depends on the specific application desired. Different dendrimers have been shown to have peculiar localizations inside cells owing to specific interactions with subcellular structures. Neutral dendrimers (for example acetylated PAMAM G4) do not show any interaction inside cells and show a diffuse localization. Thus they represent a good choice for a whole-cell imaging. On the contrary positively charged dendrimers of different generations (from G2 to G6) display electrostatic interactions with negatively charged biomolecules (*i.e.* RNA) resulting in specific cellular localization. Cationic dendrimers are thus ideal for organelle-specific imaging.

The choice of the sensing dyes is critical. Several pH indicators and pH-insensitive dyes with different color emission and H^+ affinity (pK) are available. We tried many different dye combinations and the best results were obtained with fluorescein and tetramethylrhodamine. This pair provide a good response to pH in the physiological range (pK = 6.4) and is compatible with commonly used microscopy filter setups. For different requirements, like measurements in different pH range, some optimization may be required. Notably, not all the indicators retain their properties (brightness, pK) upon dendrimer conjugation.⁵ This behavior depends both on dye and dendrimer structures; however, we were able to report several indicators where the photophysical properties of the indicators are not significantly affected.¹⁷ If this is the case we suggest conjugating the dyes through a linker in order to minimize dendrimer-dye interactions. Several reactive dyes are commercially available with alkyl spacers²² or in alternative PEG linkers can be used. Also the number of indicators and the ratio indicators-to-reference dyes on the scaffold is important. The ideal ratio depends on the spectral sensitivity of the microscopy setup used and on the biological specimen of interest^{5,17}. Although some troubleshooting may be needed, the very easy and fast synthetic procedure will greatly facilitate the process. Finally the degree of functionalization of the dendrimer, *i.e.* the percentage of end-groups of the dendrimer that are conjugated to dyes, could influence the performances of the indicators. To avoid any solubility problem we suggest functionalizing about 10%-20% of the dendrimer end groups. In the case of pegylated dendrimers however, due to PEG contribution to solubility, a complete functionalization can be achieved. This can also limit the undesired presence of FRET and other quenching phenomena among the conjugated dyes.

Dendrimers are not cell-permeable, but intracellular delivery can be achieved through electroporation. We employed DNA-transfection manufacturer protocol for the cell line of interest without further modifications. A crucial parameter is the concentration of the dendrimer in the electroporation buffer; in particular in the case of indicators with poor brightness or dendrimers that at high concentrations could be toxic to cells. Low concentrations of dendrimer will result in poor signal inside cells while high concentrations will cause toxicity and induce cell death. The ideal concentration depends on the dendrimer/indicator pair as well as on the imaging setup available and may need some optimization. If an electroporator is not available in the lab, microinjection could represent a valid alternative. However, this is a single-cell technique and will make the acquisition of a significant number of imaged cells more laborious.

The sensors imaging procedure is very flexible and can be easily adapted to several microscopy setups. We previously reported the use of the sensors under a confocal system with tunable acusto-optic filters^{5,17} but standard FITC/TRITC filter sets can also be used. When an optimal intracellular concentration is obtained, pH imaging is straightforward. We suggest optimizing laser intensity, pinhole and detectors gain over a large range of sensor intracellular concentrations before running the pH calibration with ionophores. If the same microscopy setup is used under the same conditions the calibration could be used for all experiments. Here we propose ratiometric imaging to avoid sensor concentration dependency and artifacts. Another interesting methodology to achieve that is represented by lifetime-based probes as elegantly showed by Vinogradov and coworkers¹⁵ and both options display advantages and disadvantages. Lifetime measurements do not require any correction as they are intrinsically concentration-independent and calibration procedures are often easier and more stable. Moreover, a single color is used avoiding the complications and the possible aberration induced by the green-to-red ratiometric imaging. However only few effective lifetime probes are available while a large array of intensity-based probes are commercially accessible. The equipment required for ratiometric imaging is simpler and more likely available in a standard microscopy facility. Moreover lifetime measurements are intrinsically slow and cannot be applied to follow fast biological processes. Therefore the choice between these two techniques strongly depends on the instrumentation available and on the specific biological process of interest.

Although conceptually similar, *in vivo* imaging is more challenging than in the cell culture. Signal to noise ratio is reduced by scattering and the interactions with the tissues can influence the response of the sensor. Moreover, diffusion through the tissue and thus leakage of the indicator from the region of interest represent an additional issue not present for intracellular measurements in culture. For these reasons, the choice of the indicator is crucial. While for intracellular measurements most of the dendrimers work excellently (G4, G4-Ac, G6, PAMAM-PEG hybrids), for *in vivo* sensing we recommend the use of pegylated dendrimers.¹⁷ Indeed this architecture is particularly effective for this purpose for several reasons: i) the PEG increase the size of the dendrimer and reduces its leaking from the tissue; ii) the PEG linker minimizes the quenching resulting from dye-dye and dye-dendrimer interactions and iii) the PEG shields the dyes from the tissue avoiding the loss of function often observed after tissue injection of sensors. The quality of the pH sensing strongly depends on the successful injection of a sufficient amount of dendrimer in the tissue. When this is achieved the imaging procedure and the data analysis are similar to what was already reported for the cell culture. This is a clear demonstration of sensor effectiveness *in vivo* and can be used as an internal control of indicator activity before any measurement.

We believe that these results together with the detailed procedures reported in this work will allow the application of dendrimer-based sensors to highlight physiological and pathological changes of pH in living cells and *in vivo*.

Disclosures

Default: Authors have nothing to disclose.

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