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Synthesis and application of a fluorescent probe to image and quantify total intracellular magnesium in cells

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Abstract

Magnesium plays a crucial role in many physiological functions and pathological states. Therefore, the evolution of specific and sensitive tools capable to detect and quantify this element in cells is very attractive for biological and biomedical research. We developed a Mg-selective fluorescent-dye able to identify and quantify the total Mg pool in a number of cells two order of magnitude smaller than that required by flame Atomic Absorption Spectroscopy, the reference analytical method for the assessment of cellular total metal content. This protocol reports itemised steps for the synthesis of diaza-18-crown-6-hydroxyquinoline-based fluorescent-dye (DCHQ5). We also describe its application in the quantification of intracellular total Mg in mammalian cells and its detection *in-vivo* by confocal microscopy. Remarkably, the application of DCHQ5 allowed to study the involvement of Mg in Multi Drug Resistance in human colon adenocarcinoma cells sensitive (LoVo-S) and resistant to doxorubicin, finding a higher concentration of total Mg in LoVo-R respect to LoVo-S. The time frame for DCHQ5 synthesis is 1-2 d, for its use in the intracellular total Mg quantification is 2.5 h, and 1-2 h the bioimaging.

INTRODUCTION

Magnesium is essential for a wide range of cellular functions and many biochemical reactions require it as cofactor. However, its role in the mechanisms of cellular signal transduction is not fully understood yet. Imbalance of magnesium regulation is related to various relevant human diseases, such as hypertension, cardiovascular diseases, neuromuscular disorders or diabetes (1-3). The study of magnesium homeostasis gained special attention in the last years due to the discovery and characterization of magnesium selective ion channels and the use of molecular biology tools to elucidate their physiological functions at the cellular level (3-6). On the other hand, tools capable to detect and quantify intracellular magnesium paving the way to a better comprehension of its regulation in live cells did not witness an equivalent advancement (7). In addition, either the Mg dyes commercially available or those developed by several research groups selectively detect only the free metal ions, precluding the possibility to identify the total pool of the element to analyse (7-9). This feature represents a serious restriction for magnesium whose ionic form is present at small percentage in the intracellular milieu (10).

The method commonly used to quantify total magnesium in cell or tissue is flame Atomic Absorption Spectroscopy (F-AAS) on acidic extracts. However, this technique necessitates samples of millions of cells or several milligrams of tissue representing a serious drawback in many applications. There are other more sensitive techniques as Graphite Furnace AAS and Inductively Coupled Plasma Mass Spectroscopy which require smaller sample sizes. Nevertheless, they require instrumentations not easily available in any laboratory, specific technical skills and more complex analytical procedure. A further aspect limiting a deeper comprehension of the mechanisms governing the cellular magnesium homeostasis is the lack of information about the intracellular repartition between the free and bound forms of magnesium (10). In fact, its intracellular compartmentalization have not yet been thoroughly elucidated, mainly because of the inadequacy of analytical techniques capable to map intracellular total magnesium distribution (7,11).

The absence of fluorescent dyes specific for the assessment of intracellular total magnesium presently represents a methodological trouble in the study of the intracellular content and distribution of this cation. Convincing evidences, indeed, demonstrate the peculiarity of the cellular magnesium homeostasis: as effect of different hormonal and non-hormonal stimuli, relevant fluxes of free Mg have been shown to traverse the cell membrane in both directions, resulting in significant changes in the plasma magnesium amount (10). However, this ion trafficking resulted in poor changes of free Mg intracellular concentration, albeit great variations in total Mg content have been found in subcellular organelles and tissues (12). These evidences suggest that alterations in magnesium availability, influencing several physiological functions, could be attained by modifications in the binding of magnesium among cellular ligands and in its distribution among cellular sub-compartments rather than by major modifications of the free fraction (13). It has been reported that mitochondria represents the main intracellular store of Mg (14) and that disrupting the mitochondrial membrane potential causes a release of Mg into the cytosol (11, 14). These evidences lead to hypothesize that mitochondria plays a central role in the intracellular homeostasis of this element (12).

We recently designed and developed a diaza-18-crown-6 hydroxyquinoline-based fluorescent dye with the unique analytical capability to accurately quantify the total intracellular Mg content (9). This probe is composed by a N,N'-Bis-((8-hydroxy-7-quinolinyl)methyl)-1,10-diaza-18-crown-6 ether bearing a phenyl group as a substituent in the position 5 of each hydroxyquinoline arm (15), named DCHQ5. It showed enhanced characteristics compared to the original reference DCHQ1 previously proposed (11), as greater fluorescence intensity upon cation binding, more intense membrane staining, a superior intracellular retention and the option to be excited both in the UV and visible spectrum range (8).

DCHQ5 is prepared in 60 – 65 % overall yield by a four-step linear synthetic sequence, starting from commercial 5-chloro-8-hydroxyquinoline **1** (Fig. 1). The phenyl substituent in position 5 is introduced by a palladium-catalysed Suzuki cross-coupling on protected hydroxyquinoline **2**, followed by basic hydrolysis to afford the desired 5-phenyl-8-hydroxyquinoline **4** in high yields (16). DCHQ5 is prepared through a one-pot

Mannich condensation reaction of **4** with 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane **5** and paraformaldehyde **6**, either thermally (*Method A*) or by microwave assisted reaction (*Method B*).

The photophysical properties of this class of compounds are determined by the presence of the derivatives of the well known 8-hydroxyquinoline (HQ) dye (17, 18). The absorption spectrum of DCHQ5 presents a very intense band attributed to a $\tilde{\sigma}-\tilde{\sigma}^*$ transition with a maximum at 250 nm ($\epsilon = 81000 \text{ M}^{-1} \text{ cm}^{-1}$) and a less intense, broader and non-structured one at 330 nm ($\epsilon = 9000 \text{ M}^{-1} \text{ cm}^{-1}$), that is associated with a charge transfer from the oxygen atom to the quinoline moiety (8). We had already proved that the substitution of the hydrogen in the 5 position of the quinoline structure of DCHQ1 causes a red-shift of both these peaks and in particular, introducing two aromatic groups like in DCHQ5 the overall charge delocalization causes also an increase in the molar absorption coefficients (8).

The addition of increasing amounts of Mg^{2+} induces a decrease of both bands and the appearance of a new band at 390 nm indicating the deprotonation of the hydroxyl group in the 8 position of HQ via complexation, even if in the case of Mg^{2+} binding, this deprotonation is not complete (8).

The emission spectrum of DCHQ5 presents a non-structured fluorescence band centred at 512 nm that is strongly affected by the nature of the solvent both in intensity and in energy. As for all these hydroxyquinoline-based compounds the intensity becomes higher increasing the solvent lipophilicity (19), therefore, the results obtained in MeOH–H₂O (1 : 1) solution buffered at pH 7.4 with MOPS (reported in **Table 1**) are not fully representative of the highly complex cellular environment in particular for the highly aromatic compound DCHQ5. The emission quantum yield of the free ligand is quite low (3.0×10^{-4}), as expected, since in HQ derivatives intramolecular photoinduced proton transfer (PPT) processes between the photoacid hydroxyl and the nearby photobase quinoline nitrogen quenches the emission. Moreover, in protic media, intermolecular PPT processes involving solvent molecules can also occur and further decrease it.

Upon binding of Mg^{2+} the DCHQ5 emission shifts to 517 nm and it undergoes a dramatic enhancement with respect to the free ligand (**Fig.2**), in fact, its fluorescence quantum yield becomes 3.5×10^{-2} , an attended result since both PPT and PET are inhibited by complexation (20). Remarkably, DCHQ5 fluorescence is not significantly affected by the presence of other divalent cations (**Table 1**), most importantly by Ca^{2+} , or by pH changes within the physiological range. It is worth mentioning that the complexation of Zn^{2+} and Cd^{2+} induces comparable fluorescence relative intensity changes, presenting similar binding constants. However, these ions are not expected to cause substantial interference since the intracellular Zn^{2+} concentration is around 100 times lower than that of Mg^{2+} , while cadmium is not present in non-poisoned cells.

In the Procedure, in addition to the description of the synthesis of DCHQ5, we report the experimental method, step by step, for employing DCHQ5 in mammalian cells to quantify total magnesium in a small sample size.

Intracellular Mg^{2+} assessment by DCHQ5 steady state fluorescence spectroscopy assays showed comparable results to F-AAS, which is the reference analytical method for the assessment of total metal content in cells,

as shown in **Figure 3**, where the correlation between the total intracellular magnesium assessed by AAS and DCHQ5 is reported. The data were obtained analysing cells of different histogenesis, i.e. leukemic HL60, colon adenocarcinoma HT-29 and osteoblast-like SaOS.2 and U2OS.

Moreover, this dye provides evidences on the capability to accurately quantify the intracellular total Mg in a sample of 50 thousands/ml cells, which is a size about 100 time smaller than that required by F-AAS (**Fig. 3**; ref. 9). It is interesting to underline that the Limit of Detection (LOD) of DCHQ5 obtained with this fluorimetric procedure is 0.2 μM , the same of the F-AAS (**Fig. 3**) and represents a value of great analytical interest (9).

A remarkable application of DCHQ5 was observed in the study of the involvement of Mg in Multi Drug Resistance performed in human colon adenocarcinoma cells sensitive (LoVo-S) and resistant to doxorubicin (5). In fact, we found a higher concentration of total Mg in LoVo-R respect to LoVo-S, while no differences were detected between the two cell strains in the free Mg^{2+} concentration assessed by Mag-Fura-2-AM probe (**Fig.4**; ref. 5).

The quantitative assay requires to disrupt the cellular structure by sonication, otherwise the whole cells, interacting with the methanol, increase the turbidity of the sample assay.

Although, the quantitative assay requires samples lysed by sonication and 50% of Methanol in the assays buffer, as detailed reported in the procedure part, qualitative assays by flow cytometry demonstrated that in living cells, resuspended in physiological buffers as D-PBS, the complex of DCHQ5 and Mg is efficiently retained within the cells (9); no appreciable cytotoxicity was detectable up to 60 minutes of staining (8).

Furthermore, the difference in Mg content of different samples are perfectly detectable by flow cytometry (8): for example, the ratio between the fluorescence of proliferant and differentiated HL60 cells (usually reported as mean channel) is equal to the ratio of the Mg content detected by AAS, as reported in SI **Tab.S1**.

We also tested DCHQ5 by two-photon imaging to exploit its advantageous photochemical properties in the study of intracellular total Mg distribution. Cells loaded with 5 μM of the aromatic dyes DCHQ5 (**Fig. 5**) showed a fluorescence signal diffused over the cytosolic portion with a higher intensity in the perinuclear region. These features can be ascribed to the Mg cytosolic content rather than to the un-even probe distribution, although it must be underlined that we know that DCHQ5 does not penetrate the nucleus and we do not have detailed data on the behaviour of the probe within the mitochondria (8).

These unique features, together with the other peculiar traits of this probe, such as the high fluorescence enhancement upon cation binding and the possibility to be excited both in the UV and visible region, make DCHQ5 a convenient and flexible analytical tool both for the intracellular total Mg assessment and in confocal imaging as effective total Mg indicators in living cells (**Fig.5**).

MATERIALS

REAGENTS

! CAUTION

Because most of the chemicals used for the synthesis of DCHQ5 are potentially harmful, we recommend performing all reactions in fume hood, wearing **Personal protective equipment (PPE)**: gloves, lab coat goggles.

- Milli-Q ultrapure water (Millipore Corporation)
- Dimethylsulfoxide (DMSO, Sigma-Aldrich, cat. no. 276855)
- Anhydrous magnesium sulfate (MgSO_4 ; Sigma-Aldrich, cat. no. M7506)
- Anhydrous calcium chloride (CaCl_2 ; Sigma-Aldrich, cat. no. C5670)
- 4-Morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich, cat. no. M1254)
- DPBS without calcium and magnesium (DPBS; Sigma-Aldrich, cat. no. D8537)
- RPMI 1640 (Sigma-Aldrich, cat. no. R0883)
- DMEM (Sigma-Aldrich, cat. no. D5546)
- FBS (Euroclone, cat. no. ECS0180L)
- L-Glutamine (Sigma-Aldrich, cat. no. G7513)
- Penicilline/Streptomycin, which contains 10,000 units of penicillin/ml and 10,000 μg of streptomycin/ml (Invitrogen, cat. no. 15140)
- Trypsin/EDTA ((Sigma-Aldrich, cat. no. T4049)
- Mammalian cell lines. The anticipated results described in this protocol have been obtained using LoVo (ATCC CCL-229), ROS (ATCC CRL-1661) and SaOS-2 (ATCC HTB-85) cells. However, the procedures has been successfully tested in HL60 cells, HT-29 cells, U2OS cells
- Distilled water
- 5-Chloro-8-hydroxyquinoline (Sigma-Aldrich, cat. no. C47000)
- p-Toluenesulfonyl chloride (Sigma-Aldrich, cat. no. T35955)
- Sodium hydroxide (Sigma-Aldrich, cat. no. 221465)
- Acetone (Sigma-Aldrich, cat. no. 270725)
- Toluene (Sigma-Aldrich, cat. no. 34866)
- Phenylboronic acid (Sigma-Aldrich, cat. no. 78181)
- Anhydrous *N,N*-dimethylformamide (Sigma-Aldrich, cat. no. 227056)
- Tetrakis(triphenylphosphine)palladium(0) (Sigma-Aldrich, cat. no. 216666)
- Potassium carbonate (Sigma-Aldrich, cat. no. 60110)
- Methanol (Sigma-Aldrich, cat. no. 494291)
- Ethanol (Sigma-Aldrich, cat. no. 459844)
- Anhydrous toluene (Sigma-Aldrich, cat. no. 244511)
- Paraformaldehyde (Sigma-Aldrich, cat. no. 158127)
- 1,4,10,13-Tetraoxa-7,16-diazacyclooctadecane (Sigma-Aldrich, cat. no. 295809)
- Chloroform (Sigma-Aldrich, cat. no. 528730)
- Diethyl ether (Sigma-Aldrich, cat. no. 344362)
- Cyclohexane (Sigma-Aldrich, cat. no. 179191)
- Ethyl acetate (Sigma-Aldrich, cat. no. 270989)
- Silica-gel for flash-chromatography (Merck silica gel 60, 230-400 mesh)
- Sodium sulfate anhydrous (Sigma-Aldrich, cat. no. 793531)
- Dichloromethane (Sigma-Aldrich, cat. no. 32222)
- Hydrochloric acid (Sigma-Aldrich, cat. no. 258148)

EQUIPMENT

- PTI Quanta Master C60/2000 spectrofluorimeter (Photon Technology International, Inc., NJ, USA)

- Cuvette (4.5 ml; Kartell Labware, cat. no. 81961)
- Inverted microscope (DM IRE2, Leica Microsystems GmbH)
- Microscope (Axioplan Zeiss)
- Confocal laser scanning system (TCS-SP2, Leica Microsystems GmbH) equipped with a Ti:sapphire ultrafast laser source tunable from 715 to 940 nm (Chameleon, Coherent Inc.)
- Forma direct heat CO₂ incubator (model 320, Thermo Fisher)
- Centrifuge (4236A, ALC)
- Microcentrifuge (ALC, Eppendorf)
- Centrifuge tubes (15 ml; Sarstedt, cat. no. 62554002)
- Eppendorf® Safe-Lock microcentrifuge tubes (1.5 ml; Sigma-Aldrich, cat. no. T9661)
- Sonicator (XL2020, Misonix)
- Ultrasonic bath (T420, Elma)
- Water bath (Thermomix, Braun)
- Burkert cell counting chamber (Brand, cat. no. 718920)
- pHmeter (Hanna instruments)
- Teflon-coated magnetic stirring bar (Sigma-Aldrich, cat. no. Z126969)
- Balance (Sartorius)
- Cell culture flasks T-25 (Sarstedt, cat. no. 833910)
- Cell culture flasks T-75 (Sarstedt, cat. no. 833911)
- Petri dish (100 mm; Sarstedt, cat. no. 833902)
- μ -dish (35 mm; Ibbidi GmbH, cat. no. 81156)
- Serological Pipette (2, 5, 10 ml)
- Rotary evaporator (Buchi)
- Disposable glass Pasteur pipette
- Pyrex one-necked round-bottomed flask (10, 25, 50, 100 and 250 ml)
- Pyrex two-necked round-bottomed flask (10, 25 and 50 ml)
- Pyrex three-necked round-bottomed flask (25 ml)
- Reflux condenser
- Oil bath
- Ice-water bath
- Pyrex separatory funnel (50 and 100 ml)
- Pyrex fritted filter funnel
- Pyrex flash-chromatography column
- Gas-tight volumetric syringe (50, 100 and 500 μ l, Hamilton)
- Glass luer lock syringe (1, 5 and 10 ml)
- Teflon-coated magnetic stirring bar
- Mercury-free thermometer
- Magnetic stirrer (IKA)
- Silica gel plates for thin-layer chromatography (Merck 60 F254)
- NMR spectrometer (Varian Mercury 400)
- NMR tube (Wildman, 5 mm)
- GC-MS (Agilent 6850 GC System / Agilent 5975 XL mass selective detector)
- HPLC-MS (Agilent HP1100 / Agilent MSD1100 single-quadrupole mass spectrometer)
- MS-ESI (Agilent Technologies 1100 Ion Trap Bruker)
- Milestone MicroSYNTH microwave Labstation
- Melting-point (Buchi B-540)

REAGENT SETUP

Cell culture medium Cell culture media are RPMI 1640 medium for LoVo cells and DMEM medium for ROS cells, supplemented with 2 mM L-Glutamine, 10% FBS, 100 units mL⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. These media without L-Glutamine can be stored at 4 °C for 2-3 months. Media with L-Glutamine can be stored at 4 °C for 1 month.

! CAUTION To avoid antibiotic resistance is recommended not to use routinely antibiotics in culture media
MeOH:MOPS solution, methanol:H₂O 1:1 buffered at pH 7.4 with 4 mM 3-morpholinopropane-1-sulfonic acid (MOPS) at room temperature. This solution can be stored at 4 °C for several months.

MgSO₄ solution, 1M Dissolve 12.04 g of anhydrous salt in 100 ml of Milli-Q water. This solution can be stored at 4 °C for several months.

CaCl₂ solution, 1M Dissolve 11.10 g of anhydrous salt in 100 ml of Milli-Q water. This solution can be stored at 4 °C for several months.

EQUIPMENT SETUP

Fluorescence spectroscopy. To quantify total intracellular magnesium, uncorrected emission spectra were obtained with spectrofluorimeter upon excitation at 360 nm, in the range 400 to 650 nm.

Confocal Microscopy. To monitor Mg²⁺ in cells by using DCHQ5, imaging was performed at an inverted microscope attached to a confocal laser scanning system equipped with a Ti:sapphire ultrafast laser source. Fluorescence of dye-loaded cells was excited by two-photon absorption at 750 nm and the emission signal was collected by a photomultiplier through a high magnification (40x) oil-immersion objective in a spectral window ranging from 480 to 530 nm.

PROCEDURE

Synthesis of 5-chloro-8-tosyloxyquinoline (**2**) – Timing 7-8 h

- 1) Prepare a 50-ml one-neck round-bottomed flask with a Teflon-coated magnetic stir bar.
- 2) Add 21 ml of 1 M sodium hydroxide aqueous solution (21 mmol) to the round-bottomed flask.
- 3) Dissolve 3.59 g of 5-chloro-8-hydroxyquinoline (**1**, 20 mmol) by slowly adding it to the aqueous solution.
- 4) Dissolve 3.81 g of *p*-toluensulfonyl chloride (20 mmol) in 8 ml of acetone.
- 5) Add the solution of *p*-toluensulfonyl chloride to the reaction mixture drop by drop over 5 min with stirring.
- 6) Vigorously stir the heterogeneous reaction mixture on a magnetic stirrer at room temperature for 5 h.
- 7) Add 20 ml of distilled water to the reaction mixture.
- 8) Filter the solid using a fritted filter funnel under vacuum and wash the solid twice with 10 ml of water.
- 9) Recrystallize the crude product from toluene to obtain 6.07 g of 5-chloro-8-tosyloxyquinoline (18.2 mmol, 91 % yield). Check the identity and purity of the compound by NMR and MS analyses (**Fig. S1-3**).

PAUSE POINT. Compound **2** can be stored at room temperature under air for several months without any appreciable decomposition.

Synthesis of 5-phenyl-8-tosyloxyquinoline (**3**) – Timing 8-10 h

- 10) Prepare a 25-ml three-neck round-bottomed flask, a reflux condenser with an inlet glass vacuum hose adapter and a Teflon-coated magnetic stir bar.
- 11) Connect the apparatus to a single-manifold Schlenk line attached to an inert gas supply (nitrogen or argon) using the inlet hose adapter.
- 12) Flame-dry the glassware and allow it to cool down under vacuum before refilling it with inert gas.
- 13) Add 0.668 g of 5-chloro-8-tosyloxyquinoline (**2**, 2 mmol) to the round-bottomed flask.
- 14) Add 10 ml of anhydrous *N,N*-dimethylformamide.
- 15) Add 0.116 g of tetrakis(triphenylphosphine)palladium(0) (0.1 mmol, 5 mol %) to the reaction mixture and allow it to stir for 10 min at room temperature.
- 16) Add 0.256 g of phenylboronic acid (2.10 mmol), followed by 0.58 g of K₂CO₃ (4.2 mmol).
- 17) Place the flask in a pre-heated oil bath (150 °C) on a hot-plate magnetic stirrer.
- 18) Stir the reaction mixture at reflux for 5 h under nitrogen atmosphere.
- 19) Cool the reaction mixture to room temperature and add 10 ml of dichloromethane.

- 20) Wash the organic layer twice with 10 ml of distilled water each time and twice with 5 ml of a 5 % aqueous lithium chloride solution each time, to remove most of the *N,N*-dimethylformamide.
- 21) Dry the organic layer over anhydrous Na₂SO₄ (2 g) and filter the mixture through a fritted filter funnel.
- 22) Remove the solvent using a rotary evaporator (water bath temperature: 30 °C).
- 23) Prepare a chromatography column (diameter: 2 cm; height: 15 cm) packed with silica gel by using cyclohexane:ethyl acetate 8:2 (vol/vol).
- 24) Dissolve the crude product in ~1 ml of the eluent and drip the resulting solution onto the top of the silica gel column.
- 25) Perform the purification by eluting with a 8:2 (vol/vol) mixture of cyclohexane and ethyl acetate. Collect the fractions containing the product and remove the solvent using a rotary evaporator (water bath temperature: 30 °C) to obtain 0.65 g of **3** as a faint yellow solid (1.73 mmol, 87 % yield). Check the eluted fractions by silica gel TLC with a 70:30 (vol/vol) mixture of cyclohexane and ethyl acetate (product R_f = 0.46). Check the identity and purity of the compound by NMR and MS analyses (**Fig. S4-6**).

CRITICAL The starting material and the desired product have a very similar retention factor on silica (**2** R_f = 0.47, **3** R_f = 0.46, 70:30 cyclohexane/ethyl acetate). The two compounds can be clearly distinguished since **2** appears as a darker violet spot, while **3** as a brighter violet spot, by UV absorbance at 254 nm (**Fig. S13**). If the presence of **2** in the purified product **3** is detected by NMR spectroscopy, further purify the product by recrystallization using methanol.

PAUSE POINT. Compound **3** can be stored at room temperature under air for several months without any appreciable decomposition.

Synthesis of 5-phenyl-8-hydroxyquinoline (**4**) – Timing 4-6 h

- 26) Prepare a 25-ml two-neck round-bottomed flask with a reflux condenser and a Teflon-coated magnetic stir bar.
- 27) Add 10 ml of ethanol:acetone 1:1 (vol/vol) to the round-bottomed flask.
- 28) Add 0.751 g of 5-phenyl-8-tosyloxyquinoline (**3**, 2 mmol) to the round-bottomed flask with stirring.
- 29) Add 6 mL of 1 M sodium hydroxide aqueous solution (6 mmol) to the round-bottomed flask with stirring.
- 30) Put the flask in a pre-heated oil bath (90 °C) on a hot-plate magnetic stirrer.
- 31) Stir the reaction mixture at reflux for 3 h under air.
- 32) Cool the reaction mixture to room temperature and add 10 ml of distilled water.
- 33) Adjust the pH of the solution to ~6.5 by adding a 1 M hydrochloric acid aqueous solution drop by drop.
- 34) Filter the remaining solid using a fritted filter funnel under vacuum, and wash it twice with 10 ml of distilled water.
- 35) Alternatively, instead of filtering, extract the product from the aqueous layer using dichloromethane (3 extractions with 10 ml of organic solvent each time).
- 36) Recrystallize the crude product using methanol to obtain 0.39 g of 5-phenyl-8-hydroxyquinoline (1.76 mmol, 88 % yield) as a yellow solid. Check the identity and purity of the compound by NMR and MS analyses (**Fig. S7-9**).

PAUSE POINT. Compound **4** can be stored at room temperature under air for several months without any appreciable decomposition.

Synthesis of DCHQ5 (Method A) – Timing 20-22 h

- 37) Prepare a 10-ml two-neck round-bottomed flask with a reflux condenser, an inlet glass vacuum hose adapter and a Teflon-coated magnetic stir bar.
- 38) Connect the apparatus to a single-manifold Schlenk line attached to an inert gas supply (nitrogen) using the inlet hose adapter.
- 39) Flame-dry the glassware and allow it to cool down under vacuum before refilling it with inert gas.
- 40) Add 0.066 g of 5-phenyl-8-hydroxyquinoline (**4**, 0.3 mmol) to the round-bottomed flask.
- 41) Add 2 ml of anhydrous toluene.
- 42) Add 0.039 g of 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane (**5**, 0.15 mmol) with stirring.
- 43) Add 0.009 g of paraformaldehyde (**6**, 0.3 mmol) with stirring.
- 44) Place the flask in a pre-heated oil bath (120 °C) on a hot-plate magnetic stirrer.
- 45) Stir the reaction mixture at reflux for 18 h under nitrogen atmosphere.

46) Cool the reaction mixture to room temperature and remove the organic solvent using a rotary evaporator (water bath temperature: 50 °C) to obtain a yellow powder. Check the reaction conversion by NMR spectroscopy of the crude product.

47) Recrystallize the crude product by dissolving it in hot chloroform (0.5 ml) and by adding cold methanol (2 ml) to obtain 0.093 g of pure DCHQ5 as a yellow powder (0.128 mmol, 85 % yield). Check the identity and purity of the compound by NMR and MS analyses (**Fig. S10-12**).

PAUSE POINT. DCHQ5 can be stored at room temperature under air for several months without any appreciable decomposition.

Synthesis of DCHQ5 (Method B) – Timing 5-7 h

48) Prepare a 10-ml two-neck round-bottomed flask with an inlet glass vacuum hose adapter and a Teflon-coated magnetic stir bar.

49) Connect the apparatus to a single-manifold Schlenk line attached to an inert gas supply (nitrogen) using the inlet hose adapter.

50) Flame-dry the glassware and allow it to cool down under vacuum before refilling it with inert gas.

51) Add 0.066 g of 5-phenyl-8-hydroxyquinoline (**4**, 0.3 mmol) to the round-bottomed flask.

52) Add 2 ml of anhydrous toluene.

53) Add 0.039 g of 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane (**5**, 0.15 mmol) with stirring.

54) Add 0.009 g of paraformaldehyde (**6**, 0.3 mmol) with stirring.

55) Irradiate the sample in a Milestone MicroSYNTH microwave Labstation at a power of 600 W for 4 h.

56) Cool the reaction mixture to room temperature and remove the organic solvent using a rotary evaporator (water bath temperature: 50 °C) to obtain a yellow powder. Check the reaction conversion by NMR spectroscopy of the crude product.

57) Recrystallize the crude product by dissolving it in hot chloroform (0.5 ml) and by adding cold methanol (2 ml) to obtain 0.098 g of pure DCHQ5 as a yellow powder (0.134 mmol, 90 % yield). Check the identity and purity of the compound by NMR and MS analyses (**Fig. S10-12**).

PAUSE POINT. DCHQ5 can be stored at room temperature under air for several months without any appreciable decomposition.

Quantification of total intracellular magnesium by DCHQ5

58) The following section describes how to quantify the total intracellular magnesium in sonicated cellular samples. Please proceed starting from step A for the preparation of the sonicated samples, then following step B for the preparation of the calibration curve, and ending with step C for the quantification of the total intracellular magnesium of the sonicated samples interpolating the calibration curve.

A) Preparation of sonicated cellular samples TIMING 60 min

CRITICAL This section can be performed with any mammalian cellular models, depending from readers cell system of choice. The culture conditions (kind of flask, medium, seeding and culture time) depend from the cells type and from the experimental scopes. Here we indicate the common features in our experimental condition.

i) Seed mammalian cells at 10000 cell/cm² in T25 and complete RPMI 1640 medium and grown at 37 °C in 5% CO₂ atmosphere. At the appropriate time, harvest the cells by trypsinisation.

CRITICAL this protocol is also applicable to suspension cell culture skipping the harvesting by trypsinisation.

ii) Wash the cells three times in DPBS at 250 g x 10 min.

iii) Accurately count the cells and resuspend the cells in DPBS at cellular concentration less than 1 x10⁶ cells /ml.

iv) Sonicate the samples for 1 min at 100 W.

CRITICAL We recommend to accurately count the cells and not to exceed the cellular concentration of 1 x10⁶ cells /ml to avoid problems of sonication and to remain under the critical micellar concentration of the cellular lipid content.

? TROUBLESHOOTING

B) Calibration curve preparation TIMING 80 min

B1) Dissolve 1 mg of DCHQ5 (molecular weight 728.88) in 1 ml of DMSO to obtain a solution 1.37 mM. Sonicate the solution for 20 min at 85 W in an ultrasonic bath to ensure the complete solubilization of the dye.

PAUSE POINT This DMSO DCHQ5 solution could be stored in the dark at -20 °C for several months.

B2) Blank value measurements

i) Prepare five points adding 0, 2, 6, 10, 16 μl of MgSO_4 1 mM (final concentration: 0, 1, 3, 5, 8 μM , respectively), in each cuvettes, in duplicate.

ii) Add respectively 200, 198, 194, 190, 184 μl of DPBS (up to 200 μl).

iii) Add 1.778 ml of MeOH:MOPS, mix well.

iv) Acquire the fluorescence emission spectra between 400 and 700 nm of each cuvette and record the fluorescent emission at 510 nm.

B3) Add 22 μl of the dye stock solution to each cuvette, to have a final concentration of DCHQ5 15 μM . Mix well.

i) Acquire spectra of the complex $\text{Mg}\backslash\text{DCHQ5}$ and record the fluorescence intensity at 510 nm.

ii) Add 10 μl of MgSO_4 1M to completely saturate the dye. Mix well.

iii) Acquire spectra and record the fluorescence intensity at 510 nm.

CRITICAL Acquiring the entire emission spectra allow to notice any interfering event which could affect the assay. It is possible to use a fixed wavelength instruments and record directly the fluorescence at 510 nm. The spectra of the completely saturated dye allow to check the correct amount of the dye in the cuvette.

? TROUBLESHOOTING

B4) Subtract the blank to each measurement and construct the calibration curve, plotting the Mg concentration against the fluorescence intensity. Calculate the equation of the calibration curve.

C) Quantification of the total intracellular magnesium of sonicated samples by interpolation of the calibration curve TIMING 10 min for sample

C1) Samples blank value measurements.

i) Add in cuvette the volume corresponding to 50000 cell/ml and add DPBS up to 200 μl .

CRITICAL The numbers of cells loaded can be modified depending on the cell types, from 25000 to 75000 cells/ml.

ii) Add 1.778 ml of MeOH:MOPS buffer and mix well.

iii) Acquire spectra and record the fluorescence intensity at 510 nm.

C2) Add 22 μl of the DCHQ5 stock solution to obtain a DCHQ5 concentration 15 μM . Mix well.

i) Acquire the spectra and record the fluorescence intensity at 510 nm.

ii) Add 10 μl of MgSO_4 1M to completely saturate the dye. Mix well.

iii) Acquire the spectra and record the fluorescence intensity at 510 nm.

CRITICAL This data allow to check the correct amount of the dye in the cuvette.

? TROUBLESHOOTING

C3) Calculate the micromoles of magnesium in cuvette by interpolation of the calibration curve equation.

C4) Refer the Magnesium content to the sample

CRITICAL The quantification of total intracellular magnesium can be normalized to the volume of cells if a comparison between different cell types (having different volumes) is required (Fig 3). The volume of the cells can be measured by cell volume measurement techniques such as flow cytometry, coulter counter, etc.

59) Mg imaging by confocal microscopy TIMING 1-2 h

i) Seed cells at 10000 cells/ cm^2 onto a 35 mm dish with a thin bottom for highend microscopy (μ -dish), and grow the cells in complete DMEM for 24h at 37°C in a 5% CO_2 atmosphere.

CRITICAL The researcher must optimize the seeding density for the given cell line.

- ii) Wash the cells gently twice with 1 ml of DPBS.
- iii) Add to the cells 5 ml of DPBS supplemented with 5 μ l of 1 M CaCl₂ (final concentration: 1 μ M) and incubate the cells with 18.3 μ l of 1.37 mM DCHQ5 (final concentration: 1 μ M) dissolved in DMSO for 20 min in the dark at room temperature.
- iv) At the end of the incubation, take fluorescence images of the loaded cells by using a two-photon confocal microscopy (excitation wavelength: Ti:sapphire ultrafast laser 750 nm) (**Fig. 5**).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TIMING

With the appropriate precursor in hand (**5-chloro-8-hydroxyquinoline 1**), the synthesis and purification of DCHQ5 is anticipated to require 39-46 h for Method A, and 24-31 h for Method B.

Quantification experiments are anticipated to require 70 min.

Bioimaging experiments are anticipated to require 1-2 h.

Steps 1-9, synthesis of compound **2**: 7-8 h, including purification

Steps 10-26, synthesis of compound **3**: 8-10 h, including purification

Steps 26-36, synthesis of compound **4**: 4-6 h, including purification

Steps 37-47, synthesis of DCHQ5 (Method A): 20-22 h, including purification

Steps 48-57, synthesis of DCHQ5 (Method B): 5-7 h, including purification

Step 58, quantification of total intracellular magnesium by DCHQ5

Step 58A, preparation of sonicated cellular samples: 60 min

Step 58B1, preparation and sonication of DCHQ5 solution: 20 min

Step 58B2-58B4, calibration curve preparation: 60 min

Step 58C, quantification of the total intracellular magnesium: 10 min for one sample

Step 59, Mg imaging by confocal microscopy: 1-2 h

ANTICIPATED RESULTS

Analytical data

2. Typical isolated yields 90-95 %, from white to faint colored solid. mp = 140-141 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.86 (dd, *J* = 4.2, 1.6 Hz, 1H), 8.53 (dd, *J* = 8.6, 1.6 Hz, 1H), 7.88 (d, *J* = 8.4 Hz, 2H), 7.59 (d, *J* = 8.2 Hz, 1H), 7.54 (d, *J* = 8.2 Hz, 1H), 7.51 (dd, *J* = 8.2, 3.7 Hz, 1H), 7.28 (d, *J* = 8.4 Hz, 2H), 2.43 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 151.1, 145.2, 144.4, 141.9, 132.7, 132.7, 129.8, 129.4, 128.6, 127.2, 125.8, 122.5, 122.2, 21.5; MS-ESI: 334 ([M+H]⁺); Anal. Calcd for C₁₆H₁₂ClNO₃S (333.02) C, 57.57; H, 3.62; N, 4.20. Found: C, 57.79; H, 3.68; N, 4.25.

3. Typical isolated yields 85-90 %, yellow solid. mp = 159-160 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.85 (dd, *J* = 4.2, 1.6 Hz, 1H), 8.19 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.62 (d, *J* = 7.8 Hz, 2H), 7.56 – 7.41 (m, 5H), 7.35 (dd, *J* = 8.6, 4.1 Hz, 1H), 7.31 (d, *J* = 8.2 Hz, 2H), 2.44 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 150.4, 145.0, 144.7, 141.6, 139.6, 138.3, 134.2, 133.2, 129.8, 129.4, 128.6, 128.4, 127.9, 127.8, 126.4, 121.7, 121.6, 21.6; MS-ESI: 376 ([M+H]⁺); Anal. Calcd for C₂₂H₁₇NO₃S (375.09) C, 70.38; H, 4.56; N, 3.73. Found: C, 70.62; H, 4.61; N, 3.77.

4. Typical isolated yields 85-90 %, yellow solid. mp = 88-89 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.82 (dd, *J* = 4.3, 1.6 Hz, 1H), 8.32 (dd, *J* = 8.6, 1.6 Hz, 1H), 7.58 – 7.37 (m, 7H), 7.28 (d, *J* = 8.0 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 151.7, 147.6, 139.3, 138.2, 134.6, 130.8, 130.0, 130.0, 128.4, 128.3, 128.2, 127.1, 126.6, 121.6, 109.6; MS-ESI: 222 ([M+H]⁺); Anal. Calcd for C₁₅H₁₁NO (221.25) C, 81.43; H, 5.01; N, 6.33. Found: C, 81.06; H, 5.14 N, 6.28.

DCHQ5. Typical isolated yields 90-95 %, yellow solid. mp = 158-159 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.87 (dd, *J* = 4.2, 1.6 Hz, 2H), 8.19 (broad d, *J* = 8.5 Hz, 2H), 7.50 – 7.36 (m, 12H), 7.32 (dd, *J* = 8.5, 4.1 Hz, 2H), 4.05 (broad s, 4H), 3.75 (broad s, 8H), 3.63 (s, 8H), 2.99 (broad s, 8H); ¹³C NMR (100 MHz, CDCl₃) δ 152.3,

148.4, 139.7, 139.3, 134.0, 130.1, 128.6, 128.3, 127.0, 126.5, 121.0, 118.9, 70.8, 69.5, 57.3, 53.9; MS-ESI: 729 ([M+H]⁺), 751 ([M+Na]⁺); Anal. Calcd for C₄₄H₄₈N₄O₆ (728.36) C, 72.51; H, 6.64; N, 7.69. Found: C, 72.21; H, 6.61; N, 7.73.

Mg quantification and detection in mammalian cells

The protocol for the quantification of the intracellular total magnesium has been successfully tested in HL60 cells, SaOS-2 cells, HT-29 cells, U2OS cells, obtaining overlapping results with F-AAS (Fig 3). DCHQ5 can be also used to detect intracellular magnesium in mammalian cells by using confocal microscopy. Data on the in vivo detection of magnesium on ROS and SaOS-2 cells are represented in Fig. 5.

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Table 1. Photophysical properties of DCHQ5 and its complexes in MeOH–H₂O (1 : 1) solution buffered at pH 7.4 with MOPS.

Compound	Log K_a (stoichiometry)	Absorbance		Fluorescence	
		λ_{max} / nm	ϵ / M ⁻¹ cm ⁻¹	λ_{max} / nm	Φ
DCHQ5	-	249 330	81100 9000	512	$3.0 \cdot 10^{-4}$
DCHQ5·Mg ²⁺	5.08 ± 0.06 (ML)	265 390	39600 3300	517	$3.5 \cdot 10^{-2}$
DCHQ5·Zn ²⁺	6.6 ± 0.2 (ML)	263 385	66500 7300	551	$1.4 \cdot 10^{-2}$
DCHQ5·Cd ²⁺	9.4 ± 0.2 (ML ₂)	263 390	114000 12900	544	$2.1 \cdot 10^{-2}$
DCHQ5·Hg ²⁺	10.0 ± 0.1 (ML ₂)	266 390	110000 12900	-	< 10 ⁻⁵
DCHQ5·Cu ²⁺	9 ± 1 (ML)	265 395	66000 7400	-	< 10 ⁻⁵
DCHQ5·Ni ²⁺	10.8 ± 0.1 (ML ₂)	266 395	124800 15500	-	< 10 ⁻⁵
DCHQ5·Na ⁺	<i>No complexation</i>				
DCHQ5·K ⁺					
DCHQ5·Ca ²⁺					
DCHQ5·Fe ²⁺					
DCHQ5·Fe ³⁺					

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
58 A (iv)	The solution is turbid after the sonication	The sonication is not completed	Repeat step 58 A (iv) more time until the solution remains limpid
58 B3 (iii), 58 C2 (iii)	Different fluorescence intensity values at saturation	Unappropriate dye loading	Discharge the sample and repeat starting from step 58 B2