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A novel fluorescent chemosensor allows the assessment of intracellular total magnesium in small samples.

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The present study investigated the analytical capabilities of a new fluorescent chemosensor, named DCHQ5, a phenyl derivative belonging to the family of diaza-crown-hydroxyquinolines, for the quantitative assessment of total intracellular Mg content. The results obtained were compared to the analytical performances of DCHQ1- the parent probe of the series which so far was the only suitable species for the quantitative assessment of the intracellular total magnesium and showed comparable results to atomic absorption spectroscopy. Different protocols were tested in several cell lines both by flow cytometry and by steady state fluorescence spectroscopy assays. The results obtained support the possibility to use DCHQ5 to accurately quantify the intracellular total Mg in much smaller samples than DCHQ1, also displaying an increased stable intracellular staining. These features, combined with the high fluorescence enhancement upon cation binding, and the possibility to be excited both in the UV and visible region, make DCHQ5 a valuable and versatile analytical tool for Mg assessment in biological samples.

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#### 1. Introduction

Magnesium is an abundant intracellular cation essential for many processes such as ion channel regulation, DNA and protein syntheses, membrane stabilization and cytoskeletal activity<sup>1,2</sup>. Hundreds of enzymes require MgATP<sup>2-</sup> as a cofactor or  $Mg^{2+}$  as an allosteric regulator<sup>1,3,4</sup>. As a consequence, cell functions such as proliferation and death depend on Mg<sup>2+</sup> availability<sup>5,6</sup>. Despite the concentration of Mg is in the mM range in both extracellular and intracellular milieu, many relevant pathological conditions, such as cardiovascular diseases, hypertension, diabetes and dysmetabolic syndrome, are associated with reduced Mg availability and/or increased excretion either at a systemic level or in specific tissues<sup>7-10</sup>. Furthermore, rare human genetic diseases have been recently associated with mutations of specific genes coding for magnesium channels<sup>11,12</sup>. Moreover, recent reports show that magnesium can act also by regulating expression levels and hence the ratio between pro- and anti-apoptotic proteins like Bax or Bcl2, respectively<sup>13,14</sup>. Therefore, Mg intracellular content may be important in determining apoptosis or survival through a mitochondrial pathway<sup>15</sup>, not only by regulating several molecular players but also by affecting the production of oxidative species<sup>16</sup>. It has also been shown, that a cis-platin resistant human ovarian carcinoma cell line (C13\*) presents a significantly higher Mg<sup>2+</sup> content than the sensitive parental cells<sup>17</sup>. Since mitochondria, along with nuclei and microsomes, represent preferred compartments of Mg<sup>2+</sup>accumulation<sup>18,19</sup>, mitochondria alterations acquired by C13\* cells during resistance selection may at least partly account for their elevated Mg<sup>2+</sup> concentration compared to sensitive cells<sup>19</sup>. Despite this increasing amount of evidences about the involvement of Mg in these fundamental cellular processes, the mechanisms regulating these phenomena are still under investigation. Intracellular magnesium content is regulated by

specific influx and efflux mechanisms (ion channels and antiporters), some of which have been characterized in depth 20-<sup>22</sup>. Nevertheless, magnesium distribution between free and bound forms and its intracellular compartmentalization have not yet been thoroughly elucidated, mainly because of the inadequacy of available techniques to map intracellular magnesium distribution. Some authors showed that free and total magnesium undergo different and independent regulatory mechanisms, suggesting that Mg ions move among cellular sub-compartments following mechanisms not yet fully characterized<sup>21,23-25</sup>. In addition, a further unsolved question attains the mechanisms which regulate magnesium homeostasis in different tissues, and a current open dispute is on which intracellular fraction of the element (total or free) would be more analytically and biologically relevant. To date, the most common approach to assess total cellular magnesium on cell or tissue is by flame Atomic Absorption Spectroscopy (F-AAS) on acidic extracts, a technique that requires large samples (at least millions of cells or several milligrams of tissue). Other more sensitive techniques require smaller sample sizes, such as Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) and Graphite Furnace AAS (GF-AAS). These techniques indubitably offer improved detection limits, however require instrumentations not easily available in any laboratories, more complex analytical procedures and specific technical skills and competencies<sup>26,27</sup>. On the other hand, free  $Mg^{2+}$  can be measured from the chemical shift of Mg-ATP peak by <sup>31</sup>P NMR spectroscopy<sup>28,29</sup>, by intracellular fluorescent probes derived from those used for calcium (mag-fura, mag-fluo, magindo)<sup>30-32</sup>, and by ion-selective microelectrodes<sup>31</sup>. All these techniques have different fields of application, depending on the different cells or tissues studied. More recently, in the study of the cellular magnesium homeostasis, research has focused on the development of new classes of fluorescent chemosensors, which are more sensitive and specific to magnesium than the

commercially available ones<sup>34-36</sup>. These probes showed interesting and different peculiarities in cellular Mg<sup>2+</sup> assessment and imaging. The KMG fluorescent dyes developed by Komatsu et al.34 were able to detect and image cytosolic  $Mg^{2+}$ , as well as monitor  $Mg^{2+}$  effluxes from mitochondria under uncoupling stimuli. The AMg1 fluorescent dyes developed by Kim *et al.*<sup>37,38</sup>, whose high two photon cross section makes them suitable for two photon confocal microscopy, partially discriminate between membrane and cytosolic Mg<sup>2+</sup> by two-photon confocal microscopy. We have recently described the photophysical characteristics of some derivatives of diaza-18-crown-6 ethers appended with two 8hydroxyquinoline groups (DCHQs) and proposed their potential applications to measure cell magnesium content and distribution<sup>36,39</sup>. DCHQ1, the first member of this family, binds  $Mg^{2+}$  with much higher affinity than any other available probe (having a  $K_d = 44 \mu M$ ) showing a strong fluorescence increase upon complexation. Remarkably, its fluorescence is not significantly affected either by other divalent cations, most importantly Ca<sup>2+</sup>, or by pH changes within the physiological range<sup>18,40</sup>. DCHQ1 readily permeates cells, binds intracellular Mg<sup>2+</sup>, and it has been employed to map intracellular ion distribution and movements in live cells by confocal imaging<sup>16,37</sup>. Furthermore, this probe has been shown to be a suitable analytical tool capable to quantitatively assess the total intracellular magnesium in different cell lines<sup>36</sup> providing comparable results to AAS. Besides the unquestionably analytical value of DCHQ1, some limitations of this probe have restricted its application. Indeed, its poor retention inside the cell prevents its application for monitoring Mg intracellular uptake to study the Mg-channel activity upon stimuli<sup>36</sup> or in mutant cells with impaired Mg-channel activity<sup>41</sup>. We have recently developed a new method of chemical synthesis of the DCHO compounds based on microwave heating, in order to optimize their yield and to permit us to modify their basic structure by introducing various functional groups<sup>42</sup>: the new synthetic strategy allowed to generate a variety of substituted DCHQ derivatives with improved fluorescence, uptake and localization with respect to the original reference material (DCHQ1). This method showed the possibility to obtain pure products with > 95% yields and the flexibility to synthesize a number of DCHQ derivatives that are currently object of photochemical and biological studies<sup>39</sup>. Particularly interesting features were observed for the derivative bearing a phenyl group as substituent in position 5' of each hydroxyquinoline arm, named DCHQ5 (see Figure 1), that showed improved characteristics compared to the DCHQ1 such as higher response to cation binding, membrane staining, a much higher intracellular retention and the possibility to be excited both in the UV and visible spectrum range<sup>39</sup>. However, due to its very low solubility in aqueous buffers such as phosphate buffered salines (DPBS buffers), the photophysical characterization of DCHQ5 was performed in methanol-based buffers<sup>39</sup>. This indeed precludes the possibility to use a K<sub>d</sub> for a quantitative analytical protocol for the intracellular Mg assessment like the one employed for DCHQ1<sup>18</sup>. On the other hand, in DPBS, even when incubated with cells, we found a very good staining. In fact, measurements by flow cytometry on leukemic cells both differentiated and not, showed that the distribution of fluorescence intensity of DCHQ5 strongly correlates with the total magnesium content assessed by AAS<sup>39</sup>. Nevertheless, the possibility to quantitatively assess the intracellular total Mg by DCHQ5 still remained to be established. In this study we thoroughly investigated the analytical performance of DCHQ5

for the quantitative assessment of intracellular total Mg content. To accomplish this task, we chose to compare the results obtained with DCHQ5 with the parent probe DCHQ1 and flame AAS, one of the most common routinely analytical techniques. Furthermore we tested different protocols in several cell lines both by flow cytometry and by steady state fluorescence spectroscopy assays.



DCHQ5

Figure 1. Structures of DCHQ1 and DCHQ5

#### 2. Experimental

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#### 2.1 General spectroscopic methods.

All reagents were from Sigma Aldrich (Italy), if not differently stated, and were of ultrapure grade. Dulbecco's Phosphate-Buffer Saline (DPBS) was purchased without  $Ca^{2+}$  and  $Mg^{2+}$  and the acronym DPBS in the text will indicate this formulation. The fluorescent probes were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1 mg mL<sup>-1</sup> (DCHQ1 1.7 mM, DCHQ5 1.4 mM). Aliquots were kept at 4°C in the dark.

#### 2.2 Cell culture.

All cells lines (human HL60 Promyelocytic Leukemia, human HT29 colon adenocarcinoma, human LoVo colon adenocarcinoma, human U-2 OS bone osteosarcoma) were grown at 37°C and 5% CO<sub>2</sub> in RPMI 1640 medium, supplemented with 2mM L-Glutamine, 10% FBS, 100 units mL<sup>-1</sup> penicillin and 100  $\mu$ g mL<sup>-1</sup> streptomycin.

#### 2.3 Flow cytometry.

Flow cytometric measurements were performed on a Bryte HS cytometer (BioRad, UK), equipped with a Xe-Hg lamp, using a filter set with an excitation band centered at 360 nm and two emission bands centered respectively at 500 nm (DCHQ derivatives fluorescence) and 600 nm (propidium iodide fluorescence). Before the staining, cells were washed twice in DPBS and suspended at a final concentration of  $5 \times 10^5$  cells/mL. The cells were incubated for 15 minutes in the dark at  $37^{\circ}$ C with DCHQ5 5  $\mu$ M and DCHQ1 25  $\mu$ M respectively and then analysed. Each cell sample was counterstained with 5  $\mu$ g mL<sup>-1</sup> propidium iodide (PI) to identify dead cells. Fluorescence distributions were recorded using a logarithmic scale. To assess the intracellular trapping of the dyes, the stained samples were washed in DPBS and reassayed by flow cytometry (see the flow

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cytometric assay of DCHQs staining of viable cells in Supplementary Information, Fig. S-4).

#### 2.4 Total cell magnesium measurement by AAS.

Total magnesium content was assessed by AAS on acidic cellular extracts of a sample of  $10^6$  cell/mL. Harvested cells were washed twice in cold DPBS by centrifugation and then pelleted at 250g. Ion extractions were obtained by overnight treatments of the cell pellets with 3mL of 1.0 N HNO<sub>3</sub>. After agitation and centrifugation of samples, magnesium was assayed on cell supernatants by AAS (Instrumentation Laboratory mod. S11, USA or Perkin Elmer AA200) equipped with an air/acetylene flame.

#### 2.5 Fluorescence spectroscopy.

For the fluorescence spectroscopy measurement, uncorrected emission and corrected excitation spectra were obtained with a PTI Quanta Master C60/2000 spectrofluorimeter (Photon Technology International, Inc., NJ, USA).

#### 2.6 Absorption spectra.

Absorption spectra were recorded on a Perkin-Elmer Lambda 45 spectrophotometer. Absorption spectra of DCHQ1  $25\mu$ M were acquired in three different buffer: MeOH:MOPS (methanol:H<sub>2</sub>O 1:1 buffered at pH 7.4 with 3-morpholinopropane-1-sulfonic acid at room temperature), MOPS:KCl (MOPS 30mM:KCl 100mM at pH 7.2) and DPBS upon addition of increasing amount of MgSO<sub>4</sub> (from 0 to 500  $\mu$ M). Absorption spectra of DCHQ5 10  $\mu$ M were acquired in MeOH:MOPS (See absorption spectra in Supplementary Information, Figure S-1, bottom panel for DCHQ1 and Figure S-2, on the right for DCHQ5).

#### 2.7 Stability of the probes.

For the determination of the stability of the probes, the emission wavelength was set to the value of the maximum and the samples were excited continuously for 30 minutes. Spectra were acquired at two different concentrations of magnesium, chosen to test a Mg:dye ratio of 1:2 and 100:1.

#### 2.8 Titration of Mg<sup>2+</sup> binding by fluorescence spectroscopy.

Increasing amount of a solution 100 $\mu$ M of MgSO<sub>4</sub> solution were added to a 25 $\mu$ M solution of DCHQ1, in three different buffer (DPBS, MeOH:MOPS, MOPS:KCl), and to a 10 $\mu$ M solution of DCHQ5 in MeOH:MOPS, to obtain Mg<sup>2+</sup> concentration from 0 to 500  $\mu$ M, and fluorescence spectra were recorded, upon excitation at 360 nm, in the range 400 to 650 nm. (See emission spectra in Supplementary Information, Figure Figure S-1, top panel for DCHQ1 and Figure S-2, on the left for DCHQ5).

## 2.9 Quantification of total cell magnesium by spectrofluorimetric assay.

Total magnesium content was assessed on sonicated cell samples by the two fluorescent dye DCHQ1 and DCHQ5 assay choosing a method that involves the construction of a standard curve. Briefly, DCHQ5 was dissolved to a  $15\mu$ M final concentration in a mixture which contains 10% of DPBS in a solution 1:1 of MeOH:MOPS 2 mM (pH 7.4), while DCHQ1 was dissolved to a  $25\mu$ M final concentration in DPBS.

Different amounts of  $MgSO_4$  were added and the fluorescence intensities were acquired at 510 nm for DCHQ5 and at 500 nm for DCHQ1. The Mg concentration of the unknown samples were obtained by the interpolation of their fluorescence with the standard curve (See standard calibration curves of DCHQ1 (on the left) and DCHQ5 (on the right) in Supplementary Information, Figure S-3).

#### 2.10 Limit of Detection.

The determination of LOD was performed using the following protocol: we prepared three different solutions each containing the same and known amount of Mg ions and we did this for three different increasing amounts of the analyte, always with a fixed chemosensor concentration. For each concentration we registered the fluorescence emission spectra, and we calculated the mean value of emission intensity in the maximum of the band (arithmetic mean,  $x = \frac{N - x_i}{N}$ ). We did the same for all the different concentrations of Mg2+ and the results were plotted as a function of the metal ion concentration. After performing a linear regression of this curve, we calculated the standard deviation  $\sigma x$  of the lowest concentration according to the following equation:

$$\sigma_{x} = \sqrt{\frac{\sum_{i=1}^{N} (x_{i} - \bar{x})^{2}}{N - 1}}$$

The concentration value corresponding to an intensity of  $x + 3\sigma x$  was taken as the LOD of our system. The LOD was performed for both the two probe in the assay buffer (MeOH:MOPS+10% of DPBS for DCHQ5 and DPBS for DCHQ1) and in sonicated cellular samples.

The detection limit for Mg in AAS was established by analysis of >10 blank solutions (LOD 2xSD); blank value was  $20\pm3$  ng/ml, so the detection limit resulted 6 ng/mL (0,2  $\mu$ M). Trace metals standard (MA-M-2/TM: lyophilized mussels) were analyzed every 20 samples and all values measured for reference materials were within certified limits given by the IAEA (International Atomic Energy Agency).

#### 2.11 Statistical Analysis.

Statistical significance was assessed by Student-Neuman-Keuls' tests. A probability of less than 5% (P<0.05) was considered to be significant. Details of the statistical analysis performed are reported on a excel file in the Supplementary Information.

#### 3. Results and Discussion

We have recently reported that DCHQ5 shows an appreciable increase in fluorescence intensity in the presence of Mg ions in MeOH:MOPS<sup>39</sup>. However, the buffer of choice in mammalian cellular investigations is Dulbecco's Phosphate Buffered Saline (DPBS) which mimics the extracellular environment, as far as Na<sup>+</sup>, K<sup>+</sup> and phosphate ionic species concentrations. Other simpler saline buffers are proposed in literature for other magnesium dyes, as the MOPS:KCl buffer<sup>37,38</sup>. For a complete characterization we have investigated the performance of our two probes in the three buffers mentioned above and we also acquired the absorption spectra of both dyes in all conditions (Figure S-1 and Figure S-2). MeOH:MOPS resulted to be the most suitable buffer to perform a comparison of the two probes, as they showed a solubility in the micromolar concentration range. The conjugated structure of DCHQ5, in fact, turned out to prevent the possibility to reach these concentrations in aqueous buffers. We report in figure 2 the titration profiles of the chemosensors in the presence of increasing amounts of  $Mg^{2+}$  in MeOH:MOPS and also in DPBS for DCHQ1. Due to the different luminescence quantum yields of the Mg complexes formed with our two dyes, the comparison was performed using each probe at its optimal concentration (DCHQ1 25µM and DCHQ5 10 µM) in order to optimize the signal to be measured<sup>39</sup>.

Fig 2 shows that DCHQ5, had a higher response (steeper slope) and higher fluorescent intensity for all the Mg concentrations than DCHQ1. It is worth noting that fluorescence intensities are reported normalized by the concentration of the respective probe. Therefore these results indicates that the better performance of DCHQ5 was obtained even using a much less amount than DCHQ1.



**Figure 2.** Fluorescence intensity of DCHQ1 (blue) and DCHQ5 (red) at 500 and 510 nm respectively in MeOH:MOPS reported as a function of Mg concentration. Titration of DCHQ1 in DPBS is also reported for comparison. The fluorescence intensities are normalized dividing by the concentration of the respective probe ( $25\mu$ M for DCHQ1 and  $10\mu$ M for DCHQ5).

We also evaluated the stability over time of the fluorescence of DCHQ1 and DCHQ5 at two different concentrations of magnesium, chosen to have a Mg:dye ratio of 1:2 and 100:1 in MeOH:MOPS and in DPBS.

Table 1 reports the percentage of fluorescence attenuation after 30 minutes of continuous exposure to the excitation light for the two chemosensors. The signal decrease in MeOH:MOPS is similar for the two dyes. Since the buffer employed in the standardized analytical protocol for the intracellular Mg

assessment by DCHQ1 is DPBS, we also performed the stability test of DCHQ1 in these conditions. The results showed a marked decrease of fluorescence at the Mg:dye ratio 1:2, used in the previously proposed intracellular Mg assays protocol.

Table 1. Percentage decrements of fluorescence intensity of the two probes after 30 minutes of continuous exposure to the excitation light at 500nm for DCHQ1 and at 510nm for DCHQ5.

Mg:dye	1:2	100:1
DCHQ1 in MeOH:MOPS	19%	4%
DCHQ1 in DPBS	44%	2%
DCHQ5 in MeOH:MOPS	15%	8%

However, to perform assays in cells it is required to use a physiological buffer. The presence of MeOH obviously has a detrimental effect, due to the permeabilisation effect of this solvent on cell membranes. Therefore, we decided to design a new protocol to quantitatively assess intracellular total magnesium in sonicated cellular samples by using a simple spectrofluorimetric assay, based on a standard titration curve (See standard calibration curves of DCHQ1 (on the left) and DCHQ5 (on the right) in Supplementary Information, Figure S-3). DCHQ1 was dissolved in DPBS, which is also the buffer of the sonicated samples, while DCHQ5 in MeOH:MOPS with 10% of DPBS. It is worth to underline that the assay based on DCHQ5, due to its high fluorescence intensity, requires the use of a concentration of 15µM, against the 25µM of DCHQ1. The quantitative assessment of total magnesium in different cell types was performed by comparing the fluorescence intensities of sonicated cells suspended in DPBS to the fluorescence intensities registered for the relative standard curve. Results indicated that DCHQ5 can quantitatively assess intracellular total magnesium in cellular samples of the order of 30-50x10<sup>3</sup> cells/mL, while the minimum cells/mL size in which the DCHQ1 probe can be used is  $100 \times 10^3$ .

We compared the data obtained, in a total of 24 cell samples, using DCHQ5 and DCHQ1, with ones given by AAS, chosen, as previously motivated, as the reference technique for the quantification of total intracellular magnesium (Figure 3).

Statistical analysis showed that the data could be well fitted with linear equations and we also found a good correlation between the data sets given by the two techniques for both probes, DCHQ5 presenting the closest values to the identity line. The 95% confidence interval values of linear regressions are somewhat smaller for the DCHQ5 than DCHQ1 being 0.17 and 0.26 respectively for the slope and 4.27 and 7.09 for the intercept. We also performed a paired and unpaired t-test between the dye fluorimetric assays and AAS. Results show no statistical difference in the unpaired t-test for both dyes, while DCHQ5 showed a p < 0.01 in the paired t-test (see statistical analysis in Supplementary Information). Taken together, these data demonstrate that the values of Mg concentration assessed by both dyes overlap the AAS measurements. The regression line equation of DCHQ5 has coefficients closer to the identity line than those of DCHQ1. However, the paired t-test analysis showed that DCHQ5 displays a positive systematic deviation from AAS measurements, as can be also noted by the Analyst

comparison of the correlation with the identity line (Figure 3 right panel).

We then evalueted the Limit of Detection (LOD) for both probes in the assay buffer (MeOH:MOPS+10% of DPBS for DCHQ5 and DPBS for DCHQ1) and also in sonicated cellular samples (Table 2).

The LOD of the two probes was similar for cell population of  $250 \times 10^3$  and  $100 \times 10^3$ 

As already pointed above, DCHQ1 can be used in a sample size of at least 100x10<sup>3</sup> cells/mL, while DCHQ5 can quantify Mg in

much smaller samples. Therefore, we could assess the LOD for DCHQ5 for cellular samples of even  $50x10^3$  cells/mL. It is interesting to note that the LOD of DCHQ5 for the lowest sample size, obtained with this simple fluorimetric procedure, is of the same order of the AAS one, a vale of great analytical interest (see methods).

Finally, we performed cytofluorimetric assays to evaluate the intracellular retention of DCHQ1 and DCHQ5 after washing, as well as their fluorescence stability over time.



**Figure 3** Correlation between the total intracellular magnesium assessed by AAS and DCHQ1 (on the left) or DCHQ5 (on the right). The identity line is plotted for comparison.

Table 2. Limit of detection (LOD) of DCHQ1 and DCHQ5 reported in  $\mu M$  evaluated in the buffer solution and in the cells sample.

Mg <sup>2+</sup> LOD in μM in buffer solution			Mg <sup>2+</sup> LOD in μM sample size (cells/mL)		
		$50 \times 10^3$	$100 \times 10^3$	$250\times10^3$	
DCHQ1	0.3	n.d	0.2	0.5	
DCHQ5	0.2	0.2	0.2	0.5	

Fig. 4 reports the mean channel of DCHQs fluorescence of viable population (see the flow cytometric assay of DCHQs staining of viable cells in Supplementary Information, Fig. S-4). The cytofluorimetric analyses shows that the staining is quite stable up to 60 minutes for both probes with a maximum decrease of 20% for DCHQ5. The slight intracellular fluorescence decay of the probes over time is probably due to

the suffering of cells suspended in a medium lacking of any energetic substrate leading to a decrease of intracellular magnesium. The DCHQ5 probe resulted to be much more efficiently retained within the cells than DCHQ1. It is worth noting that the DCHQ5 intracellular fluorescence change is almost negligible after the second washing. The overall fluorescence decrease of DCHQ5 is around the 30% of the

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initial value. Conversely, DCHQ1 fluorescence progressively decreases above all after the second washing, with a total decrement of 80% from the initial value. These results suggest that for DCHQ5 the first washing eliminates only the dyes loosely bound to the cellular surface, and not the probe entrapped within the cell which, unlike DCHQ1, is not removed by the second washing. Finally, we would like to underline again that the amount of DCHQ5 required for the

cytofluorimetric assays is one fifth than that for DCHQ1 (DCHQ5 5  $\mu$ M and DCHQ1 25  $\mu$ M). This indicates that DCHQ5 compared to DCHQ1 exhibits an analytical efficiency in cytofluorimetry even higher than that observed in fluorescent spectroscopy where the ratio of the dye concentration [DCHQ5]/[DCHQ1] was 1:2.5 (DCHQ5 10  $\mu$ M and DCHQ1 25  $\mu$ M).



**Figure 4:** Mean channel of fluorescence distribution of cells stained with DCHQ1 (on the left) and DCHQ5 (on the right) and after washing, data were acquired over a time lapse of 60 minutes after staining. Experiments repeated three times. Data are expressed as mean±SD.

#### Conclusions

This study showed the possibility to use the fluorescent chemosensor DCHQ5: i) to accurately quantify the intracellular total Mg with comparable performance than the reference species DCHQ1; ii) to assess intracellular total Mg in much less number of cells by loading a smaller amount of chemosensor in comparison with DCHQ1; iii) in cytofluorimetric assays by loading a smaller amount of it, with the advantage of displaying a more stable intracellular staining after washing than DCHQ1. All these features, combined with other remarkable characteristics such as the high fluorescence enhancement upon Mg<sup>2+</sup> binding, the membrane staining and the possibility to be excited both in the UV and visible spectrum<sup>39</sup>, make DCHQ5 a valuable analytical tool for Mg assessment in biological samples.

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#### Notes and references

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Electronic Supplementary Information (ESI) available: [Emission and absorption spectra of DCQH1 in the three buffer of analysis, emission and absorption spectra of DCHQ5 in MeOH:MOPS, standard calibration curves for the quantification of total intracellular magnesium, flow cytometric assay of DCHQs staining of viable cells and the complete statistical analysis are reported.]. See DOI: 10.1039/b000000x/

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