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An experimental system to detect a labeled cell monolayer in a microfluidic environment

**Abbreviated title page:** A novel experimental system to detect a labeled cell monolayer in a microfluidic environment

**Keywords:** MR Contrast Agent, Multi Turn Transmission Line Resonator, microfluidic, cell monolayer

**Abbreviations:** CA: contrast agent

MTRL : multi-turn transmission-line resonator

ICP-MS : Inductively Coupled Plasma-Mass Spectroscopy

VFA : variable flip angle

**Word count:** 4742 (include references and legends)

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ABSTRACT - 223 mots

Introduction - The development of new targeted contrast agents (CA) requires proof-of-concept studies in order to establish the detectability of the CA and to predict the role of biodistribution in its uptake mechanisms. A promising approach is to carefully mimicking the in vivo pharmacokinetical context with reduced experimental complexity compare to in vivo situations. As a first step towards this objective, this work investigates the feasibility of detecting a living cell monolayer labeled with Gd-DOTA in a microfluidic environment, by micro-MRI in a 2.35T small-animal system.

Materiel and Methods - A dedicated experimental system was built by combining a microfluidic slide and a radiofrequency probe based on a 6 mm diameter multi-turn transmission-line resonator. Adherent KB cells were incubated with different concentrations of Gd. MRI data were acquired at 2.35 T with a 3D Gradient Echo and a resolution of 12.4 µm perpendicularly to the cell layer. The longitudinal relaxation rate $R_1$ was measured as a function of the amount of Gd internalized by the cells.

Results - $R_1$ measurements for different Gd concentration per cells were performed using data with an SNR of 100. Relaxation-rate variations $\Delta R_1$ of $0.035 s^{-1}$ were measured. A quenching effect was observed at Gd concentrations above 20fmol/cell.
Conclusion - Our results suggest that this dedicated experimental system is suitable for specifically assessing new high-relaxivity targeted CA under real-time uptake conditions.
INTRODUCTION

In the past decade, a variety of innovative MRI approaches have been proposed to depict molecular processes occurring at cell level, such as the use of exogenous contrast agents (CA). In the context of molecular MRI, the sensitivity to probe molecular markers, in the nanomolar range in most pathological processes, is the critical point (1). The corresponding CA concentration is substantially lower than the detection threshold of gadolinium chelates, which is in the hundred micromolar range (2). Therefore, the detection sensitivity of routinely used gadolinium chelates is inadequate for molecular MR imaging in many cases. To overcome these limitations, CA prototypes, which are able to carry up to several thousand Gd3+ ions, are being developed (3-5). These new particles possess both longer intravascular half-lives and higher longitudinal relaxivities ($r_1$), so they are good candidates to enable receptor detection in the picomolar range and allow MR imaging of sparse binding sites (6).

In this context, new experimental tools are needed to characterize new molecular contrast agents and evaluate their efficiency. To date, results of ex vivo evaluation of most agents are applied directly to in vivo animal studies (7). Often, the former relaxation properties of novel CA measured in vitro do not correctly reflect the agent’s in vivo properties (1). Indeed, they do not match the real physiological conditions for the targeting of cellular receptors, in terms receptor concentrations and time of contact between the CA and the receptors; moreover, they are unable to predict the pharmacokinetics of the CA.
Following an intravenous injection of a CA with \textit{in vivo} animal models, many processes can occur simultaneously, such as extravasation, specific binding to receptors, or internalization inside cells. Depending on the process, the CA will have access to different numbers of water molecules and will involve different relaxation pathways (8,9). Thus the relaxation properties of new contrast agents should be initially evaluated and optimized in controlled cell culture studies prior to \textit{in vivo} animal experimentation. Such studies would allow better assessment of the efficiency of the CA as a function of its biodistribution.

This paper investigates the feasibility of detecting a living cell monolayer, labeled with Gd-DOTA (Guerbet Research®), by micro-MRI in a carefully controlled microfluidic environment. The goal of our work was to accurately assess the performance of our experimental system using a conventional Gadolinium labeling technique.

**MATERIALS AND METHODS**

\textit{CA internalization and cell monolayer preparation:}

KB cells, a human cell line derived from a nasopharynx carcinoma, were grown in RPMI 1640 medium (Bracco, Italy) supplemented with 10\% Newborn Calf Serum Heat-Inactivated and 1\% Glutamine in a humidified incubator at 37 °C and 5 \% CO$_2$. For cell labeling experiments, a protocol inspired by Terreno et al. (8) was used: 2 million cells were seeded inside a 25 cm$^2$ flask and incubated for 24 hours to make them adhere to the surface of the flask. Following this, cells were put in contact with culture
medium containing Gd-DOTA at concentrations of 0, 1, 2, 5, 10, 25, 50, 75 and 100 mM during 17 h. The Gadolinium content per cell was measured by Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS). 3.50 × 10^5 labeled cells were seeded into the microfluidic channel coated with Collagen-IV (dimensions of 0.4 × 5 × 50 mm^3) of a microscope slide (µ-slide I 0.4 Luer, Ibidi, Germany), and formed a confluent single layer as illustrated in Figure 1.c, observed by optical microscopy. The cells were left 4 hours in the microfluidic channel prior to MRI. Preliminary studies of cell viability, using an eosin exclusion test, demonstrated that incubation of the cells during 24 hours with Gd-DOTA concentrations as high as 100 mM in the labeling solution had no toxic effect. Optical microscopy confirmed that 96% of the adherent cells were still alive on the slide after the MRI exam.

**Experimental protocol:**

The relaxation rates were measured using the variable flip angle (VFA) method (11) with seven flip angles varying from 5 to 70°.

The longitudinal relaxation rate R_1 measurements were first realized in the microfluidic channel filled with 5 different concentrations of Gd-DOTA (0, 50, 100, 250 and 500 µM) diluted in water to assess the VFA method accuracy.

In a second step, R_1([Gd]) relaxation rate measurements as a function of incubated Gd were done on labeled cell monolayers in the presence of the CA-free
culture medium. The Gd concentration used for the incubation was of 0, 1, 2, 5, 10, 25, 50, 75 and 100 mM. A washing step was done at the end of the experiment on the cell layer with a flush of 5 mL of 38 g.L⁻¹ NaClO in the channel. Optical microscopy confirmed that an NaClO flush killed the whole cell layer immediately and removed the cell lysis from the microfluidic channel. The same imaging protocol was applied on the channel before (with labeled cells) and after the flush (culture medium only), to obtain a reference acquisition with exactly the same position of the slide inside the MRI scanner. R₁ values were determined on the cells, R₁,c ([Gd]), and on the CA-free culture medium, R₁,m.

The effect of the MR CA is \( R_1([\text{Gd}]) = R_1([\text{Gd}=0]) + r_1 \cdot [\text{Gd}] \) with \( R_1([0]) \) the baseline relaxation rate (free medium or cells), \( r_1 \) the CA relaxivity, of 3 mMol.s⁻¹ in our study (12).

**MRI hardware:**

Experiments were carried out on a 2.35 T horizontal scanner (Bruker Biospin, Germany), delivering a maximum gradient intensity of 200 mT/m. The microfluidic channel was manually aligned with the static magnetic field to minimize susceptibility artifacts. The quality of the alignment was then monitored by a scout scan. Warm air flowed in the gradient bore to maintain the temperature of the cells at 37 °C. To ensure a homogeneous excitation of the hydrogen nuclei, a birdcage coil (Bruker, Germany) with an inner diameter of 7 cm was used for transmission. The reception coil was a high sensitivity home-made surface coil of 6 mm mean diameter, using the MTLR
design and microfabrication process (13). It was composed of 10 circular concentric copper tracks deposited of both sides of a sapphire substrate. The MTLR was placed below the μ-slide, 180 μm away from the cell monolayer (Figure 1 a. b.) and was inductively coupled (14) to the receiver electronics using a 6 mm single-turn copper loop, parallel tuned at $f_0 = 100.53$ MHz. Orthogonality between the transmit and receive coils axis leaded to a attenuated transmission coefficient $S_{21}$ between the two coils of about – 40 dB at the Larmor frequency.

**MRI sequence:** A T$_1$-weighted 3D-FLASH (Fast Low Angle SHot) sequence was used with a field of view of $0.6 \times 1.9 \times 0.8$ cm$^3$, a resolution of 12.4 μm in the readout direction, perpendicular to the cell layer, and an in-plane resolution of $160 \times 160$ μm$^2$. To reach such high resolution in the readout direction, echo asymmetry was adjusted to 5%, meaning that only 55% of the k-space was acquired. Acquisition parameters were: TR (Time Repetition)/TE (Time Echo) of 74.7/3.7 ms, total sampling bandwidth of 12.5 kHz, 2 averages, 100 dummy scans to reach the steady state, and acquisition time of 8 min. Radiofrequency spoiling was performed with a 117° phase increment between successive excitations to reduce residual transverse magnetization effects. The alignment of the microfluidic channel onto the horizontal image axis was carefully adjusted using scout scans at the beginning of the protocol.

**Data Processing**

A cylindrical region of interest (ROI) of 5 mm diameter was centered to the sensitivity pattern of the reception coil. One-dimensional signal profiles were then
extracted with a projection signal intensity of the ROI along the 2 directions, y and z, parallel to the cell layer (5 and 50 mm). The homodyne method (15) was applied to ensure low distortion of the reconstructed profiles despite the highly asymmetric k-space coverage. To avoid truncation artifacts, a linear phase ramp correction was applied to the k-space with the appropriate sub-voxel shift before the homodyne reconstruction. The $R_1$ measurements were performed on the first non-zero voxel along the x-axis, assuming this to be the voxel containing the cells.

The signal-to-noise (SNR) measurements were extracted from magnitude images using the average signal intensity over the ROI and the standard deviation outside of the channel.

The systematic evaluation of the performance of the $R_1$ measurements was based on Cramer-Rao Bound according to the SNR and the flip angle of the experiments (16).

The relaxation rate was also simulated considering the theoretical model developed by Strijkers et al. (17), based on the Bloch equations including exchanges of water molecules among 3 different compartments (vesicles, cytoplasm and the extracellular medium). Their model allows the quantification of the decreased efficiency due to the CA internalization, depending on the location of the paramagnetic chelates. The shape of the cells highly influences the values of the water molecule exchange constants between two compartments. In the present study, cells are adherent on a surface and were assumed an oblate semi-spheroid cell shape, following
a previous work by Lee et al. (18). This modified model was applied using the parameters of Table 1 to simulate $R_{1,c}([\text{Gd}])$ measurements, assuming equal relaxation rates between the vesicle, cytosol, and extracellular compartments in the absence of CA. The number of vesicles, their radii, and their permeability were of the same order as those reported in the literature (19-21).

**Diffusion**

Based on the integration of the Bloch-Torrey equation, this system could be represented by a mean local relaxation time assuming a mono-exponential position-dependent decay (Mean Relaxation Time Approximation (22)). Due to the planar configuration of the cell monolayer (over y and z axes), the apparent relaxation rate $R_{1,\text{local}}(x)$, induced by the paramagnetic CA, is expected to vary along the $x$-direction. Considering a time integration of the Bloch-Torrey equation, from 0 to infinity, taking the bulk culture medium’s relaxation rate $R_{1,m}$, diffusion coefficient $D_m$, and assuming the cell monolayer was entirely contained in the first voxel, the local relaxation rate is then expected to vary as:

$$R_{1,\text{local}}(x)^{-1} = R_{1,m}^{-1} + \left( R_{1,\text{local}}(0)^{-1} - R_{1,m}^{-1} \right) e^{-\frac{x}{\sqrt{D_m R_{1,m}^{-1}}}} \text{ Eq. 1.}$$

Considering a Spoiled Gradient Recalled Echo sequence, with $\text{TR} = 75 \text{ ms}$, $R_{1c}$ ($x=0$) and $R_{1m}$ and respectively equal to the cells’ relaxation rate and the medium relaxation rate measured on MR acquisitions, $S_{1D,c}$ and $S_{1D,m}$, signals produced for
relaxation rate $R_{1\text{local}}(x)$ and $R_{1\text{m}}$ were calculated. The theoretical normalized difference, $(S_{1\text{D},c}(x)-S_{1\text{D},m})/S_{1\text{D},m}$ was evaluated.

RESULTS

Figure 2 displays the linear dependence with a slope of 0.499 between the amount of internalized Gd$^{3+}$ per cell and the Gd-DOTA incubation concentration, as measured by ICP-MS.

$R_1$ measurements on the slide filled with water containing different CA concentrations are shown Figure 3-a (dots); $R_1([\text{Gd}])$ were measured on data not shown here with an SNR of about 100. Water CA free relaxation rate, $R_1([0])$ was of $0.38 \pm 0.06$ s$^{-1}$. Error bars were estimated from the Cramer Rao Lower Band, an SNR of 100 and $R_1$ of water of $38$ s$^{-1}$. The theoretical curve was computed considering a Gd-Dota relaxivity of $3$ mM$^{-1}$s$^{-1}$ (black line). The extrapolation of the relaxation rate of the water CA free was $0.347 \pm 0.07$ s$^{-1}$.

Figure 3-b shows the $R_{1,c} ([\text{Gd}])$ values, $R_1$ measurements for different Gd concentration, as a function of the Gd content per cell. The measurements were made using data with an SNR of 100. The $R_{1,c}([0])$ was measured at about $0.38 \pm 0.13$ s$^{-1}$. The error bars are computed, following the Cramer Rao Lower Band and an SNR of 100 40 and $R_{1,c}([0]) = 0.38$. A saturation effect at high Gd content, up to 20 fmol/cell, was also
observed and may be attributed to compartment effect as discussed in the last section. The relaxation rate of the cell-free medium, $R_{1,m}$, was measured as $0.37 \pm 0.08$ on the slide filled with medium. The black curve in figure 3.b shows the simulated $R_{1,c}([\text{Gd}])$, based on the Strijkers model.

Figure 4.a presents the signal variation along the x-axis, i.e., the receiver coil axis. The black curve depicts the one-dimensional profile, $S_{1D,m}$, of the CA free medium. The Signal-to-Noise Ratio of the profile was about 100 40. The decrease of the signal, by less than 20% along the coil axis, is due to the $B_1$ reception field amplitude decay. This curve was used as the reference to evaluate the effect of the labeled cells. The gray curve, $S_{1D,c}$, displays the one-dimensional profile obtained for a channel containing cells after 17 h incubation with 25 mM of Gd-DOTA, corresponding to a Gd content of 15 fmol per cell, and a 20° flip angle. This profile is typically obtained with other Gd concentrations. The adherent cells are confined within the first voxel (x=0) of the microfluidic channel, corresponding to the first maximum of signal of the 1D profile. The signal enhancement was observed both at the level of the labeled cell layer and on adjacent voxels up to a distance of about 150 µm, until $S_{1D,c}-S_{1D,m}$ was equal to the noise level.

The effect of diffusion is analysed on Figure 4.b. The black points represent the measured normalized difference, $\frac{S_{1D,c}-S_{1D,m}}{S_{1D,m}}$, obtained from the experiments. The theoretical normalized difference, black line, is fitted to the experimental data considering that $D_m$ equals $2.8 \times 10^{-9}$ m$^2$.s$^{-1}$ and $R_{1,m}$ is $0.37$ s$^{-1}$. A $R_{1,local}$ (x=0) of $0.62 \pm 0.017$ s$^{-1}$ was then obtained.
DISCUSSION

In this study, we propose an experimental system to detect and measure the relaxation rate of a single cell layer by micro-MRI, a very difficult challenge given the resolution capabilities at clinical. This initial study was carried out using KB cells treated with independently determined concentrations of Gd-DOTA. The feasibility and accuracy of $R_1$ measurements in cell cultures using an MTLR resonator was assessed and confirmed with aqueous Gd-DOTA samples of known concentration. The high sensitivity of this experimental system is due to the small diameter of the receiver coil. The high spatial resolution then reached for cell detection highlighted the diffusion of $R_1$ modified water protons through the cell membranes into the culture medium. A transition zone between two compartments with two different $R_1$ values was observable in the signal profile. A comparable effect has already been observed and investigated by Kaufman et al. (23) with $T_1$-weighted high-resolution images. Thanks to the planar geometry (slice), all cells are subjected to the same conditions, which will be helpful to dissociate the several compartments involved in the theoretical model developed; here there are two compartments, the cell layer and the culture medium. A theoretical approach by Bauer et al. (24) showed that the effect of a Blood-Pool CA confined to the intravascular space was also observed in the tissue surrounding the capillaries, by transport of the water molecules through the capillary wall and diffusion throughout the extravascular space. Based on the Bloch-Torrey equation (25), they demonstrated that this system could be represented by a mean local relaxation time assuming a mono-exponential position-dependent decay (Mean Relaxation Time
Approximation (22)). In the present case, the paramagnetic CA induced such relaxation behavior in the direction orthogonal to the cell layer. This approach was in good agreement with the experimental measurements. The sensitivity of this system also allowed measurement of a detection limit ΔR₁ of 0.035 s⁻¹, considering the almost linear behavior of R₁ as a function of Gd concentration, for Gd content less than 4 fmol per cell, and a SNR of 100. This corresponds to a variation of the amount of Gd of less than 1 fmol per cell. The saturation of the R₁,c curve for Gd contents above 20 fmol [Gd] per cell is well described by a relaxivity quenching effect attributed to the CA internalization in cells via pinocytosis, a non-specific endocytosis process. This phenomenon leads to the entrapment of the CA inside vesicles within the cytoplasm, resulting in decreased CA efficiency (i.e., apparent relaxivity) compared to that of free CA (8). In this study, all R₁,c ([Gd]) values were smaller than 1 s⁻¹, which is low compared to literature values, which are in the 1.5 to 3 s⁻¹ range (8,19) for cell pellets that have internalized high amounts of Gadolinium. This difference can be explained by the shape of the cells, which are considered in the literature as spherical in cell pellets; however, in our experiments, cells are adherent and are assumed to be semi-spheroids. When we applied the Strijkers model (17) assuming spherical cells occupying half of a voxel volume, we found R₁,c values of 2 s⁻¹ for high Gd cell contents, which is in agreement with the literature data. The voxel dimension along the x-axis is small enough to be in the right range for the Strijkers model.

Cells occupied more than half of the voxel height. Since the voxels are composed of cells and water, considering the fast exchange limit, R₁ of the water and
$R_1$ of the cells will contribute to the $R_1$ measurement in the first voxel. The $R_1$ measurement of the cells will then probably be overestimated. This could explain the difference between the $R_{1c} ([25\text{mM}])$ value measured using the VFA method and the value extrapolated from the Bloch Torrey model at $x=0$. Moreover, the assumption of a perfectly known value for the angle calibration could be a source of error not considered here. Nevertheless, $R_1$ measurements of water, medium, and CA-free cells, and those extrapolated from the first experiment were consistent. Moreover, the VFA technics remained robust to the inhomogeneous reception field $B_1$ considering our experimental configuration, and the short acquisition time of 8 minutes allowed by this technique remains an obvious asset, especially for dynamic experiments. Another limitation of this study may be the small diameter of the receiver coil, which was limited here to values below 6 mm in order to match the microfluidic channel width. Decreasing the coil diameter further would result in a lower integrated signal-to-noise ratio on the 1D profile. Indeed, the coil sensitivity is expected to scale as the inverse of the coil diameter (26), while the number of detected cells in the monolayer would scale as the squared inverse of the diameter. However, combining several smaller coils to form a surface coil array with the same overall dimension as here still offers the perspective of greatly enhancing the detection level.

In conclusion, this experimental system appears suitable for monitoring the dynamic uptake of targeted contrast agents in realistically mimicked \textit{in vivo} conditions. It should allow to predict the CA kinetics to assess the relevant time for imaging. A quite similar approach was proposed by Schmalbrock et al. (10) on cell culture plates, but their
configuration was not practical for assessment of the CA dynamic uptake in a realistic and controlled environment. Here, we propose a promising approach to carefully evaluate the dynamic uptake of contrast agents by cells using *in vitro* models, in order to mimic the *in vivo* pharmacokinetic context with reduced experimental complexity.
REFERENCES

Legends

Figure 1: Description and dimensions of the complete experimental system: a. photographe of the microscope slide (µ-slide) containing a microfluidic channel in situ with MTLR placed below the µ-slide, 180 µm away from the cell monolayer. Cells adherent and forming a compact monolayer are observable on the bottom in the µfluidic channel (in orange). b - Dimensions and spatial image parameters (field of view, spatial resolution) of the microscope slice. x-axis, orthogonal to the cell monolayer is well highlighted. c - Confluent single layer observed by optical microscopy.

Figure 2: Amount of internalized Gd$^{3+}$ per cell (fmol/cell), measured by ICP-MS, as a function of the Gd-DOTA incubation concentration (black dots). Gd$^{3+}$ per cell evolved linearly (slope of 0.4996) with the Gd-DOTA incubation concentration (black line).

Figure 3: a- $R_1$ measurements as a function of the Gd-DOTA concentration. The microfluidic channel was filled with an aqueous solution of Gd-DOTA (circle). $R_1$ theoretical values (black curve) adjusted to experimental values, considering an $r_1$ of 3 mM$^{-1}$s$^{-1}$ at 2.35 T. Error bars were computed from the Cramer Rao lower bound, with an SNR of 100 and $R_{1,c}$ ([0]) of 0.38. b – longitudinal relaxation rate $R_1$ of cells ($R_{1,c}$) as a function of the Gd content per cell (fmol/cell) (circle). Error bars were computed from
the Cramer Rao lower bound, with an SNR of 40 and $R_{1,c} ([0]) = 0.38$. Black curve represented the $R_1$ adjustment using the 3-compartment Strijkers model.

Figure 4: a- 1D monocellular layer profile after Gd-DOTA incubation (25 mM) corresponding to 15 fmol/cell, $S_{1D,c}$ was represented on the gray curve. 1D medium profile without cell, $S_{1D,m}$, is represented on the black one. Both were obtained with the same sequence parameters and a flip angle of 20°. The decrease of 20 % of the signal along the coil axis on the red curve was attributed to the $B_1$ field of the reception coil. (1), (2), (3) and (4) are relative to spatial position located on the experimental system (Figure 1) b -The normalized difference, $\frac{S_{1D,c} - S_{1D,m}}{S_{1D,m}}$, black points, describes the variations of the signal as a function of the distance from the cell monolayer. The theoretical normalized difference is adjusted to the experimental data (line curve) assuming the Bloch Torrey diffusion model, considering a SPGR sequence, with $TR = 75$ ms, $R_{1,m} = 0.37$ s$^{-1}$ and $D_m = 2.8 \times 10^9$ m$^2$.s$^{-1}$. The $R_{1,app}(x=0)$ value of $0.62 \pm 0.017$ s$^{-1}$ is obtained.

Table 1: Parameters used for simulation of $R_{1,c} ([\text{Gd}])$ expected (represent by the gray curve on figure 3.b) considering the 3-compartment Strijkers model (20).
Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Gd-DOTA longitudinal relaxivity $r_1$ (mM$^{-1}$s$^{-1}$)</td>
<td>3</td>
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<tr>
<td>Cell basis radius (µm)</td>
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<tr>
<td>Cell height (µm)</td>
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<tr>
<td>Water Membrane permeability (µm.s$^{-1}$)</td>
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<td>Number of vesicles</td>
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<td>Vesicle radius (µm)</td>
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<td>Extracellular water fraction</td>
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<td>Culture medium relaxation rate (s$^{-1}$)</td>
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<tr>
<td>Cytosol relaxation rate (s$^{-1}$)</td>
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</tr>
<tr>
<td>Vesicle relaxation rate (s$^{-1}$)</td>
<td>0.38</td>
</tr>
</tbody>
</table>
Amount of Gd internalized by cells (fmol/cell) during incubation (mM)
a. 

![Graph showing $R_1$ vs. Gd concentration (μM)](image-1)

- **Experiment**
- **Theory**

b. 

![Graph showing $R_{1,c}$ vs. Gd content per cell (fmol/cell)](image-2)

- **Experiment**
- **3D Strijkers model**
b. 

(1) (2) (3)

$S_{1D,c}$ $S_{1D,m}$

down up

$0.4$ $0.3$ $0.2$ $0.1$ $0.0$

$0$ $1$ $2$ $3$ $4$ $5$

Signal (a.u.)

$S_{1D,C}/S_{1D,m}$

$0$ $200$ $400$ $600$ $800$

$x$ (µm)

Cells with Gd
Culture medium

(1) (2) (3)

$S_{1D,c}$ $S_{1D,m}$

down up

$0.4$ $0.3$ $0.2$ $0.1$ $0.0$

$0$ $1$ $2$ $3$ $4$ $5$

Signal (a.u.)

$S_{1D,C}/S_{1D,m}$

$0$ $200$ $400$ $600$ $800$

$x$ (µm)