Fluorescence eXclusion Measurement of volume in live cells

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Abstract

Volume is a basic physical property of cells; however, it has been poorly investigated in cell biology so far, mostly because it is difficult to measure it precisely. Recently, large efforts were made to experimentally measure mammalian cell size and used mass, density, or volume as proxies for cell size. Here, we describe a method enabling cell volume measurements for single living cells. The method is based on the principle of fluorescent dye exclusion and can be easily implemented in cell biology laboratories. As
**INTRODUCTION**

The role of cell volume regulation in various cellular processes has recently emerged as a topic of interest. In confined environments, it was shown that tumor cell migration depends on osmotic control and volume regulation through ion pumps’ activity and localization (Stroka et al., 2014). Recent findings also demonstrated that during cell division, mammalian cells tightly regulate their volume and exhibit a transient swelling, through osmotic processes controlled by ion pumps (Son et al., 2015; Zlotek-Zlotkiewicz, Monnier, Cappello, Le Berre, & Piel, 2015). Local or global cell volume changes could thus be a way of generating forces in cells. Therefore, osmotic regulation could be used by mammalian cells, just like in plants or yeasts, to expand or change shape (Cosgrove, 2005). The regulation of volume has been studied from a theoretical point of view (Jiang & Sun, 2013; Mitchison, Charras, & Mahadevan, 2008; Tao & Sun, 2015) but experimental test of these theoretical works is mostly lacking. At longer timescales, mammalian cell growth and cell-size homeostasis throughout the cell division cycle are currently hotly debated (Ginzberg, Kafri, & Kirschner, 2015). Part of the experimental studies suggested that cell volume is a biologically meaningful measure of cell size (Conlon & Raff, 2003; Godin et al., 2010; Son et al., 2012; Tzur, Kafri, LeBlue, & Lahav, 2009), while others favor mass (Mir et al., 2011; Park et al., 2010; Sung et al., 2013). The investigation of these questions is currently limited by the difficulty in measuring single-cell volume accurately over long periods of time.

Indeed, volume measurement on cells that constantly modify their shape is a very challenging task. For decades, the only commercially available technique was the Coulter counter. This technique yields high-throughput measurements based on impedance. It is however limited to cells in suspension and it is not possible to repeat the measurement on the same single cell (Bryan, Engler, Gulati, & Manalis, 2012; Gregg & Steidley, 1965). More recently, cell shape reconstruction from z-stacks of confocal images of labeled membrane was proposed (Boucrot & Kirchhausen, 2008). Yet, this technique requires repeated exposures to excitation light and is therefore not suitable for experiments over long time periods. Moreover, accuracy of volume measurements is limited by labeling artifacts and cell-shape reconstruction methods (Boucrot & Kirchhausen, 2008; Ernest, Habela, & Sontheimer, 2008; Habela & Sontheimer, 2007). Finally, the suspended microchannel resonator (SMR) allows very high precision measurement of cell mass, density, and volume but is limited to cells in suspension and requires very specific instrumentation (Bryan et al., 2013; Son et al., 2015).
Here, we describe a method to measure the volume of single cells using simple microfabricated cell culture chambers. This versatile technique is based on the principle of fluorescence exclusion (Bottier et al., 2011; Gray, Hoffman, & Hansen, 1983; Zlotek-Zlotkiewicz et al., 2015) and allows the measurement of volume of suspended or adherent cells with sizes ranging from few microns to several tens of microns. It allows studying phenomena ranging from seconds (drug treatments, osmotic shocks), minutes or hours (mitosis) up to several days (cell division cycles).

1. Method

1.1 Overview

Cells are cultured in a poly(dimethylsiloxan) (PDMS) chamber of controlled height with regular culture media supplemented with a fluorescent dye coupled to dextran as shown in Fig. 1A. Dextran is a polysaccharide preventing the fluorescent dye from passively entering the cells. Cells thus exclude fluorescence and appear dark on a classic fluorescence image (Fig. 1A and B). The fluorescence displaced by a cell is linearly proportional to the cell volume (Bottier et al., 2011; Zlotek-Zlotkiewicz et al., 2015). It is important to note that cells with high endocytic activity are not suitable for this method as dextran would be taken up inside the cell and the fluorescence would not be excluded. However, fluorescence exclusion measurement (FXm) can then be used to estimate the endocytosed volume, by sequentially changing the color of the excluded extracellular dye. It is also possible to flow the fluorescent dye only for short time using microfluidics, thus avoiding the accumulation of the dye in the cells, but this method is not presented here. Overall, for most cell types, minimizing the cell stress prevents fluorophore endocytosis. Careful handling of the cells is therefore important for reliable and reproducible results.

1.2 Protocol

1.2.1 General comments

This protocol describes the preparation steps for one chamber to be assembled on a glass bottom petri dish. The dish can be homemade or commercial, 6-well glass bottom plates can also be used for multiplexing.

1.2.2 Material

- Mold
- Hole puncher (diameter: 0.75 or 1 mm)
- Bottom glass petri dishes (35 mm, from WPI or Ibidi)
- PDMS and curing agent (Sylgard 184 or RTV-615)
- PBS (1×)
- Cell culture medium without phenol red
- Dextran conjugated with fluorescent dye in PBS (FITC, Alexa488, Alexa-647... from LifeTechnologies or Sigma Aldrich)
• Trypsin or EDTA
• Scalpel or razor blade
• Plasma Cleaner
• PDMS Oven between 65 and 80°C
• Cell culture hood

1.2.3 Fabrication of the PDMS chip
1.2.3.1 Rules for mold design
Molds can be fabricated by various microfabrication technics (photolithography, micromachining, …). Three major principles should be taken into account when

![FIGURE 1 Principle of volume measurement.](image)

(A) Cells are placed in poly(dimethylsiloxan) chambers of calibrated height set by pillars. Medium is supplemented with FITC-Dextran. Bottom picture: cells exclude fluorescence on epifluorescence images (Scale bar 20 µm). (B) The fluorescence profile corresponding to the dotted line in (A): maximum and minimum of fluorescence intensity correspond to chamber maximal height (background) and zero height (pillar), respectively. Right: these values are used to calibrate the signal and calculate the optical thickness of the cells. (C) Finally, cell volume is obtained by integrating the total fluorescence intensity over the cell area.
designing a chamber. First, the height of the chamber should be adapted according to the maximal size of the measured cells. Indeed to increase the contrast of the excluded fluorescence the ceiling should be as low as possible, but it should also be larger than the maximum cell height to prevent cells from being confined. Second, keeping a constant height throughout the chamber is of high importance as any error on the height will lead to the same error in volume calculation. Thus, the fabrication technique has to be chosen depending on the size of the cell/chamber and the precision required. The pillars keep the chamber height constant and their spacing should thus be adapted to the height of the chamber and the stiffness of the PDMS ceiling that depends on multiple parameters (i.e., thickness, brand, curing time). As a general rule, the distance between two pillars should not exceed 15 times the height of the chamber. Third, there should be at least one pillar in each field of view in order to calibrate the fluorescent signal, so the distance between the pillars should be adapted according to the field of view and the magnification that will be used.

1.2.3.2 Chamber fabrication

1. Mix PDMS with its crosslinker (1:10).
2. Pour onto the mold and degas to remove all air bubbles from the PDMS. The chip thickness should range between 2 and 4 mm.
3. Cure PDMS for 2 h in the oven between 65 and 80°C.
4. Punch 0.75 or 1-mm diameter inlets and outlets in the PDMS and cut the PDMS chip so that it fits in the glass part of the petri dishes. Narrow inlets (i.e., 0.75 mm) prevent fast diffusion of the fluorescent probe in the media outside the chip and are thus preferable for long imaging but the type of inlet can be adapted to the type of experiment.

1.2.3.3 PDMS chamber preparation: Day–1 (see Fig. 2)

1. Clean the PDMS chip with isopropanol and dry with an air gun (optional). Use a scotch tape to remove residual dusts.
2. Activate the structured side of the PDMS chip and the glass surface for 30 s in the plasma cleaner (Fig. 2A).
3. Put the PDMS chip on the glass surface immediately after plasma treatment and check carefully that the chamber and the pillars are properly bonded. A change in the reflection of light can be noticed by eye when PDMS and glass are bonded. If needed, use a soft tool (i.e., pipette tip or plastic tweezers) to gently press on the chamber to force the contact between the glass and the pillars. Additional 15 min incubation at 65–80°C reinforces the binding (optional).
4. Within less than 15 min after plasma treatment and under the hood, coat the chamber with fibronectin (50 μg/mL in PBS) by adding a drop of 10–20 μL over one of the inlets. Since the surfaces are still hydrophilic from the plasma activation, the drop will spread in the chamber easily (Fig. 2B). It is also possible to store the chips after plasma bonding, but then the chamber should be put under vacuum for 5 min and activated a second time with the plasma cleaner.
FIGURE 2 Preparation of volume chamber.
(A) Bind the poly(dimethylsiloxan) (PDMS) chamber to glass-bottom petri dish after plasma activation (30 s). (B) Apply surface treatment (fibronectin incubated for 30 min) directly after the bonding while the PDMS and glass are still hydrophilic. (C) Wash with PBS. (D) Inject medium and (E) cover the chip completely with medium before incubating overnight.
to make the surfaces hydrophilic and ensure a proper spreading of the fluid in the chamber. The plasma treatment step also sterilizes the dishes and the remaining steps can be performed under the hood in sterile conditions appropriate to cell culture.

5. Incubate for 30 min to 1 h in humid atmosphere to prevent fibronectin from drying out (i.e., add PBS or media around the PDMS chip or in the incubator).

6. Under the hood, wash with PBS and then with culture medium (Fig. 2C and D) and cover the chip with medium (Fig. 2E). Incubate overnight. Air bubbles can be generated along the preparation steps; if they are small enough (diameter < 1 mm), they should disappear during the incubation overnight. Using prewarmed media also reduces the appearance of bubbles when the chambers are put at 37°C.

Notes:
- This protocol can also be adapted to cells in suspension by coating the chamber with PLL-g-PEG (1 ×) rather than fibronectin to impede cell adhesion (Zlotek-Zlotkiewicz et al., 2015).
- In order to avoid mechanically stressing the cells, one should minimize the pressure applied in the chamber during the several steps of injection.
- If the experiment requires the use of hydrophobic drugs, an overnight incubation of the chip with medium already containing the drug should be performed to saturate the PDMS with the drug and limit drug depletion during the experiment. The concentration of hydrophobic drugs should be optimized within the chamber since an unknown amount of drugs could still be depleted from the chamber due to hydrophobicity and porosity of the PDMS material.

1.2.4 Cells’ injection: Day 2 (Fig. 3)

1. Prewarm the culture medium and the Trypsin or EDTA. In case of CO2-dependent medium, it is even better to preincubate the medium in the incubator to allow its equilibration.

2. Remove all medium around the chambers in the dish and dry the surface of the chamber with an aspirating pipette and inject medium to wash the chamber (Fig. 3A).

3. Detach cells in culture with TrypLE, Trypsin, or EDTA depending on the cell type and the time required for adhesion, using EDTA results in shorter readhesion times for most cell types.

4. Resuspend cells with warm media and centrifuge them to remove the trypsin and resuspend well to a final concentration ranging from approx. 0.5 to 2 × 10^6 cells/mL (Fig. 3B). Cell concentration has to be adapted to the final density required for the experiment. Obtaining single-cell volumes using this method can be done only on cells that are clearly separated. The cell density should thus be adapted to the requirement of the cell line/experiment (i.e., some cell lines require high density to properly proliferate while fast migrating cells...
FIGURE 3 Cells’ injection into the chamber.

(A) Remove medium on top and wash the chamber with fresh medium. (B) Harvest cells, spin and resuspend in fresh medium to the concentration needed. Collect a small volume of cells (typically 10 μL). (C) Inject cells, cover with medium, and incubate few hours for adhesion. (D) Change the medium to the medium supplemented with FITC-dextran. (E) Wash the medium on top of the chip and cover completely with the medium without FITC dextran.
at too high density will constantly bump into each other or migrate out of the observation field). Moreover, it is important to resuspend well the cells in order to avoid cellular aggregates that cannot be measured.

5. Inject the cells slowly to prevent mechanical stress. Check cell density under a bench microscope. The solution of cells can be concentrated or diluted according to the density observed in the chamber and reinjected.

6. Once the cell density is correct, cover completely the chamber with medium to stop the flow and incubate a few hours for the cells to adhere properly (Fig. 3C). The incubation time depends on the approach used to detach the cells and on the cell line itself but is typically ranging from 1 to 4 h.

7. For one chip: prepare 10 μL of medium supplemented with FITC-Dextran to a final concentration of 1 g/L. This solution can be prepared when cells are injected and stored in the incubator to equilibrate the medium.

8. Remove the medium above the chip and gently inject the prewarmed and equilibrated medium in the chip (Fig. 3D). One should see the dextran-colored medium getting out of the outlet.

9. Suck out all medium in the petri dish and replace by new medium completely covering the chamber (Fig. 3E). This step is very important: first, to wash dextran remaining outside of the chamber and avoid parasite fluorescence signals coming from outside the chamber; second, to completely cover the chip with the medium to prevent evaporation during the image acquisition under the microscope since PDMS is porous to gas.

1.3 IMAGING

The imaging should be performed with a simple epifluorescence microscope, adapted for live cell imaging, with a chamber with controlled temperature, humidity, and CO₂. For long experiments, keeping the atmosphere humid is particularly important in order to prevent evaporation that could lead to changes of osmolarity in the volume measurement chamber.

The acquisition can be performed with various objectives with different magnifications and numerical apertures (NA). However, it is important to keep in mind that the FXm method is based on the collection of the light over the entire height of the chamber (see data analysis), which depends on the depth of field provided by each objective. The depth of field of an objective, which defines the range of its acceptable focus, scales with the inverse square of its numerical aperture. Nevertheless, we could demonstrate theoretically and experimentally that the volume measurement does not depend much on the depth of field or on the axial position of focalization (as shown respectively in Figs. 4 and 5A). Indeed, with an epifluorescence microscope, all fluorescence light is integrated on the camera, so even the out-of-focus information can be retrieved in the signal. The out-of-focus fluorescence is simply diffracted on several pixels of the camera around the real position of the object. If the area over which fluorescence intensity is integrated is large enough (see Section 1.4), one can therefore collect all the information coming from this
FIGURE 4 Simulated thickness and volume measured with FXm.

Results of a home-made 2D simulation showing the effect of a change of the depth of field on the calculated thickness and volume of the cell. The curves show the simulated thickness of a round theoretical cell of 16 μm in diameter in a 22-μm high chamber at multiple foci. Simulations were performed for 10× 0.3NA objective (A) and 20× 0.75NA objective (B). Grey disk represent the simulated object. (C) Volume calculation for a sphere of radius Rc = 8 μm from 2D simulations. The surface of the 2D cell was calculated using an infinite integration distance and with a finite distance of 20 μm. The surface was multiplied by 4/3 × Rc to obtain the volume.
cell. However, having a depth of field too small with respect to the object or the chamber height will have two main effects. First, the accuracy on a single pixel height measurement drastically reduces, since the out-of-focus fluorescence has diffracted on the neighbor pixels (Figs. 4 and 5A). Second, it is then possible to integrate information from a neighboring out-of-focus cell, dust, or pillar, which will lead to an overestimation of the calculated volume.

Fig. 4 presents the results of a home-made simulation in two dimensions of a spherical cell (diameter: 16 \(\mu m\)) in a volume measurement chamber (height: 22 \(\mu m\)). The thickness of the cell obtained by FXm was simulated for multiple

![Graphical representation of the simulation results]

**FIGURE 5 Experimental validation of the setup.**

(A) Volume measured using FXm of the same cell obtained as a function of Z-position for objectives with different magnification and numerical aperture. Objectives with lowest numerical aperture yield robust volume measurement over a 20-\(\mu m\) range below or above the focal plan. (B) On round cells, comparison of volumes measured with FXm versus volumes calculated from the measurement of the cell diameter. The robust linear fit shows a good correlation between the two measurements. Measurements were made in a 25.2-\(\mu m\) high chamber with an objective LD 20\(\times\) NA0.4.
Z-positions. It shows that the calculated thickness at a given pixel can be largely mis-
estimated when part of the cell or the chamber is out of focus. In any case, the 2D
volume calculated over infinite integration distance is constant and equal to the theo-
retical value of 201 \( \mu m^2 \), which is the theoretical surface of the cross section of this
round cell (Fig. 4C). The simulations show that for a finite (20-\( \mu m \)) integration dis-
tance, the calculated volume drops for largely out of focus images (Fig. 4C), as
observed experimentally (Fig. 5A).

In conclusion, fluorescence exclusion yields accurate measurements of volumes
if calculated over large enough areas containing no other object than the cell
measured, while it can be wrong for single-pixel height measurements, depending
on the objective’s depth of field.

In order to experimentally validate a given setup (height of the chamber and
objective), control measurements comparing FXm with another volume measure-
ment method can be made. So far, four different objectives were validated using
giant unilamellar vesicles (GUVs) as references: 10\( \times \) NA0.3, 10\( \times \) NA0.5, 20\( \times \)
NA0.5, and 20\( \times \) NA0.75 (Zlotek-Zlotkiewicz et al., 2015) and three others have
been validated with AFM measurements: 40\( \times \) NA0.75 air, 40\( \times \) NA 1 Oil, and
100\( \times \) NA1.25 Oil (Bottier et al., 2011). Fig. 5 shows two examples of controls
that can be rapidly made to estimate the accuracy and the limitations of a given setup
(i.e., chamber height, objectives).

The homogeneity of the fluorescence illumination is also important for the preci-
sion of the measurement, as it will ease image segmentation and signal calibration.
The light path must be clean, without any dusts obstructing the fluorescence (see
Section 1.4).

1.4 DATA ANALYSIS (FIG. 6)

1.4.1 Principle of volume calculation

The FXm measurement is based on the inverse linear relationship between intensity
of fluorescence and the thickness or height of the sample over which fluorescence is
measured:

\[
I_{\text{max}} - I_{\text{min}} = \alpha \cdot h_{\text{chamber}}
\]  

\( I_{\text{max}} \) corresponds to the intensity measured where fluorescence is present over the
entire height of the chamber whereas \( I_{\text{min}} \) corresponds to the minimum intensity
at the center of the pillar of the chamber. The linearity of this relationship has
been verified in Zlotek-Zlotkiewicz et al.. The extraction of \( \alpha \) enables the calculation
of the optical thickness of an object excluding the fluorescence at each pixel. As
discussed in the Section 1.3, this optical thickness can misestimate the real local
thickness, but integrating this signal over the area \( S \) around the cell gives accurate
volumes:

\[
V_{\text{cell}} = \iint_S \frac{I_{\text{max}}(x,y) - I(x,y)}{\alpha}
\]
1.4.2 Description of the Matlab software from QuantaCell for image analysis

Image analysis is performed using a Matlab software first designed in the lab and optimized by QuantaCell (a company specialized in quantitative image analysis for life sciences). The software proceeds through two main steps. First, it processes raw images and normalizes them. Second, manual or automated cell tracking is performed through a user-friendly and versatile interface that is suitable to a wide range of measurements (i.e., short time on multiple cells or lineage tracking over long periods of time).

1.4.2.1 Background normalization

The accuracy of volume calculation highly depends on the accuracy of the estimation of $I_{\text{max}}$ and $I_{\text{min}}$ that yields $\alpha$ (Eq. 1). A good estimation of $I_{\text{max}}$ first depends on the acquisition of good images with a clean background (devoid of dusts), homogeneous fluorescent probe in the chamber and homogeneous fluorescence illumination.
A good estimation of $I_{\text{min}}$ depends on the correct and even binding of pillars to the glass bottom of the chamber. However, when all these precautions have been taken before image acquisition, one source of inhomogeneity will remain: the image of the lamp. For this reason, a good posttreatment protocol to normalize background intensity is crucial. Importantly, it seemed that in our setup, the image of the lamp can vary a bit through time or through positions in the chamber (possibly because of the PDMS structure). Thus, we chose to calculate the image of the lamp from our images for each frame rather than from a reference image made in an empty field. This alternative method might however give satisfying results in other experimental setups. Briefly, the different steps of the background normalization protocol in our Matlab software are, for each frame, $I_0$ as shown (Fig. 6A):

1. A first smoothing of $I_0$ is obtained using a morphological closing. From the resulting image $I_1$, a mask $M_1$ is calculated where dark pillars or dusts are excluded by filtering pixels below a threshold $T_{h1}$. The value of $T_{h1}$ is calculated as follows: $T_{h1} = \text{percentile}(I_1, 0.1) \times 0.8 + \text{percentile}(I_1, 50) \times 0.2$. The percentile ($I_0, 0.1$) and percentile ($I_0, 50$) corresponds to the 0.1 and 50 percentiles of the fluorescence intensity distribution, respectively.

2. From this mask $M_1$, the mean value $I_{\text{min}}$ of the pillar intensity is calculated over the center of all segmented pillars (to avoid border effects, an erosion step is applied and pillar intensity is estimated only at the center of the pillar on a minimum of 1000 pixels).

3. $B_1$ is obtained by applying $M_1$ to $I_1$ and filling in the holes through performing an iterative Gaussian blur of the background signal around each hole. $B_1$ thus corresponds to a first estimation of the background.

4. $I_2$ is then obtained as follows: $I_2 = (I_1 - I_{\text{min}})/B_1$. This image corresponds to a cleaned image of $I_0$ and is used to calculate a new mask.

5. From $I_2$, a second mask $M_2$ excluding the pillars and the cells is calculated, using the same filtering strategy as in Step 1 with the threshold $T_{h2}$. It is indeed only possible to reliably detect cells on a normalized $I_2$ image. The threshold $T_{h2}$ is calculated as follows: $T_{h2} = \text{percentile}(I_2, 50) \times 0.5 + \text{percentile}(I_2, 1) \times 0.5$. Then $M_2$ is a merge of $M_1$ and $M_2$: $M_2 = \max(M_1, M_2)$ (Fig. 6B).

6. $B_2$ is obtained by applying $M_1$ to $I_0$ with the same iterative Gaussian blur used as in Step 2 to fill in the holes.

7. The final renormalized image $I_3$ is then obtained: $I_3 = I_0/B_2$ (Fig. 6C).

1.4.2.2 Cell tracking and volume measurement

For each cell, the calibration of fluorescence intensity as a function of thickness is performed as in Eq. (1) with a value of $I_{\text{max}}$ estimated locally (Fig. 6D). It is however true that after the normalization procedure that produces a very flat background, this additional precaution might be considered optional.

To calculate cell volume, the tracking software performs the following steps:

1. The user or the automated tracking protocol defines a region of interest (ROI) around each cell to be analyzed.
2. $I_{\text{max}}$ is calculated locally around each cell: a fixed-sized window $S$ centered on the ROI is defined (see Fig. 6D). This area has been set to 200 × 200 pixels around the cell for a 10× binning one image. $M_2$, the mask excluding cells and pillars, is applied to $S$. The median value of the remaining background fluorescence intensity is then calculated. This step ensures that median is estimated on a large enough number of background pixels.

3. $I_{\text{min}}$ has been previously calculated and stored as explained in Step 2 of the background normalization procedure.

4. Using these calibration parameters, cell volume is then calculated by integrating fluorescence intensity on the ROI defined (see Eq. 2 and Fig. 6D).

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2. **FXm PITFALLS AND LIMITATIONS**

The FXm method is excellent for relative volume measurements such as those performed during mitosis (Zlotek-Zlotkiewicz et al., 2015). However, in order to ensure good absolute measurements, one must pay attention to five categories of parameters.

- The accuracy of the calibration of the optical thickness of a pixel will depend on both the flatness of the roof of the chamber and the even binding of the pillars to the bottom of the chamber. These two parameters must be even throughout the device and the time of the experiment.

- Absolute volume measurement requires that fluorescence intensity signal is homogeneous in the chamber, which can be ensured by a good diffusion of the fluorescent probe in the chamber and a homogeneous fluorescence illumination. To correct for the inevitable remaining inhomogeneity of illumination, a good image analysis protocol is important.

- The axial/height resolution will depend on the signal-to-noise ratio, which depends here on the maximal fluorescence intensity. The three adjustable parameters for this are: the illumination parameters, the concentration of the fluorescent probe that will yield a homogeneous background signal, and the difference between the height of the chamber and the height of the object measured.

- As explained in Section 1.3, the single-pixel thickness is often misestimated, and the volume can be as well when the depth of field is too small compared to the object’s size. The acceptable height over which fluorescence intensity extraction is reliable depends on the objective used.

- In addition to these parameters, a final limitation to the method is cell-type dependent: cell types that tend to uptake the fluorescent probe or attach to each other will be poorly adapted to the method.

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3. **DISCUSSION**

Fluorescence exclusion measurement (FXm) allows for measurement of volume of single cells over time periods from seconds up to several days. Calibrations of
volume measurement using FXm compared with geometrically obtained measurement of giant unilamellar vesicles or circular cells (Fig. 5B) yields an accuracy of approximately 10% (Zlotek-Zlotkiewicz et al., 2015). This estimation is however limited by the accuracy of the geometrical measurement itself. Among currently available single-cell volume measurement techniques, the SRM technique is probably the most accurate one with errors estimated to less than 1%, but is limited to suspended cell types (Son et al., 2015). For the 3D reconstruction-based method, accuracy of measurement is limited temporally by the time of acquisition of all the stacks and moreover the precision on volume calculation of such a method has not been demonstrated yet; furthermore, repeated illumination is probably the main limitation for measurement through time due to its toxicity. FXm is however a good compromise between accuracy of measurement and throughput of the experiment as it is the only method that allows parallel measurements on multiple single cells through time.

FXm shows several unique advantages: (1) it is easy to use and adapted to many different cell types, both adherent and suspended; (2) it is largely compatible with classical cell biology tools such as fluorescence imaging or transient drug treatment thus enabling direct investigation of cell volume regulation; (3) it is adapted to timescales ranging from seconds to several days and is therefore suitable for a wide range of biological questions such as short-time response to external perturbations (i.e., osmotic shocks), mitosis, or cell growth through the cell division cycle (see Fig. 7). All these make FXm the most versatile technique currently available to study cell volume regulation.

**FIGURE 7 Cell volume throughout a complete cell cycle.**

(A) Volume trajectory of a HeLa cell. The two volume overshoots at the beginning and the end correspond to transient volume increase in mitosis with the first one corresponding to the mother cell and the second one to the daughter cell. (B) Raw Fluorescence images of the cell in (A) with FXm. Scale bar 50 µm.
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