Fluorescence exclusion: a rapid, accurate and powerful method for measuring yeast cell volume

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SUMMARY STATEMENT

Fluorescence exclusion provides a unique method to accurately measure the volume of yeast cells at both the population and single-cell levels.

ABSTRACT

Cells exist in an astonishing range of volumes across and within species. However, our understanding of cell size control remains limited, due in large part to the challenges associated with accurate determination of cell volume. Much of our comprehension of size regulation derives from models such as budding and fission yeast, but even for these morphologically stereotypical cells, assessment of cell volume has relied on proxies and extrapolations from two-dimensional measurements. Recently, the fluorescence exclusion method (FXm) was developed to evaluate the size of mammalian cells, but whether it could be applied to smaller cells remained unknown. Using specifically designed microfluidic chips and an improved data analysis pipeline, we show here that FXm reliably detects subtle differences in the volume of fission yeast cells, even for those with altered shapes. Moreover, it allows for the monitoring of dynamic volume changes at the single-cell level with high time resolution. Collectively, our work reveals how coupling FXm with yeast genetics will bring new insights into the complex biology of cell growth.

KEYWORDS

Cell volume, yeast, fluorescence exclusion

RUNNING TITLE

Measuring yeast cell volume
INTRODUCTION

Regulation of cell volume is central to the biology of living organisms. It is a dynamic and complex trait that is modulated by both genetic and environmental components (Amodeo and Skotheim, 2016; Cook and Tyers, 2007; Lloyd, 2013; Marshall et al., 2012; Mueller, 2015). It has an impact on a host of processes, ranging from cytoskeletal dynamics (Pedersen et al., 2001) to the scaling of global transcription rates (Zhurinsky et al., 2010). In metazoa, cell volume contributes to the overall size and shape of an organism and plays important roles in the development and architecture of tissues (Amodeo and Skotheim, 2016; Cook and Tyers, 2007; Lloyd, 2013; Marshall et al., 2012; Mueller, 2015). At the single-cell level, volume and surface area not only define how cells sense external stimuli and interact with their environment but also determine their intracellular chemistry and organization. Moreover, cell size has a profound impact on basic nuclear functions. Indeed, in a number of organisms, nuclear size scales with cell size, and the nucleo-cytoplasmic ratio was shown to be constant in a range of conditions (Jorgensen et al., 2007; Neumann and Nurse, 2007). This is poised to affect chromatin-related processes as well as events that require highly regulated nuclear mechanics such as chromosome segregation. Strikingly, deregulation of cell size has dramatic consequences and is a hallmark of aging and cancer development (Li et al., 2015; Pietras, 2011; Yang et al., 2011).

In proliferating cells, volume is intimately linked to the processes that control cell cycle progression. The dynamics of cell growth are complex across species, and specific stages of the cell cycle are associated with differential changes in cell size and morphology. For instance, while cells of the fission yeast Schizosaccharomyces pombe stop growing and maintain their overall shape during mitosis, mammalian cells undergoing division show a transient phase of rounding and volume alteration (Lancaster et al., 2013; Zlotek-Zlotkiewicz et al., 2015). In addition, size thresholds have been proposed to act as key checkpoints during the fission yeast cell cycle, allowing for the coordination of growth and division (Fantes, 1977; Fantes and Nurse, 1978; Fantes and Nurse, 1977). These corrective mechanisms reduce the cell-to-cell heterogeneity that can result from variability in cell size at birth or asymmetric cell division, thereby maintaining size homeostasis in a growing population. Interestingly, increases in cell volume were frequently observed during long-term experimental evolution in bacteria and were proposed to contribute to improved proliferation (Grant et al., 2021; Lenski and Travisano, 1994; Mongold and Lenski, 1996). This highlights how the volume of dividing cells may result from the necessary balance between growth advantages and evolutionary trade-offs.

Alterations in cell size are triggered by changes in the environment, such as upon modulation of nutritional conditions, and cell cycle exit is often associated with a decrease in cell volume. For example, yeast cells exposed to nitrogen or glucose starvation enter quiescence with a significantly reduced size (Sun and Gresham, 2021). However, the coupling of growth and proliferation is more complex, playing a key role in organismal structure. In adult C. elegans where total cell number is
constant, changes in cell volume directly impact the size of the animal (Cook and Tyers, 2007; Watanabe et al., 2007). In contrast, alteration of proliferation in *Drosophila* can be compensated by modulation of cell size (Neufeld et al., 1998; Su and O'Farrell, 1998; Weigmann et al., 1997).

The principles and mechanisms underlying the control of cell size have been the focus of in-depth investigation in a broad range of organisms. While several pathways have been described to play a role in regulating this critical parameter (Amodeo and Skotheim, 2016; Cook and Tyers, 2007; Lloyd, 2013; Marshall et al., 2012; Mueller, 2015), whether they represent the key mechanisms that ensure size homeostasis remains unclear. Furthermore, how cells measure and control their geometric characteristics is surprisingly poorly understood. As a result, multiple models that are not mutually exclusive have been proposed. According to these models, cells may divide 1) when they have reached a specific size (sizer), 2) after a specific time following a landmark cell cycle event (timer) and 3) after having produced a fixed amount of mass, at a rate that relies on birth size (adder). While all three possibilities are supported by experimental and theoretical studies (Fantes and Nurse, 1977; Mueller, 2015; Soifer et al., 2016; Sveiczer et al., 1996; Taheri-Araghi et al., 2015), general conclusions are difficult to draw. This may be due in part to the diversity of measurements used to describe cell size, including volume, surface area, cell length or dry mass. Indeed, the question remains whether one or a combination of these characteristics is more relevant for understanding size regulation and whether different cell types rely on the same or on distinct parameters. Thus, while the sizes and shapes of individual cell types are commonly accepted as fundamental hallmarks of cell identity, no unifying principles for size control have emerged.

Experimentally, the regulation of cell size has been difficult to study in multicellular eukaryotes, due to the complexity of modulating the characteristics of cells that are integrated in tissues with relatively stable local chemical and physical properties. Even in mammalian cell lines, the challenges of advanced genetic manipulation and the lack of simple methods for accurate measurement of parameters such as volume and surface area have been an obstacle to investigating the molecular mechanisms of cell size control. Therefore, unicellular eukaryotes such as the budding and fission yeasts remain the models of choice for deciphering the bases of this critical cellular feature. In these organisms, regulation of cell size is intimately linked to the operation of the cell cycle machinery. However, how this coupling operates, how cells monitor their dimensions and how variation from the target size triggers a corrective response are not fully understood. Several concepts have emerged that may provide robust ways of regulating size (Amodeo and Skotheim, 2016). For example, titration mechanisms involving a factor that scales with cell size and a constant reference may suffice for size control. Geometric parameters including cell length or surface area may also be involved. This may be particularly appropriate for cells with a stereotyped shape such as the fission yeast (Facchetti et al., 2019), but it is more complex to envisage in other situations.
In the fission yeast *S. pombe*, the existence of cell size checkpoints was initially suggested at both S phase and, more importantly, mitosis (Fantes, 1977; Fantes and Nurse, 1978; Fantes and Nurse, 1977). While an elegant geometric mechanism based on an intracellular gradient of a cell cycle regulator was subsequently proposed (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009), recent evidence challenged this model (Amodeo and Skotheim, 2016; Pan et al., 2014a; Saunders et al., 2012), and the pathways controlling fission yeast cell size remain unclear. Moreover, the commonly-employed approach of using cell length at division as a proxy for size may not be sufficient for investigating size regulation. Indeed, slight changes in the diameter of these rod-shaped cells are usually ignored, although they have a stronger impact on cell volume than similar alterations of cell length: considering a fission yeast cell as a cylinder with half spheres at both ends, the volume of the cell 1) is linearly dependent on the length of the cylinder and dependent on the square of its radius and 2) involves the cube of the radius for each end. This is particularly relevant, as cell volume is a parameter that is more critical to cell biology and biochemistry than 2-dimensional cell length. In addition, morphology mutants or cells grown in conditions that induce alteration of cellular dimensions and scaling become complex to study and to compare with reference strains. Finally, size at division restricts the analysis to only a small fraction of a proliferating population.

To investigate the control of yeast cell volume in a more unbiased manner, different techniques have been applied. These include volume calculation from geometrical assumptions, 2D imaging data that allow 3D reconstruction or the integration of cellular sections extrapolated from the cell outlines, and complex approaches using advanced micromechanical devices (Baybay et al., 2020; Bryan et al., 2010; Bryan et al., 2014; Facchetti et al., 2019; Model, 2018; Pan et al., 2014b; Zegman et al., 2015). However, these strategies are either low-throughput, difficult to establish or rely on various assumptions and complex processing of 2D measurements. For instance, accurate evaluation of cell volume based on automated recognition of cell outlines requires this detection to be highly precise in both wild type and mutant strains (Baybay et al., 2020; Zegman et al., 2015), which is crucial given the impact of cell diameter on cell volume. Therefore, while these methods represent important steps in our capacity to address the regulation of cell volume, a more direct and simple methodology that does not introduce the biases of 2D data acquisition and multiple steps of image segmentation and reconstruction is necessary. This will allow us to precisely determine single cell volume in a large number of yeast cells irrespective of cell morphology, throughout the cell cycle and in successive rounds of division.

The impact of cell morphology on volume measurement was recently addressed in mammalian cells through the development of a fluorescence exclusion method (FXm) for evaluating cell volume (Cadart et al., 2017). FXm relies on microfluidic devices in which cells are imaged in the presence of a fluorescently-labelled dextran that does not cross the plasma membrane. Thus, using chambers of known height, the local reduction in fluorescence due to the presence of a cell allows for the determination of the volume of this cell with high accuracy (Fig. 1A). This technique is simple and only
requires single-plane imaging with low magnification objectives. It is entirely uncoupled from any
global or local morphological alterations and is compatible with the monitoring of high numbers of
cells. Although perfectly adapted to large mammalian cells, whether this new approach could be used
for smaller cells such as yeast remained unclear, as this would require microdevices with significantly
lower internal chambers. For example, while HeLa cells are around 20-40 µm in diameter with a volume
ranging from ~1500 µm$^3$ at birth to ~3000 µm$^3$ at division (Cadart et al., 2018; Cadart et al., 2019), wild
type fission yeast cells divide at a length of ~14 µm, with a diameter of ~4-4.5 µm. This gives an
approximate calculated volume at division, depending on the conditions and exact diameter, between
~120 and 200 µm$^3$ (Navarro and Nurse, 2012; Nurse, 1975).

Here we use the fission yeast *Schizosaccharomyces pombe* as a model system and demonstrate that
the fluorescence exclusion method is a unique tool for measuring the volume of such small cells. We
fabricate specific microdevices for these cells, assess the reproducibility and sensitivity of the system,
and provide an improved way to perform large-scale analyses. Furthermore, we demonstrate the
compatibility of the approach with multi-color imaging for evaluating single-cell volume in specific
subpopulations. We then show how time-lapse experiments using these devices are a powerful strategy
to couple advanced yeast genetics with volume measurements for deciphering growth dynamics and
size modulation. Interestingly, comparing results from length vs. volume measurements suggests that
re-evaluating previous models and conclusions based on the former will be essential for understanding
how cell volume is regulated in this unicellular organism, with implications for the general principles
that govern cell size control in more complex eukaryotes.

RESULTS

Microfluidic devices for determining the volume of yeast cells by fluorescence exclusion

FXm was developed to accurately measure the volume of mammalian cells in an unbiased manner,
irrespective of the cell type and despite complex cell morphologies (Cadart et al., 2017; Cadart et al.,
2018). This method has the advantage of being simple and potentially compatible with any type of
microscopic object, making it an ideal approach for investigating the regulation of cell volume in
genetically amenable models such as yeast. However, to ensure an optimal and broad dynamic range
for accurate measurements, the internal height of the microfluidic chamber must be of the same order
of magnitude as that of the cells. Indeed, in the presence of a cell, the exclusion of fluorescent molecules
in a microsystem several times higher than the cell would only marginally affect the total fluorescence
intensity in the region of interest, impacting the reliability of the results. Microfluidic devices employed
to determine mammalian cell volume are therefore not adapted to small fission yeast cells, which require
chambers of ~ 4-7 µm in height, depending on the strain studied. Interestingly, evaluation of FXm
suggests that such reduced heights represent an advantage for the accuracy of these types of measurements (Model, 2020).

This design constraint led us to build specific and optimized devices for our yeast model. When made using the most common elastomer for microfabrication, PDMS (see Materials and Methods), microfluidic chips of such reduced height require the integration of vertical structures to support the chamber ceiling, preventing its collapse during the bonding of the chip to a glass coverslip. Cells in close vicinity of these pillars must be excluded from the FXm measurements, due to the bias introduced in their volume determination by the presence of a neighboring PDMS structure (Cadart et al., 2017). The density of pillars, which also serve as normalization references during the image analysis (Cadart et al., 2017), must therefore be sufficient to ensure chamber integrity while maintained as low as possible to increase the throughput of the technique. To establish the most appropriate design for our experiments, we tested chambers with inter-pillar distances of 120, 180 and 270 µm (Fig. 1B). We found a distance of 180 µm between pillars coupled with an optimized cell concentration and preparation procedure to be the most reliable combination for the measurement of high numbers of individual cells in a single frame while preventing chamber collapse and limiting cell aggregation (Fig. 1C, see Materials and Methods).

**Measuring the volume of individual fission yeast cells using FXm**

The necessity to use microfluidic chambers of reduced height may represent a challenge for the precise measurement of the volume of individual yeast cells. Indeed, in these conditions, the difference between the minimum (pillar) and maximum (no cell) fluorescence intensities (Fig. 1A), as well as the extent of fluorescent molecule displacement in the presence of a cell, are inherently low. This may render the technique less sensitive and more susceptible to noise in fluorescence measurements, with consequences for its accuracy and reproducibility.

In order to evaluate whether FXm provides high quality results for yeast, we measured the size of wild type fission yeast cells, comparing both the median volumes and volume distributions from independent experiments (see Materials and Methods). To this end, we used a 5.53 µm high chamber design and analyzed five replicates performed in separate devices built from the same master mold. Remarkably, we obtained highly reproducible data, with a median volume for the pooled datasets of 104.8 ± 24.5 µm³ (standard error for the 5 replicates, s.e.m.: 1.7 µm³), and comparable population profiles (Fig. 2A). This value for wild type cells is consistent with previous results established by complex image processing (Baybay et al., 2020), validating FXm for cell size monitoring in yeast. In contrast to cell size at division, which only focuses on a small subpopulation, our method describes the sizes of the cells over the entire population, from birth to division. It therefore integrates the normal changes in volume that occur during the division cycle. In addition, our experiments suggest that several
repeats may be needed when evaluating median volume differences of less than 10 %, as such alteration
is in the range of the observed experimental variability (Fig. 2A). However, when considering a
geometric cell model consisting of a cylinder of with two half spheres at both ends and a constant cell
diameter of 4 μm, an increase of 10 % in cell volume at division is the result of an increase in cell length
at division from 14 μm to ~ 15 μm. Conversely, a similar change in cell volume at constant length is
brought about by an increase in cell diameter of ~ 0.2 μm. In both cases, these differences in 2D
parameters are at the limit or below the resolution of the strategies routinely used for measuring cell
length or width in S. pombe, respectively. This demonstrates the high sensitivity of FXm for yeast cell
volume measurement, even when applying a conservative threshold of 10 % volume change.

Next, as master molds deteriorate over time when heavily used and given that the standard
microfabrication procedures commonly found in cell biological laboratories make it difficult to generate
highly reproducible chamber heights, we set out to evaluate how changes in the dimensions of the
devices may impact volume determination. We therefore produced microfluidic chips of various
heights, ranging from 4.85 to 6.95 μm, and measured the size of wild type fission yeast cells by FXm.
Again, we observed remarkably similar median volumes and volume distributions between
measurements (Fig. 2B – median volume of the pooled datasets: 106.8 ± 24.4 μm³; s.e.m. for the 5
replicates: 1.9 μm³). This shows that data obtained from assays performed with different devices can
be reliably compared.

Finally, we further validated the approach by assessing the volumes of known fission yeast size
mutants as well as wild type diploid cells. In particular, we measured the size of well-described
temperature-sensitive cell cycle mutants: wee1-50° (small cells) and cdc25-22° (large cells) (Fantes,
1979; Fantes and Nurse, 1978; Nurse, 1975). As anticipated, wee1-50° and cdc25-22° cells grown at
the permissive temperature of 25 °C showed significantly smaller and larger volumes than wild type,
respectively (Fig. 2C, Fig. S1A). Note that the apparent broader distribution observed in cdc25-22° is
the result of their increased overall size, as the volume profiles of these different strains are similar
when the data are normalized to their respective median sizes (Fig. S1C). In the case of wild type diploid
cells, we found that the median population volume is 1.84-fold higher than of haploid cells (Fig. 2D,
Fig. S1B).

Altogether, these experiments demonstrate that FXm with specifically adapted microfluidic chips
is compatible with the analysis of yeast cell volume and produces highly reproducible results.
Furthermore, in contrast to measurements of cell length and width, this method does not rely on 2D
parameters that can be difficult to accurately determine. It will therefore allow us to identify changes in
cell volume that result from limited global or local alterations in cell diameter. Finally, our data also set
a first conservative threshold of 10 % for the estimation of differences in cell volume between cell
populations.
Improving FXm image analysis

FXm image analysis consists of three main steps (Cadart et al., 2017): normalization, cell selection and volume calculation. However, as this technique requires the use of a low magnification objective, the size of yeast cells and the high number of cells in a frame can make it time consuming and tedious to manually select individual cells using the code established for larger mammalian cells. Interestingly, during the normalization procedure, a mask is generated that separates pillars and cells from the background (Cadart et al., 2017). Since the majority of objects extracted at this step corresponds to valid individual cells, we took advantage of the normalization mask and developed a semi-automated Python script to assist the user in cell selection (see Supplementary information). Thus, instead of manually delineating the multiple regions of interest (Fig. 3A), this tool offers two distinct and intuitive strategies to the user:

First, a manual cell selection mode can be chosen. In the context of this new code, the cells pre-selected by the normalization mask are magnified and displayed (Fig. 3A), allowing the user to rapidly exclude incorrect objects such as dust particles or cell aggregates. This reliable approach makes the analysis of a high number of cells easier and faster.

Second, an automated and non-discriminatory selection can be applied. In order to exclude aberrant measurements (e.g. large cell aggregates, abnormally small dead cells), thresholds are then automatically set to remove outliers based on the standard deviation (s.d.) of the measurements in the population. This is more appropriate than the use of absolute cut-offs, which may need to be adjusted from strain to strain. After testing different s.d. thresholds (data not shown), we found that excluding values outside of 2 s.d. (1 IQR for a normal distribution) for the first and third quartiles was optimal for obtaining results similar to those established with the manual method, correctly selecting between 87 and 92% of the objects in our experiments (Fig. 3B).

Finally, our script provides the option of separating the selected cells in different groups. This is particularly useful when determining the volumes of distinct subpopulations, for instance based on additional fluorescence markers (see below).

Collectively, the improvements brought by our analysis code facilitate FXm analysis and make it more powerful, providing more options to the user for in-depth and complex studies of cell populations.

Interestingly, while manual selection is likely to be the most accurate approach, we show that automated analysis with outlier exclusion allows a rapid assessment of high numbers of cells while remaining sufficiently accurate to be used when screening the volume of several different strains.
Small variations in cell dimensions and population heterogeneity

Our initial volume measurements in wild type cells and the evaluation of the experimental variability associated with the use of FXm with yeast cells suggest that 10% changes in volume and above can be reliably detected by this technique (Fig. 2).

To further assess the sensitivity of FXm, we first determined the volume of cells showing a marginal difference in cell length at division compared to wild type. To this end, we took advantage of a fission yeast strain whose proliferation relies on a minimal cell cycle control network (MCN). In this background, cell proliferation is solely dependent on the oscillation of a single qualitative cyclin-dependent kinase (CDK) activity between two thresholds (high for mitosis and low for S phase onset) (Coudreuse and Nurse, 2010). This is achieved through the expression of a fusion protein between cyclin B/Cdc13 and Cdk1/Cdc2, in the absence of all other cell cycle cyclins (Coudreuse and Nurse, 2010). Interestingly, while behaving similarly to wild type (Coudreuse and Nurse, 2010), MCN cells show a minor increase of ~7% in their length at division in our experimental conditions (15 µm for wild type and 16.1 µm for MCN cells, Fig. 4A). This alteration is predicted to contribute to a similar increase in volume according to the geometric model of S. pombe cells (see above). Unexpectedly, our FXm measurements showed a median volume difference of 17% between these strains (Fig. 4A, Fig. S2; compare WT and MCN). This suggests that the change in size between wild type and MCN cells is not solely the result of an increase in cell length. Importantly, it demonstrates that FXm is a promising approach for investigating the regulation of yeast cell volume, providing novel and more in-depth information on cell size compared to the standard measure of cell length at division.

Next, we focused on establishing whether FXm is able to reliably detect volume changes below our initial and conservative threshold of 10%. We started by evaluating whether increasing the number of independent replicates is sufficient to render FXm compatible with the monitoring of small alterations in cell size. For this, we built on the results in Fig. 2A and Fig. 2C, which indicate a difference in cell volume of less than 10% between wild type cells grown at 32 vs. 25°C. Comparison of the volume profiles and median values in these conditions for 5 independent experiments showed that such small differences can be robustly established (Fig. 4B and Fig. S3A). Next, we investigated whether even smaller alterations in cell volume at the population level could be detected when monitoring progressive changes in cell size over time. As a proof-of-concept, we used the MCN cells described above, as they harbor an additional mutation in the ATP-binding pocket of the Cdc2 moiety of the fusion module. This makes these cells sensitive to dose-dependent and reversible inhibition of CDK activity by small non-hydrolysable ATP analogues such as 3-MBPP1 (Bishop et al., 2000; Coudreuse and Nurse, 2010). This allowed us to block cells in G2 using the inhibitor, let them synchronously re-enter the cell cycle upon inhibitor wash off (Fig. S3B, C) and monitor volume changes in the population throughout the following G2 phase with high time resolution (Fig. 4C). Remarkably, even considering
the experimental variability observed at certain time points, very small increases in volume (<5%) and
changes in growth rates could be measured, demonstrating the high sensitivity of FXm in this context.

In addition to the median cell volume, another critical parameter in our understanding of cell size
homeostasis is the size distribution in the population. Indeed, this reflects the cell-to-cell heterogeneity
in cell cycle progression and the strength of the size control system operating in these cells. To date,
most studies evaluating this feature in fission yeast have focused on variability in cell length at division.
However, not only does this approach exclude the majority of the population, but our results above
suggest that the evaluation of cell volume distribution in the entire population may lead to different
conclusions. To establish whether FXm measurements are sufficiently sensitive for monitoring
alteration of cell size distribution, we took advantage of MCN cells in which the target residues of the
conserved Wee1/Cdc25 feedback loop on Cdc2 are mutated (T14A Y15F, MCN-AF) (Coudreuse and
Nurse, 2010). Loss of this mitotic switch was previously shown to result in an increase in cell-to-cell
variability in length at division (Coudreuse and Nurse, 2010), and in the non-supplemented minimal
medium used in our experiments, we also found that these dividing cells are slightly smaller than wild
type (Fig. 4A). As anticipated, we observed a broader distribution of cell volume in MCN-AF compared
to MCN and wild type cells (Fig. 4A, D). Interestingly, the relative changes in median volume vs.
average length at division between MCN-AF, MCN and wild type provide insights into the dimensions
that are altered in these strains (Fig. 4A, Table S1): while MCN-AF and MCN appear to differ only in
length (same ratios for volume vs. size at division), both strains are likely to show an increase in cell
diameter compared to wild type (differing ratios).

Altogether, these results demonstrate that FXm is easier and more sensitive than previously used
methods, allowing for the monitoring of small alterations in cell volume and changes in size
homeostasis populations of yeast cells. Coupling these data with more traditional cell length
measurements will shed light on how changes in cell volume are mediated and whether modulations of
cellular geometry occur.

Measuring the size of cells with altered geometries and morphologies

As discussed above, FXm allows for the identification of small changes in size that are either
difficult to accurately evaluate from cell length measurements or result from below-detection alterations
in cell diameter. The use of cell length as a proxy is even more problematic when studying mutants or
conditions where dimension scaling or cell shape is altered. In the latter, cell length at division becomes
generally irrelevant. Furthermore, strategies using automated cell outline detection to extrapolate cell
volume may not be sufficiently reliable, given the impact of minor changes in diameter on cell volume.

As a proof-of-concept, we determined the volumes of various strains known to show defects in
their morphology and shape. First, we assessed the size of strains lacking Rga2, Rga4 or Rga6, which
are members of the RhoGAP family. These factors are involved in the regulation of polarized growth, and their loss results in changes in cell geometry (Das et al., 2007; Revilla-Guarinos et al., 2016; Soto et al., 2010; Villar-Tajadura et al., 2008). When ranking these strains according to their length at division, we found that Δrga2 cells are the longest, followed by Δrga6 and Δrga4 (Fig. 5A). Strikingly, FXm measurements, which integrate changes in both morphology and geometry, led to the opposite conclusion, with Δrga4 and Δrga2 cells having the largest and smallest median volumes, respectively (Fig. 5B, Fig. S4). These data show that cell length measurement is not reliable as a proxy for cell size when studying such mutants. They also demonstrate the advantages of FXm for establishing size differences between strains and determining the influence of diverse pathways on cell volume regulation.

Next, we used FXm to evaluate the size of cells for which length can be difficult to measure or inherently inaccurate due to strong alterations in their morphology. To this end, we used bent cells (Δknk1, Δtea1) as well as cells with significant shape defects and irregular diameters (Δmal3) (Beinhauer et al., 1997; Mata and Nurse, 1997; Scheffler et al., 2014) (Fig. 5C). As anticipated, while cell length at division could not be determined without excluding a significant fraction of the cells, volume measurement by FXm allowed us to establish the profile of cell volume in the population and compare it with that of wild type rod-shaped cells. This again highlights the power of this method for studying size control mechanisms, independently of cell morphology (Fig. 5D, Fig. S5). Interestingly, in cells with morphological defects, both the median volume and size distribution may change (Figs. S4, S5), potentially reflecting alterations of their cell cycle organization and associated growth patterns. This would open the door to investigating how cell shape may delineate the complex interplay between cell growth and division cycle at the single-cell level.

Finally, we evaluated data obtained by FXm for significantly smaller cells that have stopped dividing. Upon glucose exhaustion, fission yeast cells exit the cell cycle and enter a non-proliferative quiescent state, which is associated with a dramatic reduction in cell size. Given the shape of the cells in these conditions (Fig. 5E), determining their length and width to extrapolate cell volume is difficult and unreliable. In contrast, FXm measurements established high-quality profiles of cell volume in these populations (Fig. 5F, Fig. S6). This will allow for investigating the impact of cell size on cell physiology and aging in this critical cellular state.

Collectively, this set of experiments demonstrates the versatility of FXm, which provides an unprecedented ability to measure the size of any strain, irrespective of cell morphology and physiological status. Our results also suggest that previous conclusions on size control in fission yeast may need to be re-evaluated using FXm.
Measuring the volume of specific subpopulations

All the experiments presented so far consider two aspects of cell volume: its median value and its distribution in the entire population. The former provides a way of rapidly comparing different strains while the latter brings insights into cell size homeostasis. However, extracting the volume of specific subpopulations of interest is critical for understanding how different biological processes may interact with the mechanisms underlying the regulation of cell size and the coupling of cell growth and proliferation. Thus, associating FXm with the use of other intracellular fluorescent markers in yeast cells would represent a particularly powerful method to take advantage of advanced yeast genetics and explore the complexity of size regulation in different conditions and throughout the cell cycle. Similarly, this could allow for discriminating between genetically distinct strains expressing specific markers in a complex population, opening the door to exploring non-cell autonomous processes that may feed into volume regulation. The only critical consideration for such experiments resides in the choice of the secondary markers, whose emission spectra should not bleed into the FXm channel (FITC in our experiments), as this would alter the accuracy of the volume measurements.

To evaluate these approaches, we set out to determine cell volume at division, using the non-histone chromatin-associated protein Nhp6 coupled to the red fluorescent marker mCherry. First, we validated that the presence of Nhp6::mCherry in the nucleus did not induce any significant signal in the FITC channel under FXm conditions (Fig. S7A). This also showed that the slightly reduced volume of these cells compared to wild type (Fig. 6A, Fig. S7B) is the result of an alteration of Nhp6 function due to the mCherry tag rather than an experimental artifact from mCherry fluorescence. Using our improved FXm analysis code, we segmented the population of binucleated cells (~11% of the total number of cells) and determined their median volume (126.3 ± 18.2 µm³ vs. 93.5 ± 21.3 µm³ for the mononucleated cells, Fig. 6B). The partial overlap between mono- and binucleated cells is consistent with the interruption of growth during mitosis: cell size does not change throughout nuclear division, and growth only resumes in G1. Note that the volume of binucleated cells measured in our assay is consistent with previous calculations of cell volume at division (Nurse, 1975) but significantly differs from calculations presented in a more recent study (Navarro and Nurse, 2012). This further demonstrates that fission yeast cell size cannot be reliably and reproducibly determined using single measurements of cell length and diameter. Collectively, our experiments validate the possibility of combining FXm with additional markers in yeast for more complex investigation of cell size control and dynamics.

Dynamic changes in fission yeast cell volume at the population and single-cell levels

Next, we set out to assess whether FXm can be reliably used to assess dynamic changes in yeast cell size. First, we tested how time course experiments coupled with FXm may allow for following alterations in cell volume over time in asynchronous populations. Indeed, a wide range of growth
conditions have been shown to strongly influence size \( (e.g. \) heat stress \( \text{Vjestica et al., 2013\), osmotic stress \( \text{Millar et al., 1995\), change in nutrient availability \( \text{Fantes and Nurse, 1977; Petersen, 2009\). In addition, experimentally induced modulation of cellular pathways using conditional mutants, chemical genetics, or inducible/repressible promoters may result in cell size changes, and understanding these dynamics may be key to determining the implication of these processes in cell size homeostasis. To evaluate the use of FXm for these approaches, we took advantage of the sensitivity of MCN cells to the 3-MBPP1 ATP analogue. Indeed, reducing Cdc2/CDK activity in fission yeast using this method leads to an increase in cell size \( \text{Chen et al., 2016; Coudreus and Nurse, 2010\). We therefore measured cell volume in asynchronous MCN cells at 40-minute intervals \( \sim1/4\) of the cell cycle) after treatment with 0.05 µM of the 3-MBPP1 analog (Fig. 7). Our data show that MCN cells progressively increase their volume, up to 1.7-fold after one doubling time. Strikingly, analysis of the changes in cell length at division and volume throughout the experiment \( \text{comparison of the volume vs. size at division ratios, Table S1\) suggests complex dynamics in the respective modulations of length and diameter upon inhibition of CDK activity. This demonstrates that FXm 1) can be used to easily follow short-term alterations in median yeast cell size and size distribution in a population of asynchronous cells and 2) provides new perspectives in our understanding of how the geometrical dimensions of these cells contribute to overall cell volume.

The experiments above only analyze population snapshots at different time points and do not monitor changes in cell size over time at the single-cell level. This type of strategy is therefore incompatible with evaluating immediate responses to environmental perturbations and determining the behaviors of separate cell lineages. In addition, it does not allow for relating volume at birth, growth rate and volume at division within single cells. Following cell length in an entire population during time-lapse experiments is tedious and introduces the same bias as discussed earlier. We therefore tested whether FXm provides high quality data when performing time-lapse experiments with yeast cells. First, we evaluated the experimental noise for single cells in our chips by comparing 20 successive measurements of 10 individual cells over a period of \( \sim6\) seconds \( (\text{Fig. 8A}\)\). Making the assumption that no volume changes occur in such a short time, this showed that the experimental noise inherent to the method when using chips of reduced height remains very limited. Interestingly, it also suggests that when monitoring single cells, volume alterations as low as \( \sim3\%\) can be detected. Next, to perform time-lapse experiments of several hours, we began by using modified chips in which the cell injection inlets were made bigger \( \text{from 2.5 to 4 mm\), acting as nutritional reservoirs to maintain growth conditions as stable as possible. As fission yeast cells are non-adherent, this also required pre-coating of the chip coverslip with lectin. In this context, we observed a linear increase in cell volume during each cell cycle and a plateau in size around the time of cell division, corresponding to the well-described growth arrest at mitosis \( \text{Mitchison, 1957; Mitchison and Nurse, 1985\) (Fig. 8B). We also found that in these experiments, cells divide at smaller sizes in succeeding cell cycles, suggesting that the built-in reservoirs...
are insufficient to ensure a favorable growth environment throughout our assay. We therefore applied a constant flow of fresh medium containing FITC-Dextran (~3-5 µL.min⁻¹) for the entire duration of the experiments. Remarkably, in this setup, we did not detect any consistent reduction in cell volume at division after each cycle (Fig. 8C). Furthermore, in the presence of flow, the growth rate (see Materials and Methods) was significantly higher than without medium renewal (Fig. 8B, C). This demonstrates that FXm is a unique tool for monitoring volume dynamics of small cells with a high time resolution and for detecting changes in growth rate at the single-cell level. Coupled with additional markers (see previous section), this technique offers an unprecedented entry into the investigation of cell size control and its interplay with complex biological processes.

**DISCUSSION**

Regulation of cell volume is critical for eukaryotic organisms, but the mechanisms underlying this process are still unclear. Yeast models have played a pioneering role in deciphering this complex trait and remain at the forefront of this very active research field. However, the difficulty of accurately measuring cell volume at both the population and single-cell levels in these small unicellular organisms represents an obstacle for our understanding of size control and homeostasis. In fact, most studies in yeast have relied on approaches that are inherently biased, as they are based on indirect proxies or 2D measurements of cell dimensions and extrapolation of cell volume. Unravelling the complexity of this essential cellular feature thus requires direct and more reliable methods to probe cell volume in these genetically amenable models.

Here we show that the fluorescence exclusion method (FXm), which was initially developed for ascertaining the size of larger mammalian cells, is ideal for evaluating the size of small yeast cells in diverse contexts. We show that it provides high quality data on the volume profiles of yeast populations irrespective of cell morphology and physiological state, allows for single-cell analysis of volume changes with high time resolution and is compatible with complex studies when coupled with additional fluorescent markers. Our experiments in fission yeast also demonstrate that in contrast to the measurement of cell length at division, a commonly-used proxy for cell size in this organism, FXm gives researchers unique access to in-depth analyses of yeast cell volume dynamics and their coupling with cell cycle progression, integrating the size of cells at all stages of their division cycle. Remarkably, combining FXm with time-lapse imaging provides an unprecedented tool to explore the changes in growth rate and cellular geometry that occur not only during proliferation or upon major transitions such as quiescence entry and exit, but also when cells respond to acute alteration in the environment. Thus, provided the ease of FXm implementation and the improved software that we have developed, we believe that this technique will become a new standard for investigating cell size control in yeast, with implications for our knowledge of size regulation in complex eukaryotes.
Interestingly, our work in fission yeast led to a number of observations that may question existing models of cell size regulation. First, research on *S. pombe* size control and its interplay with cell cycle progression has mostly been based on two key assumptions: the diameter of proliferating haploid fission yeast cells is 1) constant from one cell to another and 2) homogenous along the growing axis of the cells. Thus, despite the critical impact of cell diameter on cell volume, most studies generally ignore this parameter. Strikingly, our results clearly show that these assumptions can lead to incorrect conclusions when comparing strains, as shown using various mutants (Figs. 4, 5). In this context, FXm has the unparalleled advantage of being a direct method for volume measurement that does not rely on any pre-conceived idea of cell geometry. The combination of FXm with the measurement of cell length also offers an additional level of understanding of cell size dynamics in different strains and conditions.

Indeed, assessing the changes in volume vs. length in distinctive strains or upon specific perturbations gives a new entry into the complexity of the regulation of cellular dimensions. For example, our data suggest that when cells are arrested in their division cycle through CDK inhibition, the geometric changes that occur over time are surprisingly complex, with length and volume alterations showing different kinetics (Table S1, MCN + 3-MBPP1). The controls of cell length and diameter may therefore operate independently in these conditions. Similarly, we find that simplifying the architecture of cell cycle control or growing cell in different environments has unanticipated impacts on cell geometry, affecting not only cell length but also cell diameter (Table S1).

Focusing on subpopulations, as is the case when using cell length at division as a proxy for cell size, may also hinder our comprehension of important processes linking cell growth, morphology and proliferation. For instance, our analyses of morphology mutants and the apparent changes in size distribution at the population level suggest that the interplay between growth rate and the phases of the cell cycle may be altered in these cells, again shedding light on potentially novel inputs and mechanisms that modulate cell size dynamics. In this context, comparing the volume vs. cell length at division ratios between distinctive *rga* mutants and wild type cells (Table S1) also indicates different cell geometries in these backgrounds that may represent a promising tool to decipher the key parameters used by the cells to “monitor” their size.

Finally, the use of natural isolates of yeast cells has become more and more widespread for investigating various biological processes, and in fission yeast, these strains are well-known to be less homogenous in morphology and overall size (Jeffares et al., 2015). These characteristics are largely incompatible with traditional methods for volume estimation. FXm may therefore represent a unique solution to fully exploit this promising resource and take advantage of advanced population genetics and quantitative trait loci strategies to decipher the regulation of cell size.

Altogether, our work demonstrates that previous models that describe the way cell size is regulated in fission yeast need to be revisited using FXm. Coupled to the wealth of knowledge on size control in this organism, this approach will provide unique insights into the processes that underlie the biology of
cell growth. While we use fission yeast as a proof-of-concept model, our results also show that FXm can be applied to any type of cells over a broad range of sizes. Our studies therefore establish the versatility and power of FXm for investigating cell volume homeostasis and its regulation in small cells and model organisms, contributing to our general understanding of the modulation of cellular dimensions and scaling in eukaryotes.

**MATERIALS AND METHODS**

**Fission yeast strains and methods**

Standard methods and media were used (Hayles and Nurse, 1992; Moreno et al., 1991). All the strains described in this study are detailed in Table S2. The deletions of *rca2, rca4, rca6, mal3, knk1* and *tea1* have already been described (Soto et al., 2010; Villar-Tajadura et al., 2008) (Beinhauer et al., 1997; Mata and Nurse, 1997; Revilla-Guarinos et al., 2016; Scheffler et al., 2014). All experiments were carried out in non-supplemented minimal medium (EMM) at 32 ºC, except otherwise noted. To inhibit CDK activity in analog-sensitive MCN strains, the 3-MBPP1 inhibitor (A602960, Toronto Research Chemicals, Inc.) was dissolved in DMSO at stock concentrations of 10 or 0.4 mM and added to liquid cultures at a final concentration of 1 or 0.05 µM. The percentage of binucleated cells (Fig. S3C) was determined from heat fixed samples on microscope slides (70 ºC for 5 min) stained with a 11:1 solution of Blankophor (1 mg/mL): DAPI (1 µg/mL).

**Microfabrication of FXm chips**

PDMS microfluidic chips for FXm were prepared following standard microfabrication protocols (McDonald and Whitesides, 2002). In brief, master molds were made by spin-coating SU-8 2005 resin (MicroChem Corp., USA) on silicon wafers using a spincoater (Laurell Technologies, USA) according to manufacturer’s instructions. Microstructures were then generated using high-resolution chrome masks (JD phototools, UK) and 365nm UV exposure (UV KUB 3, Kloe, France) followed by PGMEA (Sigma-Aldrich) development. For each mold, the height of the structures was determined as the average of three measurements perpendicular to the long axis of the design (Fig. 1B) using a Veeco Wyko NT9100 optical profilometer (Veeco Instruments Inc., USA). Note that molds showing significant variability between the three height measurements should be either discarded or used considering local rather than global averaged height. To produce chips for FXm, a 10:1 mixture of PDMS (Sylgard 184, Dow Corning, USA) was cast on the SU-8 master mold and allowed to cure at 70 ºC for 2 hours. Inlets were then made using 2.5 or 4 mm biopsy punches, and chips were bonded to microscopy-grade coverslips by plasma activation (Harrick Plasma, USA).
Preparation of fission yeast cells for FXm

To perform FXm experiments, exponentially growing fission yeast cells were sampled at an optical density between 0.25 and 0.45 OD_{595}. To limit the formation of cell aggregates, a mild sonication cycle of 5 s at 10 % amplitude (Branson 450 Digital Sonifier, Emerson Electric Co.) was applied. This treatment had no effect on the results we obtained by FXm (data not shown). In order to optimize the number of cells that can be measured on a single FXm image in our chip design, cells were concentrated to ~ 5.5 x 10^7 cells/mL in their own conditioned minimal medium (note that to prevent bias in our volume measurements, we did not use autofluorescent rich medium). FITC-Dextran was then added to the sample (FD10S, Sigma Aldrich) at a final concentration of 1 mg/mL, and cells were loaded into the chip shortly before image acquisition. For evaluating the volume of quiescent cells, cells were sampled 24 hours after they reached 0.4 OD_{595}.

Microscopy

All microscopy experiments were carried out using an inverted Zeiss Axio Observer (Carl Zeiss Microscopy LLC) equipped with a Lumencor Spectra X illumination system and an Orca Flash 4.0V2 sCMOS camera (Hamamatsu Photonics). Acquisition was performed using the VisiView software (Visitron Systems GmbH). A Plan-Apochromat 63X/1.4 NA immersion lens and a Plan-Apochromat 20X/0.8 NA Ph2 lens (Carl Zeiss Microscopy LLC) were used for cell length measurements and FXm, respectively. For routine volume measurements, the acquisition parameters were 100 ms exposure at 20 % illumination power, and 30 to 40 images were taken across the whole chambers to limit potential bias due to local changes in chamber height. For the time-lapse experiments in Fig 8, 100 ms exposure at 10 % illumination power was used.

Image analysis

Cell length at division was determined from Blankophor images (1 mg/mL Blankophor solution, A108741, Ambeed Inc.) using FIJI (National Institutes of Health) and the Pointpicker plug-in. For the blankophor images in Figs. 2, 4, 5, 6 and 7, the brightness and contrast were adjusted for display purposes. For routine volume measurements, images were first normalized using a custom Matlab software (Cadart et al., 2017). Cell selection and volume calculation were subsequently performed using the normalization mask with a new, specifically-developed Python interface (see Results). For all Figures, cell selection was done using the manual mode, except for Fig. 3B and 4C where automated cell identification was used. Note that except for the time-lapses in Fig. 8B and C, we systematically excluded cells that were undergoing cytokinesis but that had not fully separated into two individual daughter cells; this is similar to studies of cell length at division. While this category can be integrated as newly-born pairs of cells using the original Matlab software (Cadart et al., 2017), manually delineating the division site would introduce measurement errors. Finally, our Python code is not adapted to tracking the volume of individual cells in long time-lapse experiments (Fig. 8B, C), as slight
cell movements make it difficult to reliably follow single cells over time when using the normalization mask pre-selection. For these assays, we used the original Matlab software (Cadart et al., 2017).

**FXm time-lapse and analysis using fission yeast**

As fission yeast cells are non-adherent, FXm time-lapses required coverslips to be coated in order to prevent cells from moving inside the chamber, which would hamper data acquisition and analysis. To this end, 10 µl of 1mg/ml filtered lectin was spread in a rectangle of ~10 x 5mm at the center of the coverslip and allowed to dry at 37 °C. The coverslip was then washed once with filtered ultra-pure water and dried at 37 °C. For experiments without medium flow (Fig. 8B), Ø 4 mm inlets were made in the PDMS chip using the appropriate biopsy punch. 5 µl of cells prepared as described above were loaded in one inlet, and both inlets were then filled with 45 µl EMM containing the FITC-Dextran dye. Cells were allowed to flow into the chamber prior to imaging. For time-lapse experiments using medium flow (Fig. 8C), two successive layers of lectin were applied as above and inlets of Ø 0.75 mm were fabricated. Cells were then loaded by depositing a drop of cells prepared as above over one of the inlets, and a mild vacuum was applied at the other inlet using the tip of a 10 mL pipet connected to a vacuum pump. The loaded chip was then connected to a flow control system (pressure generator and flow controller, Elvesys, France), and a flow of medium (~ 3-5 µL/ min) containing the FITC-Dextran dye was used throughout the experiment. For the analysis of the time-lapse experiments, the two daughter cells after a division were manually delineated during the analysis and only one of them was followed for further investigation (Fig. 8B, C). The growth rate of each individual cell was determined by calculating the slopes of the traces during each cycle. This was obtained by linear regression considering the volume at birth as the first time point (when an invagination is detected) and excluding the mitotic plateau: entry into mitosis was assigned to the last point prior to strong reduction of growth (when two consecutive points on the graph decrease the slope).

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**COMPETING INTERESTS**

No competing interests declared.
FUNDING

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FIGURE LEGENDS

Figure 1. Microfluidic chips for FXm. A. Schematic of the principles of FXm (Cadart et al., 2017). Left panel: cells are injected in a microfluidic chip designed for FXm (see B) in the presence of FITC-Dextran. Pillars are integrated in the chip for ceiling support and image normalization. A low-magnification objective (20X) is used to acquire single-plane images (see C). Right panel: After normalization, the volume of the cell can be determined from the loss of fluorescence intensity (grey area) in the region analyzed (the precise internal height of the microfluidic chip must be determined (Cadart et al., 2017)). B. Schematic of the chamber design used for fission yeast cells. Left panel: design and dimensions (schematic not to scale). Right panel: all pillars used are 60 µm in diameter and separated from other pillars by 180 µm. C. Image of fission yeast cells injected in the presence of FITC-Dextran in the chip (prior to image normalization) in the conditions used for all our experiments.

Figure 2. Measuring the volume of fission yeast cells by FXm. A-D, graphs are histograms with box and whiskers plots (indicating the min, Q1, Q3 and max, with outliers determined by 1.5 IQR (interquartile range). White dot indicates the median volume). The median volume values with standard deviations are shown. A. Reproducibility of volume measurements of fission yeast cells by FXm. Measurements of wild type cells in five independent experiments (grey) and the total pooled dataset (black) are shown (chamber height: 5.53 µm; n≥316 for each replicate, n=2266 for the total dataset; values outside of the plot range (n*) were excluded: n*≤ 1 for each replicate). B. Impact of chamber height on the reproducibility of FXm. Measurements of wild type cells from a single culture using five distinct devices of the indicated heights (grey) and the total pooled dataset (black) are shown (n≥225 for each replicate, n=1777 for the total dataset; no values were outside of the plot range (n*≥0 for each replicate). C. Top panel: volume measurement by FXm using known cell size mutants. Wild type (WT), wee1-50 and cdc25-22 temperature-sensitive strains were grown exponentially at the permissive temperature of 25 °C and their volumes were evaluated by FXm. Pooled datasets (n≥1144) of three independent experiments (n≥337 for each replicate –Fig. S1A) are shown. Values outside of the plot range were excluded (n*≤ 6 for each replicate). Bottom panel: blankophor images with cell length at
division of the corresponding strains (averages with standard deviations for pooled datasets of three independent experiments, $n\geq 100$ for each replicate). Scale bars = 10 µm. At 25 °C, WT cells have a reduced volume and cell length at division compared to 32 °C (see A, B and Figs. 4-7). D. Top panel: comparison of the volume distributions in populations of wild type haploid and diploid fission yeast cells as determined by FXm. Cells were grown in supplemented EMM6S at 32 °C. Pooled datasets ($n\geq 1073$) for three independent experiments ($n\geq 207$ for each replicate – Fig. S1B) are shown. Values outside of the plot range were excluded ($n^*\leq 1$ for each replicate). Bottom panel: blankophor images with cell length at division of the corresponding strains (averages with standard deviations for pooled datasets of three independent experiments, $n\geq 100$ for each replicate). Scale bars = 10 µm. In EMM6S, WT cells have a volume that is similar to that in EMM (compare with A, B and Figs. 4-7) but a reduced cell length at division (compare with Fig. 4A, 5A and 7), suggesting a difference in cell width between these conditions.

Figure 3. Analysis pipeline for FXm data. A. Top panel: representative image showing the selection of individual cells (multiple individual regions of interest are indicated) using the original FXm analysis code. Each cell must be visually identified from the FITC image (see Fig. 1C for full frame image; the area displayed here has been magnified for display purposes) and manually selected. Bottom panel: representative image showing the tool that we developed, taking advantage of the image normalization mask for facilitating cell selection. All objects from the FITC image are automatically identified and displayed to the user at a higher magnification. Cell aggregates (bottom left), recently divided cells (bottom right) or artifacts can then be rapidly and reliably excluded from the analysis by the user (red-barred). B. Comparison of the results obtained by FXm using manual (black) and automated (grey, 2 s.d. filter) cell selection. Data for manual selection are from Fig. 2C. For the automated selection, 1) the same images as for the manual method were used, pooling the data from all replicates, and 2) a filter was applied that excludes entries that are 2 standard deviations above and below Q3 and Q1 ($n^*$), respectively (unfiltered selected objects: $n\geq 1390$, excluded objects $n^*\leq 200$). The overlap between the two approaches for both correctly assigned cells and outliers is between 87 and 92 %. Graphs are as in Fig. 2.

Figure 4. FXm allows for the detection of subtle changes in cell volume and size distribution. A. Top panel: volume measurement of wild type (WT), MCN and MCN-AF cells by FXm. Pooled datasets ($n\geq 913$) of three independent experiments ($n\geq 172$ for each replicate – Fig. S2) are shown. Values outside of the plot range were excluded ($n^*\leq 1$ for each replicate). Graphs are as in Fig. 2. Bottom panel: blankophor images with cell length at division of the corresponding strains (averages with standard deviations for pooled datasets of three independent experiments, $n\geq 100$ for each replicate). Scale bars
= 10 µm. B. Comparison of the median volumes for 5 independent replicates of wild type cells at 25 and 32 °C. The full datasets are presented in Fig. S3A. For each condition, the values are sorted in descending order. Difference in median cell volume: 8.8 %. C. MCN cells grown in EMM6S at 32 °C were G2-arrested using 1 µM 3-MBPP1 for 160 min and allowed to synchronously re-enter the cell cycle by washing off the inhibitor (Coudreuse and Nurse, 2010) (Fig. S3B). Cells were then injected in a microfluidic chip, and FXm images were acquired at 5 min intervals throughout the next G2, after cells had undergone a first round of mitosis and cell division (60 min, Fig. S3C). Top panel: median population volumes at the indicated time points (black line – automated cell selection) with IQR (grey area) are shown. Note that the higher starting volume for MCN cells compared to other Figures results from the initial G2 block, during which cells grow without dividing. The phases of the cell cycle during which cell volume was measured (first G2 and mitosis/M after release – Fig. S3C) are indicated. G2 growth rate: 0.59 µm³.min⁻¹ (linear regression from 60 to 110 min, prior to the mitotic plateau). From 125 to 140 min, the automated cell selection does not separate cells that are newly divided (17.4%, 25.9% and 25.5% of the selected objects at 130, 135 and 140 min, respectively, are pairs of cells; the remainder of the cells have not undergone cytokinesis at these time points), resulting in an apparent increased growth rate. Note the slight delay in cell cycle progression in this experiment compared to Fig. S3C, which may be due to the temporary temperature downshift when cells are injected in the microfluidic chip and the growth conditions when no medium flow is applied (see Fig. 8B). For each time point, 214≤n≤278 (unfiltered automatically selected objects). Outliers were removed from the automatic analysis using the 2 s.d. filter (see Fig. 3B; 28≤n̂∗≤49, representing a maximum of 18% of the number of selected objects). Bottom panel: representative images for cells in the FXm chips at 85 min (G2), 120 min (M) and 135 min (pairs of sister cells are not excluded by the automated analysis and represent a significant subset of the population at this time point, see above). D. Volume distribution for the indicated strains as a percentage of the median volume of the population. Data are as in A. Values outside of the plot range were excluded (n̂∗≤10).

**Figure 5.** Measuring the volume of yeast cells with altered morphologies. A, C, E. Blankophor images with cell length at division of the indicated strains (averages with standard deviations for pooled datasets of three independent experiments, n̂≥100 for each replicate). n.d.: not determined. Scale bars = 10 µm. B. Volume measurement by FXm of the strains in A. Pooled datasets (n̂≥832) of three independent experiments (n̂≥268 for each replicate – Fig. S4) are shown. Values outside of the plot range were excluded (n̂∗≤1 for each replicate). D. Volume measurement by FXm of the strains in C. Pooled datasets (n̂≥940) of three independent experiments (n̂≥230 for each replicate – Fig. S5) are shown. No values were outside of the plot range (n̂∗=0 for each replicate). Data for wild type (WT) are as in Fig. 2D (haploids). E. Blankophor images of wild type cells in exponential growth and quiescence. For quiescent cells, samples were taken 24 h after the culture reached an OD₅₉₅ of 0.4. For proliferating
cells, cell length at division is shown (average with standard deviation for pooled dataset of three independent experiments, \( n \geq 100 \) for each replicate). n.d.: not determined. Scale bars = 10 µm. F. Volume measurement by FXm of the cells in \( E \). Pooled datasets (\( n \geq 965 \)) of three independent experiments (\( n \geq 238 \) for each replicate – Fig. S6) are shown. No values were outside of the plot range (\( n^* = 0 \) for each replicate). C, D: Experiments were carried out in EMM6S due to the presence of auxotrophies in some of the mutant strains (see Table S2). B, D, F: Graphs are as in Fig. 2.

**Figure 6.** FXm is compatible with the use of fluorescent markers to study subpopulations. A. Top panel: volume measurement of wild type (WT) and cells expressing \( nhp6::mCherry \) by FXm. Pooled datasets (\( n \geq 1568 \)) of three independent experiments (\( n \geq 499 \) for each replicate – Fig. S7B) are shown. No values were outside of the plot range (\( n^* = 0 \) for each replicate). The median volume values with standard deviations are shown. Bottom panel: blankophor images with cell length at division of the strains above (averages with standard deviations for pooled datasets of three independent experiments, \( n \geq 110 \) for each replicate). Scale bars = 10 µm. B. Volume comparison between mono- (grey) and bi-nucleated (black) cells in the population of \( nhp6::mCherry \) cells as in A. The percentages and median volumes for the mono- (\( n = 1619 \)) and binucleated (\( n = 196 \)) subpopulations are indicated below the graph. Binucleated cells include both septated (dashed line) and non-septated cells. A, B: Graphs are as in Fig. 2.

**Figure 7.** Dynamic changes in cell volume at the population level. Left panel: FXm volume measurement of MCN cells after treatment with 0.05 µM of the ATP analog 3-MBPP1. Untreated wild type cells (WT) were used as a control. For MCN, samples were collected and analyzed every 40 min. after addition of the inhibitor (\( n \geq 265 \) at each time point. \( n = 528 \) for WT). Values outside of the plot range were excluded (\( n^* \leq 2 \) for each dataset). Graphs are as in Fig. 2. Note that the mild potential bimodal distribution observed in this experiment may not be relevant, as it can also be observed in WT cells but does not appear to be systematically present (Fig. 2A, B). Right panel: blankophor images with average cell length at division and standard deviation for the experiment in A. \( n \geq 100 \) for WT and MCN (0, 120 and 160 min). For the 40 and 80 min time points, the initial delay of mitosis due to the inhibitor treatment leads to a reduction in the number of dividing cells, making it difficult to obtain similar numbers for the calculation of cell length at division (\( n = 45 \) and 22, respectively). Septation indices: WT: 11.5 %, MCN 0 min: 20.4 %, MCN 40 min: 1 %, MCN 80 min: 2 %, MCN 120 min: 15.5 %, MCN 160 min: 26.9 %; \( n \geq 400 \) for each time point. Scale bars = 10 µm.

**Figure 8.** Determination and real-time monitoring of single-cell volume by FXm. A. Evaluation of FXm experimental noise using the chips designed for yeast cells. 20 consecutive images were acquired...
in the standard FXm setup (see Materials and Methods; 100 ms exposure per image for a total acquisition time of 6 s due to shutter delay) and the volumes of 10 individual cells were followed throughout the experiment. This assay makes the assumption that no significant volume changes occur during this 6 s time window (the generation time of wild-type fission yeast cells in these growth conditions is ~140 min). The coefficients of variation are between 0.3 and 3.1%. B. FXm time-lapse experiment using wild type cells in EMM at 32 °C without medium flow. C. FXm time-lapse experiment using wild type cells in EMM at 32 °C with a constant flow of medium (3-5 µL.min⁻¹) containing FITC-Dextran dye. B, C: individual traces represent the volume dynamics of single-cell lineages (at each division, only one of the two daughter cells is further monitored). Volumes were determined every 10 min. For display purposes, the individual traces are aligned to the first division event (see Materials and Methods). Calculated average growth rates (with standard deviations) are shown for each cycle. Note that the growth rate in the presence of a flow is consistent to that obtained from the bulk analysis of a synchronized population (Fig. 4C).

REFERENCES


FIGURE 1

A

Microfluidic chip

FITC-Dextran

Pillar

20X lens

B

15 mm

0.5 mm

1 mm

2.6 mm

140 µm

60 µm

C

Fluorescence intensity

Max

Min

Area

FITC-Dextran

2.6 mm

15 mm

1 mm

5.0 mm
FIGURE 2

A

Cell volume (µm³)

% 20

0

10

20

Total

102.6 ± 25.4 µm³

99.3 ± 24.8 µm³

109.8 ± 23.0 µm³

103.9 ± 24.6 µm³

103.8 ± 23.1 µm³

104.8 ± 24.5 µm³

0 50 100 150 200 250

Cell volume (µm³)

B

h = 6.95 µm

104.4 ± 24.3 µm³

h = 6.55 µm

100.8 ± 22.7 µm³

h = 5.60 µm

109.9 ± 25.4 µm³

h = 5.53 µm

111.4 ± 24.8 µm³

h = 4.85 µm

108.3 ± 23.3 µm³

h = 4.85 µm

108.6 ± 24.5 µm³

0 50 100 150 200 250

Cell volume (µm³)

C

Wee1-50ts - 25 °C

72.5 ± 19.2 µm³

Cdc25-22ts - 25 °C

142.5 ± 35.1 µm³

WT - 25 °C

95.9 ± 23.4 µm³

0 50 100 150 200 250

Cell volume (µm³)

D

diploids

200.0 ± 48.0 µm³

haploids

108.6 ± 24.5 µm³

0 50 100 150 200 250 300 350

Cell volume (µm³)

WT (haploids)  WT (diploids)
FIGURE 3

A

Cell: 12 (99 µm³)

Cell: 13 (89 µm³)

Cell: 17 (292 µm³)

Cell: 18 (150 µm³)

B

wee1-50Δ, 2 SD filter

75.8 ± 21.7 µm³

wee1-50Δ, manual

72.5 ± 19.2 µm³

cdc25-22ts, 2 SD filter

146.4 ± 37.7 µm³

cdc25-22Δ, manual

142.5 ± 35.1 µm³

WT, 2 SD filter

98.4 ± 25.0 µm³

WT, manual

95.9 ± 23.4 µm³

Cell volume (µm³)
FIGURE 4

A

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<tr>
<th></th>
<th>MCN-AF</th>
<th>MCN</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell volume (µm³)</td>
<td>102.3 ± 31.4</td>
<td>122.1 ± 29.6</td>
<td>104.2 ± 25.1</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>25 °C</th>
<th>32 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell volume (µm³)</td>
<td>99.1 ± 21.8</td>
<td>109.8 ± 23.0</td>
</tr>
<tr>
<td></td>
<td>97.8 ± 21.2</td>
<td>103.9 ± 24.6</td>
</tr>
<tr>
<td></td>
<td>94.2 ± 20.4</td>
<td>103.8 ± 23.1</td>
</tr>
<tr>
<td></td>
<td>93.6 ± 23.7</td>
<td>102.6 ± 25.4</td>
</tr>
<tr>
<td></td>
<td>93.3 ± 20.5</td>
<td>99.3 ± 24.8</td>
</tr>
</tbody>
</table>

Full datasets: 96.3 ± 21.9 µm³, 104.8 ± 24.5 µm³

C

G2

M

Time after release (min)

<table>
<thead>
<tr>
<th></th>
<th>85 min</th>
<th>120 min</th>
<th>135 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell volume (µm³)</td>
<td>97.8 ± 21.9</td>
<td>109.8 ± 23.0</td>
<td>103.8 ± 24.6</td>
</tr>
<tr>
<td></td>
<td>94.2 ± 20.4</td>
<td>93.6 ± 23.7</td>
<td>93.3 ± 20.5</td>
</tr>
<tr>
<td></td>
<td>99.3 ± 24.8</td>
<td>104.8 ± 24.5</td>
<td>96.3 ± 21.9</td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th></th>
<th>MCN-AF</th>
<th>MCN</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell volume (µm³)</td>
<td>102.3 ± 31.4</td>
<td>122.1 ± 29.6</td>
<td>104.2 ± 25.1</td>
</tr>
</tbody>
</table>

Full datasets: 96.3 ± 21.9 µm³, 104.8 ± 24.5 µm³
FIGURE 5

A

\[\Delta \text{rna4} \]

\[\Delta \text{rna6} \]

WT

\[13.1 \pm 0.9 \text{ µm} \]

\[14.3 \pm 1.0 \text{ µm} \]

\[14.9 \pm 1.0 \text{ µm} \]

\[17.4 \pm 1.4 \text{ µm} \]

B

\[\text{Arga6} \]

\[106.7 \pm 26.6 \text{ µm}^3 \]

\[\text{Arga4} \]

\[116.2 \pm 27.7 \text{ µm}^3 \]

\[\text{Arga2} \]

\[98.8 \pm 25.8 \text{ µm}^3 \]

\[\text{WT} \]

\[103.8 \pm 25.1 \text{ µm}^3 \]

C

\[\Delta \text{mal3} \]

\[\Delta \text{knk1} \]

WT

\[\text{n.d.} \]

\[\text{n.d.} \]

\[14.0 \pm 0.9 \text{ µm} \]

\[\text{n.d.} \]

D

\[\Delta \text{knk1} \]

\[103.5 \pm 24.8 \text{ µm}^3 \]

\[\Delta \text{mal3} \]

\[104.7 \pm 27.7 \text{ µm}^3 \]

\[\Delta \text{tea1} \]

\[121.1 \pm 28.6 \text{ µm}^3 \]

\[\text{WT} \]

\[108.6 \pm 24.5 \text{ µm}^3 \]

E

Proliferating

Quiescent

\[14.9 \pm 1.3 \text{ µm} \]

\[\text{n.d.} \]

F

Quiescent

\[52.7 \pm 14.8 \text{ µm}^3 \]

Proliferating

\[105.7 \pm 24.5 \text{ µm}^3 \]
FIGURE 6

A

\[
\begin{align*}
\text{nhp6::mCherry} & : 96.7 \pm 23.6 \, \mu m^3 \\
\text{WT} & : 103.2 \pm 25.4 \, \mu m^3 \\
\end{align*}
\]

B

\[
\begin{align*}
\text{nhp6::mCherry} & : 14.6 \pm 1.1 \, \mu m \\
\text{WT} & : 14.9 \pm 1.0 \, \mu m \\
\end{align*}
\]

Cell volume (\(\mu m^3\))
FIGURE 7

Cell volume (µm$^3$)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>MCN, 0 min</th>
<th>MCN, 40 min</th>
<th>MCN, 80 min</th>
<th>MCN, 120 min</th>
<th>MCN, 160 min</th>
<th>WT</th>
<th>MCN, 0 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>108.4 ± 25.7 µm$^3$</td>
<td>126.7 ± 29.5 µm$^3$</td>
<td>136.0 ± 33.1 µm$^3$</td>
<td>161.5 ± 42.2 µm$^3$</td>
<td>182.3 ± 41.6 µm$^3$</td>
<td>218.5 ± 45.5 µm$^3$</td>
<td>25.6 ± 2.1 µm</td>
<td>26.1 ± 3.2 µm</td>
</tr>
</tbody>
</table>

MCN, 0 min to MCN, 160 min

0 to 20%
FIGURE 8

A

B

C

Cycle 1

0.36 ± 0.05 µm³/min

0.43 ± 0.15 µm³/min

Cycle 2

0.28 ± 0.03 µm³/min

0.54 ± 0.07 µm³/min

Frame (every 100 ms)

Time (min)

Cell volume (µm³)

Time (min)

Cell volume (µm³)