Characterizing Cellular Biophysical Responses to Stress by Relating Density, Deformability, and Size

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Characterizing cellular biophysical responses to environmental stresses by relating density, deformability and size

Running title: Cellular density, deformability and size

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Abstract

Cellular physical properties are important indicators of specific cell states. Although changes in individual biophysical parameters, such as cell size, density and deformability, during cellular processes have been investigated in great detail, relatively little is known about how they are related. Here, we use a suspended microchannel resonator (SMR) to measure the single-cell density, volume, and passage time through a narrow constriction of populations of cells subjected to a variety of environmental stresses. Osmotic stress significantly affects density and volume, as previously shown. In contrast to density and volume, the effect of an osmotic challenge on passage time is relatively small. Deformability, determined by comparing passage times for cells with similar volume, exhibits a strong dependence on osmolarity, indicating that passage time alone does not always provide a meaningful proxy for deformability. Finally, we find that protein synthesis inhibition, cell cycle arrest, protein kinase inhibition, and cytoskeletal disruption result in unexpected relations between deformability, density, and volume. Taken together, our results suggest that measuring multiple biophysical parameters can detect unique characteristics that more specifically reflect cellular behaviors.

Keywords: suspended microchannel resonator, cell stiffness, cell density, deformability, osmotic stress
Introduction

Cellular biophysical properties reflect aggregate effects of particular cellular activities, such as malignant transformation, differentiation, cell cycle progression, disease response, and apoptosis. Studying these properties can help provide insight into the underlying molecular mechanisms governing cellular behavior. For example, increased metastatic potential of cancer cells has been correlated with increased cell deformability (1–6). Studies on differentiation have also reported that the deformability decreases as stem cells become more differentiated (7, 8). Cell cycle stage is known to be associated with changes in cell shape and deformability (9, 10). Red blood cells (RBCs) affected by malaria show decreased deformability and density (11, 12). Finally, previous studies have reported that apoptosis is related to volume shrinkage and changes in cell deformability (13, 14).

Another common physiological source for changes to cellular biophysical properties is shifts in extracellular osmolarity. Certain tissues, such as the kidney, are regularly exposed to dramatic osmolarity shifts, and adjust their membrane surface area to allow for increases or decreases to cell volume to maintain a constant cortical tension (15). Articular cartilage in the synovial joints, such as the knee and hip, is subjected to both static and dynamic mechanical compression during articulation, resulting in changes in interstitial osmolarity of the cartilage tissue (16). This, in turn, can affect the biosynthesis rate, deformability, and volume of chondrocytes in the tissue (17–19). Moreover, the inability to respond to an osmotic challenge can result in impaired function, as is the case with T lymphocytes lacking the osmosensitive transcription factor NFAT5/TonEBP. Eliminating NFAT5/TonEBP expression prevents normal cell proliferation and development of adaptive immunity, likely due to an inability to adapt to the hyperosmolar conditions present in many lymphoid tissues (20).

Although most studies focus on the measurement of individual biophysical parameters, increasing evidence shows that combining information from measurements of multiple parameters can improve prediction of cell state. RBCs from patients with thalassemia, a genetic disorder which leads to a measurable but not significant decrease in RBC volume, can be distinguished from healthy RBCs based on a simultaneous comparison of both mass and density, but not from either parameter taken individually (12). The differentiation potential of stem cells can be accurately predicted by measuring the deformability of stem cells along with size, but not by measuring size alone (21, 22). Measuring deformability and diameter also enables prediction of four types of malignant diseases from a pleural effusion, whereas the identification based on a single parameter only provides an incomplete prediction (8). Measuring deformability and friction of cancer cells reveals that reduced friction may play a role in further facilitating the passage of more deformable metastatic cancer cells through tight spaces (23).

Here we used a murine pro-B cell line as a model system and measured multiple biophysical parameters of individual cells with a suspended microchannel resonator (SMR). First, to fully understand how cells respond to osmotic stress, we measured volume, density, and passage time through a narrow constriction. Increasing the media osmolarity
leads to increased density and decreased volume as expected. Interestingly, we found that the density change following an osmotic challenge cannot be accounted for by water exchange alone, suggesting that the dry mass must shift as well. In contrast to density and volume, passage time is nearly independent of osmolarity even though deformability changes considerably, indicating that passage time should be measured together with cell volume. Finally, we compared relationships between deformability and density for cells treated with various pharmacological perturbations, including latrunculin B, staurosporine, cycloheximide, rapamycin, and Torin 1.

Materials and Methods

Cell culture and preparation

FL5.12 cells, a murine pro-B lymphoid cell line, were cultured as previously described (24). Briefly, cells were cultured in RPMI media (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) FBS (Sigma-Aldrich, St. Louis, MO), 100 IU penicillin, 100 µg/mL streptomycin (Gemini, West Sacramento, CA) and 0.02 µg/mL IL-3 (R&D Systems, Minneapolis, MN) at 37°C. FL5.12 cells were exposed to an osmotic challenge or biochemical agent before each measurement. To maintain each condition during the experiment, the media used in the SMR were also supplemented with the same stimuli. For the osmotic challenge, cells were collected from the culture flask, centrifuged at 150 g for 5 min, resuspended in hypertonic or hypotonic media, and incubated for 30 min before the measurements. D-mannitol (182.17 g/mol, Sigma-Aldrich) and deionized H₂O (dH₂O) were added to the media for the hyperosmotic and hypoosmotic conditions, respectively. For the isoosmotic control group (300 mOsm/L), cells were resuspended in the untreated culture medium. For chemical perturbations, cells were resuspended and incubated in the media supplemented with 5 µg/mL latrunculin B for 30 min (Sigma-Aldrich), 2 µM staurosporine for 2 h (Enzo Life Sciences, Farmingdale, NY), 1 µg/ml or 10 µg/ml cycloheximide for 3 h (Sigma-Aldrich), 100 nM rapamycin (Santa Cruz Biotechnology, Dallas TX) for 24 h, or 250 nM Torin 1 for 24 h (generously provided by Prof. David M. Sabatini, Massachusetts Institute of Technology). Control groups for these conditions were cells resuspended in the culture media supplemented with DMSO as the vehicle. The SMR measurement data from the start of the measurement was compared to the data at the end of the measurement to ensure that the data showed no time dependence.

Experimental systems

SMRs were fabricated at Innovative Micro Technology (Santa Barbara, CA). Overall instrumentation for measurement and data acquisition have been previously reported in detail (25). Schematics of experimental approaches are shown in Fig. 1. Density and volume were measured by a separate SMR system from that measuring passage time. To measure density and volume, each cell was weighed in the SMR in two fluids as previously described (12). Briefly, a cell immersed in a fluid of low density (Fluid 1, ~1.01 g/mL) is flowed from a bypass channel through a channel embedded in a resonating cantilever, and trapped in an
opposite bypass filled with a fluid of high density (~1.1 g/mL). The direction of flow is reversed, and the same cell is passed through the cantilever a second time, but now in Fluid 2 (~1.08 g/mL), which is a mixture of the high density fluid with a residual amount of Fluid 1. Each time the cell flows through the cantilever, a buoyant mass measurement is recorded, along with a corresponding fluid density value. The two buoyant masses are plotted versus their respective fluid densities to obtain a line with a slope corresponding to cell volume and x-intercept corresponding to cell density (Fig. 1C). As the value of cell density approaches that of the fluid density, the cell buoyant mass becomes progressively smaller, and therefore more difficult to accurately determine. Thus, using our current system, we were unable to quantify the density of cells exposed to very high osmotic stresses (> 500 mOsm/L), due to the value of cell density approaching that of the Fluid 2 density. For all density measurements, a 7:3 mixture of cell media:iodixanol solution (OptiPrep Density Gradient Medium, Sigma-Aldrich) was used as the high density fluid. In experiments involving osmotic stress, the osmolarity of this solution was adjusted with either mannitol or water to match that of Fluid 1.

To measure passage time, single cells were measured in an SMR with a constriction as previously described (23). Briefly, a cell is flowed into the embedded microfluidic channel of the SMR, and deformed as it flows into the constriction. The geometry of the constriction is a rectangular channel 6 μm wide, 15 μm deep, and 50 μm long. The cross-sectional area of the constriction is 90 μm² (6 μm wide × 15 μm deep) and the average diameter of FL5.12 cells is approximately 12 μm (untreated control), indicating a maximum cross-sectional area of approximately 113 μm². The narrow width of the constriction ensures the deformation of a cell as it squeezes into the constriction’s entrance (entry) and then passes through the constriction channel (transit). The resonant frequency response of the SMR, which depends on cell’s buoyant mass and position in the microfluidic channel, is tracked in real time as the cell passes through the channel. The passage time includes the total time required for the cell to slow down as it deforms to enter the constriction (entry time), and speed up as it travels through and exits the constriction (transit time). Here we measured the passage time as a metric for cell deformability, which is defined as the total time required for the cell to deform into and then transit through the constriction. The relative contribution of surface friction to the passage time can be estimated by comparing the cell’s velocities during the entry (entry velocity) and the transit (transit velocity). Typically, the passage time is dominated by the entry time since the transit velocity is significantly faster than the entry velocity (23). Dominance of the entry time in the passage time was consistently observed in our previous study, in which we measured seven adherent cell lines, including mouse embryonic fibroblasts, mouse lung cancer cell lines, human lung cancer cell lines, as well as a mouse lymphoblastic leukemia cell line that was grown in suspension (23). We have also measured cells in various conditions, such as treating cells with latrunculin B, and nocodazole, or coating the microchannel surface with positively charged poly-L-lysine. In all of these cell lines and treatments, the passage time was dominated by the entry time (23). Therefore, here we assume that the friction can affect but does not dominate the differences in passage times. The fluidic channel is coated with poly(ethylene glycol) [1 mg/mL; PLL(20)-g[3.5]-PEG(2); Surface Technology] to reduce non-specific adhesion of cells to the walls of the constriction. All measurements were
acquired at room temperature under a constant applied pressure drop across the microchannel (0.15 psi) established by pressure regulators.

**Fig. 1.** Schematic diagrams of the approaches for measuring deformability, density and volume, and examples of the data extracted from the measurement. (A) A SMR with a constriction measures the passage time and buoyant mass as a cell flows into an embedded microfluidic channel and transits through the constriction. (B) Passage time versus buoyant mass for the FL5.12 cell line shows the change in passage time induced by staurosporine (STS). (C) Measuring the buoyant mass of a single cell in two fluids of different densities allows the cell density and volume to be determined. (D) Cell density versus volume of FL5.12 cells treated with STS. Treatment with STS leads to an increase in density and a slight decrease in volume.

**Data analysis**
SMR frequency data was converted to buoyant mass, passage time, volume and density using methods previously described (12, 23). Data plotted in Figs. 2C, 2E, 3A, and 3C represent volume, water content, passage time, and buoyant mass, respectively. These parameters are plotted with a logarithmic scale, which we considered to be the form that most accurately represents the data. Cell size follows a log-normal distribution, as has been previously reported (26). Passage time is expected to follow a log-normal distribution as well, since passage time has a strong power-law dependence on buoyant mass, as shown in Fig. 1B (23, 27).

As previously presented, the SMR can be used to measure biophysical properties with high precision (12, 23, 25). The buoyant mass and the position of the center of mass of a particle passing through a constriction in the SMR can be measured with a precision near 1 pg and sub-micron, respectively (23). Additionally, the density and volume can be measured with a resolution of 0.001 g/ml and 3 fL, respectively (12). We can therefore attribute the variability observed in our measurement to inherent biological variations rather than experimental artifacts. For example, one source of biological variation in size is related to a distribution of cells across the cell cycle. Interestingly, in our measurements, cells of similar buoyant mass showed a significant variation in passage time (Fig. 1B), suggesting that, in addition to biological variation such as a cell cycle (10), the orientation of the cell upon entry into the constriction can cause a wide distribution (23, 28).

The buoyant mass obtained during passage time measurements was converted to volume using the following equation,

\[ V = \frac{b_m}{\rho_{cell} - \rho_{fluid}} \]

where \( V \) is the cell volume, \( b_m \) is the cell buoyant mass, \( \rho_{cell} \) is the average of the cell density obtained from a corresponding SMR density measurement, and \( \rho_{fluid} \) is the fluid density. The uncertainty contributed to the volume distribution resulting from converting the buoyant mass using an average cell density was determined to be not significant based on a Monte Carlo estimate (Fig. S1 in the Supporting Material).

Statistical significance between density measurements was determined using a non-parametric rank-sum analysis (Wilcoxon rank sum) implemented in MATLAB. Changes in passage time were estimated and the statistical significance of those changes were tested by fitting data sets to a linear model in R. For all statistical tests, a \( p \)-value less than or equal to 0.05 was considered significant. In particular, since most statistical tests showed very small \( p \)-values (\(10^{-50} - 10^{-5} \)), we indicated those small \( p \)-values by “\( p < 0.0001 \”).

**Results**

**Characterizing the density and volume of cells exposed to osmotic stress.**
To characterize the biophysical effects of osmotic stress, we first measured the changes to
density and volume of FL5.12 cells exposed to a range of osmolarities (Fig. 2). We varied
the osmolarity of the cell media from 250 to 600 mOsm/L by diluting with dH$_2$O or
concentrating with D-mannitol. Due to limitations described in Materials and Methods, we
were not able to measure the density and volume at 600 mOsm/L, though we did obtain
passage time measurements. Cell density increases following exposure to hyperosmotic
media and decreases following exposure to hypoosmotic media (Fig. 2A); the effect of
osmotic stress on cell volume follows an opposite trend (Fig. 2B). We expect that the
primary source of this trend is the change in cellular water content (29, 30). Water has a
lower density than most non-aqueous cellular components, particularly proteins, nucleic
acids, and certain lipid conjugates (31–33). Thus, when an osmotic challenge leads to a
change in volume, the ratio of water to non-aqueous components will change as well. If the
cellular water fraction increases, the density will decrease; this is the case with
hypoosmotic swelling, in which the influx of water is associated with both a larger volume
and a lower density. Similarly, the loss of water in the hyperosmotic case leads to both a
decreased volume and an increased density. We estimated the change to water content
using a previously described method (Supporting Material), and confirmed that
hypoosmotic stress leads to an increase in water content, while hyperosmotic stress leads
to a decrease in water content (Fig. 2E).

Interestingly, the variability in cell density appears to be much smaller than that of volume;
we found this to be true both among a population of cells from single measurement (Fig. 2A
and C) and when comparing the means of multiple measurements (Fig. 2B and D). For the
300 mOsm/L measurement shown in the box plot in Fig. 2A and C, the interquartile range
of density is approximately 0.005 g/mL, or approximately 0.5% of the mean, while the
interquartile range of volume is approximately 500 fL, or approximately 25% of the mean.
Similarly, the CV of density is approximately 0.3%, while that of volume is approximately
50%. We can attribute the wide volume range in part to differences in cell cycle stage in the
population; the much narrower range of density could suggest that density remains
relatively constant for a majority of the cell cycle. The variability for multiple experiments
is shown in Fig. 2B and D where each point represents the geometric mean of a single
measurement, and the green bar represents the mean across multiple measurements. In
this case, the CV of volume is approximately 10%, whereas that of density is 0.065%. This
outcome further reinforces the notion of a biological mechanism to maintain a narrow
density distribution. As a possible explanation, we can consider density to be a reflection of
the crowding of intracellular macromolecular components; previous work has shown that
the cytoplasm is extremely crowded, and that the level of crowding affects protein stability,
adsorption to surfaces, and reaction rates (34–36). Thus, we can hypothesize that tight
regulation of density results from a requirement to maintain a uniform level of crowding.
An important consequence of the tight distribution of density relative to volume is that
cells of similar densities will not always have the same volumes. Thus, simultaneous
measurement of both density and volume is necessary to more accurately describe cell
state.
Fig. 2. Effect of osmotic stress on density and volume. FL5.12 cells are incubated in hypo- and hyperosmolar media for 30 min before and during the measurement. (A) Box plots of density from a representative experiment. Each data point represents the density of an individual cell (n = 83-170). (B) Density changes resulting from osmotic stress across multiple replicates. A single point represents the geometric mean of one replicate and the green line indicates the mean from multiple replicates (3-8 for each condition). (C) Box plots of volume from a representative experiment. Data shown was measured simultaneously with density in A. Each data point represents the volume of an individual cell. (D) Volume changes resulting from osmotic stress across multiple replicates. A single point represents the geometric mean of one replicate and the green line indicates the mean from all replicates (3-8 for each condition).
Characterizing the passage time and deformability of cells exposed to osmotic stress.

Next, we determined how osmotic stress affects the passage of a cell through a narrow constriction by comparing the median passage times for osmotically-stressed cells to an isoosmotic control. In contrast to cell density and volume, the effect of an osmotic challenge on passage time is relatively small (Fig. 3). Changes in passage time following osmotic challenges of 250, 350 and 400 mOsm/L are 0.68, 0.66, and 5.1%, respectively (Fig. 3B). However, increasing the media osmolarity to 500 and 600 mOsm/L results in a ~ 30% increase in passage time. Similarly, following exposure to osmotic stress, cellular buoyant mass does not deviate significantly from its control (3.6, -0.79, and -3.6% changes, respectively, for 250, 350 and 400 mOsm/L, Fig. 3D); exposure to 500 and 600 mOsm/L media results in a further decrease in buoyant mass (-9.2%, and -16% respectively), suggesting that buoyant mass decreases as osmolarity increases.

The relatively small changes to passage time and buoyant mass following osmotic stress between 250 and 400 mOsm/L can be attributed to simultaneous, compensatory changes in density and volume. For example, as a cell is compressed with an osmotic challenge, its density increases while its volume decreases. As a cell becomes denser by water loss, cell deformability decreases by molecular crowding within cytoplasm (30), which, in turn, increases the passage time (23). Therefore, though a denser cell should exhibit a longer passage time due to its decreased deformability (Fig. 4), its smaller volume concurrently decreases the passage time (23, 27). As a result, the overall change in passage time caused by osmotic stress is relatively small. These results demonstrate that passage time reported independently of size may not always predict cell deformability, and that passage time must be compared for cells of similar size to decouple effects of varying size and deformability (23).

Buoyant mass is a convenient size metric since it is simultaneously measured with passage time for individual cells (Fig. 1, and Data Analysis). However, buoyant mass depends on cell volume and density, and, like passage time, is susceptible to compensatory changes in these parameters. Buoyant mass is defined as the product of the volume and the difference between the cell density and the density of the surrounding fluid; thus, the increase in density following osmotic compression would lead to an increase in buoyant mass, while the decrease in volume would lead to a decrease in buoyant mass. Indeed, we find that the buoyant mass remains relatively unaffected by osmotic compression (350, 400 mOsm/L) (Fig. 3C, D). Thus, we define deformability as the passage time of a cell accounting for its volume, thereby eliminating the dependence of passage time on size (Fig. 4) (23). By using an average value for population density, which has a variance 100-fold smaller than that of both buoyant mass and volume, we can convert buoyant mass to volume with only minimal error contributions (Fig. 2A, C, Fig. S1) (12, 37). This is an improvement over previous methods, in which we performed the same conversion but starting instead with a volume distribution from a commercial Coulter counter (23, 38). A plot of each data set with a log-log scale is subsequently fit to lines with a fixed slope and variable intercepts (Fig. 4A). The deformability is determined by the ratio of passage times given the same cell volume,
which is acquired from the difference between the two intercepts (green arrow), and is then converted to a percentage (Fig. 4B).

**Fig. 3.** Effect of osmotic stress on passage time and buoyant mass. FL5.12 cells are incubated in hypo- and hyperosmolar media for 30 min before and during the measurement. Passage time and buoyant mass are relatively unaffected by osmotic stress at 250, 350, and 400 mOsm/L. (A) Box plots of passage time scaled by the median of the control (300 mOsm/L). Each data point represents passage time of an individual cell (n = 972-1101). (B) Percentage change in the median of passage time. The median passage time from each condition is normalized by the median of the control. A single point represents one replicate and the green line indicates the mean from multiple replicates (3-6 for each condition). (C) Box plots of buoyant mass scaled by the median of the control. Data shown was measured simultaneously with passage time in A. Each data point represents the buoyant mass of an individual cell. (D) Percentage change in the median buoyant mass. The median buoyant mass from each condition is normalized by the median of the control. A single point represents one replicate and the green line indicates the mean from multiple replicates (3-6 for each condition).
Fig. 4. Determining deformability from passage time by accounting for cell volume. Volume is obtained by converting single cell buoyant mass data using the population average density. (A) Passage time versus volume of the two datasets (from Fig. 3, isoosmotic and hyperosmotic conditions) in a log-log scale are fitted to the linear models (black lines) with a fixed slope and variable intercepts corresponding to the two conditions. The deformability is determined by the ratio of passage times given the same cell volume, which is acquired from the difference between the two intercepts (green arrow). (B) Dependence of deformability (percentage change in passage time based on cell volume) on osmolarity of media. The data used is the same as shown in Fig. 3.

After accounting for volume, we can identify a significant difference between two cells of the same volume exposed to differing values of media osmolarity (-6.1, 8.5, and 21% changes, respectively, for 250, 350 and 400 mOsm/L), which demonstrates that cells become stiffer by hyperosmotic compression and more deformable by hypoosmotic swelling. In other words, though the population of cells as a whole still exhibits similar a passage time to the isoosmotic control (Fig. 3B), the viscoelasticity of an individual cell is changed by osmotic stress (Fig. 4B). The observed decrease in deformability caused by hyperosmotic compression and determined by a longer passage time is consistent with previous studies, one of which links the change to cytoplasmic crowding resulting from water loss (30, 39, 40). The increase in deformability by a hypoosmotic challenge has also been reported (19, 39, 41), but is not consistent among all studies (40), likely due to discrepancies in measurement techniques and cell lines. Our results indicate that volume should be taken into consideration when relating passage time through a constriction to cellular deformability.

**Characterizing cell states by deformability, density, and volume**

When cells are exposed to an osmotic challenge, changes in density or volume correlate with changes in deformability due to the dependence of all three parameters on the
osmolarity of the surrounding fluid (Fig. 2A). However, a general relationship between
density, volume and deformability cannot be established a priori, since cellular activity or
external stimuli can affect cellular composition and cytoskeletal proteins, which can affect
density, volume and deformability independently of each other (1, 12). Similarly, a change
in volume alone cannot predict density or deformability, except when cells change their
volume only by water exchange. Thus, we next sought to investigate representative
relationships of deformability versus density and volume (Fig. 5), which would allow us to
demonstrate how cell states can be characterized by multiple biophysical properties. In
addition to an osmotic challenge, FL5.12 cells were also exposed to the following
conditions: latrunculin B, staurosporine, cycloheximide (1 µg/ml, 10 µg/ml), rapamycin,
and Torin 1. A summary of the effects of these drugs is provided in Table S1 in the
Supporting Material. Density, volume, and deformability were measured using the same
methods as previously described. Passage time accounting for volume is then plotted
versus density (Fig. 5A) and volume (Fig. 5B).

Increases in osmolarity lead to increases in density and decreases in volume and
deformability, represented by the black solid lines in Fig. 5A and 5B. Latrunculin B,
however, leads to an increase in deformability accompanied by a slight increase in density
and insignificant change in volume (green line, Fig. 5A and 5B). A small increase in volume
by latrunculin B has been previously reported (42). This outcome likely results from the
relatively specific behavior of latrunculin B, which complexes with actin monomers to
inhibit actin polymerization (43). These changes may result in a slight change in cellular
water content, due to shifting hydration layers in the proteins; this shift may be sufficient
to change density, but not volume (44).

Treating with staurosporine (red line) maintains the same relationship between density
and deformability as we observe with osmotic stress but with a greater change in
deformability. A cell undergoing apoptosis by staurosporine typically shows an apoptotic
volume decrease and denser cytoplasm, which is consistent with our measurements of
volume and density (45). Staurosporine can also lead to cell cycle arrest (46), inhibit
protein synthesis independently of caspase activation (47), and delocalize myosin II (48).
Therefore, a decrease in deformability after treating with staurosporine may be related to
impaired myosin activity, which may explain why staurosporine leads to a much greater
change in deformability than does hyperosmotic compression.

Treating with 10 µg/ml cycloheximide decreases density similarly to what we observe
under a hypoosmotic condition (250 mOsm/L), but a greater increase in deformability. The
increase in deformability caused by treating with cycloheximide, which inhibits protein
synthesis, is consistent with a previous study (49). Interestingly, the biological effects of
cycloheximide depend on the concentration and cell type. For example, while
cycloheximide is cytotoxic at low concentrations to Jurkat cells, it has no effect on CEM C7
cells at low or high concentrations, even though both are human leukemic cell lines (50).
Moreover, additional studies of different cell types exposed to low levels of cycloheximide
have shown that it may have cytoprotective effects (51). This concentration dependence
may explain the slight discrepancy between the changes to density and volume at 1 µg/mL
and 10 µg/mL (5A and red arrows, Fig. 5B). Since cycloheximide can also induce the elongation or arrest of cell cycle, which may affect the protein content and cell volume (52, 53), the relationship between volume and density is difficult to predict. Thus, while density and volume are inversely correlated at the higher concentration, they are directly correlated at the lower concentration.

Similarly to the case with cycloheximide, we also notice that Torin 1 and rapamycin induce decreases in both density and volume, providing another example of how volume is not always inversely related to density (arrows in Fig. 5B). This outcome may be attributed to the biological effect of Torin 1 and rapamycin, which block mTOR, inducing cell cycle arrest at the G1/S transition and inhibiting protein synthesis (54, 55). Previous studies have shown that cell volume and deformability depend on the cell cycle (10). The slight decrease in population volume may thus be attributed to arrest in S phase. A decrease in density could be due to lower levels of both protein and DNA. In addition, Torin 1 and rapamycin are also known to affect actin polymerization and protein synthesis (56–59). Although previous studies often demonstrated that Torin 1 and rapamycin can inhibit actin polymerization (57), rapamycin can also increase actin polymerization in RBL-2H3 cells (56), suggesting that the slight decrease in cell deformability by these mTOR inhibitors may be induced by the change in actin structure.

We would also like to note that, the drugs used in our study, typically cause a more significant change in deformability than does osmotic stress. For example, staurosporine induces more than a 200% change in passage time, and the deformability of FL5.12 cells is increased by cycloheximide regardless of changes in density or volume. This suggests that the integrity of cytoskeletal structure can play a more important role on deciding deformability than the changes driven by water exchange. However, Zhou et al. previously showed that latrunculin A-induced weakening of the cytoskeletal rigidity is overwhelmed by stiffening of the cytoplasm induced by very high osmotic stress (~ 1000 mOsm/L), suggesting that the change in cytoskeletal structure does not always dominate cell deformability (30).

Conclusion

We have shown the value of measuring multiple biophysical parameters. Although volume is the most commonly identifiable cell size metric, density can reveal additional information for understanding the mechanical properties of cells. Density may represent changes in structure or composition as well as the crowdedness in the cytoplasm which may not be directly related to volume, but may still affect deformability. Grover et al. previously found that measuring density enables the identification of cell states which were not detectable by other cell size metrics, such as volume and mass (12). However, changes to deformability may not necessarily lead to changes in any size metric, particularly if the deformability change is associated with changes to structural proteins. Thus, by combining measurements of deformability and density, we can detect more specific biophysical characteristics that refine our representation of cell state.
Fig. 5. Deformability versus density and volume for various conditions: osmotic challenge (250, 350, 400, 500 mOsm/L), latrunculin B (LatB), staurosporine (STS), 1 µg/ml and 10 µg/ml cycloheximide (CHX), rapamycin (Rap), and Torin 1 (Tor). Changes in deformability, density, and volume after treatments are quantified based on the isomotic control (untreated, 300 mOsm/L) in each experiment. Plots are divided into four quadrants, defined by two grey dotted lines. (A) The percentage change in passage time accounting for volume is plotted versus the change in density. The correlation between changes in deformability and density depends on the mechanism associated with each treatment. (B) The percentage change in passage time accounting for volume is plotted versus the change in volume. Rap, Tor, and CHX (1 µg/ml) are located in different quadrants compared to A (arrows). Vertical error bars represent standard deviation of the mean. Horizontal error bars (density, volume) represent standard error of the mean. All treatments induce a significant change in the density ($p < 0.0001$, Wilcoxon rank sum). All treatments, except Torin 1 ($p = 0.0501$), induce a significant change in the passage time ($p < 0.0001$, linear model). The data used for osmotic challenge is the same data as shown in Fig. 4 and for the other conditions, we measured ~ 200 cells and ~ 1000 cells for density and deformability, respectively.
Author Contributions
S.B., V.C.H., and S.R.M. designed research; S.B. and V.C.H. performed research; S.B. and V.C.H. analyzed data; and S.B., V.C.H., and S.R.M. wrote the paper.

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Supporting Citations
Reference (60) appears in the Supporting Material.
References


