Trends in
Biochemical Sciences

CellPress

Review

A physicochemical perspective on cellular ageing

Sara N. Mouton,[1,*] Arnold J. Boersma,[2] and Liesbeth M. Veenhoff[1,*]

Cellular ageing described at the molecular level is a multifactorial process that leads to a spectrum of ageing trajectories. There has been recent discussion about whether a decline in physicochemical homeostasis causes aberrant phase transitions, which are a driver of ageing. Indeed, the function of all biological macromolecules, regardless of their participation in biomolecular condensates, depends on parameters such as pH, crowding, and redox state. We expand on the physicochemical homeostasis hypothesis and summarise recent evidence that the intracellular milieu influences molecular processes involved in ageing.

Highlights

Ageing is a complex, multifactorial process which cannot be fully explained by molecular mechanisms.

The physicochemical intracellular environment provides a backdrop to all molecular mechanisms.

Yeast replicative ageing is associated with changes in multiple physicochemical parameters, including the pH of the cytosol, vacuole, and cell cortex, as well as redox state and organellar crowding.

Changes in physicochemical parameters impinge on aspects of the molecularly defined hallmarks of ageing, including loss of protein homeostasis, genome instability, and mitochondrial function.

A change in physicochemical parameters during ageing is an aspect that emerges as a potential contributor, or even driver, of age-related diseases.

Exploring the physicochemical perspective of ageing

What molecular mechanisms underlie cellular ageing is one of the major questions remaining unanswered in biology. Several molecular mechanisms implicated in ageing have been identified [1] and categorised into 12 ageing hallmarks [2,3]. However, a unifying molecular theory of ageing is currently lacking [4–6]. Such a single theory may not exist because what extends lifespan dramatically in some species only has a moderate effect on lifespan extension in others [7]. This is, for example, the case with the insulin/IGF-I signalling (IIS) pathway where a single-gene mutation leads to a tenfold increase in the lifespan of *Caenorhabditis elegans* worms [8], whereas the effects in flies and mice vary between 20% and 40% [9–12].

Interestingly, organisms from **isogenic populations** (see Glossary) that live in the same environment can have very different lifespans and ageing phenotypes [13]. This implies that they follow individual ageing trajectories driven by different underlying mechanisms. Thus, although the majority of multicellular and some unicellular organisms are subject to ageing, it is evident that there is not a single molecular pathway that is central to this process. At a molecular level, ageing is indeed a complex multifactorial biological phenomenon that is very challenging to uncover.

A valid question is whether there are complementary ways of describing ageing that would help to understand this process better. A physicochemical perspective may provide such an additional perspective. An essay by Alberti and Hyman in 2016 highlights the importance of the physicochemical parameters of cells and how age-dependent changes in such parameters have the potential to drive aberrant transitions in biomolecular condensates and subsequent loss of intracellular organisation [14]. Such transitions are now well linked to the pathology of age-related neurodegenerative diseases, suggesting that a decline in homeostatic mechanisms and changes in physicochemical parameters might explain several of the observed ageing phenotypes [15].

All biological macromolecules have evolved to function in an environment characterised by parameters such as finite volume, viscosity, **macromolecular crowding**, internal pH, ionic strength, water activity, osmotic pressure, temperature, redox potential, and ATP availability. Cells employ mechanisms that regulate many of these parameters, counteract fluctuations, or repair dysfunction arising from environmental stressors. For example, upon exposure to external stressors such as

[1]European Research Institute for the Biology of Ageing, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[2]Cellular Protein Chemistry, Bijvoet Centre for Biomolecular Research, Faculty of Science, Utrecht University, Utrecht, The Netherlands

*Correspondence:
s.mavrova@rug.nl (S.N. Mouton) and
l.m.veenhoff@rug.nl (L.M. Veenhoff).

suboptimal temperature, proteins unfold and form aggregates which are dealt with by the proteostasis network. Because the proteostasis network is altered in ageing, this renders cellular processes more sensitive to the environment in which they take place (e.g., suboptimal temperature) [16]. In addition, some cellular processes may, by nature, be more sensitive to small changes in environmental conditions; for example, the thermal stability of proteins varies broadly [17]. In the event of weakened, deregulated, or overburdened protective mechanisms, we speculate that the most sensitive processes would be most impacted by fluctuations in the surrounding milieu.

In this review we expand on the physicochemical homeostasis hypothesis and explore broadly how age-related changes in the physicochemical parameters of cells have consequences for cellular physiology. Such a perspective is, at present, only beginning to emerge for the process of replicative ageing in yeast. We therefore primarily focus on yeast replicative ageing and additionally discuss findings from different organisms when relevant.

Yeast replicative ageing

The budding yeast, *Saccharomyces cerevisiae*, is a single-cell organism that reproduces through **asymmetric division**. At the start of its lifespan, a single yeast cell buds off a daughter cell about every 1.5 h. Towards the end of the replicative lifespan, it enters a slow-dividing mode, termed **senescence entry point**, and eventually dies [18]. The average number of daughters produced by a single mother cell is ~25, although there is a large variability within a single population. The number of divisions a mother cell completes determines its **replicative lifespan (RLS)** [19,20]. Yeast cells can also survive in nutrient-depleted conditions without dividing. These cells also age and die after a period of time, which is referred to as the chronological lifespan [21], but this is not the focus of the current review. The lifespan of baker's yeast cells is malleable by genetic interventions, and many genes that expand lifespan in yeast also do so in more complex organisms [22].

During their RLS, mother cells accumulate ageing phenotypes: for instance, cellular and vacuolar sizes increase dramatically, division frequency slows down, and extrachromosomal rDNA circles (ERCs) and protein aggregates accumulate (summarised in [16–22]). Because of asymmetric division, budded-off daughter cells generally do not inherit **ageing factors** (Figure 1A). Their division frequency and lifespan are fully reset; daughter cells are therefore considered to be rejuvenated and born at the replicative age of zero. This asymmetry breaks down late in the lifespan of mother cells when daughters are 'born' with reduced lifespan [23].

Why do yeast cells from isogenic populations have different lifespans? Studies using microfluidic devices [18,24–29] to follow individual yeast cells throughout their life have shown that cells with identical genomes, grown under the same environmental conditions, have different lifespans. This spread in lifespan is, in fact, similar to the spread in human population with its wide variety of genomes and environmental conditions (Figure 1B). The heterogeneity of lifespans may originate from the stochastic nature of biochemical reactions that drive variability of gene expression, which in turn causes phenotypic differences [30]. The most convincing and exciting explanation for the seemingly stochastic nature of yeast replicative ageing currently comes from the finding that ERC excision from the ribosomal RNA gene array (rDNA) on the genome is a stochastic event that sets off an ageing trajectory leading to cell death in a fixed number of divisions [31].

We conclude that, even in the simple baker's yeast system, the genes and molecular pathways involved in ageing so far described can account for only a part of the ageing process, but cannot fully explain it. A physicochemical perspective may provide a complementary view, and we discuss this aspect based on reports addressing pH [32–36], redox potential [32], ATP levels [37], crowding [38,39], and cellular [40] and organellar size [26,33].

Glossary

Ageing factors: damaged and lifespan-limiting cellular components which accumulate in ageing cells. In yeast they are asymmetrically retained in the ageing mother cell.
Asymmetric division: a cell division that produces two unequal cells. In budding yeast the mother cell retains the ageing factors and ages progressively while the daughter cell is 'born' with a full lifespan.
Cellular: the ratio of dry cellular mass to total cell volume, usually expressed in mg/ml.
Excluded volume effect: arises from the fact that two macromolecules cannot occupy the same place at the same time owing to nonspecific steric repulsion. The part of the total volume that cannot be occupied by a particular macromolecule is the excluded volume, and the remaining part is the available volume. The excluded volume effect is present in all cases where the macromolecules freely diffuse and is modulated by attractive or repulsive chemical forces.
Fermentative growth: cultures grown in the abundant presence of glucose, which yeast cells catabolise to obtain energy in the form of ATP.
Isoelectric point (pI): the pH at which the net charge of a protein equals zero. At pH values below their pI, proteins have a positive charge, and above the pI they have a negative charge.
Isogenic population: a population of individuals with identical genomes. Yeast cultures represent an isogenic population because these cultures originate from a single yeast cell.
Macromolecular crowding: refers to the fact that all macromolecules together (protein, RNA, DNA, glycogen, etc.) occupy a significant fraction of the cell volume.
***Petite* phenotype:** the absence of mitochondrial DNA (mtDNA), or mutations in mtDNA or the nuclear genome, that drive loss of mitochondrial membrane potential $\Delta\Psi$ and cause respiratory deficiency. These mutants were named *petite* after the small appearance of their colonies and reduced cell size.
Replicative lifespan (RLS): a measure of the total number of mitotic divisions a mother cell can undergo before death.
Respiratory growth: if glucose is not present in the growth media, yeast use other non-fermentable carbon sources such as ethanol or glycerol. The respiratory chain and the tricarboxylic

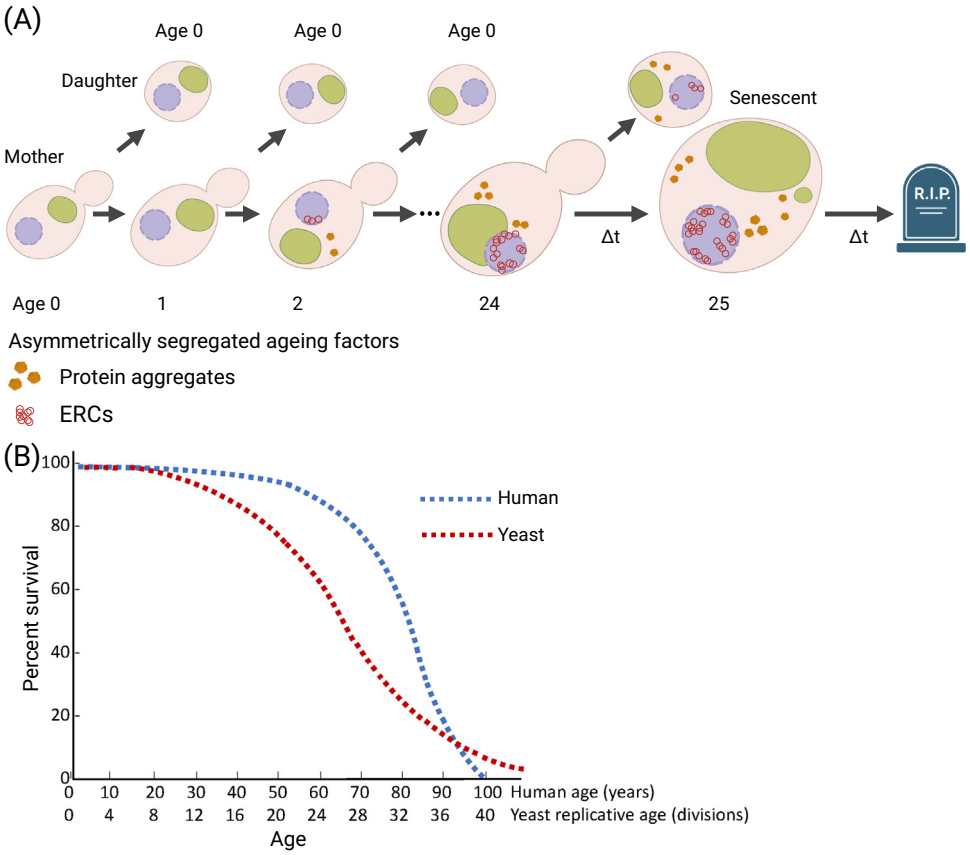

Figure 1. The yeast replicative ageing model. (A) Mitotic divisions in yeast occur through the outgrowth of a daughter cell in a process termed budding. The replicative age of a yeast cell is defined by the number of individual daughter cells that it has produced. Baker's yeast divides asymmetrically such that ageing factors, such as extrachromosomal rDNA circles (ERCs; red circles) and protein aggregates (orange diamonds), are retained in the ageing mother cell. The daughter cell is rejuvenated and has a full replicative lifespan potential. The asymmetry breaks down later in the lifespan when daughter cells are born prematurely aged. Most cells become senescent (stop dividing) before they die; the senescence period can last up to 20–30 h. Yeast cell populations have a wide spread of replicative lifespans, but many laboratory strains have an average lifespan of ~25 divisions. Nucleus and vacuole are indicated in blue and green, respectively. (B) Survival curves of humans (blue, http://www.mortality.org, Survival in 2020) and baker's yeast (red) [126]. Humans are genetically diverse and live under variable conditions, and this contributes to the variability of lifespan. The yeast cells are genetically identical and they live under identical conditions, but their lifespans are also variable.

acid (TCA) cycle in mitochondria are essential for this process.

Senescence entry point: the timepoint when yeast cells enter a slow division mode before they completely stop dividing, become senescent, and eventually die.

pH homeostasis in yeast replicative ageing

The systems that regulate cellular pH

Almost every biological process is pH-dependent, and changes in pH homeostasis have been reported in ageing [32–36]. In addition, cytosolic pH acts as a signal to activate key pathways in ageing – Ras/PKA and TORC1 [41]. We provide a brief introduction to pH regulation and homeostasis in yeast; extensive review of this topic can be found elsewhere [42].

In yeast, two ATP-dependent proton pumps, Pma1 [43,44] and the vacuolar ATPase (V-ATPase) [45], are responsible for active pH regulation in the cytosol [46]. Pma1 is a P_2-type H^+-ATPase that is located at the plasma membrane (PM) and is one of the most abundant proteins there. It translocates protons out of the cytosol at a rate of 1 H^+ per hydrolysed ATP molecule [47]. The vacuolar-type H^+-ATPases are localised on the membranes of organelles in the

secretory pathway and also pump protons out of the cytosol, thus increasing cytosolic pH and acidifying the vacuole (the yeast lysosome), the late Golgi apparatus, and endosomes [45]. Both ATPases are regulated by the availability of glucose and are responsive to cytosolic pH [41]. In addition, metabolites such as free phosphates and glutathione, and millions of ionisable groups on the surface of proteins, provide a strong buffering capacity against changes in cellular pH [33,48].

pH derailment with ageing

All three contributors to pH homeostasis discussed above change in ageing. A whole-cell proteome analysis shows that the components of the V-ATPase become substoichiometric in ageing [49]. The levels of Pma1 increase in ageing cells due to asymmetric inheritance between the mother and the rejuvenated daughter cell [34,50]. The proteome of ageing cells also has a lower **isoelectric point (pI)** [33], implying that the buffering capacity may alter in ageing.

pH values are measured so far with pH-sensitive GFP derivatives or small-molecule dyes. The earliest pH changes occur in the vacuole; indeed, alkalisation of the vacuole of the ageing mother is already observable after three divisions [34,35]. The pH near the PM, at the cell cortex, also changes early in the lifespan, and mother cells have an increasingly alkaline pH [34]. The pH of the cytosol becomes more acidic [32], and following single cells in microfluidic devices throughout the lifespan revealed the kinetics of this change [33]. The cytosolic pH decreases progressively early in ageing, and declines sharply after senescence [33]. Together, this paints a picture in which the cell cortex and vacuole of ageing yeast cells become more alkaline while the cytosol increases in acidity (Figure 2).

How these pH changes are related was best addressed for the PM and the vacuole. The use of mutants in which the expression or activity of Pma1 and V-ATPase was altered allowed measurements of the effects on vacuolar pH. Clearly, the activities of both are connected because reduced Pma1 activity results in a lower vacuolar pH, and the function of the V-ATPase also regulates Pma1 [34,46]. A mechanistic explanation that has been put forward is competition

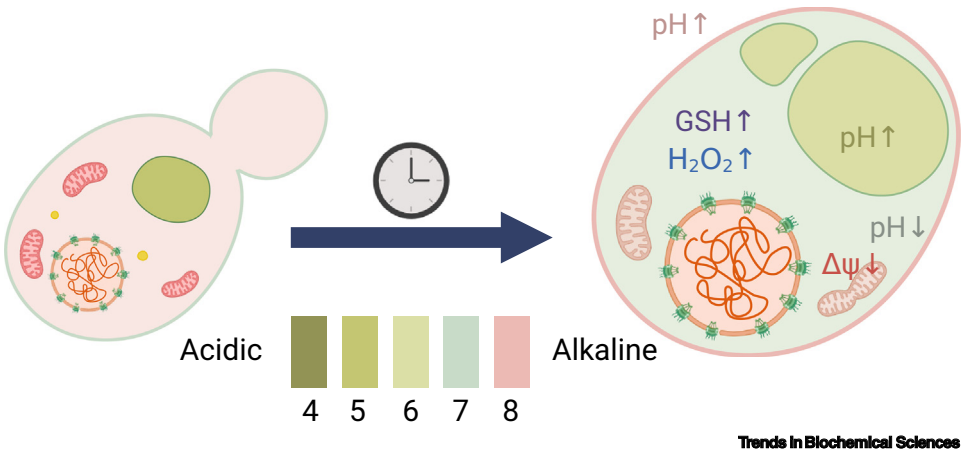

Figure 2. pH and redox status in yeast replicative ageing. The vacuole and cell cortex already become more alkaline (pH increases) at the third division. The cytosol progressively becomes more acidic (pH decreases) during the mitotic lifespan and acidifies sharply after senescence. Cytosolic acidification leads to an increase in the function of glutathione reductase Glr1, thus promoting a more reducing glutathione potential despite increasing hydrogen peroxide levels. The age-related increase in function of the plasma membrane ATPase Pma1 has been proposed to impair vacuolar acidification, which in turn is proposed to be linked to mitochondrial dysfunction and genomic instability.

for protons [34]. However, this is difficult to reconcile with the measured decrease in cytosolic pH [32,33] and the large buffering capacity of the cytosol. An alternative explanation may relate to ATP levels [32]: the function of both proton pumps is fuelled by ATP hydrolysis, and Pma1 may therefore limit the function of the V-ATPase by lowering cellular ATP levels to below a threshold [32]. V-ATPase is indeed sensitive to cellular energy levels, and the complex disassembles rapidly after glucose deprivation [51]. Data from a population-based study shows that ATP levels decrease in mid-aged cells but consecutively increase again [37] (discussed in the ATP section below). How the cortical and cytosolic pH relate has not been experimentally addressed. Simultaneous imaging of all three subcellular locations will be necessary to resolve this dissimilarity in local pH (Box 1).

Can this physicochemical description help to connect multiple ageing hallmarks?

Work by Gottschling and colleagues was one of the first studies connecting the chemical properties of the cell to the hallmarks of ageing – mitochondrial dysfunction and genomic instability [35,52,53]. In an elegant model, pH homeostasis was proposed to be causally related to mitochondrial dysfunction and to the production of iron–sulfur clusters required by many DNA repair enzymes. This model thus connects multiple aspects of biology – protein homeostasis (related to Pma1 and V-ATPase levels, stoichiometry, and asymmetric inheritance), through metabolism (mitochondrial function), to genome instability (production of iron–sulfur clusters). The model is supported by experiments, which show that mitochondrial morphology and membrane potential $\Delta\Psi$ are rescued when vacuole acidity is maintained [35], and that improved mitochondrial function (iron uptake) can rescue the respiratory deficiency in cells with impaired V-ATPase activity [36,54]. Even though there are some inconsistencies that require additional experimental work (Box 1), changes in cellular pH homeostasis appear throughout the lifespan of yeast cells, and improving pH maintenance seems to have the potential to extend lifespan by influencing multiple hallmarks of ageing.

Box 1. Experimental challenges to better resolve the relationship between pH homeostasis, mitochondria, and genome stability

Although a large number of experiments indicate that pH homeostasis is causally involved in ageing and connects the molecular ageing hallmarks of mitochondrial dysfunction and genome instability, there are ambiguities that need to be addressed in future studies. First, the current evidence connecting loss of pH homeostasis to mitochondrial phenotypes derives in part from strains that more readily lose their mitochondrial DNA, the so-called ***petite*** **phenotype** [18,35,54,121,122]. Compared to other strains, such cells may more frequently follow an ageing trajectory that is dominated by the effects of mitochondrial dysfunction. It was previously shown that loss of vacuolar acidity causes mitochondrial dysfunction, most likely through increased cytosolic amino acid concentrations [35]. Recent studies show that loss of vacuolar acidity leads to reduced iron bioavailability [36,54] owing to impaired vacuolar import of amino acids and increased cytosolic cysteine concentrations. High cytosolic cysteine then exerts toxic effects through increased oxidative stress which disrupts iron–sulfur clusters in proteins [54]. Indeed, in vacuole-impaired and aged cells, iron supplementation [36] or overexpression of the amino acid transporter AVT1 can rescue mitochondrial dysfunction and extend lifespan [54]. However, there are other causes of mitochondrial dysfunction in ageing [122], suggesting that the vacuole–mitochondria axis is a major contributor to age-related mitochondrial dysfunction in yeast cells with a particular genetic background, but is unlikely to be the only one. In addition, vacuolar dysfunction likely impacts on ageing cell physiology beyond its effects on mitochondrial fitness [123]. Further experiments will be necessary to prove that lifespan extension through improved vacuolar acidity is mediated by preserved mitochondrial membrane potential in yeast strains that are not S288C-derived. For example, the lifespan-extending effect of improved V-ATPase function should be lost in mutants lacking mitochondrial DNA. In addition, key experiments showing that improved pH homeostasis extends lifespan through reducing age-associated loss of mitochondrial $\Delta\Psi$ and a subsequent decrease in DNA damage have not yet been done. Finally, the reported difference in cortical and cytosolic pH needs to be better experimentally addressed where cortical, cytosolic, and vacuolar pH are monitored simultaneously in single cells at high temporal resolution combined with interventions at the Pma1 and V-ATPase level. Such simultaneous monitoring of multiple parameters at the single-cell level has now become within reach given the dramatically improved methods of imaging and image analysis for tracking thousands of individual cells over their lifespan [126].

Redox homeostasis in yeast replicative ageing

The oxidising or reducing potential of the intracellular environment, termed redox potential, is influenced by several molecular species including reactive oxygen species, antioxidant enzymes, and redox couples. Cellular redox systems are enzymatically regulated and are not in equilibrium with each other [55], and some redox reactions are pH-dependent [56]. Disrupted redox homeostasis is implicated in several age-related diseases such as cancer and cardiovascular and neurodegenerative diseases [57,58]. As discussed, in replicatively aged yeast cells undergoing **fermentative growth** (on glucose), the pH decreases towards the end of the RLS [32,33]. Concomitantly, the ageing cytosol also becomes more reducing [32], as measured using the ratiometric roGFP-Grx1 sensor [59] to determine the glutathione redox potential. The direction of this change may come as a surprise because H_2O_2 levels increase with ageing [32] (Figure 2). The explanation given is that the function of the main glutathione reductase Glr1 reaches its optimal activity in the acidifying ageing cytosol. Thus, as pH decreases, the cytosol of old cells becomes more reduced, rendering old yeast cells more resistant to oxidative stress. This is an excellent example of how age-related changes in chemical parameters of the cell (i.e., pH) regulate protein activity, culminating in a change in redox state and impinging on resistance to oxidative stress. The physiological consequences of such regulation during ageing at the global proteome level are largely unknown.

Another redox couple – NAD^+/NADH – has been shown to play an important role in ageing [60]. The oxidised form of nicotinamide dinucleotide (NAD^+) is consumed by NAD^+-dependent enzymes, most prominently by poly(ADP-ribose) polymerases (PARPs) and sirtuins. Although the exact role of the sirtuin Sir2 in lifespan extension has been debated over the past 20 years, it is clear that its functions are tightly connected to redox homeostasis and ageing [61–63]. Sir2 is a NAD^+-dependent deacetylase that regulates a multitude of cellular functions and acts as a metabolic sensor in the cell [64]. Its functions related to the genome include acetylation of histones and a wide range of transcriptional regulators, as well as repression of recombination in the rDNA locus [62]. Yeast *Δsir2* mutants have a shorter lifespan due to, among other causes, increased accumulation of ERCs [31,62]. In addition, Sir2 is repressed by NADH, and lowering the levels of NADH has the potential to extend lifespan [61]. Finally, yeast cells undergoing **respiratory growth** have longer lifespans, higher division frequency, and enter more slowly into senescence compared to cells grown under fermentative conditions [32]. Although there might be other reasons for these phenomena, it is noteworthy that respiration favours higher NAD^+/NADH ratios [65].

Despite the many open questions, current knowledge of redox state regulation in ageing yeast illustrates connections between pH homeostasis in the cytosol and redox homeostasis that impinge on oxidative damage and genome stability.

ATP levels in yeast replicative ageing

ATP is traditionally thought of as the main energy currency of the cell that fuels many cellular processes. In a very different perspective, more recent findings highlight the ability of ATP to maintain protein solubility [66–68]. In a study from 2017, Patel and colleagues showed that ATP has the characteristics of a biological hydrotrope [66]. How ATP works to solubilise proteins and whether it should be considered to be a hydrotrope or a biological aggregation suppressor has been further explored and debated [69–74]. Regardless of the exact mechanism, the ability of ATP to enhance the solubility of endogenous and pathological proteins [68], to modulate properties of biomolecular condensates [66,75], and its millimolar concentration in the cell (much higher than is required for enzymatic activity) [67] make ATP a crucial parameter for the physicochemical state of the cell. Furthermore, cellular ATP levels have the ability to impact on other physicochemical parameters or/and their effects in the cell [51,69,76,77].

As pH homeostasis declines [32,33] and protein aggregation occurs [78], it is relevant to ask whether ATP levels are stable during ageing. Currently, single-cell data on ATP levels in RLS are not available. However, a population-based metabolome analysis of batch-aged yeast cells grown in minimal media shows a substantial decrease in ATP of up to 80% in mid-aged cells, followed by an increase towards the end of lifespan [37]. In such population-based studies it is difficult to judge whether these changes occur in cells during the mitotic phase of the RLS or after senescence. In addition, the late-life increase in ATP levels in these data may represent a long-lived subset of cells. From single-cell studies we know that such cells do not enter senescence and generally remain healthy for the entirety of their lifespan [29,33]. In contrast to replicative ageing, a study on chronological ageing showed that ATP levels are maintained [79].

In mammals, the ATP levels in aged brains from an Alzheimer's disease (AD) mouse model are reduced compared to wild-type mice [80], and high ATP concentrations in the eye lens are necessary to keep the high protein content soluble [69,77]. Interestingly, aggregation-induced cataractogenesis is a common condition associated with ageing, and ATP levels in this specific organ might decrease in an age-dependent manner. We speculate that a decrease in the ATP concentration will have widespread effects on cellular function through its solubilising effects and by fuelling enzymatic conversions including disaggregases and proton pumps, and it may therefore connect to all hallmarks of ageing.

Crowding homeostasis and yeast replicative ageing

Understanding macromolecular crowding and cellular density

Macromolecular crowding is an inherent property of the cell. Cells are highly crowded with macromolecules which occupy up to 40% of the total cell volume [81,82]. Single molecular species do not occur at very high concentrations in cells, but all macromolecules together (protein, RNA, DNA, glycogen) occupy a significant fraction of the cell volume. The crowding effect is also known as the **excluded volume effect** which arises from the fact that two macromolecules cannot occupy the same place at the same time owing to nonspecific steric repulsion. How much of the total volume is available to a particular solute species depends on its concentration, size, and shape relative to the rest of macromolecules occupying the same space (termed background species) [83]. A relevant explanation of crowding effects in biology can be found elsewhere [84].

Cellular density is a term that is related to crowding and it refers to the ratio of dry cellular mass to total cell volume. Various methods are available to estimate crowding or density levels. For density measurements, perhaps the best and most direct method is refractive index tomography, although other methods have been applied [85,86]. Macromolecular crowding can be estimated from diffusion coefficients with labelled dextrans [87] and globular proteins [88], total biopolymer fractions [89] or using Förster resonance energy transfer (FRET)-based sensors [90]. Crowding on the scale between 10 and 100 nm was termed mesoscale crowding [38], the degree and effects of which have been determined by tracking genetically encoded fluorescent foreign particles with sizes between 20 and 100 nm [38,76,91,92]. Exposing cells to hyper- or hypo-osmotic conditions is the most accessible way to induce a change in macromolecular crowding, and a hyperosmotic shock leads to intracellular water loss and cell shrinkage, thus increasing crowding. In addition to osmotic stress, it has been inferred that mechanical pressure [93], carbon starvation [92,94], modulation of the mTORC1 pathway [38], and gene dosage [39] can alter crowding in yeast.

How is crowding regulated in cells?

Macromolecular crowding plays an essential role in the process of phase separation [95], and mismanaged phase-separated compartments have been implicated in many age-related

diseases [15]. However, how eukaryotic cells regulate crowding, which genes are involved in its regulation, and the range of optimal crowding levels remain largely unknown. Evidence from several recent studies shows that crowding-induced condensate formation can be part of the mechanism for regulating crowding homeostasis in mammalian cells [96–99]. Most recently, it was shown that WNK1 [with no lysine (K)1] forms condensates upon hyperosmotic stress [96]. Observable cluster formation results in the regulation of ionic concentrations and cell volume, making WNK1 a sensor and a regulator of macromolecular crowding. WNKs are a unique type of kinases that are conserved in multicellular organisms but are not present in most unicellular eukaryotes such as yeast. All cells down to prokaryotes, however, have the ability to restore cell volume and osmolyte imbalances after exposure to hyperosmotic conditions [100,101]. Altogether, it is likely that crowding is genetically regulated across different organisms.

Crowding derailment with ageing

What do we currently know about the homeostasis of crowding in ageing yeast cells? A study from Neurohr and colleagues [39] addressed cellular density in cell-cycle arrested and aged yeast cells, as well as in senescent human fibroblasts. This study reports that when yeast cells grow beyond a particular volume (>200 fl) they suffer dilution of the cytoplasm, which leads to a reduced transcriptional and translational capacity and difficulties in re-entering the cell cycle. This relationship between cell size, density, and the cell cycle is proposed to also apply to ageing yeast cells as they also reach this proposed critical volume threshold of 200 fl and also have reduced cell density. Although this is an attractive interpretation, there may be additional considerations to take into account to fully resolve the relationship between cellular density and ageing (Box 2). We followed single yeast cells in a microfluidic device [24], expressing a FRET-based sensor which reports on macromolecular crowding [33,90,102]. This showed that, although macromolecular crowding on average remains the same between the young and old cell population, there is an increased spread in crowding levels in old cells. We observed a 4% increase in crowding read-out in short-living cells, whereas longer-living cells maintained their crowding levels [33]. Compared to the ~25% increase induced by severe osmotic shock, we speculate that 4% may actually be a significant change to the physiology of the cells. Further characterisation of the dynamic range of the crowding sensor will be necessary to confirm the scope of such changes.

Overall, a description of crowding in ageing is beginning to appear, but a definitive answer to the question of how crowding is regulated and how it plays a role in the molecular aspects of ageing

Box 2. Experimental challenges to better resolve the relationship between cell size, density and senescence entry

A recent study has linked increased cell size to **cellular density** and entry into senescence in both yeast and mammalian cells [39]. In this study, the authors performed a series of experiments showing that yeast cells larger than a threshold of 200 fl have a lower density, have difficulties in re-entering the cell cycle, and are inefficient in mounting transcriptional responses, phenotypes that are also characteristic of aged cells. Indeed, aged cells that become larger than 200 fl are senescent. The hypothesis is that DNA becomes limiting in aged cells and, as a result, the cytoplasm becomes diluted. However, interpreting cytosolic volume from cell size is complex because large and fragmented vacuoles of old cells occupy a significant fraction of the cell volume [26,33], potentially leading to an overestimation of cytosolic volume. Furthermore, aged cells have a larger fat content [33,124,125] – lipid droplets increase 7.2-fold in replicatively aged yeast cells [33,125] – and the contributions of vacuoles and lipid droplets that are low-density structures [48] will lead to an underestimation of the cytosolic density. Several experiments could provide answers. Accurate cytosolic volume measurements in a large ageing cell population at high temporal resolution could pinpoint whether increasing cytosolic volume precedes entry into senescence. In addition, crowding measurements in aged cells were so far only performed with a FRET sensor [33]. Single-particle tracking of fluorescently labeled foreign particles such as genetically encoded multimeric nanoparticles (GEMs) [38] or viral non-structural protein assemblies GFP-μNS [76,91] may also be used in ageing. However, owing to the organellar crowding that occurs in ageing [37], such experiments may report on confinement rather than on crowding.

awaits further experimentation. Uncovering which mechanisms regulate crowding will be an important first step.

Cellular and organellar volume

Volume derailment in aging

Differences in cell size and its regulation have been topics of interest in the field of cell biology for more than a century [103]. Extensive reviews of recent findings on cell size regulation in yeast and other organisms can be found elsewhere [104,105]. Cytosolic volume has so far been mostly extrapolated directly from the cell volume, without taking into account the contribution of other organelles occupying intracellular space. In the case of yeast, a large vacuole is one of the most prominent features of aged cells. Other types of cells have different subcellular compartments that take up cytoplasmic volume, such as yolk in oocytes or large lipid droplets in adipocytes. Thus, cytosolic volumes have rarely been estimated with the necessary precision in ageing research or otherwise.

Vacuoles and nuclei of aged yeast cells grow larger such that the cell becomes more crowded with organelles, which we termed 'organellar crowding' [33]. Based on our estimates we find that the cytosolic volume, relative to total cell volume, decreases by 25% in mid-aged cells [33]. The exact impact of the relative increase of membrane surface area was not studied, but may include the formation of aberrant organellar membrane contact sites. The smaller distance between organellar membranes is also predicted to particularly slow down the diffusion of larger cellular components and particles such as messenger ribonucleoproteins (mRNPs) and lipid droplets. There is generally a strong correlation between nuclear and cellular size [106–108], and ageing yeast cells are not an exception [33]. However, it is predominantly vacuolar volume that increases with ageing. Based on studies on yeast mutants [109], we speculate that the growing vacuoles could be a driver of increasing cell size with ageing.

Connection to other ageing hallmarks

The currently described changes in cell size, macromolecular crowding, and organellar crowding are cartooned in Figure 3. Future studies will need to clarify the impact of these changes on molecular aspects of yeast replicative ageing. We speculate that increased organellar crowding [33] may intersect with the loss of vacuolar acidity and TORC1 signalling, as follows. Early in ageing, mTOR activity could contribute to loss of vacuolar function, leading to vacuolar expansion [110], and vacuolar expansion leads to increased organellar crowding [33]. This may induce more PM–vacuole contacts, which in mammals are known to promote high mTORC1 activity [111,112]. The increased mTOR activity then further inhibits vacuole acidification through V-ATPase disassembly [110]. Such TOR hyperactivity is a mechanism universal to cellular ageing across species [113].

In humans, it has been shown that myocytes in men but not women grow much larger to compensate for the age-related decrease in myocyte numbers and thus maintain ventricular wall thickness [114,115]. Cardiovascular disease is one of the most prevalent causes of death in ageing men. In addition, human and murine haematopoietic stem cells (HSCs) increase in size during ageing, and enlarged HSCs have decreased stem cell potential [116].

Overall, volume alterations are a prominent and widespread phenotype of aged cells in different tissues and species, but the physiological consequences of volume alterations have not yet been extensively studied. Future studies will need to address questions specifically about cytosolic volume, limiting pools of cytoplasmic components, and aberrant membrane contact sites.

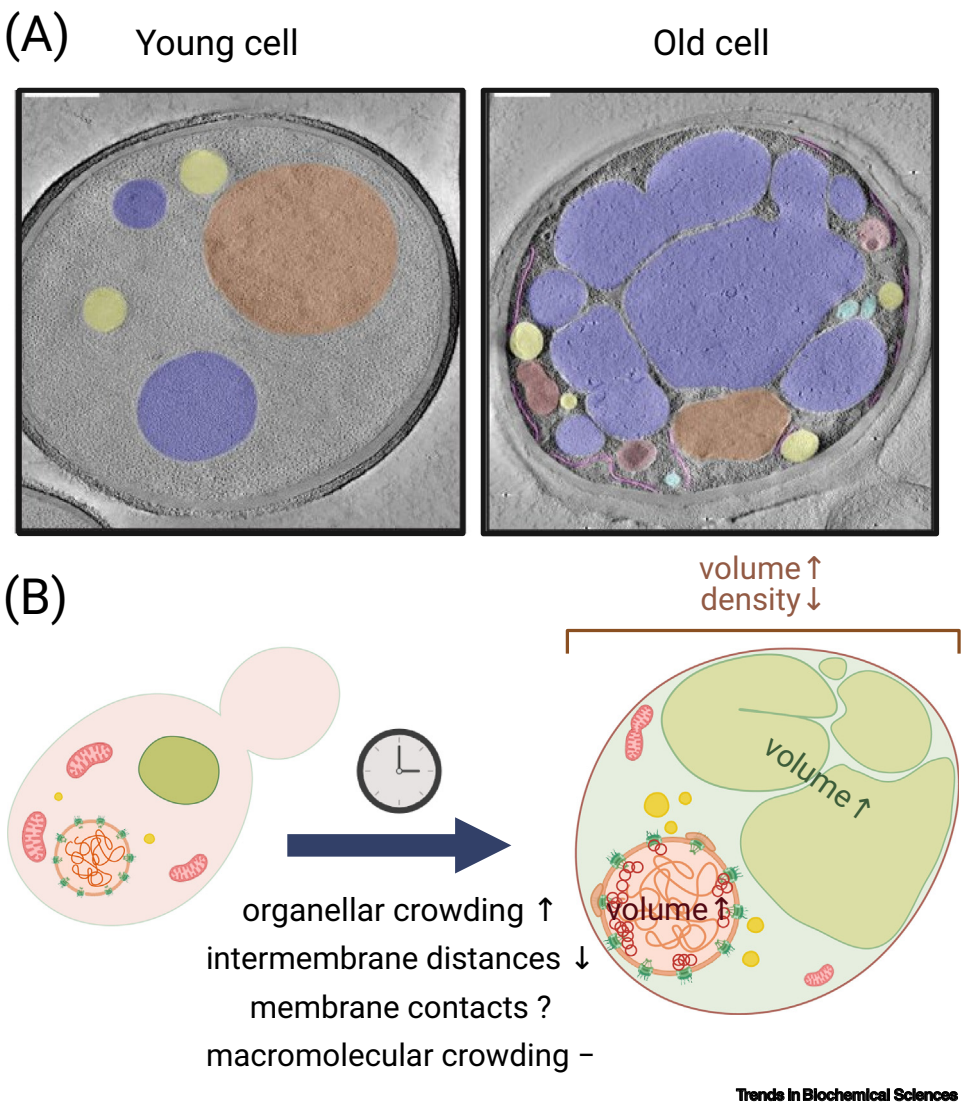

Figure 3. Volume and crowding in yeast replicative ageing. (A) Images of a young yeast cell and an old yeast cell that shows a particularly strong increase in organellar crowding obtained through correlative light and electron microscopy (CLEM). Reproduced, with permission, from [33]. The old cell comes from a population with a replicative age of ~13 divisions. Nuclei (N) are coloured in orange, vacuoles (V) in blue, mitochondria (M) in red, and lipid droplets (LDs) in yellow. Scale bars are 500 nm. (B) In yeast replicative ageing the total cell volume steadily increases. In aged cells most of the intracellular space is occupied by large and fragmented vacuoles, which results in an increase in organellar crowding and possibly extended membrane contacts. Macromolecular crowding is maintained remarkably well in the majority of cells. Oversized old cells have reduced cellular density, possibly owing to the accumulation of low-density structures such as vacuoles, membranes, and lipid droplets.

Concluding remarks

In this review we provide a summary of the important physicochemical parameters for cellular function and examine the experimental evidence for age-related changes in these parameters. Theoretically, a change in such parameters will have a global impact on cellular physiology, and indeed in several cases the data support this.

Outstanding questions

Are age-related changes in the physicochemical aspects of aging, as observed in yeast, conserved in human cells?

How do single-cell perspectives on yeast age-related changes in organellar crowding, viscosity, ionic strength, osmotic pressure, redox potential, and ATP availability complement those already provided for pH and macromolecular crowding?

How do the age-related alterations in physicochemical parameters interrelate with each other?

Can the physicochemical perspective more broadly unite seemingly unrelated molecular ageing phenotypes?

An overview of the physicochemical parameters in the context of yeast replicative ageing are cartooned in Figure 4. In short, vacuoles first become excessively large [26,33], more alkaline, and dysfunctional [35,36]. Whereas the vacuole loses acidity early in ageing, the cytosol acidifies moderately during the mitotic lifespan and sharply after senescence [33]. This promotes the function of Glr1, thus creating a more reducing environment in replicatively aged fermenting cells [32]. The decline of vacuolar acidity was proposed to drive loss of mitochondrial $\Delta\Psi$ through cysteine-mediated toxicity [54], and mitochondrial dysfunction in turn is implicated in nuclear genome instability [35,36]. Old cells grow larger, and growth rate correlates negatively with lifespan [40], but the relationship between this growth in cell size, the crowding or density of the cytosol, and ageing is not fully resolved [33,39].

In the future, it will be relevant to address other important parameters at a single-cell level, namely cytosolic ATP, viscosity, and ionic strength in ageing yeast cells, as well as to obtain precise measurements of cytosolic volume. These parameters impinge on many fundamental cellular properties and functions such as the formation of membraneless organelles, phase transition, diffusion, and metabolism.

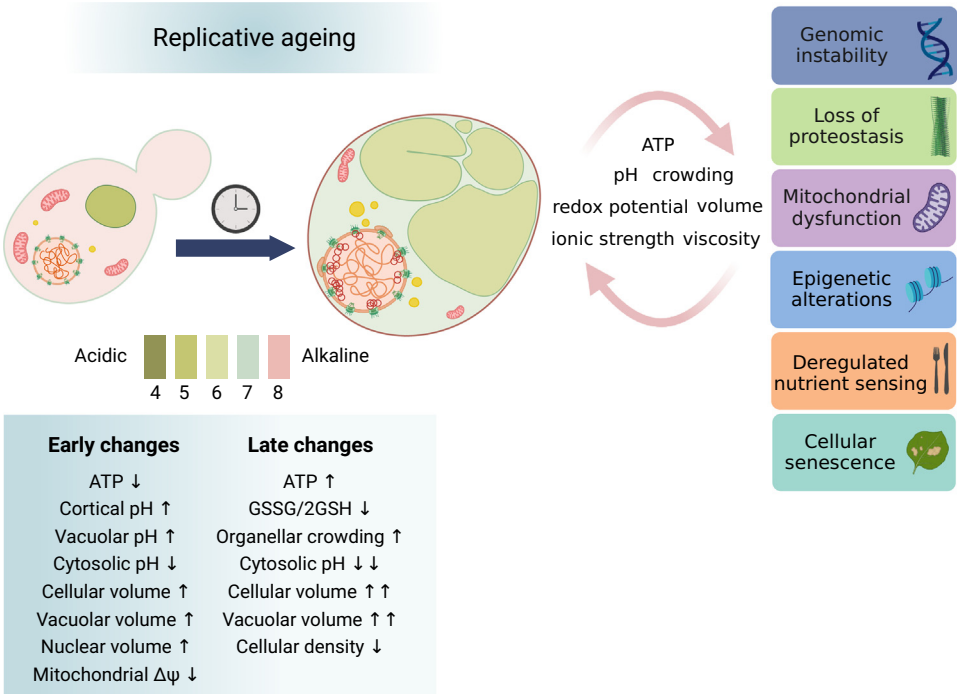

Figure 4. The physicochemical aspect of ageing – an overview. Graphical representation of currently known age-related changes in key physicochemical parameters and how they interplay with the molecularly defined ageing hallmarks. In yeast replicative ageing, vacuoles become excessively large, more alkaline, and dysfunctional, whereas the cytosol becomes more acidic. The vacuole loses acidity early in ageing, but the cytosol acidifies moderately during the mitotic lifespan and the pH decreases sharply after the cells become senescent. This cytosolic acidification contributes to a more reducing environment in replicatively aged cells. The decline of vacuolar acidity and function has been proposed to drive loss of mitochondrial membrane potential ($\Delta\Psi$) through cysteine toxicity; mitochondrial dysfunction, in turn, is implicated in decreasing nuclear genome stability maintenance [52]. The total cell volume increases dramatically; however, most of the intracellular space is occupied by large and fragmented vacuoles. Macromolecular crowding, on the other hand, is maintained remarkably well in the majority of cells. However, oversized old cells have reduced cellular density [39], possibly due to the accumulation of low-density structures such as vacuoles, membranes, and lipid droplets. Abbreviations: GSH, reduced glutathione; GSSG, oxidised glutathione.

Are the physicochemical aspects of ageing valid for the study of human ageing? It is likely that different parameters are derailed in different types of cells and tissues. For example, cells of the eye lens might struggle with a decline in ATP levels [69,77], whereas neurons might be subject to lysosomal dysfunction [117]. Ageing stem cells also increase in cell size [116], and the maintenance of stemness is dependent on functional lysosomes and mitochondria [118–120].

From the relatively small number of studies reviewed here we can conclude that it is important to consider intracellular physicochemical parameters, especially in the context of ageing, because homeostatic mechanisms decline. This will provide a more global approach than the conservative strategy of looking at separate molecules and pathways. Furthermore, incorporating a physicochemical description into previously established ageing mechanisms has the potential to unite the currently rich but fragmented knowledge in the ageing field. Indeed, changes in the physical and chemical parameters of the cell will impinge on all hallmarks of ageing, and mapping this using novel experimental approaches could be transformative for the field (see Outstanding questions).

Acknowledgments

We thank Anton Steen for discussion and feedback. All figures were created with BioRender.com

Declaration of interests

The authors declare no competing interests.

References

1. Kenyon, C.J. (2010) The genetics of ageing. *Nature* 464, 504–512
2. López-Otín, C. *et al.* (2013) The hallmarks of aging. *Cell* 153, 1194–1217
3. López-Otín, C. *et al.* (2023) Hallmarks of aging: an expanding universe. *Cell* 186, 243–278
4. Blagosklonny, M.V. (2010) Why men age faster but reproduce longer than women: mTOR and evolutionary perspectives. *Aging (Albany NY)* 2, 265–273
5. Magalhães, J. and Church, G. (2006) Cells discover fire: employing reactive oxygen species in development and consequences for aging. *Exp. Gerontol.* 41, 1–10
6. Gems, D. and de Magalhães, J.P. (2021) The hoverfly and the wasp: a critique of the hallmarks of aging as a paradigm. *Ageing Res. Rev.* 70, 101407
7. Vijg, J. and Campisi, J. (2008) Puzzles, promises and a cure for ageing. *Nature* 454, 1065–1071
8. Ayyadevara, S. *et al.* (2008) Remarkable longevity and stress resistance of nematode PI3K-null mutants. *Aging Cell* 7, 13–22
9. Blüher, M. *et al.* (2003) Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science* 299, 572–574
10. Selman, C. *et al.* (2008) Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. *FASEB J.* 22, 807–818
11. Broughton, S.J. *et al.* (2005) Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands. *Proc. Natl. Acad. Sci. U. S. A.* 102, 3105–3110
12. Bartke, A. (2005) Minireview: role of the growth hormone/insulin-like growth factor system in mammalian aging. *Endocrinology* 146, 3718–3723
13. Kirkwood, T.B.L. *et al.* (2005) What accounts for the wide variation in life span of genetically identical organisms reared in a constant environment? *Mech. Ageing Dev.* 126, 439–443
14. Alberti, S. and Hyman, A.A. (2016) Are aberrant phase transitions a driver of cellular aging? *BioEssays* 38, 959–968
15. Alberti, S. and Hyman, A.A. (2021) Biomolecular condensates at the nexus of cellular stress, protein aggregation disease and ageing. *Nat. Rev. Mol. Cell Biol.* 22, 196–213
16. Hipp, M.S. *et al.* (2019) The proteostasis network and its decline in ageing. *Nat. Rev. Mol. Cell Biol.* 20, 421–435
17. Leuenberger, P. *et al.* (2017) Cell-wide analysis of protein thermal unfolding reveals determinants of thermostability. *Science* 355, eaai7825
18. Fehrmann, S. *et al.* (2013) Aging yeast cells undergo a sharp entry into senescence unrelated to the loss of mitochondrial membrane potential. *Cell Rep.* 5, 1589–1599
19. Steinkraus, K.A. *et al.* (2008) Replicative aging in yeast: the means to the end. *Annu. Rev. Cell Dev. Biol.* 24, 29–54
20. Crane, M.M. and Kaeberlein, M. (2018) The paths of mortality: how understanding the biology of aging can help explain systems behavior of single cells. *Curr. Opin. Syst. Biol.* 8, 25–31
21. Longo, V.D. *et al.* (2012) Replicative and chronological aging in *Saccharomyces cerevisiae*. *Cell Metab.* 16, 18–31
22. Wasko, B.M. and Kaeberlein, M. (2014) Yeast replicative aging: a paradigm for defining conserved longevity interventions. *FEMS Yeast Res.* 14, 148–159
23. Kennedy, B.K. *et al.* (1994) Daughter cells of *Saccharomyces cerevisiae* from old mothers display a reduced life span. *J. Cell Biol.* 127, 1985–1993
24. Crane, M.M. *et al.* (2014) A microfluidic system for studying ageing and dynamic single-cell responses in budding yeast. *PLoS One* 9, e100042
25. Koschwanez, J. *et al.* (2005) Automated lifetime analysis of a single yeast cell. In *IEEE International Conference on Automation Science and Engineering 2005*, pp. 13–18, IEEE
26. Lee, S.S. *et al.* (2012) Whole lifespan microscopic observation of budding yeast aging through a microfluidic dissection platform. *Proc. Natl. Acad. Sci. U. S. A.* 109, 4916–4920
27. Xie, Z. *et al.* (2012) Molecular phenotyping of aging in single yeast cells using a novel microfluidic device. *Aging Cell* 11, 599–606
28. Liu, P. *et al.* (2015) Yeast replicator: a high-throughput multiplexed microfluidics platform for automated measurements of single-cell aging. *Cell Rep.* 13, 634–644
29. Jacquel, B. *et al.* (2021) Monitoring single-cell dynamics of entry into quiescence during an unperturbed life cycle. *eLife* 10, e73186
30. Elowitz, M.B. *et al.* (2002) Stochastic gene expression in a single cell. *Science* 297, 1183–1186
31. Morlot, S. *et al.* (2019) Excessive rDNA transcription drives the disruption in nuclear homeostasis during entry into senescence in budding yeast. *Cell Rep.* 28, 408–422

32. Knieß, R.A. and Mayer, M.P. (2016) The oxidation state of the cytoplasmic glutathione redox system does not correlate with replicative lifespan in yeast. *NPJ Aging Mech. Dis.* 2, 16028
33. Mouton, S.N. *et al.* (2020) A physicochemical perspective of aging from single-cell analysis of pH, macromolecular and organellar crowding in yeast. *eLife* 9, e54707
34. Henderson, K.A. *et al.* (2014) Mother–daughter asymmetry of pH underlies aging and rejuvenation in yeast. *eLife* 3, e03504
35. Hughes, A.L. and Gottschling, D.E. (2012) An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast. *Nature* 492, 261–265
36. Chen, K.L. *et al.* (2020) Loss of vacuolar acidity results in iron-sulfur cluster defects and divergent homeostatic responses during aging in *Saccharomyces cerevisiae*. *Geroscience* 42, 749–764
37. Leupold, S. *et al.* (2019) *Saccharomyces cerevisiae* goes through distinct metabolic phases during its replicative lifespan. *eLife* 8, e41046
38. Delarue, M. *et al.* (2018) mTORC1 controls phase separation and the biophysical properties of the cytoplasm by tuning crowding. *Cell* 174, 338–349
39. Neurohr, G.E. *et al.* (2019) Excessive cell growth causes cytoplasm dilution and contributes to senescence. *Cell* 176, 1083–1097
40. Janssens, G.E. and Veenhoff, L.M. (2016) The natural variation in lifespans of single yeast cells is related to variation in cell size, ribosomal protein, and division time. *PLoS One* 11, e0167394
41. Dechant, R. *et al.* (2014) Cytosolic pH regulates cell growth through distinct GTPases, Arf1 and Gtr1, to promote Ras/PKA and TORC1 activity. *Mol. Cell* 55, 409–421
42. Orij, R. *et al.* (2011) Intracellular pH is a tightly controlled signal in yeast. *Biochim. Biophys. Acta* 1810, 933–944
43. Ferreira, T. *et al.* (2001) The yeast Pma1 proton pump: a model for understanding the biogenesis of plasma membrane proteins. *J. Biol. Chem.* 276, 29613–29616
44. Serrano, R. *et al.* (1986) Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+ + K^+), K^+- and Ca^{2+}-ATPases. *Nature* 319, 689–693
45. Kane, P.M. (2006) The where, when, and how of organelle acidification by the yeast vacuolar H^+-ATPase. *Microbiol. Mol. Biol. Rev.* 70, 177–191
46. Martínez-Muñoz, G.A. and Kane, P. (2008) Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. *J. Biol. Chem.* 283, 20309–20319
47. Perlin, D.S. *et al.* (1986) H^+ATP stoichiometry of proton pumps from *Neurospora crassa* and *Escherichia coli*. *Arch. Biochem. Biophys.* 248, 53–61
48. Milo, R. and Phillips, R. (2015) *Cell Biology by the Numbers*, Garland Science
49. Janssens, G.E. *et al.* (2015) Protein biogenesis machinery is a driver of replicative aging in yeast. *eLife* 4, e08527
50. Thayer, N.H. *et al.* (2014) Identification of long-lived proteins retained in cells undergoing repeated asymmetric divisions. *Proc. Natl. Acad. Sci. U. S. A.* 111, 14019–14026
51. Parra, K.J. and Kane, P.M. (1998) Reversible association between the V1 and V0 domains of yeast vacuolar H^+-ATPase is an unconventional glucose-induced effect. *Mol. Cell. Biol.* 18, 7064–7074
52. Veatch, J.R. *et al.* (2009) Mitochondrial dysfunction leads to nuclear genome instability via an iron–sulfur cluster defect. *Cell* 137, 1247–1258
53. Gottschling, D.E. and Nyström, T. (2017) The upsides and downsides of organelle interconnectivity. *Cell* 169, 24–34
54. Hughes, C.E. *et al.* (2020) Cysteine toxicity drives age-related mitochondrial decline by altering iron homeostasis. *Cell* 180, 296–310
55. Jones, D.P. (2006) Redefining oxidative stress. *Antioxid. Redox Signal.* 8, 1865–1879
56. Warren, J.J. and Mayer, J.M. (2015) Moving protons and electrons in biomimetic systems. *Biochemistry* 54, 1863–1878
57. Waris, G. and Ahsan, H. (2006) Reactive oxygen species: role in the development of cancer and various chronic conditions. *J. Carcinog.* 5, 14
58. Go, Y.-M. and Jones, D.P. (2011) Cysteine/cystine redox signaling in cardiovascular disease. *Free Radic. Biol. Med.* 50, 495–509
59. Gutscher, M. *et al.* (2008) Real-time imaging of the intracellular glutathione redox potential. *Nat. Methods* 5, 553–559
60. Ying, W. (2008) NAD^+/NADH and $NADP^+$/NADPH in cellular functions and cell death: regulation and biological consequences. *Antioxid. Redox Signal.* 10, 179–206
61. Lin, S.-J. *et al.* (2004) Calorie restriction extends yeast life span by lowering the level of NADH. *Genes Dev.* 18, 12–16
62. Kaeberlein, M. *et al.* (1999) The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev.* 13, 2570–2580
63. Lu, S.-P. and Lin, S.-J. (2010) Regulation of yeast sirtuins by NAD^+ metabolism and calorie restriction. *Biochim. Biophys. Acta* 1804, 1567–1575
64. Houtkooper, R.H. *et al.* (2012) Sirtuins as regulators of metabolism and healthspan. *Nat. Rev. Mol. Cell Biol.* 13, 225–238
65. Bakker, B. (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 25, 15–37
66. Patel, A. *et al.* (2017) ATP as a biological hydrotrope. *Science* 356, 753–756
67. Sridharan, S. *et al.* (2019) Proteome-wide solubility and thermal stability profiling reveals distinct regulatory roles for ATP. *Nat. Commun.* 10, 1155
68. Takaine, M. *et al.* (2022) High and stable ATP levels prevent aberrant intracellular protein aggregation in yeast. *eLife* 11, e67659
69. He, Y. *et al.* (2020) ATP antagonizes the crowding-induced destabilization of the human eye-lens protein γS-crystallin. *Biochem. Biophys. Res. Commun.* 526, 1112–1117
70. Kang, J. *et al.* (2019) A unified mechanism for LLPS of ALS/FTLD-causing FUS as well as its modulation by ATP and oligonucleic acids. *PLoS Biol.* 17, e3000327
71. Pal, S. and Paul, S. (2020) ATP controls the aggregation of Aβ 16–22 peptides. *J. Phys. Chem. B* 124, 210–223
72. Kurisaki, I. and Tanaka, S. (2019) ATP converts Aβ42 oligomer into off-pathway species by making contact with its backbone atoms using hydrophobic adenosine. *J. Phys. Chem. B* 123, 9922–9933
73. Mehringer, J. *et al.* (2021) Hofmeister versus Neuberg: is ATP really a biological hydrotrope? *Cell Rep. Phys. Sci.* 2, 100343
74. Sarkar, S. and Mondal, J. (2021) Mechanistic insights on ATP's role as a hydrotrope. *J. Phys. Chem. B* 125, 7717–7731
75. Marini, G. *et al.* (2020) Reorganization of budding yeast cytoplasm upon energy depletion. *Mol. Biol. Cell* 31, 1232–1245
76. Munder, M.C. *et al.* (2016) A pH-driven transition of the cytoplasm from a fluid- to a solid-like state promotes entry into dormancy. *eLife* 5, e09347
77. Greiner, J.V. and Glonek, T. (2020) Hydrotropic function of ATP in the crystalline lens. *Exp. Eye Res.* 190, 107862
78. Erjavec, N. *et al.* (2007) Accelerated aging and failure to segregate damaged proteins in Sir2 mutants can be suppressed by overproducing the protein aggregation-remodeling factor Hsp104p. *Genes Dev.* 21, 2410–2421
79. Choi, J.-S. and Lee, C.-K. (2013) Maintenance of cellular ATP level by caloric restriction correlates chronological survival of budding yeast. *Biochem. Biophys. Res. Commun.* 439, 126–131
80. Zhang, C. *et al.* (2015) Characterization of ATP alternations in an Alzheimer's disease transgenic mouse model. *J. Alzheimers Dis.* 44, 375–378
81. Zimmerman, S.B. and Trach, S.O. (1991) Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of *Escherichia coli*. *J. Mol. Biol.* 222, 599–620
82. Spitzer, J. and Poolman, B. (2013) How crowded is the prokaryotic cytoplasm? *FEBS Lett.* 587, 2094–2098
83. Minton, A.P. (2001) The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J. Biol. Chem.* 276, 10577–10580
84. Minton, A.P. (2006) Macromolecular crowding. *Curr. Biol.* 16, R269–R271
85. Model, M.A. and Petruccelli, J.C. (2018) Intracellular macromolecules in cell volume control and methods of their quantification. In *Current Topics in Membranes*. *Cell Volume Regulation* (Vol. 81) (Levitane, I. *et al.*, eds), pp. 237–289, Elsevier

86. Neurohr, G.E. and Amon, A. (2020) Relevance and regulation of cell density. *Trends Cell Biol.* 30, 213–225
87. Weiss, M. *et al.* (2004) Anomalous subdiffusion is a measure for cytoplasmic crowding in living cells. *Biophys. J.* 87, 3518–3524
88. Arrio-Dupont, M. *et al.* (2000) Translational diffusion of globular proteins in the cytoplasm of cultured muscle cells. *Biophys. J.* 78, 901–907
89. Cayley, S. *et al.* (1991) Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity. Implications for protein–DNA interactions in vivo. *J. Mol. Biol.* 222, 281–300
90. Boersma, A.J. *et al.* (2015) A sensor for quantification of macromolecular crowding in living cells. *Nat. Methods* 12, 227–229
91. Parry, B.R. *et al.* (2014) The bacterial cytoplasm has glass-like properties and is fluidized by metabolic activity. *Cell* 156, 183–194
92. Joyner, R.P. *et al.* (2016) A glucose-starvation response regulates the diffusion of macromolecules. *eLife* 5, e09376
93. Alric, B. *et al.* (2022) Macromolecular crowding limits growth under pressure. *Nat. Phys.* 18, 411–416
94. Xie, Y. *et al.* (2023) Increased mesoscale diffusivity in response to acute glucose starvation. *MicroPubl. Biol* Published online February 21, 2023. http://dx.doi.org/10.17912/micropub.biology.000729
95. Woodruff, J.B. *et al.* (2017) The centrosome is a selective condensate that nucleates microtubules by concentrating tubulin. *Cell* 169, 1066–1077
96. Boyd-Shiwarski, C.R. *et al.* (2022) WNK kinases sense molecular crowding and rescue cell volume via phase separation. *Cell* 185, 4488–4506
97. Watanabe, K. *et al.* (2021) Cells recognize osmotic stress through liquid–liquid phase separation lubricated with poly (ADP-ribose). *Nat. Commun.* 12, 1353
98. Jalihal, A.P. *et al.* (2020) Multivalent proteins rapidly and reversibly phase-separate upon osmotic cell volume change. *Mol. Cell* 79, 978–990
99. Jalihal, A.P. *et al.* (2021) Hyperosmotic phase separation: condensates beyond inclusions, granules and organelles. *J. Biol. Chem.* 296, 100044
100. van den Berg, J. *et al.* (2017) Microorganisms maintain crowding homeostasis. *Nat. Rev. Microbiol.* 15, 309–318
101. Liu, B. *et al.* (2019) Decreased effective macromolecular crowding in *Escherichia coli* adapted to hyperosmotic stress. *J. Bacteriol.* 201, e00708
102. Liu, B. *et al.* (2018) Influence of fluorescent protein maturation on FRET measurements in living cells. *ACS Sensors* 3, 1735–1742
103. Conklin, E.G. (1912) Cell size and nuclear size. *J. Exp. Zool.* 12, 1–98
104. Xie, S. *et al.* (2022) Eukaryotic cell size control and its relation to biosynthesis and senescence. *Annu. Rev. Cell Dev. Biol.* 38, 291–319
105. Zatulovskiy, E. and Skotheim, J.M. (2020) On the molecular mechanisms regulating animal cell size homeostasis. *Trends Genet.* 36, 360–372
106. Jorgensen, P. *et al.* (2007) The size of the nucleus increases as yeast cells grow. *Mol. Biol. Cell* 18, 3523–3532
107. Mukherjee, R.N. *et al.* (2020) The perinuclear ER scales nuclear size independently of cell size in early embryos. *Dev. Cell* 54, 395–409
108. Neumann, F.R. and Nurse, P. (2007) Nuclear size control in fission yeast. *J. Cell Biol.* 179, 593–600
109. Han, B.-K. *et al.* (2003) The G1 cyclin Cln3p controls vacuolar biogenesis in *Saccharomyces cerevisiae*. *Genetics* 165, 467–476
110. Ratto, E. *et al.* (2022) Direct control of lysosomal catabolic activity by mTORC1 through regulation of V-ATPase assembly. *Nat. Commun.* 13, 4848
111. Carosi, J.M. *et al.* (2022) The mTOR–lysosome axis at the centre of ageing. *FEBS Open Bio* 12, 739–757
112. Korolchuk, V.I. *et al.* (2011) Lysosomal positioning coordinates cellular nutrient responses. *Nat. Cell Biol.* 13, 453–462
113. Gems, D. (2022) The hyperfunction theory: an emerging paradigm for the biology of aging. *Ageing Res. Rev.* 74, 101557
114. Olivetti, G. *et al.* (1991) Cardiomyopathy of the aging human heart. Myocyte loss and reactive cellular hypertrophy. *Circ. Res.* 68, 1560–1568
115. Olivetti, G. *et al.* (1995) Gender differences and aging: effects on the human heart. *J. Am. Coll. Cardiol.* 26, 1068–1079
116. Lengefeld, J. *et al.* (2021) Cell size is a determinant of stem cell potential during aging. *Sci. Adv.* 7, eabk0271
117. Carmona-Gutierrez, D. *et al.* (2016) The crucial impact of lysosomes in aging and longevity. *Ageing Res. Rev.* 32, 2–12
118. Arif, T. (2022) Lysosomes and their role in regulating the metabolism of hematopoietic stem cells. *Biology (Basel)* 11, 1410
119. Mansell, E. *et al.* (2021) Mitochondrial potentiation ameliorates age-related heterogeneity in hematopoietic stem cell function. *Cell Stem Cell* 28, 241–256
120. Liang, R. *et al.* (2020) Restraining lysosomal activity preserves hematopoietic stem cell quiescence and potency. *Cell Stem Cell* 26, 359–376
121. Dimitrov, L.N. *et al.* (2009) Polymorphisms in multiple genes contribute to the spontaneous mitochondrial genome instability of *Saccharomyces cerevisiae* S288C strains. *Genetics* 183, 365–383
122. Hughes, A.L. *et al.* (2016) Selective sorting and destruction of mitochondrial membrane proteins in aged yeast. *eLife* 5, e13943
123. Colacurcio, D.J. and Nixon, R.A. (2016) Disorders of lysosomal acidification – the emerging role of v-ATPase in aging and neurodegenerative disease. *Ageing Res. Rev.* 32, 75–88
124. Kovacs, M. *et al.* (2021) Lipid droplets protect aging mitochondria and thus promote lifespan in yeast cells. *Front. Cell Dev. Biol.* 9, 774985
125. Beas, A.O. *et al.* (2020) Independent regulation of age associated fat accumulation and longevity. *Nat. Commun.* 11, 2790
126. Aspert, T. *et al.* (2022) DetecDiv, a generalist deep-learning platform for automated cell division tracking and survival analysis. *eLife* 11, e79519