CHAPTER 2

Exploring the physicochemical perspective on cellular ageing

- An introduction

Sara N. Mouton1, Arnold J. Boersma2, Liesbeth M. Veenhoff1

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

This chapter is adapted from Trends in Biochemical Sciences 2023, Nov; 48(11): 949-962.
How do cells age? – The molecular perspective is dauntingly complex.

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 and categorized into twelve ageing hallmarks. However, a unifying molecular theory of ageing is currently lacking. Such a single theory may not exist because what extends lifespan dramatically in some species only has a moderate lifespan extension in others. This is for example the case with the insulin/IGF-I signalling (IIS pathway of Caenorhabditis elegans, where a single-gene mutation leads to a 10-fold increase in lifespan. In contrast, the lifespan extension in flies and mice caused by single-gene mutations related to IIS varies between 20% and 40%.

Interestingly, organisms from isogenic populations (see Glossary) that live in the same environment have very different lifespans and ageing phenotypes. This implies that they follow individual ageing trajectories driven by different underlying mechanisms. Furthermore, diversity is also observed within the individual organism, where some types of tissues age faster while others show little signs of ageing. Thus, while 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. On a molecular level, ageing is indeed a complex multifactorial biological phenomenon that is very challenging to uncover.

How do cells age? – A physicochemical perspective is emerging.

A valid question is thus if there are complementary ways of describing ageing that help to understand this process better. A physicochemical perspective may provide such an alternative. An essay from Alberti and Hyman in 2016 highlights the importance of the physicochemical parameters of cells and how age-dependent change in such parameters has the potential to drive aberrant phase transitions in biomolecular condensates and a subsequent loss of intracellular organization. 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 a variety of ageing phenotypes observed.

Here, we expand on this perspective and explore how the age-related changes in physicochemical parameters of cells have consequences on the cellular physiology including and beyond biomolecular condensation. We explore if taking this different perspective on ageing has the potential of connecting multiple molecular hallmarks of ageing. Such a perspective is, at present, only beginning to emerge for the process of replicative ageing in yeast.
An approach to study the physicochemical aspect of ageing.

To measure multiple physicochemical parameters and their relation to ageing is not trivial. One will have to obtain a sufficient amount of aged samples and be able to measure parameters in a non-invasive way. This kind of research therefore requires a convenient unicellular model system with large experimental power. The yeast replicative ageing model has been a valuable tool in the ageing research field and many fundamental aspects of ageing biology are conserved between yeast and mammals. Different possibilities for obtaining old yeast cells exist (discussed below) and this combined with the powerful genetic tools the yeast field has developed over several decades and the remarkable tolerance of yeast cells to variable growth conditions makes this model system a suitable candidate for further elucidating the physicochemical perspective on ageing. Here, we 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. A single yeast cell will start budding off a daughter cell about every 1.5 hours. At the end of the replicative lifespan, yeast cells enter into a slow dividing mode, termed senescence entry point (SEP), and eventually die. The average number of daughters produced by a single mother cell is ~25, albeit there is a large variability within a single population. The number of divisions a mother cell completes determines her replicative lifespan (RLS). Yeast cells can also survive in nutrient depleted conditions without undergoing any divisions. These cells will also age and die after a certain period of time, which is referred as chronological lifespan and will not be the focus of this thesis.

There are several interventions and 238 mutants known to increase yeast lifespan. The lifespan of baker’s yeast cells is thus malleable by genetic interventions and many genes that expand lifespan in yeast also do so in more complex organisms. During their RLS, mother cells accumulate ageing phenotypes: for instance, the cellular and vacuolar sizes increase dramatically, division frequency slows down, extrachromosomal rDNA circles (ERCs) and protein aggregates accumulate. Age-related phenotypes in ageing yeast have been previously summarized and found in the stochastic nature of biochemical reactions which can drive variability in gene expression, which in turn causes phenotypic differences. In yeast ageing, it seems that the protein biogenesis machinery drives uncoupling of the proteome from the transcript levels, potentially accounting in part for protein aggregation.

Obtaining old yeast cells.

As mentioned above budding yeast divide asymmetrically and every division results in two non-identical cells. Every mother cell will, on average, produce about 25 daughter cells and each one of these daughter cells will produce on average another 25 daughter cells and so on. Therefore, when age is determined based on number of divisions, in an exponentially growing culture the predominant population of cells will be young. The fraction of the population at an age of $n$ divisions is about $1/2^n$. Therefore, cells that approach the average lifespan of 25 division contain an insignificant fraction of the cell population. This is a confounding issue for isolating individual cells and several methods exist to overcome it, namely: micromanipulation, MEP genetic programme, magnetic columns that retain cells coated with iron beads, and microfluidic devices. The micromanipulation method was the first to be used for isolation of ageing yeast cells where daughter cells are removed after every division. Micromanipulation is not a very convenient method, because it is constrained by the very small number of cells that can be analysed. Later, an inducible genetic program called the mother enrichment program (MEP) was introduced where the proliferative potential of daughter cells is eliminated. The use of the MEP is limited to a certain laboratory background and still not very high numbers of cells due to daughter cells being able to escape the cell cycle arrest caused by spontaneous mutations. In addition, these methods also experience cell cycle arrest. Very large samples of aged cells can be obtained through the use of binding biotinylated cells to streptavidin-conjugated iron beads. These cells are then placed in magnetic columns, where a constant flow of media ensures stable nutrients and environment. Daughter cells do not inherit the cell wall thereby being released from the column and flushed away by the media stream. Finally, microfluidic devices have been designed to retain single cells in “traps” while daughter cells are flushed away from the media stream. These devices allow for the cells to age in a constant environment with ample nutrient supply while permitting microscopic analysis of many aspects of cell physiology during the entire course of the cells’ lifespan.

Why do yeast cells from isogenic populations have different lifespans?

From microfluidic devices, we know that cells with identical genomes, grown under the same environmental conditions, have different lifespans. Some cells will complete forty divisions or more, and others just five. This spread in lifespan is in fact similar to the spread in human population with its wide variety of genomes and environmental conditions. An explanation for this heterogeneity in lifespans can be found in the stochastic nature of biochemical reactions which can drive variability in gene expression, which in turn causes phenotypic differences. In yeast ageing, it seems that the protein biogenesis machinery drives uncoupling of the proteome from the transcript levels, potentially accounting in part for protein aggregation.
and decreased function of protein complexes. The most convincing and exciting explanation for the seemingly stochastic nature of yeast replicative ageing currently comes from the description that the excision of ERCs 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.

We conclude that even in the simple baker’s yeast system, the so far described genes and molecular pathways involved in ageing can only account for some part of the ageing process, but cannot fully explain it.

![Diagram of the yeast replicative ageing model](image)

**Figure 1.** The yeast replicative ageing model. (A) Mitotic divisions in yeast occur through the outgrowth of a daughter cell in a process called budding. The replicative age of a yeast cell is defined by the number of individual daughter cells that it has produced. The hallmarks of loss of proteostasis and genomic instability are two of the best described molecular hallmarks of ageing in yeast. Baker’s yeast divides asymmetrically where ageing factors, such as 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 hours. Yeast cell populations have a wide spread of replicative lifespans but many laboratory strains have an average lifespan of approximately 25 divisions. Nucleus and vacuole are indicated in blue and green, respectively. (B) Survival curves of humans (http://www.mortality.org, Survival in 2020) and baker’s yeast. Humans are genetically diverse and live under variable conditions and this contributes to the variability in lifespan. The yeast cells are genetically identical and they live under identical conditions, yet their lifespans are also variable.

**The physicochemical perspective.**

All biological macromolecules exert their function in an optimal environment, with defined parameters such as finite volume, viscosity, macromolecular crowding, internal pH, ionic strength, osmotic pressure, temperature, redox potential, and ATP availability. Cells employ mechanisms that regulate the intracellular milieu, counteract fluctuations in it, or repair dysfunction arising from environmental stressors. For example, in the occasion of suboptimal temperatures, proteins unfold and form aggregates, and these are resolved most often by ATP-dependent disaggregases. Proteins that are beyond repair are degraded. However, in ageing, the efficiency of these protective mechanisms decreases, which renders cellular processes more sensitive to the environment in which they take place. Additionally, some cellular processes are by nature more sensitive to small changes in environmental conditions. In the event of weakened protective mechanisms, these sensitive processes would be most impacted by fluctuations in the surrounding milieu. For example, a stably folding maltose binding protein will maintain its structure over a wide temperature range, whereas a stress granule may form or even evolve into an aggregate upon small temperature changes.

Several studies showed that some of the physicochemical parameters in yeast cells change as a function of age. So far, cytosolic, cortical and vacuolar pH, cellular and organellar size have been addressed. Furthermore, mesoscale macromolecular crowding was shown to be regulated by the TOR pathway, which is central to the ageing process. Finally, it has been suggested that reduced cytoplasmic density is a driver of cellular senescence. Below we introduce some of the most important parameters and how they impact cellular physiology.

**PH HOMEOSTASIS IN YEAST REPLICATIVE AGEING**

Almost every biological process has a pH-dependency. Among other things, pH is important for protein stability, solubility, folding, and condensate or complex formation, which impact enzyme activities such as the phosphorylation of metabolites and proteins, the establishment and use of proton gradients for transport, ATP production, and the maintenance of redox potential. Furthermore, it has been shown that cytosolic pH (pHc) has a signalling function in regulating cell growth through
the Ras/PKA and TORC1 pathways\textsuperscript{44}. Here, we provide a brief introduction to pH regulation and homeostasis in yeast, however, an extensive review on the topic can be found elsewhere\textsuperscript{45}.

**The systems that regulate cellular pH.**

In yeast, two ATP-dependent proton pumps – Pma1p\textsuperscript{46,47} and the vacuolar ATPase (V-ATPase)\textsuperscript{48}, are responsible for active pH regulation in the cytosol\textsuperscript{49}. Pma1p is a P\textsubscript{2}-type H\textsuperscript{+}-ATPase, located at the plasma membrane and is one of the most abundant proteins there. It translocates protons out of the cytosol at a rate of 1 H\textsuperscript{+} per hydrolysed ATP molecule\textsuperscript{50}. The vacuolar-type H\textsuperscript{+}-ATPases are localized on the membranes of organelles in the secretory pathway\textsuperscript{48} and also pump protons out of the cytosol, thus reducing pH and acidifying the vacuole (the yeast lysosome), the late Golgi apparatus and endosomes. Both ATPases are regulated by the availability of glucose and are responsive to cytosolic pH. Next to proton pumps, 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 the cellular pH\textsuperscript{51}.

**pH homeostasis is altered in ageing.**

It was shown that vacuolar pH increases (becomes more alkaline) early in yeast replicative ageing\textsuperscript{52}, potentially leading to mitochondrial dysfunction and genome instability\textsuperscript{52,53}. Later, it was proposed that the increasing copy numbers of Pma1 due to its asymmetric retention in the mother cells depletes the cytosol of free protons thus limiting the function of the V-ATPase and hampering proper vacuolar acidification. Simultaneously, an increase in pH in the cell cortex, a region in an immediate proximity to the plasma membrane where Pma1 exerts its function, is observed. Finally, an overexpression of the V-ATPase components has the potential to extend lifespan and limiting Pma1 function improves vacuolar pH\textsuperscript{54}.

In a different study, the cytosolic pH was addressed and shown to remain relatively stable early in the replicative lifespan of yeast cells. However, later in the lifespan, cytosolic pH decreases contributing to a more acidic environment\textsuperscript{55}. These findings are somewhat contradictory to the Pma1 - V-ATPase competition model, since it appears that the cytosol is not depleted from free protons. While it is possible that the Pma1 creates a local H\textsuperscript{+} gradient at the cell cortex as observed in other cases\textsuperscript{56,54–57}, the competition model needs to be revised. Another study from the Veenhoff laboratory shows that transcript and proteome levels of different subunits of the V-ATPase protein complex become substoichiometric in ageing, which at least in part can explain the decline of function of the V-ATPase\textsuperscript{58}. Possible alternative explanations are proposed in the General Discussion (Chapter 8 of this thesis).

**REDOX HOMEOSTASIS IN YEAST REPLICATIVE AGEING**

The oxidising or reducing potential of the intracellular environment, termed redox potential, is influenced by a number of molecular species including reactive oxygen species, antioxidant enzymes and redox couples. The redox systems in the cells are enzymatically regulated and not in equilibrium with each other\textsuperscript{58}, and some redox reactions are pH-dependent\textsuperscript{59}. Disrupted redox homeostasis is implicated in several age-related diseases such as cancer, cardiovascular, and neurodegenerative diseases\textsuperscript{60,61}. The redox homeostasis is maintained by thioredoxin, glutathione, and NADPH regenerating mechanisms and their associated enzymes\textsuperscript{62}. Up to our knowledge only the glutathione redox system has been addressed in yeast replicative ageing so far.

**The glutathione redox system and redox potential in ageing.**

The glutathione redox system is a central protective mechanism against ROS in the cell and is the most abundant low molecular weight thiol in the majority of aerobic organisms\textsuperscript{63}. Glutathione (GSH) is a tripeptide, γ-L-glutamyl-L-cysteinylglycine, harbouring a sulphydryl group (SH) that enables GSH to scavenge the intracellular environment from free radicals, thus protecting macromolecules from oxidative damage. When oxidized, glutathione forms glutathione disulphide (GSSG), which is converted back to GSH by glutathione reductase, Grl1\textsuperscript{64}. For a more complete overview on redox homeostasis in baker’s yeast, the reader is directed to a review from Wheeler and Grant\textsuperscript{64}.

The synthesis of glutathione is transcriptionally regulated and feedback inhibited by the presence of glutathione in the cell, thus making GSH a strictly controlled system. Interestingly, cytosolic acidification in ageing stimulates the function of glutathione reductase, decreasing the GSSG/2GSH ratio, thus creating a reducing environment in the cytosol of old cells\textsuperscript{65}. However, higher levels of H\textsubscript{2}O\textsubscript{2} have been reported in ageing cells in respiring and fermenting conditions. Both increased levels of GSH and H\textsubscript{2}O\textsubscript{2} have been shown to have little effect on the replicative lifespan, but the upregulated function of Grl1 leads to improved oxidative stress resistance in aged mother cells\textsuperscript{66}. The increased activity of Grl1 resulting in higher GSH levels as a consequence of cytosolic acidification in ageing shows that beyond transcriptional, translational and post-translational regulation, physicochemical parameters in the cell can provide an additional layer of regulation which has not been considered so far when looking at age-associated changes in the cell.
ATP LEVELS IN YEAST REPLICATIVE AGEING

*ATP as a chemical property of the cell.* ATP is the main energy currency of the cell since it drives and supports most cellular processes. ATP fuels the maintenance of cellular pH and drives the function of chaperones, it powers metabolic reactions and it is a precursor of RNA. In a very different perspective, more recent findings highlight the ability of ATP to maintain protein solubility. In a study from 2017, Patel and colleagues identified ATP to have characteristics of a *biological hydrotrope*. How ATP works to solubilize proteins and if it should be considered a hydrotrope or rather a biological aggregation suppressor has been further explored and debated. A study using in vitro and in silico approaches argues that ATP is better described as a biological aggregation suppressor rather than a hydrotrope. The results show that the solubilizing effects of ATP are a consequence of specific ion effects and π-π interactions and not due to hydrotrope-type mechanism. Another study utilizing molecular dynamics (MD) in a bottom-up approach, however supports the notion that ATP indeed possesses key characteristics of a biological hydrotrope. Regardless of the exact mechanism, the ability of ATP to enhance the solubility of endogenous and pathological proteins, to modulate properties of biomolecular condensates, and its millimolar concentration in the cell (much higher than required for enzymatic activity), make ATP a crucial parameter for the physicochemical state of the cell. Furthermore, cellular ATP levels have the ability to impact other physicochemical parameters and/or their effects in the cell.

**Incentives for ATP measurements in ageing.**

As pH homeostasis declines and protein aggregation occurs, it is relevant to ask whether ATP levels are stable in ageing. Currently, single cell data on ATP levels in RLS are not available in the published literature. 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. In such population-based studies it is difficult to judge whether these changes occur in cells during the mitotic phase of the RLS or rather post senescence. Additionally, 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 into senescence and generally remain healthy for the entirety of their lifespan. In contrast to replicative ageing, a study on chronological ageing shows that ATP levels are maintained.

ATP levels in aged brains from an Alzheimer’s disease (AD) mouse model are reduced compared to wild type and high ATP concentrations are needed in the eye lens to keep the high protein content soluble. Interestingly, aggregation-induced cataractogenesis is a common condition associated with ageing, so it could be that in this specific organ ATP levels decrease in an age-dependent manner.

Until recently, the role of ATP in maintaining cellular function has been appreciated from a metabolite point of view. However, the aggregation suppressing function of ATP puts it at the centre stage in the physicochemical makeup of the cell. Decreasing the ATP concentrations will have widespread effects on cellular function through its solubilizing effects and through fuelling enzymatic conversions including, disaggregases and proton pumps.

**CROWDING HOMEOSTASIS AND YEAST REPLICATIVE AGEING**

**Macromolecular crowding as an inherent property of the cell.**

Cells are highly crowded with macromolecules which occupy up to 40% of the total cell volume. Single molecular species do not occur in very high concentrations in cells, but all macromolecules together (protein, RNA, DNA, glycogen) occupy a significant fraction of the cell volume. To describe this feature of intracellular media, the term macromolecular crowding is used. 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 due to nonspecific steric repulsion. Thus, 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. 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). The excluded volume effect is present in all cases where the macromolecules freely diffuse and is modulated by attractive or repulsive forces. A short explanation of relevant crowding effects in biology can be found elsewhere.

**Measurements of macromolecular crowding.**

Various methods are available to estimate crowding levels. Crowding can be estimated from diffusion coefficients with labelled dextrans and globular proteins, total biopolymer fractions or using a FRET-based sensor. Crowding on the scale between 10 and 100 nm was termed mesoscale crowding, the degree and effects of which have been determined by tracking genetically encoded fluorescent foreign particles with sizes between 20 and 100 nm.

Exposing cells to hyper- or hypo-osmotic conditions is the most accessible way to induce a change in macromolecular crowding. Here, a hyperosmotic shock leads to...
intracellular water loss and cell shrinkage, thus increasing crowding. Next to osmotic stress, it has been inferred that, e.g., mechanical pressure, carbon starvation, modulation of the mTORC1 pathway, and gene dosage, can alter crowding in yeast.

**Regulation of macromolecular crowding.**

How eukaryotic cells regulate crowding, which genes are involved in its regulation, what the range of optimal crowding levels is, or whether crowding is stable in a disease and ageing context is largely unknown. Evidence from recent studies shows that crowding-induced condensate formation can be part of the mechanism for regulating crowding homeostasis in mammalian cells. Most recently, it was shown that WNK1 (With No lysine(K)) forms condensates upon hyperosmotic stress. Observable cluster formation results in the regulation of ionic concentrations and cell volume, making WNK a sensor and 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. Thus, it is likely that crowding is genetically regulated across different organisms.

**CELLULAR AND ORGANELAR VOLUME**

Cell size has been proposed to play a role in cellular metabolism and cell cycle and proliferation. While a lot of research efforts have been dedicated to these areas the notable cell size increase during aging in different organisms and tissues remains an observation with mostly unexplained underlying biology and physiological consequences. Cell size increase is observed in aging and age-related disease both in yeast and mammals. The rate at which cell size increases in yeast replicative aging has a negative correlation with lifespan and cell size increase beyond a certain threshold has been proposed to trigger senescence.

**Subcellular volume regulation.**

Cell size increases in aging, but whether organelar volumes scale along with the cell size has not been extensively studied. Nuclear size has been shown to be well regulated and tightly linked to the cell size, but up to our knowledge there are no reports addressing this relationship in aging. Of note, nuclear shape changes in ageing and is tightly linked to the cell size increase beyond a certain threshold has been proposed to trigger senescence.

Observational studies in yeast have shown that old vacuoles grow significantly in size with ageing, where towards the end of lifespan the vacuole/cell volume ratio increases.

Apart from yeast, human senescent cells also have increased lysosomal content with a higher pH, two factors that lead to a better senescence associated-β-galactosidase staining, one of the most predominantly used markers for the identification of senescent cells. How the increase in vacuolar and cell size impact cytosolic volume is unknown. Studies with cytoplasmic extracts show that for determining the size of the mitotic spindle, not cellular size, but cytoplasmic volume is the most important determinant. It is therefore reasonable to assume that not cellular volume per se, but volume of subcellular compartments is what may provide further insight into the mechanistic relationship between cell size and functionality in ageing.

**MEASUREMENTS OF PHYSICOCHEMICAL PARAMETERS IN LIVE CELLS AND AGEING**

While there are different ways to measure physicochemical parameters not all methods are applicable to ageing cells. As previously mentioned, one particular difficulty is obtaining samples with sufficient amount of aged cells. At the moment, microfluidic devices allow for following single cells throughout their replicative lifespan in constant conditions with minimal perturbation of the cell function. Currently, the most accessible and non-invasive method to measure physicochemical parameters during the course of replicative ageing is the use of genetically encoded fluorescent sensors. Different types of fluorescent biosensors exist, most prominently used in cell biology are sensors that alter the fluorescence of a single GFP derivative and FRET-based sensors. Single fluorescent protein (FP) sensors can be ratiometric where the FP has a bimodal excitation spectrum, such as pH sensor pHluorin or redox sensor roGFP. Single FP ratiometric sensors usually shift the efficiency of excitation between two wavelengths dependent on the surrounding environment. These ratiometric FPs are also sometimes coupled to a second FP which is fairly insensitive to the parameter of choice and thereby serves as a reference (e.g., as Rosella pH sensor). Alternatively, the degree of exposure of the chromophore to the solvent is modulated through attaching protein domains onto the FP β-barrel which upon ligand binding change the conformation of the fluorescent protein and as a consequence, the fluorescence intensity increases (e.g., G-CaMP Ca^{2+} sensor). There are also sensors that utilize Förster Resonance Energy Transfer (FRET), such as crGE2 crowding sensor series or ATP sensor ATeam. Here, two fluorescent proteins – a donor and an acceptor, are connected with a linker that is the core sensing part of the sensor. The conformation of the linker will change due to e.g., ligand binding thus shortening the distance between the donor and acceptor and increasing
the FRET efficiency. In the case of a crowding sensor, the linker is compressible thus resulting in shorter distance between the FRET pair (Figure 2). When this distance is less than 10 nm, FRET occurs. This phenomenon is very sensitive to small changes in distance as the FRET efficiency is inversely proportional to the sixth power of the distance between the donor and acceptor. The energy transfer is due to long-range dipole-dipole coupling where no actual photon is being exchanged between the donor and acceptor. Apart from the distance, the efficiency of the transfer depends on the overlap between the donor emission and acceptor excitation spectra, as well as the relative dipole orientation of the two chromophores. For a more extensive overview on fluorescent sensors the reader is directed to the following review.`131.

Figure 2. (A) Graphical representation of the structure of crowding sensor (crGE2 probe). The linker of the crowding sensor consists of 2×6 EAAK repeats forming two rigid alpha-helical blocks, separated by flexible 3×6 GSG regions. The alpha helical blocks separate the acceptor and donor in the absence of crowding. The flexible GSG repeat regions allow for the conformational change of the linker and facilitate favourable orientation of the donor and acceptor fluorescent domains for FRET. (B) Fluorescence emission spectra of the crowding sensor upon addition of increasing Ficoll PM70 concentrations. Upon high FRET the donor is partially quenched and a higher signal can be detected in the FRET channel. Image and data obtained from 88.

CONCLUDING REMARKS

In this introduction we provided an overview of important physicochemical parameters for cellular function and examine the experimental evidence for the age-related change in these parameters. Theoretically, a change in such parameters will have a global impact on cellular physiology.

Yeast cells are remarkably agile with changing growth conditions and are able to adapt to different temperatures, growth media, desiccation, oxidative stress, UV and ionizing radiation, and are able to utilize toxic substances such as ethanol and methanol as energy sources. To be able to withstand so many different conditions of life, yeast cells must have very robust systems to maintain the physicochemical parameters of their interior. Thus, by studying the maintenance systems that yeast cells employ, one can potentially learn a lot about the fundamental mechanisms that maintain physicochemical homeostasis. Such knowledge may lead to future development of anti-ageing interventions that simultaneously impact several molecularly defined ageing phenotypes.

Addressing the physicochemical status of aged cells is challenging. For some parameters, such as ionic strength, the right tools are still missing, while for others, the available tools and techniques need to be optimized to meet the requirements of long-term imaging. In the experimental chapters that follow, we tackle several of these technical challenges in order to provide robust methods for future studies (Chapters 3, 4, and 5). We then take the first strides towards providing a roadmap for a physicochemical perspective on ageing by addressing cytosolic pH, macromolecular crowding, organellar volume (Chapter 6), and ATP (Chapter 7) during yeast replicative ageing.

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.

Biological hydrotrope
Small molecules that can solubilize hydrophobic molecules in water. ATP can act as a hydrotrope when present at concentrations of 5-10 mM.

Fluorescence quenching (in the case of FRET)
Decrease in the fluorescence of the donor fluorophore due to a molecular interaction with an acceptor fluorophore, resulting in non-radiative energy transfer between the two.

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 – 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.

Replicative lifespan (RLS)
A measure of the total number of mitotic divisions a mother cell can undergo before death.

Senescence entry point
The time point when yeast cells enter into a slow division mode before they completely stop dividing – are senescent- and eventually die.
DEVELOPING ROBUST FLUORESCENCE-BASED BIOSENSORS FOR USE IN DERAILED AND EXTREME INTRACELLULAR ENVIRONMENTS

ABSTRACT

Fluorescent proteins have become an essential tool in many branches of biological research. Apart from visualization, fluorescent proteins can be used for quantitative purposes, especially in the context of genetically encoded fluorescent biosensors. It is important that a biosensor reports only on one parameter of interest e.g. pH, Ca$^{2+}$, ATP, etc. and is not influenced by other physiological parameters. In practice, this can be challenging because fluorescent proteins are sensitive to the intracellular environment, and this sensitivity is fluorophore specific. For example, red fluorescent proteins are generally resistant to acidic conditions and preserve their signal in stereotypical cellular environment. In contrast, yellow fluorescent proteins are usually sensitive to pH. This is a problem for fluorescence-based biosensors in general, but especially for those which harbour two different fluorescent proteins, such as FRET-based sensors. The challenge increases when FRET sensors are used for long-term imaging, especially in derailed or extreme cellular environments, such as in disease models or in ageing. Particularly challenging conditions are changes in cellular physiology due to toxic effects of disease-associated proteins, or ageing effects on pH, division frequency, transcriptional levels, and others.

The ideal FRET pair, therefore, should be optimized for the target organism, cell type, subcellular localization, and metabolic and health state. In Part I, we address the optimization of FRET-based sensors, particularly for their expression in yeast in both normal and disrupted intracellular environments.