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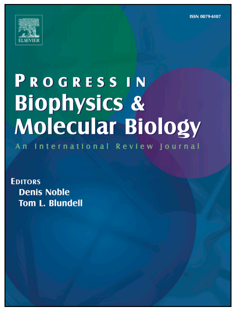

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Cell aging - a relevant factor in live cell microscopy (mini-review)

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Abstract

Live-cell microscopy is gaining importance for studying cellular behavior in response to environmental cues. However, cell aging can result in modifications of various cellular structures and functions, affecting or distorting microscopy-based readouts. These changes include gene expression, nuclear architecture, energy metabolism, or changes in the mechanical properties of cell membranes and microtubules. In this mini-review, we briefly discuss how cell aging affects critical subcellular compartments and alters live-cell imaging outcomes. In contrast to many papers available on cell aging, here we are focusing on the influence of cell aging on the performance and outcome of advanced microscopy techniques such as super-resolution imaging, fluorescence lifetime imaging (FLIM), variable-angle total internal reflection fluorescence microscopy (VA-TIRFM), as well as micromanipulation techniques such as laser-assisted optoporation. Our findings highlight the importance of considering cell passage number and senescence markers in experimental design and data interpretation.

Keywords: living cells, senescence, cell passage number, light microscopy, fluorescence microscopy

1. Introduction

Research in Biomedical Optics is often based on *in vitro* experiments using cell cultures, solutions, or tissue models. Employing cultivated cell lines is an indispensable part of preclinical research and development, providing valuable information about cellular physiology, metabolic pathways, disease-related processes , and assessing the effect of pharmaceutical agents (Errington et al., 2005; Kaniyala Melanthota et al., 2022; Saari et al., 2018). Furthermore, cells can be manipulated in order to examine or modify specific properties, e.g., through lasers in optical tweezer (Whitley et al., 2017; Wolfson et al., 2015) or optoporation systems (Patskovsky et al., 2020; Stracke et al., 2005). However, the results obtained may differ depending on several factors. These factors include cell type (e.g. fibroblasts, epithelial cells, or various organ-specific cells), cell growth in two or three dimensions and cell density, which may have a great impact due to different cell-cell contact or different availability of nutrient fluid (Graf and Boppart, 2010; Yao et al., 2022). Of considerable importance is cell aging (or senescence), which is often an underestimated variable. This can arise from genetic predisposition or be induced by external stress factors including ionizing radiation (Chen et al., 2019; Li et al., 2018; Nguyen et al., 2018), UV exposure (Lee et al., 2021; Shin et al., 2023), hypoxia, or toxic agents (Coradduzza et al., 2023).

A variety of microscopy techniques are considered to be relevant for studying cellular aging. These techniques include transmission microscopy (particularly phase contrast or interference contrast microscopy), elastic and non-elastic scattering microscopy, and fluorescence-based microscopy. Phase contrast microscopy is sensitive to morphological changes, making it a suitable tool for detecting alterations in cell shape upon aging. Similarly, elastic light scattering microscopy can detect changes in cell size and subcellular structures by analyzing angular or spectral behavior of scattered light, which is also sensitive to apoptosis and senescence (Allen et al., 2007; Richter et al., 2015). Inelastic scattering techniques, particularly Raman scattering microscopy, give detailed information about relevant molecular vibrations. Shifts in Raman spectra can correlate with biochemical changes happening during cellular aging (Li et al., 2021). Among all, fluorescence microscopy is known as the most widespread technique in life sciences. It is adaptable to both label-free detection techniques as well as methods including fluorescent dyes or genetically encoded fluorescent proteins (Cubitt et al., 1995). Important fluorescence-based approaches include super-resolution microscopy (SRM), micro-spectral analysis, fluorescence lifetime imaging microscopy (FLIM), Förster resonance energy transfer (FRET) (Förster, 1948; Masters, 2014; Schneckenburger, 2019), and Total Internal Reflection Fluorescence Microscopy (TIRFM) (Axelrod et al., 1983; Schneckenburger, 2005). In addition, laser-assisted micromanipulation techniques provide valuable information on both cell analysis and targeted interventions (Greulich, 2017).

Many review papers have addressed the effect of cell aging on cell physiology and behavior. Here we specifically focus on how cell aging affects and often distorts experimental outcomes in biomedical optics, particularly in the context of light and fluorescence microscopy. We highlight the application of advanced imaging methods to study nuclear architecture, energy metabolism, membrane dynamics, or drug response in cultured cells at different passage numbers, emphasizing the effect of cell aging on key microscopy readouts.

2. Live-cell microscopy and cell aging

2.1 Cell aging effects on subcellular compartments

Aging or senescence can affect various domains of the cell, including the cell nucleus, mitochondria, cytoskeleton, and/or cell membrane (Zhu et al., 2021). This can cause various changes in the structure and function of cell compartments, influencing cell morphology, intracellular organization, mechanical properties, and intra- or intercellular protein interactions. Here, we briefly discuss the impact of cell-aging on cellular components, the microscopy techniques used for detecting these changes, and their influence on microscopy experiments.

2.1.1. Cell nucleus

One effect of cell aging is the arrest of cell growth where the cells halt their cycle. This can happen due to different reasons including contact inhibition, lack of growth factors, or DNA damage, which is considered a defense mechanism against tumor progression (Cho and Hwang, 2011; Kumari and Jat, 2021). Change in the nuclear architecture and chromatin microstructure is another indication of cell senescence (Heckenbach et al., 2022).

Since gene regulation, genomic stability, and the ability of cells to respond to environmental cues, undergoes significant alterations during aging (Stegeman and Weake, 2017), correlating modifications of chromatin nanostructure (Cremer et al., 2020; Dekker et al., 2024) are to be expected (Chen et al., 2014). For example, age-related modifications of histone acetylation and methylation patterns may result in genome structure changes such as chromatin relaxation or heterochromatin modifications, which hence may be studied by appropriate microscopy methods (Larson et al., 2012; Lee et al., 2020). Furthermore, heterochromatic foci (SAHF) coincide with the recruitment of heterochromatin senescence regulators (Narita et al., 2003). Irregular nuclear morphology like nuclear envelope invaginations and loss of nuclear envelope integrity (NEI) is associated with reduced Lamin function (Kim, 2023; López-Otín et al., 2013; Pathak et al., 2021; Scaffidi and Misteli, 2006; Shumaker et al., 2006). These changes result in mechanical instability and increased nuclear fragility.

For a better understanding of the correlation between chromatin nanostructure and gene activity, various theories have been put forward. While some models claim that transcriptionally silent genes are organized in a chromatin compartment distinguished by a DNA density sufficiently high to restrict or even prevent the access of transcription factor complexes (TFCs) to target sequences in their interior, other observations using conventional resolution microscopy suggest that the nuclear DNA density is so low that TFCs may screen effectively the entire DNA: Live-cell measurements (HeLa) performed with fluorescence correlation microscopy (FCS) indicated that the absolute DNA density (Mbp/μm^3) of heterochromatin in the nuclear interior was too small for density-controlled accessibility (Weidemann et al., 2003).

For further analyses, methods with enhanced optical and structural resolution (super-resolution microscopy/SRM) are required. While until a few decades ago, such an enhancement beyond the conventional resolution limit of ca. 200 nm appeared to be impossible, presently various types of SRM (Birk, 2017; Cremer et al., 2017; Cremer and Masters, 2013; Hell, 2007; Lelek et al., 2021; Schmidt et al., 2021; Schneckenburger, 2019) allow to overcome this fundamental limit.

In contrast to the absolute nuclear DNA density data achieved by conventional resolution microscopy cited above, super-resolution measurements at high optical and structural resolution (Gelléri et al., 2023) revealed that in the cell types studied (HeLa cervix carcinoma cells, human and murine fibroblasts), the total amount of nuclear DNA having an absolute density (Mbp/μm^3) sufficiently high for restriction of TFC accessibility, made up a substantial part of the total nuclear volume and DNA content. On the other side, a major percentage of the nuclear volume of the same cells also had a low absolute DNA density, indicating a facilitated accessibility of the genes localized in those regions. As a consequence, both modes of accessibility describe essential partial features of nuclear organization, and may be combined in the active/inactive nuclear compartment (ANC/INC) model of functional nuclear genome organization (Cremer et al., 2020; Gelléri et al., 2023). Within this model, aging-related shifts in the nuclear landscape of high/low-density DNA domains are expected to be correlated with appropriate shifts in transcription rates, including age-relevant genes.

In other studies, SRM was used to monitor and analyze changes in chromatin compaction or fragmentation upon ionizing or UV radiation. Using single-molecule localization microscopy (SMLM), X-radiation induced conformational changes of nuclear chromatin associated with DNA single and double strand breaks (DSBs) were investigated (Hausmann et al., 2018): Since hetero- and euchromatin are differentially accessible to DNA repair pathways, local chromatin rearrangements and structural modifications are among the consequences of an activated DNA damage response. Upon this damage, clusters of γ-H2AX molecules are formed in correlation with the irradiation dose, peaking at 30 minutes and returning to baseline after 8 hours, thus indicating that the original nanoscale structure of chromatin was reestablished. In other studies, a combination of SMLM and structured illumination microscopy (SIM) was used to analyze the nanostructure of DSB repair foci induced by carbon-ion radiation (Lopez Perez et al., 2016). The results showed that the conventionally detected γH2AX foci (Ø700-1000 nm) were composed of elongated subfoci with a size of ∼100 nm, themselves consisting of even smaller subfoci elements (Ø 40-60 nm). The structural organization of the subfoci suggested that they could represent the local chromatin structure of elementary repair units at the DSB damage sites. Such methods will allow detailed studies of age-related chromatin nanostructure modifications, particularly due to the accumulation of DBS in non-dividing cells or changes in methylation (Buitrago et al., 2021; Klutstein et al., 2016).

At the present state, nuclear genome organization has been studied mostly at the level of fixed non-senescent cells. For a better understanding of its dynamics in aging cells, live cell microscopy approaches at enhanced resolution (Ren et al., 2024) should be highly desirable. Due to the high photon burden connected with prolonged microscopic observation, this remains a major challenge (Schneckenburger and Cremer, 2025). To reduce such problems, single-particle tracking techniques have been successfully used to study chromatin movements relevant for nuclear nanostructure analysis (Lee et al., 2025) and transcription (Shimazoe et al., 2025). In such studies, age-related changes in the nucleus may distort live-cell imaging by affecting efficiency of fluorescence labeling, chromatin accessibility or repair kinetics.

In summary, the emerging correlation between gene transcription and chromatin nanostructure opens new avenues to analyze aging effects not only biochemically, but also to combine these with direct microscopic measurements at the single cell/single molecule level (Hübner et al., 2013; Jiang et al., 2013; Kirmes et al., 2015; Senapati et al., 2025). Novel high-throughput SRM approaches (Barentine et al., 2023) and very large field-of-view imaging techniques (von Hase et al., 2023), together with oligopaint-based approaches (Beckwith et al., 2025; Bintu et al., 2018) will allow such measurements on a large scale, even within tissue sections and organoids (up to millions of individual cells). Beyond diagnosis, such techniques are envisaged to contribute substantially also to the monitoring of cellular drug distribution to alleviate or even reverse the effects of aging on gene regulation (Zhu et al., 2024).

2.1.2. Mitochondria

Aging effects on mitochondria may influence their function, morphology, as well as the efficacy of the respiratory chain and dynamics (Mohamad Kamal et al., 2020; Seo et al., 2010). This can cause changes in the optical properties of relevant molecular species due to a decrease in the membrane potential, mtDNA mutations, and changes in reactive oxidative stress (ROS) (Rottenberg, 2023; Xu et al., 2025). Approaches to explore dynamic characteristics of mitochondria at the nanoscale have also been established by various SRM methods, such as stimulated emission depletion (STED) microscopy, SMLM, or SIM (Jörg et al., 2024; Li et al., 2024; Stephan et al., 2019; Yang et al., 2020). Fluorescent dyes like JC-1 are being used to assess changes in the membrane potential of the mitochondria (Wang et al., 2024). Age-related changes in mitochondria can alter the fluorescence intensity, the localization of fluorophores, and cause an increase of autofluorescence, thus affecting the measurement and reducing signal specificity (König et al., 2017).

2.1.3. Endoplasmic reticulum

Aging in endoplasmic reticulum (ER) can cause structural disorganization and decreased function, leading to the accumulation of unfolded or misfolded proteins and increased stress level. As a result, cellular signaling can be affected, leading to abnormal vesicle formation and endocytic dysfunction (Brown and Naidoo, 2012; Estébanez et al., 2018). Super-resolution microscopy techniques have been utilized to visualize the structure of ER network and protein misfolding in live cells (Han et al., 2023; Hao et al., 2022). Additionally, phase contrast microscopy and fluorescence-based microscopy are sensitive enough to capture changes in ER refractive index or fluorescence quenching, caused by accumulation of misfolded proteins (Chen et al., 2024; Zhong and Fang, 2012). Such age-related changes in ER can influence fluorophore binding to specific targets and distorting brightfield contrast which can affect the visualization and quantification of microscopy readouts.

2.1.4 Cytoskeleton

Due to aging, the cytoskeletal network can undergo disorganization of actin filaments, instability of microtubules and changes in the expression level of intermediate filaments (Kim et al., 2022; Lai and Wong, 2020). These changes can influence cell shape, mechanical properties, and motility, caused by rigidity in microtubules and impaired intracellular transport (Singam et al., 2024). Live-cell imaging, e.g. 3D imaging and live-cell tracking, have been used to monitor cytoskeletal stiffness, structural changes and migration dynamics (McKayed and Simpson, 2013). Aging cytoskeletons display increased stiffness, decreased migration, and slower response to external stimuli. Furthermore, strong autofluorescence expressed due to the accumulation of cytoplasmic inclusions in aging cells can interfere with fluorescence-based imaging systems (Kim et al., 2022; Lai and Wong, 2020).

2.1.5. Membrane

Cell-aging can significantly affect the membrane stiffness. More specifically, increased membrane stiffness and reduced fluidity is proven upon aging, due to alteration in lipid composition, including increased cholesterol content (Luo et al., 2016; Singam et al., 2024; Weber et al., 2010). Cholesterol-rich micro-domains in cell membranes, often referred to as lipid rafts, play a major role in membrane organization and function and appear to be a primary target in cell aging (Nakamura et al., 2003). Furthermore, cell senescence can affect the behavior of membrane proteins, activation of signaling pathways, and endocytosis, influencing cellular response to pharmaceutical substances, such as drugs or nanomaterials (Einem et al., 2012; Kolodziejczyk et al., 2020; Shaw et al., 2011; Torres et al., 2021), as well as for the uptake of metabolites, or pharmaceutical drugs (Brueckner et al., 2018; Varma et al., 2022; Xiang et al., 2018). Reduced membrane integrity leads to increased permeability and reduced ability to regulate ion flux, thus contributing to osmotic stress and leading to mechanical damage in aged cells (Tabibzadeh and Brown, 2024).

To assess membrane stiffness and fluidity, polarity-sensitive dyes, such as the membrane marker laurdan (6-dodecanoyl-2-dimethylamino naphthalene) have been widely used (Bagatolli, 2015; Kerch, 2023; Klymchenko and Mely, 2013; Loura et al., 2010; Weber et al., 2010). Laurdan shows two characteristic fluorescence bands around 440 nm and 490 nm. While the first band dominates in a more rigid gel-phase cell membrane, mostly visualized at lower environmental temperature, the latter one dominates in a more fluid liquid crystalline phase at higher temperature, when laurdan gets into contact with water molecules of the adjacent cytoplasm (~40°C). Previously, the "generalized polarization” (GP) parameter, an appropriate parameter for membrane stiffness, was introduced (Parasassi et al., 1991):

$$GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$$

where I_{440} and I_{490} are the fluorescence intensities measured at 440 nm and 490 nm respectively. GP showed a pronounced decrease with an increasing temperature between 24°C and 41°C, as well as an increase with cell aging, indicating higher membrane stiffness. Additionally, GP is consistently higher in the plasma membrane than within intracellular membranes (Schneckenburger et al., 2004). Further parameters for membrane stiffness include nanosecond fluorescence lifetime (Schneckenburger et al., 2004) and rotational correlation time (Weber et al., 2010), both of which decrease with increasing temperature.

An interesting finding was the cellular distribution of fluorescence lifetimes (Fig. 1). Younger subcultures (12-23 passages) indicate heterogeneous lifetime domains (Fig. 1C), whereas aging subcultures (35-38 passages) show a more homogeneous lifetime pattern (Fig. 1D). This proves that quantitative experiments, like cholesterol-dependent pathologies (including cancer, atherosclerosis or neurodegenerative diseases), require cell cultures of similar age (Arnim et al., 2008; Jerome and Yancey, 2003). Application of an appropriate raft marker may give additional information about the mechanisms involved (Sheriff et al., 2004).

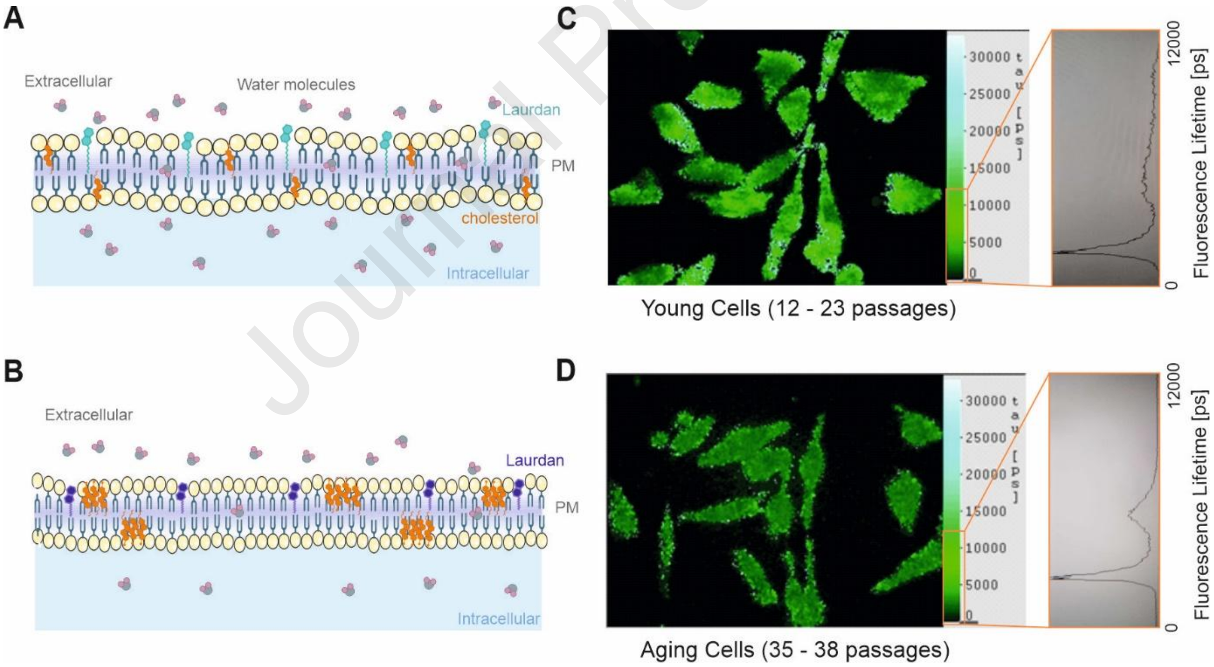

Fig. 1. Assessment of age-related changes in the membrane fluidity using FLIM of laurdan. Schematic presentation of plasma membrane in young (12-23 passages) (A) and aging (35-38 passages) (B) cells. A) Young cells have relatively more fluid membrane structure with lower cholesterol content and stronger interaction between laurdan molecules and adjacent water dipoles resulting in a fluorescence band around 490 nm. B) In aged cells, as a result of reduced membrane fluidity, caused by higher cholesterol accumulation and tighter lipid packing, laurdan shows less interaction with water dipoles, and its fluorescence is dominant at 440 nm. C and D) Exemplary images result from fluorescence lifetime patterns at 35 °C for young (C) and aging (D) CHO cells incubated with laurdan (8 µM; 60 min). Young cells (C) show a heterogeneous pattern, indicating a dynamic lipid environment, whereas aged cells (D) show a more homogeneous pattern revealing a stiffer membrane structure. This is also proven by the attached histograms, which in addition to a dark background peak below 2 ns show a broad distribution of fluorescence lifetimes between 3 ns and 10 ns for young cells, but a distinct peak around

4 ns for aging cells. Excitation wavelength: 391 nm; detection range: 420-800 nm; image size: 220 µm x 160 µm, time scale: 0-30 ns in each case). Reproduced from (Schneckenburger et al., 2004) with modifications.

2.2 Cell aging effects on drug response

It is reported in the literature that chemotherapeutic drugs can induce apoptosis as well as senescence of cells (Buttiglieri et al., 2011; Rhim, 2003). However, only in individual study cases has the influence of cell aging on the efficacy of cytotoxic drugs been described (Fourie et al., 2019), and almost no standardized parameters for cell aging characterization have been defined. Furthermore, cellular senescence has an impact on the uptake of various drugs or nanoparticles, which is mainly due to changing elastic properties of their membranes or their cytoskeleton and may provoke different cell responses (Kolodziejczyk et al., 2020). Cell aging also has an influence on the properties of nucleoli, including biogenesis of ribonucleoprotein particles (Carotenuto et al., 2019), as well as mitochondrial integrity, where measurement of the mitochondrial membrane potential may be affected (Sugrue et al., 1999).

Advanced imaging techniques allow for the detection of subtle changes in drug uptake, caused by cell aging. In an article concerning cellular responses to the cytostatic drug doxorubicin, we investigated the distances between the surface of Chinese Hamster Ovary (CHO) or MCF-7 human breast cancer cells and an adjacent glass substrate, on which the cells were growing. Using variable-angle TIRFM (VA-TIRFM), we observed a reduced cell-substrate distance, up to a factor of 2, when sub-cultures with a larger number of passages were used (Krecsir et al., 2022). The measurements were performed with an axial resolution in the nanometer range and fluorescence staining (or transfection with a green fluorescent protein (GFP) encoding plasmid) of the plasma membrane. 2- or 24-hour doxorubicin treatment caused an increase in cell-substrate distances up to two times. However, the evaluation of the impact of the drug on cell-substrate distances requires a controlled cell age.

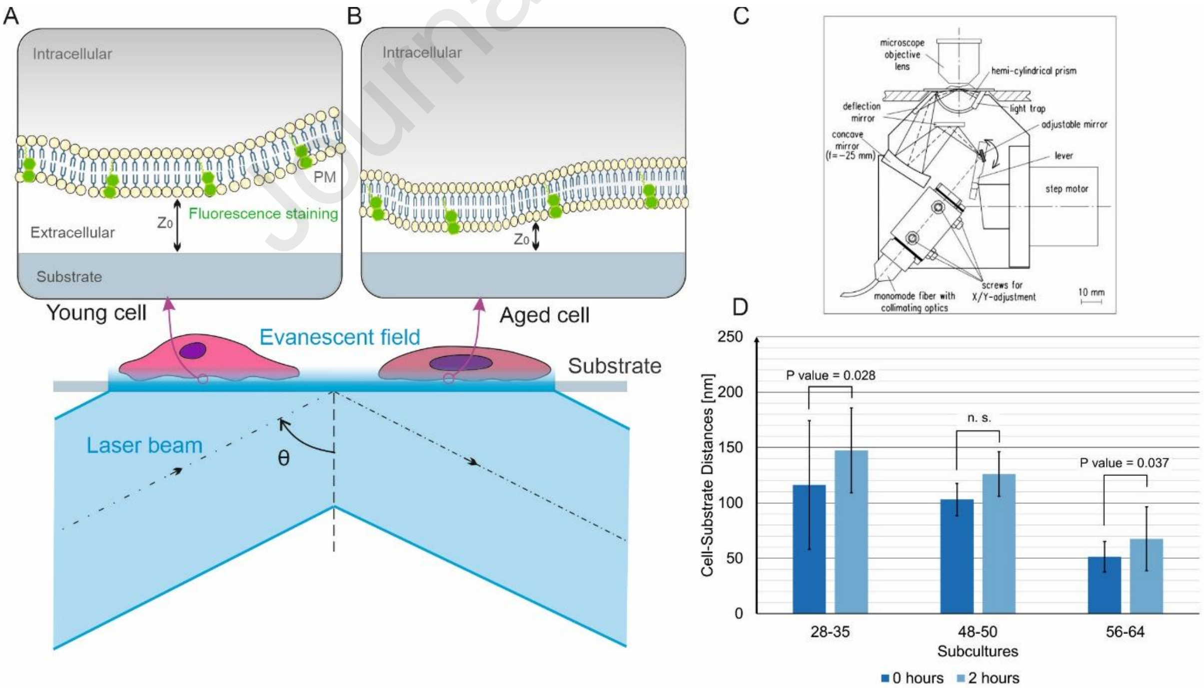

Fig. 2. Effect of cell aging on cell-substrate distance in the presence of doxorubicin. A, B) Schematic illustration of the plasma membrane (PM) of young (A) and aged (B) CHO cells transfected with a membrane-associated GFP encoding plasmid (CHO-pAcGFP1-Mem). VA-TIRFM was used to assess the vertical distance (Z_0) between the membrane and the substrate (bottom panel). C) Microscope condenser to record fluorescence intensity as a function of the angle of incidence (VA-TIRFM) and to calculate Z_0 from this relation (Stock et al., 2003). D) Quantification of cell-substrate distances of CHO subcultures with different passage numbers (28-35, 48-50, and

56-64) at 0- and 2-hour treatment with 2 µM doxorubicin. Mean value ± standard deviation including the p-values for statistical significance ($p \leq 0.05$: statistically significant; n.s.: non-significant). The results show how the effect of doxorubicin on the membrane-substrate distance varies with the passage number of subcultures. Reproduced from (Krecsir et al., 2022) with modifications.

2.3 Cell aging effects on energy metabolism

Cell aging has a significant impact on energy metabolism, mainly due to changes in redox balance and mitochondrial dysfunction (Xu et al., 2025). Most studies on cell energy metabolism are mainly focused on ATP synthesis and fluorescence measurements of coenzyme turnover involved in oxidoreductases (Covarrubias et al., 2021; Rigoulet et al., 2020). Among these, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), as well as nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) play a predominant role in metabolic redox reactions (see e.g. (Kalinina et al., 2021)). Upon excitation with near-UV spectral range, NADH and NADPH fluoresce in their reduced state but are almost non-fluorescent in their oxidized state. Fluorescence measurements can therefore give valuable insight into the efficacy of relevant metabolic processes, particularly glycolysis and respiration.

It is reported that glycolysis and respiration (oxidative phosphorylation) are mainly related to free NADH and protein-bound NADH molecules. Both molecular species differ by their emission maxima around 480 nm for free NADH and 440 nm for bound NADH (Fig. 3) (Galeotti et al., 1970; Salmon et al., 1982) and their fluorescence lifetimes in the (sub)nanosecond range (Morone et al., 2020; Schneckenburger and König, 1992; Trinh et al., 2017; Weber et al., 2012). In previous studies, tumor cells and less malignant cell lines have been distinguished based on these spectral and lifetime data. As an example, based on 140 individual measurements evaluated by multi-variate data analysis, a predominance of the 480 nm band in U251-MG glioblastoma cells was observed, whereas the 440 nm band was more pronounced in less malignant cells with activated tumor suppressor genes (Weber et al., 2012).

It is an open question whether the relative composition of NADH species changes upon cell aging. In Fig. 3B, the intrinsic fluorescence of small clusters of 5-10 3T3 mouse fibroblasts with different cell passage numbers (46, 54, and 130) is compared. The results suggest that while the spectra are dominated by emission bands at 440 nm and 480 nm, their relative intensity remains unchanged. Although no age-related changes of metabolic pathways can be deduced from the indicated results, a possible correlation between NADH fluorescence and cell aging, including mitochondrial dysfunction, has been discussed in the literature (Croce and Bottiroli, 2017; Dong et al., 2019; Heikal, 2010; Song et al., 2024). Moreover, a decrease in (oxidized) NAD^+ levels has been linked to numerous aging-associated diseases (Covarrubias et al., 2021).

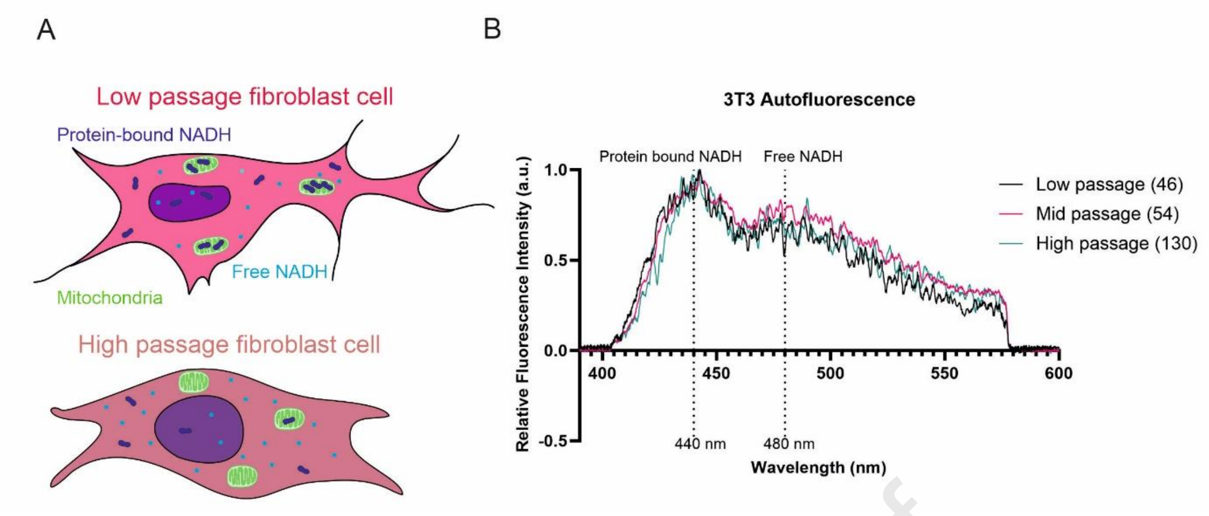

Fig. 3. Intrinsic fluorescence of 3T3 fibroblasts from subcultures with a variable number of cell passages. A) schematic representation of fibroblast cells at low (top) and high (bottom) passage numbers. A different free NADH/protein-bound NADH ratio reflects different metabolic stages. B) Autofluorescence spectra of 3T3 fibroblast subcultures at low (46), mid (54), and high (130) passage numbers. Each graph represents an average measurement of cell clusters with 5-10 individual cells each after subtraction of a cell-free background and normalization to the maximum fluorescence value. 40×/0.75 objective lens; excitation wavelength: 365 nm; spectral resolution: ≤ 10 nm (H. Schneckenburger, unpublished data).

2.4 Cell aging effects on laser microbeam responses

In addition to optical microscopy, numerous applications of the laser microbeam technique, including selective illumination of cell compartments or organelles, structural and functional studies, optical trapping and manipulation or microdissection, have been reported and summarized (Ashkin, 1997; Berns, 2007; Greulich, 1999; Nüsslein-Volhard et al., 1980; Zorn et al., 1979). While laser microbeams are often used for measurement or imaging of biological parameters, as well as for trapping or moving of cells in an optical tweezer system, micromanipulation or optoporation techniques have been used to temporarily increase cell membrane permeability to introduce molecules or small particles into cells. These techniques compete with injection via micro-needles (microinjection), with the added advantage of maintaining cell viability as induced perturbations are reversible (McAllister et al., 2000; McCaffrey et al., 2015).

Laser-assisted optoporation is based on photochemical, photothermal, or opto-mechanical interactions, depending on laser parameters, and may be used to introduce specific dye molecules or genes of a foreign organism into the cell (known as transfection) (Fig.4). While opto-mechanical interactions are related to the generation of small holes in the plasma membrane due to ablation by picosecond or femtosecond laser pulses (Schinkel et al., 2008; Stracke et al., 2005), photothermal interactions include a transient warming-up of small spots in the membrane, possibly with a phase transition of membrane lipids from the rigid gel phase to the more fluid liquid crystalline phase (Parasassi et al., 1991), typically in the 35-41 °C range (Schneckenburger et al., 2002). Following the application of a GFP-encoding plasmid, we demonstrated an increase in transfection rate (ratio of fluorescent cells versus non-fluorescent controls) in younger CHO cell subcultures (10-21 passages), from 6.5 ± 2.5% to about 29% (Fig. 4C). When including all subcultures (12-37 cell passages), the transfection efficiency was around 13% (Schneckenburger et al., 2002). These results are associated with age-related membrane rigidity probably due to increased cholesterol content, so that the phase transition was expected to occur at a higher temperature, and uptake of molecules, such as GFP encoding plasmids, was impeded.

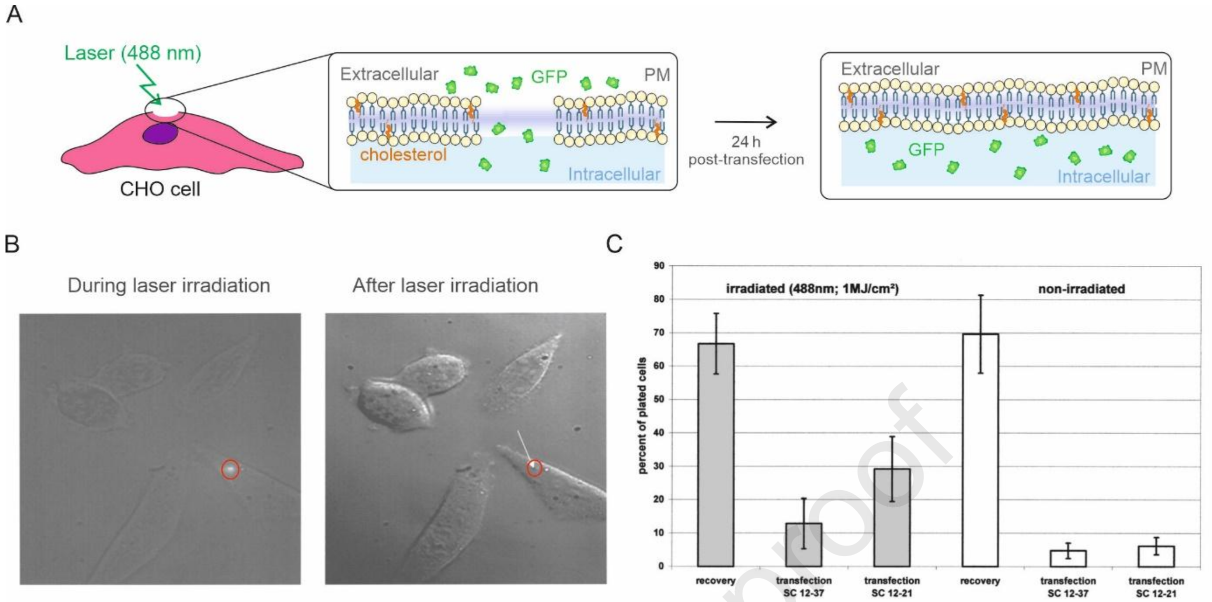

Fig. 4. Effect of cell aging on membrane permeability and GFP transfection efficiency of CHO cells upon laser-assisted optoporation. A) Schematic illustration of laser-assisted optoporation. A 488 nm laser beam induces a transient opening in the membrane, providing an entry for the GFP encoding plasmid. After 24 hours, GFP expression is visible in transfected cells. Changes in membrane composition upon aging affect membrane permeability, resulting in a reduced transfection rate. B) Phase contrast microscopy of CHO cells during and interference contrast microscopy after focused laser irradiation. The irradiation spot is marked with a red circle. C) Quantification of cell recovery and transfection efficiency based on a colony formation experiment (laser irradiation: 488 nm; 1 MW/cm^2; 2.5 s; culture medium enriched with 40 µM phenol red). Bars represent the percentage of recovery or successful transfection of plated cells. The data compares irradiated cells and non-irradiated controls of subcultures (SC) with 12-37 or 12-21 passages. Younger subcultures show a higher transfection rate post-irradiation. Reproduced from (Schneckenburger et al., 2002) with modifications.

Beyond transfection, laser microbeam techniques have been used to irradiate endothelial cells of the vein, thus inducing stiffening, a hallmark of high blood pressure, particularly associated with the aging population. Furthermore, in the context of DNA damage and repair with laser microbeams, accumulation of incorrectly repaired DNA strands over time was reported, suggesting that these techniques prove to be valuable tools in ageing research (Grigaravicius et al., 2009). More recent studies were focused on the viscous and viscoelastic properties of different cell types under stress and aging-inducing conditions to gain a deeper understanding of the aging process and aging-related diseases (Singam et al., 2024). For instance, optical tweezer micro-rheology was used for rapid phenotyping of intracellular material properties and protein blends in view of cell aging studies, with further biomedical and drug-screening applications (Català-Castro et al., 2025).

3. Conclusion and future directions

Nuclear genome architecture, coenzymes related to energy metabolism as well as mechanical properties of the cell membrane and cytoskeleton may be sensitive to cell aging, influencing a wide range of microscopy-based readouts. Passage number of the cells is usually overlooked despite its clear influence on fluorescence intensity, spectral properties, mechanical characteristics, and molecular uptake. Therefore, age-matched cells, e.g. with similar numbers of passages, should be used for comparative *in vitro* experiments. Implementing cell age metrics standardization, such as passage number and replicative age markers, especially in long-term imaging and drug testing assays, will help to ensure data reproducibility and eliminate data variability (Ogrodnik et al., 2024). This can be achieved by developing unified age-reporting guidelines and integrating age-matched control experiments. Alternatively, strategies can be employed to avoid or delay cell aging effects, e.g. by use of antioxidants or by depletion of cholesterol. In the first case, DNA damage by reactive oxygen species or mitochondrial dysfunction may be prevented (Marí et al., 2009; Vickridge et al., 2022), whereas in the second case, an increased membrane stiffness of aging cells can be avoided using e.g. methyl-β-cyclodextrine (MβCD) (Weber et al., 2010). Maintaining comparable cell age or minimizing cell senescence plays a key role in highly sensitive experiments in biomedical optics, including live cell microscopy.

In case of a long measuring time, phototoxicity is known to be a limiting factor in live cell imaging, especially in super-resolution imaging (Ren et al., 2024). Further optimization of imaging techniques, such as FLIM, SRM (e.g. SIM, MINFLUX, Airy Disc scanning microscopy), TIRFM, and VA-TIRFM, with adaptive optics, high-efficiency detectors, and AI-assisted image reconstruction can help in improving the imaging resolution and reduce phototoxicity damage to cells. Additionally, emerging high-resolution imaging techniques with machine-learning-guided phenotype classification can assist in distinguishing aging phenotypes at single-cell level.

From the biological perspective, further studies exploring the relationship between organelle aging and microscopy-based structural and functional readouts can enhance our understanding of metabolic aging and its effects on cellular features such as redox state, chromatin nanostructure, and mechanical behavior. Such information improves the experimental design and provides the possibility to predict and avoid cell aging effects on cell-based assays.

Furthermore, integrating high-throughput systems with super-resolution imaging techniques allows analyzing thousands of cells simultaneously. This approach can provide the opportunity to characterize aging phenotypes across large cell populations and can be applied to various research or diagnostic fields including drug screening, aging diagnostics, and cellular rejuvenation strategies (Mahecic et al., 2019). Similarly, advanced microscopy-guided imaging tools can be expanded to microscopy-guided manipulation techniques. This includes the application of laser-based optoporation in age-selective transfection or drug delivery for rejuvenation, investigating membrane fluidity or cytoskeletal tension by photo-biomodulation or activation of aged cells in co-cultures using targeted ablation. Such manipulation techniques can contribute to advanced therapeutic approaches and provide better insight into regenerative medicine and age-related disease therapy.

CRediT authorship contribution statement

Tina Karimian: Writing – original draft, Writing – review & editing, Visualization, Conceptualization. **Christoph Cremer:** Writing – original draft, Writing – review & editing. **Julian Weghuber:** Writing – review & editing, Funding acquisition. **Herbert Schneckenburger:** Writing – original draft, Writing – review & editing, Conceptualization, Visualization, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

Aβ	Amyloid-beta
ANC/INC	Active/Inactive nuclear compartment
CHO	Chinese hamster ovary
DSB	Double strand break
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide
FCS	Fluorescence correlation microscopy
FLIM	Fluorescence lifetime imaging microscopy
FMN	Flavin mononucleotide
FRET	Förster resonance energy transfer
GFP	Green fluorescent protein
MßCD	methyl-ß-cyclodextrine
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEI	Nuclear envelope integrity
ROS	Reactive oxidative stress
SAHF	Senescence-associated heterochromatic foci
SC	Subculture
SIM	Structured illumination microscopy
SMLM	Single molecule localization microscopy
SRM	Super-resolution microscopy
STED	Stimulated emission depletion
TFC	Transcription factor complex
TIRFM	Total internal reflection fluorescence microscopy

VA-TIRFM Variable-angle total internal fluorescence microscopy

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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