Small-molecule fluorescence-based probes for aging diagnosis

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ABSTRACT

Aging is a time-dependent decline in physiological function that affects most organisms and is the major risk factor for many non-communicable diseases. The early diagnosis of aging is critical for the treatment of aging and aging-related diseases. In recent years, extensive efforts have attempted to accurately diagnose aging. To date, multiple types of fluorescent probes for various age-related biomarkers have been developed, with the aims of achieving rapid, precise diagnosis of the aging process and evaluating the efficacy of anti-aging drugs. This review summarizes recent research progress in small-molecule fluorescence-based probes for aging diagnosis, and further discusses the challenges and opportunities in this field.

Keywords: fluorescent probes, aging, senescence, diagnosis for aging, age-related biomarkers

1. INTRODUCTION

Aging is a time-dependent decline in physiological integrity that leads to physical function, a high risk of chronic diseases and vulnerability to death [1]. Several aging pathways and processes, such as the insulin-like signaling pathway, oxidative stress, chronic inflammation, mitochondrial dysfunction and cellular senescence, are associated with a myriad of age-related diseases including type 2 diabetes, neurodegenerative diseases, cardiovascular disease and cancer frailty [2, 3]. During the past 40 years, the demand for anti-aging medicine has shifted from sick care after occurring of diseases to preventive healthcare applications, and the risk factors for disease development are more concerning before disease onset [3]. As such, early identification and interventions for aging and age-related diseases are the basis for “healthy aging” in the general population. Aging research has achieved great success in elucidating the genetic pathways that underlie aging phenotypes, and the number of candidate

anti-aging drugs discovered in model organisms has substantially grown [4]. However, no anti-aging drugs have currently been approved, largely because of a lack of reliable drug targets and effective methods for evaluating anti-aging efficacy. Thus, in the field of aging research, an urgent need exists to develop effective methods to monitor the aging process, assess the efficacy of interventions and predict individuals' healthy lifespans.

With the development of aging research, many aging-related biomarkers originating from multiple signaling pathways have emerged [5]. Typically, the activity of senescence-associated β -galactosidase (SA- β -gal) enhances in senescent cells as the number of lysosomes increases [6]. The persistent DNA-damage response induces the phosphorylation of histone H2AX and p53; therefore, γ H2AX nuclear foci and phosphorylated p53 are commonly used as age-related biomarkers [7, 8]. Cyclin-dependent-kinase inhibitors, including p16, p15 and p21, are the main drivers of the cell-cycle arrest in cellular senescence [9–11]. Additionally, senescent cells secrete senescence-associated

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secretory phenotype (SASP) proteins, including IL-1A, IL-1B, IL-6, IL-8, and MMP-1, which contribute to dysfunction in several biological processes [12]. On the basis of those age-related biomarkers, common methods for the diagnosis of aging include SA- β -gal staining by X-gal, and the detection of proteins and mRNAs by western blotting and qPCR, respectively. However, these methods are complicated, time-consuming and particularly inappropriate for *in vivo* biological imaging. Because of the advantages of time savings, high sensitivity, flexible molecular design strategies and imageable features, various fluorescent probes for age-related biomarkers have been developed for the diagnosis of aging.

Herein, we review recent advancements and insights in the development of small-molecule fluorescence-based

probes for the diagnosis of aging (Table 1). These probes were constructed on the basis of different biomarkers, including small active molecules, microenvironmental parameters and various enzyme types. Furthermore, several probes based on unique detection strategies for aging have been designed. We hope that this review will help researchers design more precise and valuable molecular tools for the diagnosis of aging.

2. DIAGNOSIS OF AGING

2.1 Fluorescent probes for aging diagnosis based on enzymes and other markers

Sialic acid (SIA) is a type of anionic monosaccharide, which is usually located at the termini of glycoconjugates

Table 1 | Summary of optical properties and biological applications of small-molecule fluorescence-based probes for aging diagnosis

Probe	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	Detection approach	Imaging applications	Biomarkers	Reference
Sia-RQ	580/610	Turn-on	Cells	Sialidases	[14]
TP-SS	435/570 550/675	Ratiometric	Cells, mice	GPx	[17]
MitoCy-NH ₂	730/770-810 (650/700-740)	Ratiometric	Cells, mice	MAO-B	[19]
QM-NH α fuc	543/586	Turn-on	Cells, mice	α -L-fucosidase	[22]
NIR-BG2	675/709	Turn-on	Cells, mice	β -gal	[25]
BOD-L- β Gal	488/574 (533/727)	Ratiometric	Cells, mice	β -gal	[26]
YDGAL	500/585	Turn-on	Cells, tissues and organs of mice	β -gal	[27]
HeckGal	488/550	Turn-on	Cells, tumors and kidneys of mice	β -gal	[28]
KSL11	425/662	Turn-on	Cells	β -gal	[31]
KSL0401	435/706	Turn-on	Cells	β -gal	[32]
TR-G	550/590 (638)	Ratiometric	Cells, mice	β -gal	[33]
Comp. 1	488/624	Turn-on	Cells	β -gal	[34]
SRP	495/545	Turn-on	Cells	β -gal	[35]
1a	365/510	Ratiometric	Cells	H ₂ O ₂	[38]
QX-B	725/772	Turn-on	Cells, mice	H ₂ O ₂	[39]
Mito-Bor	700/730	Turn-on	Cells, mice	H ₂ O ₂	[40]
KSLP1	560/700	Light-up	Cells, <i>C. elegans</i>	Polarity	[44]
SGJ	380/480	Light-up	Cells	pH	[47, 48]
CyBC9	649/670	Light-up	Cells	Membrane potential	[49]
KSA01	460/536	Ratiometric	Cells, tissues of mice	Two-dimensional: pH, β -gal	[50]
KSA02	460/534				
ROKS	350/534; 350/534(602)	Turn-on; Ratiometric	Cells, <i>C. elegans</i>	Stress-response capability	[51]

on the surfaces of mammalian cells. The terminal SIA residues can be hydrolyzed by sialidase. Desialylation mediates a variety of biological processes, such as pathogenicity and cell inflammation [13]. To investigate the relationship between desialylation and cellular senescence, Han et al. have developed a fluorescent probe (**Sia-RQ**) for detecting sialidases [14]. **Sia-RQ** is composed of the very bright fluorophore rhodamine-X and a self-immolative moiety of 4-hydroxymandelic acid combined with a SIA as the recognition group for sialidase (Figure 1). After desialylation, **Sia-RQ** undergoes self-immolation and releases a fluorescent, reactive receptor (quinone methide-rhodamine-X dyad), which further binds sialidases. SDS-PAGE and in-gel fluorescence imaging have indicated that **Sia-RQ** can covalently label sialidase after enzymatic activation. More importantly, **Sia-RQ** has been successfully used to assess the expression level of sialidase in senescent Huh7 cells incubated with palbociclib for 0–14 days. The red-channel fluorescence of senescent cells dramatically increased at 3 d and plateaued at 7–14 d after palbociclib stimulation, thus indicating that the expression of sialidase was upregulated with senescence.

Glutathione peroxidase (GPx), a selenocysteine (Sec)-containing protein, is a crucial protease in the front-line antioxidant defense system, which is involved in the regulation of redox homeostasis and the pentose phosphate pathway [15]. Oxidative stress has been demonstrated to become more severe during the aging process. GPx, a central factor in the GPx/GSH redox pool, plays a crucial role in this process [16]. Chen et al. have reported the two-photon fluorescent probe **TP-SS** for the ratiometric detection of GPx [17], which consists of the two-photon

chromophore **TP-NH** and a 2,2'-dithiodiethanol (response site) (Figure 2A). **TP-SS** exhibits a rapid response, high selectivity and stability for the detection of Sec which serves as the active site for GPx *in vitro*. The probe has been used to monitor the fluctuation of GPx in senescent cells and the catalytic cycle of the GPx/GSH redox pool in live cells (Figure 2B). Thus, this probe can be used to monitor the catalytic cycle of the GPx/GSH redox pool and evaluate oxidative stress in living cells. Importantly, using **TP-SS**, the authors have monitored changes in GPx in mice at different ages (Figure 2C) and observed a decrease in GPx activity with aging.

Monoamine oxidase (MAO) is a flavoenzyme that is located in the outer membrane of mitochondria and catalyzes the oxidative deamination of neurotransmitters, thus producing aldehydes and hydrogen peroxide. MAOs have two isoforms: MAO-A and MAO-B. The latter generates reactive oxygen species (ROS), which cause the oxidation of biogenic amines to the corresponding aldehydes [18]. MAO-B levels are higher in young than old individuals, and consequently are a potential biomarker for senescence detection. Chen et al. have reported two probes (**MitoCy-NH₂** and **MitoHCy-NH₂**) to synergistically monitor MAO-B and its contribution to oxidative stress [19]. Both probes consist of heptamethine cyanine, propanamide and a triphenylphosphonium cation. As shown in Figure 3A, **MitoCy-NH₂** displays ratiometric fluorescence responses toward MAO-B. In contrast, **MitoHCy-NH₂** enables logic-based detection of O₂^{•-} (a by-product of oxidation of biogenic amines catalyzed by MAO-B) and MAO-B. **MitoHCy-NH₂** has been used to monitor the activity of MAO-B and ROS (O₂^{•-}) in replicative-senescent HepG2. The level of MAO-B

Figure 1 | The chemical structure of **Sia-RQ** and the proposed detection mechanism.

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Figure 2 | (A) The chemical structure of **TP-SS**. (B) **TP-SS** fluorescence imaging of GPx in living cells. Cells in group a were incubated with 10 μ M **TP-SS**; cells in group b were incubated with 10 μ M Hg^{2+} and 10 μ M **TP-SS**; cells in group c were incubated 10 μ M Hg^{2+} , 10 μ M Sec and 10 μ M **TP-SS**. The fluorescence intensity ratio of **TP-SS**-loaded cells in different groups is shown. (C) **TP-SS** fluorescence images of GPx in aged mice and in fresh brain slices. Adapted with permission from Ref. [17]. Copyright 2021 American Chemical Society.

increases with senescence, and the generated ROS may exacerbate apoptosis (Figure 3B). In addition, the probe **MitoCy-NH₂** has successfully enabled selective identification of MAO-B in H_2O_2 -induced senescent cells and in aged mice in a ratiometric near-infrared (NIR) fluorescence response (Figure 3B,C).

α -Fucosidase (α -Fuc) is a lysosomal glycosidase encoded by *FUCA1*, which is involved in the decomposition of glycoproteins, oligosaccharides and glycolipids. In all canonical types of cellular senescence (replicative, oncogene-induced and DNA-damage-induced senescence), the mRNA expression levels and enzyme activity of α -Fuc are up-regulated; consequently, this enzyme is an emerging biomarker for senescence [20, 21]. Kim et al. have reported the aggregation-induced emission (AIE) probe **QM-NH α fuc** for detecting α -Fuc (Figure 4A). The probe comprises a quinolone-malononitrile scaffold and AIEgen core and a hydrophilic α -fucopyranoside group as the response site of α -Fuc [22]. The α -Fuc expression in several cellular senescence models has been evaluated with chromogenic X-fuc assays in multiple types of senescent cells overexpressing α -Fuc. The ability of **QM-NH α fuc**

to detect α -Fuc has been verified in those senescent cell types (Figure 4B). Moreover, the ability of **QM-NH α fuc** to track aging has been evaluated in drug-induced aged xenograft-tumor mice (Figure 4C).

SA- β -gal, a lysosomal enzyme encoded by the *GLB1* gene, is widely used as a biomarker to track senescence [23]. The activity of SA- β -gal is directly associated with cellular senescence and has been regarded as the "gold standard" for monitoring senescence [24]. Recently, Cui et al. constructed the self-immobilizing NIR fluorogenic probe **NIR-BG2** [25]. A difluoromethyl group was incorporated in the probe, so that the electrophilic quinone methide species can be released and trapped by surrounding nucleophilic proteins after activation by SA- β -gal, thus retaining NIR signals (Figure 5). **NIR-BG2** produces a marked fluorescence enhancement at 709 nm after reaction with SA- β -gal. The results of fluorescent western blot assays have shown that the NIR fluorescence signal absolutely overlaps with the green fluorescence signal, thus indicating that **NIR-BG2** can be activated and further labeled with β -galactosidase (β -gal). **NIR-BG2** can be used to monitor endogenous

Figure 3 | (A) Chemical structures and the sensing mechanism of both probes. (B) Synergistic imaging of MAO-B and O₂^{•-} in senescent cells. (C) **MitoCy-NH₂** fluorescence imaging of brains and various organs in mice at different ages. Adapted with permission from Ref. [19]. Copyright 2021 American Chemical Society.

β-gal in CT26.CL25 cells and SA-β-gal in camptothecin-induced senescent Hela cells. Notably, *in vivo* fluorescence imaging experiments have indicated that **NIR-BG2** is suitable for the long-term tracking of SA-β-gal activity *in vivo*, owing to the prolonged signal retention.

Gu et al. have developed the nanoprobe **BOD-L-βGal-NPs** for the detection of β-gal. The authors first designed the NIR ratiometric probe **BOD-L-βGal** [26] by introducing a β-D-galactosyl group into boron dipyrromethene fluorophore (BODIPY) via a self-elimination linker (Figure 6A). **BOD-L-βGal** enabled the real-time detection of β-gal in senescent cells (Figure 6B). However, because **BOD-L-βGal** exhibited poor cellular uptake, the authors incorporated the probe into polymeric nanoparticles consisting of poly (lactic-co-glycolic) acid to construct the nano-probe **BOD-L-βGal-NPs**. With **BOD-L-βGal-NPs**, senescent cells and the vascular system of atherosclerotic mice have successfully been visualized (Figure 6C). Therefore, the probe may have great potential in the

early diagnosis of atherosclerosis and other age-related diseases.

Lin et al. have developed the two-photon fluorescent probe **YDGAL** for monitoring the activity of β-gal [27]. The probe has good affinity ($K_m = 12.35 \mu\text{M}$), a rapid response (stable within 10 min), high selectivity and an extremely low limit of detection ($2.185 \times 10^{-6} \text{ U/mL}$) (Figure 7A,B). **YDGAL** has also been used to evaluate the anti-aging therapeutic effects of a combination of dasatinib (D) and quercetin (Q). As shown in Figure 7C, the cells in the control group showed weak fluorescence and exhibited strong red fluorescence because of the high SA-β-gal expression. The red-channel fluorescence of senescent cells markedly decreased after treatment with anti-aging drugs (D + Q). In addition, the probe can be used to track aging process and assess the efficacy of anti-aging drugs at the organ level.

Ramón et al. have presented the new two-photon naphthalimide-styrene probe **HeckGal**, which

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Figure 4 | (A) The chemical structure and detection mechanism of **QM-NHafuc**. (B) Fluorescence imaging of the probe in different types of senescent cells. (C) **QM-NHafuc** fluorescence imaging of senescent cells in mice. Scale bar: 50 μ m. Adapted with permission from Ref. [22]. Copyright 2021 The Royal Society of Chemistry.

Figure 5 | Chemical structure of the probe **NIR-BG2** and schematic diagram of the detection of senescent cells *in vivo* by using this probe. Adapted with permission from Ref. [25]. Copyright 2021 American Chemical Society.

consists of a fluorophore (naphthalimide-stryene) and an acetylated β -galactose (Figure 8A) [28]. HeckGal has poor emission performance and a low fluorescence

quantum yield of 0.074. However, after hydrolysis with β -gal, the detection system exhibits an 11.8-fold enhancement in fluorescence quantum yield (0.8754).

Figure 6 | (A) Molecular design of the fluorescent probe **BOD-L-βGal** and its sensing mechanism. (B) Ratiometric images of VSMCs including Ang II-induced senescent VSMCs, serum-free-treated VSMCs and primary VSMCs. (C) **BOD-L-βGal-NPs** fluorescence imaging of atherosclerotic mice and control mice. (B) and (C) Adapted with permission from Ref. [26]. Copyright 2021 American Chemical Society.

Figure 7 | (A) Chemical structure of **YDGAL**. (B) Fluorescence spectra of probe after the addition of β -gal. (C) Use of the probe to evaluate the therapeutic effects of anti-aging drugs. (B) and (C) Adapted with permission from Ref. [27]. Copyright 2021 American Chemical Society.

The ability of **HeckGal** to detect β -gal in cells has been evaluated in normal human fibroblasts and senescent cancer cells including SK-Mel-103, 4T1, A549 and BJ cells. Importantly, the authors used two different models of

senescence-related disease to investigate the ability of **HeckGal** to track the aging process *in vivo*, including an orthotopic breast cancer mouse model (BALB/cByJ mice) stimulated with palbociclib and a renal fibrosis mouse

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Figure 8 | (A) Molecular design of the probe and the application of the probe in aged mice. (i) Kidney fibrosis in C57BL/6J male mice was induced by treatment with folic acid. (ii) BALB/cByJ female mice bearing 4 T1 breast cancer tumors were stimulated with palbociclib. The emission spectra of **HeckGal** and **Heck** are shown. (B) Images of tumors stained with the SA- β -Gal assay; immunohistochemical detection and fluorescence images of organs and tumors in BALB/cByJ female mice (ii) are shown. (C) Images of p21 immunostaining in kidney slides and fluorescence images of kidneys from C57BL/6J mice in the indicated groups (i). Scale bar: 200 μ m. Adapted with permission from Ref. [28]. Copyright 2021 American Chemical Society.

model (C57BL/6J mice) induced by folic acid. As shown in **Figure 8B,C**, only senescent tumors emitted significant fluorescence in BALB/cByJ mice, and substantial fluorescence emission was observed in only the fibrotic senescent kidneys of FA-treated mice. Therefore, **HeckGal** enables unambiguous detection of senescence *in vitro*, as well as in tissues and tumors *in vivo*.

E. coli β -gal, a *lacZ*-encoded enzyme from bacteria, is frequently used as the SA- β -gal model protein *in vitro* [29, 30]. However, unlike human β -gal, bacterial β -gal is not associated with senescence. To distinguish human

SA- β -gal from the β -gal from other species (such as *E. coli* β -gal), Li et al. have proposed a molecular-design strategy to build fluorescent probes by using structure-based steric hindrance adjustment to cater to various enzyme pockets [31]. The authors designed and synthesized a series of probes (**KSL01–KSL12**) differing in their steric hindrance around the β -D-galactosyl group (recognition group), thus providing a wide range of green-to-NIR color options for monitoring β -gal. Among those probes, **KSL08–KSL12** exhibits desirable responses to only *A. oryzae* β -gal, thus enabling the first species-specific

Figure 9 | (A) Chemical structure of the species-selective chemical probe **KSL11** and schematic diagram of the application of this probe to distinguish human SA-β-gal from *E. coli* β-gal. (B) Representative images of HepG2 cells and Hep3B cells co-stained with 10 μM **KSL11** and Hoechst. Scale bar: 20 μm. (C) Confocal images of kidney sections contaminated by *E. coli* β-gal, incubated with **KSL04** and **KSL11**. Adapted with permission from Ref. [31]. Copyright 2021 The Author(s).

identification of β-gal. **KSL11** was selected as the representative probe among the first-generation species-selective probes (Figure 9A) and further investigated in senescent cells (MRC-5 cells), *lacZ*-transfected cells (293T and HepG2 cells) and tissue sections contaminated by *E. coli* β-gal (Figure 9B,C). **KSL11** has been used to selectively monitor SA-β-gal without interference from bacterial β-gal in these samples. Importantly, with this probe, the authors have discovered that the content of SA-β-gal is gradually enhanced with age, and the kidney undergoes the most severe aging among organs during the aging process.

Li et al. have reported the NIR fluorescent probe **KSL0401** for monitoring SA-β-gal activity [32]. This probe was constructed by grafting the coumarin-dicyanoisophorone hybrid fluorophore **KSL0401-OH** with a β-D-galactosyl group (β-gal-activable trigger) (Figure 10A). **KSL0401** releases the fluorophore **KSL0401-OH**, which exhibits a long NIR fluorescence wavelength (706 nm) and a large Stokes shift (218 nm) after being hydrolyzed by SA-β-gal (Figure 10B). Notably, **KSL0401** displays a highly rapid response to β-gal (< 80 s) (Figure 10B), which is better than that of most previously reported SA-β-gal probes. Additionally, **KSL0401** shows good biocompatibility and has been used to monitor SA-β-gal activity in various senescent

cell types, including H₂O₂-induced senescent MRC-5 cells, MLN4924-induced senescent HepG2 cells and A549 cells (Figure 10C), thus indicating the feasibility of using **KSL0401** to evaluate the degree of senescence.

Lin et al. have developed the fluorescent probe **TR-G** for the detection of β-gal [33]. The authors first synthesized the new fluorophore **Rho** by replacing the phenethylamine moiety of rhodamine B with naphthol, then introduced a β-D-galactose group into the fluorophore to build the probe **TR-G** (Figure 11). After the addition of β-gal, **TR-G** exhibited a marked increase in fluorescence intensity at 638 nm. **TR-G** also exhibits two-photon properties, thereby indicating that the probe is suitable for monitoring β-gal in deep tissues. The activity of β-gal in living cells, organs and tissues has been detected effectively with **TR-G**. More importantly, **TR-G** has been successfully used to monitor β-gal in tumor tissue with deep penetration (up to 120 μm in tissues) through two-photon microscopy. **TR-G** has also been applied to monitoring the activity of β-gal *in vivo* in tumors with β-gal overexpression in a mouse model.

Yang et al. have developed the β-gal-responsive molecule **Comp. 1** (TPE-ETH-R-GFFY(gal)-ERGD), which is based on the novel AIEgen TPEETH-R (Figure 12), emits strong red fluorescence and generates cytotoxic ROS under light excitation in the aggregated state [34].

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Figure 10 | (A) Molecular design of **KSL0401** and its mechanism for SA-β-gal detection. (B) Emission spectra and kinetic analysis of **KSL0401** after the addition of β-gal. (C) Representative confocal images of young cells and drug-induced senescent cells after incubation with **KSL0401**. Scale bar: 30 μm. Reprinted with permission from Ref. [32]. Copyright 2021 Elsevier.

Figure 11 | Molecular design and the proposed detection mechanism for SA-β-gal of **TR-G**.

Comp. 1 can be transformed into **TPE-Eth-R-GFFYERGD** after hydrolysis by β-gal, thus leading to the production of a supramolecular assembly with remarkable fluorescence and ROS generation. **Comp. 1** can specifically identify cisplatin-induced senescent HeLa cells with strong red fluorescence. Additionally, **Comp. 1** under light irradiation selectively produces ROS, thereby removing senescent HeLa cells under light irradiation. Consequently, **Comp. 1** has the potential to be used to monitor and remove senescent cancer cells.

Bhuniya et al. have developed the β-gal fluorescent probe, **SRP**, to track senescence (Figure 13) [35]. **SRP** displays faint fluorescence in spirocyclic form. After

the addition of β-gal, a marked increase (~27-fold) in fluorescence intensity at 545 nm relative to the probe has been observed. **SRP** exhibits high reactivity to β-gal within a pH range of 5–9 and consequently has the potential to evaluate β-gal activity in cell lysosomes. In addition, the probe can target lysosomes. More importantly, **SRP** has high biocompatibility and can distinguish senescent from young human umbilical vein cells, thus confirming the feasibility of using **SRP** to assess cellular senescence.

ROS, such as the hydrogen peroxide (H_2O_2), the hydroxyl radical ($\text{OH}\cdot$), the superoxide anion ($\text{O}_2^{\cdot-}$) and hypochlorous acid (HOCl), act as intracellular signals

Figure 12 | Chemical structure of the probe **Comp. 1** and the proposed sensing mechanism.

Figure 13 | Molecular design of **SRP** and its proposed detection mechanism.

and growth stimulants. However, excess ROS cause oxidative damage to DNA and further promote stress-induced premature senescence in cells [36]. Typically, the ROS levels increase in various types of cancers and cellular senescence [37]. Mizuta et al. have developed two fluorescent probes to detect oxidative stress and the activity of β -gal to evaluate stress-induced premature senescence [38]. The authors first synthesized six candidate fluorescent probes **1a–f** for ROS detection and screened the optimal probe **1a** (Figure 14A,B). Probe **1a** displayed a ratiometric response to ROS (H_2O_2 , $\text{OH}^\bullet$ and $\text{O}_2^{\bullet-}$) and was further used to image the accumulation of ROS in living cells. In addition, the authors designed and synthesized the fluorescent probe **CBT- β -Gal 4** to monitor β -gal activity. **CBT- β -Gal 4** was used to monitor β -gal activity during oxidative-stress-induced senescence, and HeLa cells stimulated by H_2O_2 exhibited marked fluorescence. Thus, probes **1a** and **CBT- β -Gal 4** can be used to monitor the increased levels of ROS and β -gal activity in drug-induced senescent cells (Figure 14C).

Li et al. have developed the NIR fluorescent probe **QX-B** for the detection of H_2O_2 [39]. The probe was constructed on the basis of a quinolinium-xanthene fluorophore with long absorption (725 nm) and emission (772 nm) wavelengths. Borated ester-caged

QX-B is non-fluorescent, because the intramolecular charge-transfer process was blocked (Figure 15A). After reaction with H_2O_2 , the borate ester unit of the probe was removed, and the intramolecular charge-transfer process was restored, thus leading to a pronounced fluorescence signal. The probe was used to monitor exogenous and endogenous H_2O_2 in various cells, including HCT116, 4T1 and HeLa cells. Furthermore, the probe successfully enabled visual tracking of H_2O_2 in zebrafish and in mice (Figure 15B,C).

Yu et al. have developed a mitochondria-targetable NIR fluorescent probe (**Mito-Bor**) for tracking of H_2O_2 *in vitro* and *in vivo* [40]. The probe consists of three moieties comprising an NIR fluorophore (azo-BODIPY), a H_2O_2 -responsive unit (aryl borate) and a mitochondrial targeting group (triphenylphosphonium cation) (Figure 16A). **Mio-Bor** is highly sensitive to H_2O_2 , and the limit of detection has been calculated to be 23 nM. Moreover, the probe specifically targets mitochondria and can be used to monitor H_2O_2 in live cells (Figure 16B,C). Using **Mio-Bor**, the authors have successfully monitored the changes in H_2O_2 levels in mouse models of pulmonary fibrosis induced by bleomycin (Figure 16D).

Many studies have documented that cellular senescence can cause significant changes in the intracellular

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Figure 14 | (A) Chemical structures of **1a** and **CBT-β-Gal 4**. (B) Emission spectra of **1a** after the addition of H_2O_2 and of **CBT-β-Gal 4** after reaction with β -gal. (C) Images of **1a** and **CBT-β-Gal 4** in HeLa cells incubated with or without doxorubicin (Dox). Adapted with permission from Ref. [38]. Copyright 2021 American Chemical Society.

microenvironment [41, 42]. Polarity, a microenvironmental factor, is considered to play an important role in multiple cellular processes including proliferation, cell differentiation and regulation of the immune system [43]. Li et al. have developed six curcumin-based fluorescent probes (**KSLP1–6**) for the detection of polarity [44]. Thru analysis of the detection ability of all probes, **KSLP1** was identified for its high selectivity to polarity and high biocompatibility. The probe **KSLP1** exhibited a significant enhancement (42-fold) in fluorescence intensity at ~ 700 nm, as the polarity changed from $\Delta f = 0.397$ to $\Delta f = 0.367$ (Figure 17A,B). The polarity in various cells, including 293T, HL-7702, A549, HepG2 and Hep3B cells, has been evaluated with **KSLP1**. Moreover, **KSLP1** has been used to monitor lysosomal polarity in LY3177833-induced senescent Hep3B cells and replication-induced senescent MRC-5 cells. The polarity in lysosomes increases with senescence (Figure 17C–E). Furthermore, the polarity of *Caenorhabditis elegans* (*C. elegans*) at various ages

(from 2 days old to 10 days old) has been visualized with **KSLP1**, and the overall body polarity has been found to increase with age. The authors have proposed a potential mechanism for the variation in polarity in lysosomes with senescence, in which the activity of pH-dependent hydrolases is decreased by the de-acidification of lysosomes, thereby promoting proteolysis of these lysosomal enzymes and resulting in further production of hydrophilic substrates (Figure 17F).

Lysosomes, ubiquitous and dynamic organelles, receive and degrade macromolecules from various pathways including secretory autophagic, endocytic and phagocytic membrane-trafficking pathways [45]. The pH value of lysosomes ranges from 4.5 to 5.5 in the normal cellular state, which is maintained by proton-pumping vacuolar ATPases. Studies have indicated altered v-ATPase activity and lysosomal pH dysregulation in cellular senescence [45, 46]. Miao et al. have reported the small-molecule fluorescent probe **SGJ**, which can selectively and sensitively monitor pH with a

Figure 15 | (A) Chemical structure of **QX-B** and sensing mechanism. (B) Confocal images of zebrafish stained with **QX-B**. Scale bar: 200 μm . (C) Fluorescence imaging of mice from the indicated groups after the injection of **QX-B**. (B) and (C) Adapted with permission from Ref. [39]. Copyright 2021 American Chemical Society.

rapid response [47, 48]. **SGJ** was designed and synthesized on the basis of a novel fluorophore comprising an imidazo[1,5-a]pyridine derivative, which has shown good biological activity in anti-inflammation and has been widely used to develop medicines (Figure 18A). The probe shows a rapid response, good reversibility, high selectivity and sensitivity to pH and is able to target lysosomes (Figure 18B). Interestingly, the authors have discovered that **SGJ** suppresses cellular senescence and improves the viability of senescent cells. After treatment with **SGJ**, the acidic vacuoles in senescent cells significantly increase, on the basis of staining with acridine orange, an indicator of changes in lysosomal pH (Figure 18C). Furthermore, **SGJ** inhibits the decrease in H^+ concentrations in lysosomes; increases the expression of the lysosome-associated membrane proteins LAMP1 and LAMP2 (Figure 18D); and restores lysosomal activity in senescent cells, thereby suppressing senescence.

Oh et al. have performed high-throughput screening based on the diversity-oriented fluorescence library

approach [49]. Using this platform to screen 6417 compounds (fluorescent probes), the authors found that the fluorescent probe **CyBC9** (Figure 19A,B) specifically stains senescent human mesenchymal stromal cells (MSCs). **CyBC9** exhibits high cellular uptake, and high sensitivity and specificity in monitoring senescent MSCs in both early and late stages of senescence. Moreover, **CyBC9** stains live senescent MSCs in microcarrier-based culture. To study the mechanism of **CyBC9**'s detection of senescent cells, the authors performed colocalization assays using **CyBC9**, which revealed that the probe accumulates in the mitochondria, according to the high Pearson correlation coefficient values. Furthermore, using carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) to decrease the membrane potential of mitochondria by inducing uncoupling of mitochondria in living cells, the authors found that CCCP-treated cells and senescent cells are specifically stained by **CyBC9**, thus suggesting that **CyBC9** selectively stains senescent cells because of the decrease in membrane potential of mitochondria with senescence.

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Figure 16 | (A) Chemical structure of **Mito-Bor** and the sensing mechanism. (B) Co-localization experiments with A549 cells stained by Mito tracker and **Mito-Bor**. Scale bar: 2 μ m. (C) Confocal images of A549 cells and PC9 cells after incubation with **Mito-Bor**. Scale bar: 10 μ m. (D) *In vivo* fluorescence imaging of mice (model of pulmonary fibrosis) treated with the probe via an intratracheal spray. (B–D) Adapted with permission from Ref. [40]. Copyright 2021 American Chemical Society.

2.2 Fluorescent probes for precise diagnosis of aging, in the time and space dimensions

Most evaluations of senescence and diagnosis of aging have focused on monitoring the levels of single age-related biomarkers in the resting state. However, the non-specificity of current senescence-associated biomarkers and the existence of different senescence programs have strongly limited these methods.

Previously described fluorescent probes for monitoring SA- β -gal to track cellular senescence have reported on only a single dimension: the accumulation of SA- β -gal in lysosomes. However, endogenous β -gal is closely associated not only with senescence but also with several other biological processes, and thus single-marker levels are insufficient to fully evaluate aging. Recently, Guo et al. have developed two-dimensional fluorescent probes (**KSA01** and **KSA02**) to precisely track senescence [50]. De-acidification has been reported as a unique feature of lysosomes in senescent cells. Thus, the authors have proposed a two-dimensional detection strategy that relies on the monitoring of SA- β -gal activity in combination with the detection of pH in the lysosomal microenvironment. Both probes were designed on the basis of merocyanine-based fluorescent dyes modified by carboxyl groups to achieve sensitivity within the pH range of lysosomes. These pH-sensitive dyes contain a β -D-galactosyl group as the β -gal-responsive site (Figure 20A). The ability to simultaneously monitor β -gal activity and the local microenvironment of the probes was first verified *in vitro* (Figure 20B). Using the probes,

the authors have successfully distinguished the SA- β -gal in senescent HL-7702 cells from cancer-related endogenous β -gal in SKOV-3 cells. As shown in Figure 20C, **KSA02**-stained senescent cells mainly display red fluorescence, whereas **KSA02**-stained SKOV-3 cells exhibit green fluorescence, thus indicating the ability of **KSA02** to distinguish SA- β -gal from cancer-associated β -gal. Furthermore, the visual detection of SA- β -gal by **KSA01** and **KSA02** in kidney tissues with different degrees of aging has been achieved (Figure 20D).

Examination of the stress response capacity (SRC) holds promise in the dynamic assessment of aging. Thiophenol (PhSH), a highly toxic chemical substance, can induce cellular oxidative stress and lead to increased levels of ROS, such as HOCl. Accordingly, PhSH and HOCl act as the stressor and the stress product, and the sequential and logical detection of PhSH and HOCl is crucial to detect the evolution of stress and evaluate the SRC. Very recently, Guo et al. developed the first SRC fluorescent probe, **ROKS** (Figure 21A), a dual-channel fluorescent probe enabling sequential, logical detection of PhSH and HOCl [51]. **ROKS** displays faint fluorescence, whereas a significant emission peak at 534 nm is observed upon reaction with PhSH. After continued addition of HOCl, this emission peak (534 nm) disappears, and simultaneously a red emission peak at 602 nm forms (Figure 21B,C). Thus, the fluorescence ratio of both channels (I_{red}/I_{green}) can be used to precisely evaluate the SRC in individuals. With the dual-channel fluorescent probe **ROKS**, the changes

Figure 17 | (A) Chemical structure of **KSLP1**. (B) Emission spectra of **KSLP1** in mixtures of dioxane and water (dioxane from 0% to 70%) and the relationship between the fluorescence intensity at ~ 700 nm and the orientation polarizability (Δf). (C) Confocal images of Hep3B cells and senescent Hep3B cells incubated with **KSLP1**. Scale bar: 25 μ m. (D) Confocal images of MRC-5 cells incubated with **KSLP1**. Scale bar: 25 μ m. (E) Fluorescence images of *C. elegans* at different ages (from 2 days old to 10 days old), stained with **KSLP1**. Scale bar: 200 μ m. (F) Schematic illustration of proposed variations in lysosomal polarity in aging. (B–F) Reprinted with permission from Ref. [44]. Copyright 2021 Elsevier.

in SRC, as a function of aging within *C. elegans* at different ages, has been monitored. As shown in **Figure 21D**, the SRC of young *C. elegans* is higher than that of aged *C. elegans* after stimulation with PhSH, thereby suggesting that SRC decreases with aging. These results have confirmed that the SRC-based strategy is particularly useful to precisely track the aging process from a dynamic perspective.

3. CONCLUSIONS AND PERSPECTIVES

As described above, fluorescent probes for the diagnosis of aging are important in aging research and healthspan evaluation. We have summarized the advances in various types of small-molecule fluorescence-based probes used for the diagnosis of aging. These optical probes can be divided into two categories according to various age-related biomarkers, including enzymes (β -gal, α -Fuc, GPx and SIA) and other

markers (small active molecules and microenvironmental factors), and the novel detection strategies have expanded from the time dimension to the space dimension. Among these probes, **TP-SS**, **YDGAL**, **HeckGal** and **TR-G** are two-photon probes for aging diagnosis, which can be used for *in vivo* biological imaging. **KSL11**, the first-generation species-selective fluorescent probe for SA- β -gal, has enabled the first species-selective identification of human SA- β -gal. In addition, **Sia-RQ** and **NIR-BG2** can label proteins after activation, thereby allowing for *in situ* imaging of age-related biomarkers. Moreover, compared with probes that monitor the single-dimensional markers to evaluate the aging process, **KSA01-02** and **ROKS**, designed to detect the dimensions of space and time, can be used to track aging more precisely. Generally speaking, researchers have developed diverse fluorescent probes based on different biomarkers and strategies to diagnose aging, and have made exciting progress.

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Figure 18 | (A) Chemical structure of the probe **SGJ**. (B) **SGJ** co-located with lysosome trackers. (C) Fluorescence images of young BMSCs (population doubling level, PDL 5) and senescent BMSCs (PDL 20) stained with acridine orange (AO). (D) Expression levels of LAMP1 and LAMP2, analyzed by western blotting with β -actin as a loading control. BMSCs were treated with **SGJ** (20 μ M) for different times.

Figure 19 | (A) Senescence-specific probes for MSCs screened through a diversity-oriented fluorescence library approach and (B) chemical structure of **CyBC9**.

Figure 20 | (A) Schematic diagram of the molecular design of two-dimensional fluorescent probes for tracking aging and their proposed sensing mechanism. (B) Emission spectra of the probe **KSA02** after the addition of *A. oryzae* β-gal (0–1 U) and *E. coli* β-gal (0–1 U). (C) Representative images of senescent (Sct) HL-7702 cells and SKOV-3 cells incubated with **KSA02**; Western blot analysis of human β-gal and γH2AX expression levels in Sct HL-7702 and SKOV-3 cells. A schematic illustration of the distributions of SA-β-gal in Sct senescent HL-7702 cells and SKOV-3 cells is shown. Scale bar = 5 μm (C, column 1 and column 2); scale bar = 10 μm (C, column 3). (D) Representative images of kidney sections of mice without or with treatment with doxorubicin (DOX, 38 days), stained by **KSA01** and **KSA02**. Scale bar = 100 μm. Adapted with permission from Ref. [50]. Copyright 2021 Wiley.

Although great progress in developing fluorescent probes for the diagnosis of aging has been made, some challenges remain to be addressed. Given the high heterogeneity and the dynamics of aging *in vivo*, no consensus exists regarding aging biomarkers; consequently, progress has been slow in the accurate diagnosis of aging and the discovery of anti-aging drugs. Thus, developing more accurate and trustworthy strategies for aging evaluation beyond the identified age-specific biomarkers should shed light on a new paradigm for the precise

diagnosis of aging. Additionally, an urgent need exists to establish an analytic platform for rapid, high-throughput screening of anti-aging drugs by using desirable small-molecule fluorescent probes. This screening platform could decrease the time and costs for discovering novel anti-aging drugs, and promote the development of anti-aging research. Ideally, in the future, new fluorescent probes will enable more accurate, sensitive and efficient diagnosis of aging, and significantly contribute to improving the human healthspan.

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Figure 21 | (A) Schematic diagram of the dynamic evaluation of aging by using **ROKS**. (B) The sensing mechanism and reaction scheme of **ROKS** for logical detection of PhSH and HOCl. (C) Emission spectra of **ROKS**, **ROKS** after the addition of PhSH or the mixture of PhSH and HOCl; emission spectra of **ROKS** after addition of PhSH; emission spectra of **ROCL** after the addition of HOCl. (D) Representative ratio images of *C. elegans* of different ages (6 days old and 12 days old). Young and aged *C. elegans* were incubated with **ROKS** and then treated with PhSH for various times (0–60 min). Adapted with permission from Ref. (51). Copyright 2021 The Author(s).

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