

A GUIDE TO...

A guide to assessing cellular senescence *in vitro* and *in vivo*

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Cellular senescence is a physiological mechanism whereby a proliferating cell undergoes a stable cell cycle arrest upon damage or stress and elicits a secretory phenotype. This highly dynamic and regulated cellular state plays beneficial roles in physiology, such as during embryonic development and wound healing, but it can also result in antagonistic effects in age-related pathologies, degenerative disorders, ageing and cancer. In an effort to better identify this complex state, and given that a universal marker has yet to be identified, a general set of hallmarks describing senescence has been established. However, as the senescent programme becomes more defined, further complexities, including phenotype heterogeneity, have emerged. This significantly complicates the recognition and evaluation of cellular senescence, especially within complex tissues and living organisms. To address these challenges, substantial efforts are currently being made towards the discovery of novel and more specific biomarkers, optimized combinatorial strategies and the development of emerging detection techniques. Here, we compile such advances and present a multifactorial guide to identify and assess cellular senescence in cell cultures, tissues and living organisms. The reliable assessment and identification of senescence is not only crucial for better understanding its underlying biology, but also imperative for the development of diagnostic and therapeutic strategies aimed at targeting senescence in the clinic.

Introduction

The field of cellular senescence has witnessed a marked explosion in the last decades as a multitude of novel roles in both physiology and disease have been

attributed to this intriguing phenomenon. In 1961, Hayflick and Moorhead first described cellular senescence as an irreversible nondividing state where cells

Abbreviations

ARF, alternative reading frame; BrdU, 5-bromo-2'-deoxyuridine; CDKIs, cyclin dependent kinase inhibitors; cfDNA, cell-free DNA; DAPI, 4',6'-diamidino-2-phenylindole; DDR, DNA damage response; DNA-SCARS, DNA segments with chromatin alterations reinforcing senescence; EdU, 5-ethynyl-2'-deoxyuridine; EVs, extracellular vesicles; FACS, fluorescence-activated cell sorting; HP1, heterochromatin protein 1; IR, infrared; *LacZ*, structural gene encoding for β -galactosidase in *E. nonbreaking space coli*; MRI, magnetic resonance imaging; NIR, near infrared; OIS, oncogene-induced senescence; PET, positron emission tomography; PTM, post-translational modification; RB, retinoblastoma protein; ROS, reactive oxygen species; SAHF, senescence-associated heterochromatic foci; SASP, senescence-associated secretory phenotype; SA- β -gal, senescence-associated β galactosidase; SBB, Sudan Black-B; TIF, telomere induced foci; TPP, tri-phenyl-phosphonium; γ -H2AX, phosphorylated H2A histone family member X.

remain metabolically active after reaching their replicative potential *in vitro* [1]. After extensive research, we now know that cellular senescence can be triggered in response to a variety of stressors, including telomere shortening, oxidative stress, DNA damage and oncogene activation [2], among others, and that the cell cycle arrest is only one of its hallmarks (Fig. 1). Consequently, cellular senescence has been redefined as a mechanism whereby a dividing cell enters a stable cell cycle arrest upon a damaging or stressing stimulus and generally exerts a complex secretion of factors (known as Senescence-Associated Secretory Phenotype, or SASP) that impacts the nearby tissue, while remaining metabolically active and unresponsive to mitogenic and apoptotic signals.

Remarkably, depending on the context, senescence can result in both beneficial and detrimental effects to the tissue or organism. It is generally believed that in young individuals or upon acute damage, senescence contributes to tumour suppression, wound healing and tissue homeostasis, primarily due to the arrested cell cycle and the secretion of specific factors and cytokines via the SASP. This secretion informs the immune system to initiate the clearance of senescent cells and stimulates the damaged tissue to heal [3–11]. However, in older individuals or upon consistent and chronic damage, the process of immunosurveillance is deregulated and senescent cells accumulate, contributing to tissue dysfunction, chronic inflammation and a variety of age-associated disorders, including cancer [12–15], cardiovascular diseases [16,17], fibrosis [18,19], diabetes [20,21], neurological disorders [22,23] and osteoarthritis [24], among many others [2,25]. Given these antagonistic effects, cellular senescence is regarded as a double-edge sword in health and disease.

As our knowledge of senescence advances at a staggering speed, so does our awareness of the complexity and heterogeneity of the senescent programme itself. It is very likely that the term cellular senescence comprises different phenotypes and cellular states, owing to the evidence that the programme implemented significantly depends on the stressor or insult, the cell type and the physiological context [26]. This complexity is exacerbated by the fact that the biomarkers used to detect cellular senescence are generally nonspecific and sometimes unreliable, and a real consensus on how to best detect and describe cellular senescence still remains to be determined. This is highlighted in a recent meta-review, where the authors found a positive correlation between senescence and ageing in different tissues, but the findings varied substantially depending on the identification

technique employed [27]. These barriers have hampered our ability to detect senescent cells confidently and, as a consequence, a complete picture of the role that cellular senescence plays within tissues and living organism remains uncovered.

Despite these difficulties *in vivo*, a set of general hallmarks of cellular senescence has been defined (Fig. 1). In addition to the cell cycle arrest and the SASP, senescent cells also exhibit an enlarged and flattened morphology, expanded lysosomal compartment and particular chromatin and epigenetic alterations, among others. However, many of these features are not exclusive nor essential for the implementation of the programme. Consequently, in order to validate the presence of cellular senescence with greater confidence, a multimarker approach has been proposed [27,28]. Here, we refine the existing knowledge and describe a practical guide to assess and detect cellular senescence both *in vitro* and *in vivo*. We compile and organize the different methods and strategies that have been described thus far, and present an optimized multifactorial strategy for the definition of cellular senescence in cell culture and in tissues. Lastly, we outline novel techniques and features that can potentially play a role in defining the senescent state and identifying it in living organisms in the near future.

Assessing cellular senescence *in vitro*

Because no single trait can solely define cellular senescence, the concurrent validation of multiple hallmarks is needed to confirm the senescent phenotype *in vitro*. As depicted in Fig. 1, these hallmarks include a variety of structural, epigenetic and signalling alterations. However, such changes may sometimes be only relative as compared to nonsenescent counterparts, and they may not be present in some senescent phenotypes. We therefore suggest the verification of at least three different traits, including (a) the halt of the cell cycle progression, (b) a relevant structural change (such as the increased lysosomal mass, multinucleation, senescence-associated heterochromatic foci (SAHFs), increased number of vacuoles, the loss of lamin B1 and a flattened and enlarged shape) and (c) an additional trait that is known to be specific for that subtype of senescence being tested (for instance, DNA damage-related markers, increased ROS levels, upregulation of specific SASP factors, etc.) (Fig. 2A). Here, we outline each of these hallmarks and present the most informative-rich techniques employed for their detection *in vitro*, which are also thoroughly compiled in Table 1. We describe primary markers as the most commonly used for senescence identification, which

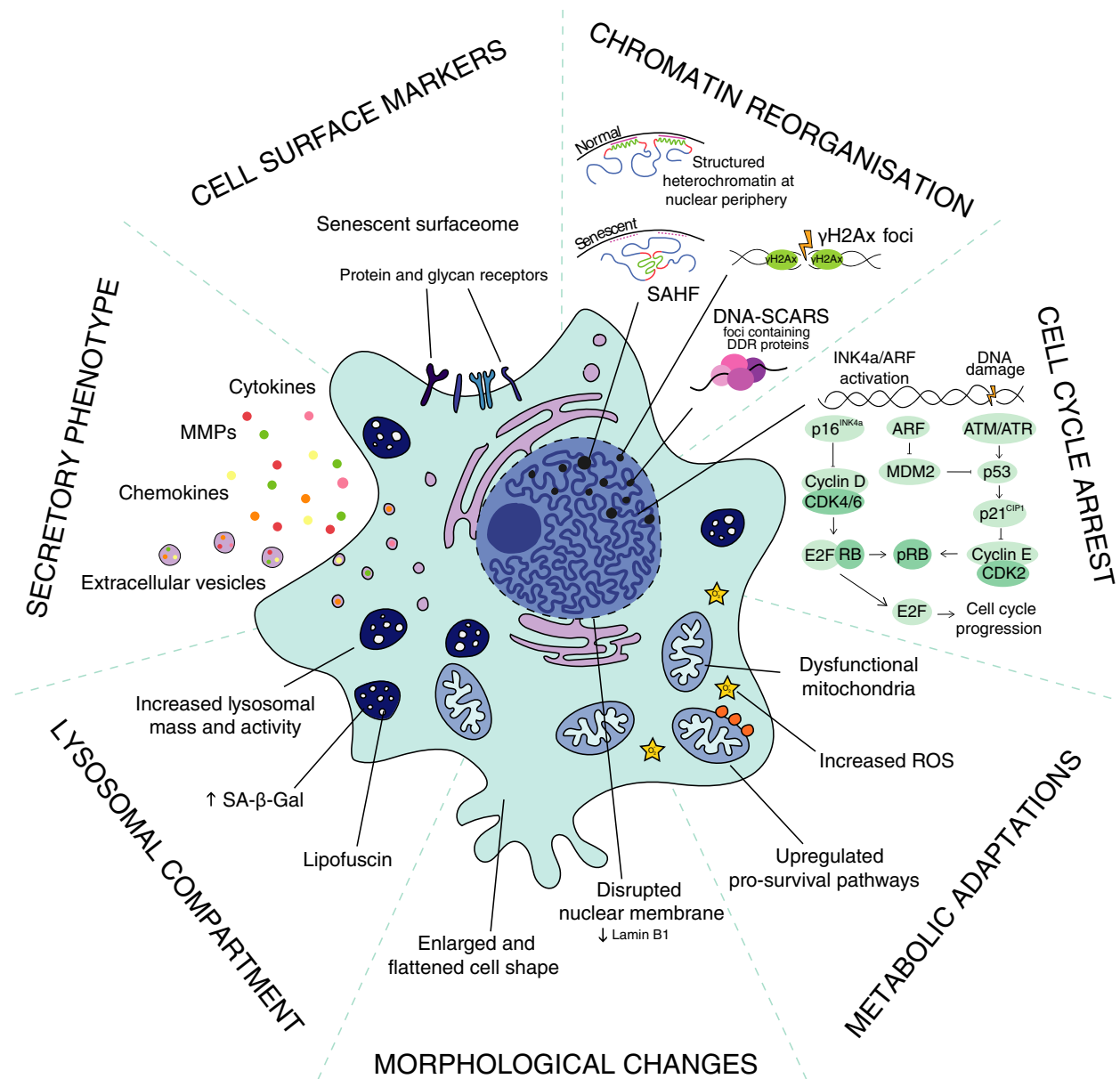


Fig. 1. General hallmarks of cellular senescence. A number of cellular mechanisms and alterations constitute the so-called hallmarks of cellular senescence. These include a stable cell cycle arrest, driven by the action and cooperation of several proteins implicated in the p16^{INK4a}/Rb and p21^{CIP1}/p53 axes, depending on the senescence driver; chromatin alteration and reorganization, which includes SAHFs and DNA-SCARS marks and also results in the disruption of the nuclear envelope; macromolecular damage and metabolic changes, including increased ROS levels and dysfunctional mitochondria; resistance to apoptosis, resulting from the upregulation of pro-survival pathways; increased lysosomal compartment, which is characterized by the well-known overexpression of SA-β-gal, and the implementation of a strong paracrine secretion termed SASP. While these markers are strongly associated to the senescent phenotype, they are not exclusive nor indispensable for the implementation of the programme (except for the cell cycle arrest), and may not always be detected in senescent cells. Senescent cells *in vitro* also generally present a characteristic enlarged and flattened morphology. In addition, further features have been described, including the presence of more than one nucleus (multinucleation), cytoplasmic DNA and the expression of what is now surging as the senescent surfaceome.

include those needed for the verification of steps 1 and 2 (Fig. 2A), and secondary markers as those used with less frequency, whose presence or expression might

depend on the specific senescent phenotype and are required for step 3 of our proposed assessment approach (Fig. 2A).

Detection of primary senescence markers

Cell cycle arrest

The arrested cell cycle is one of the most defining hallmarks of cellular senescence and is characterized by the protein markers p16, p21, p53 and a decrease in phosphorylated Retinoblastoma protein (pRB). The two major pathways that govern this cell cycle arrest include the p16^{Ink4a}/RB and p53/ p21^{CIP1} axes. In both networks, the final downstream target is RB as its hypophosphorylated state binds to E2F, a transcription factor that promotes the progression of the cell cycle and the transition into S phase. However, when bound with RB, E2F cannot target its typical gene promoters, which prevents the transcription of replicative genes, and as a result, the cell cycle is halted.

In the p16^{Ink4a}/RB pathway, upon activation of *INK4a/ARF* genetic locus, the protein p16^{Ink4a}, a cyclin-dependent kinase inhibitor (CDKI), directly inhibits the CDK4-CyclinD complex, which allows for the dephosphorylation and stabilization of the RB-E2F complex, and thus the inhibition of the cell cycle (Fig. 1A) [29]. In the p53/ p21^{CIP1} pathway, p53 is activated via phosphorylation (p-p53) after a DNA-damaging stimulus and then upregulates the transient expression of the CDKI p21^{CIP1}. p21^{CIP1} then inhibits CDK2-cyclin E, which allows the dephosphorylation of RB leading to the sequestering of E2F to arrest the cell cycle (Fig. 1A).

The p53/ p21^{CIP1} pathway is observed in replicative senescence, DNA damage response (DDR)-induced senescence, Reactive Oxygen Species (ROS)-induced senescence and oncogene-induced senescence (OIS) and is thought to be activated early on in the senescence programme [30,31]. On the other hand, p16^{Ink4a}/RB pathway is usually activated in replicative senescence, ROS-induced senescence and OIS, but not during DDR-induced senescence, and it is thought to play a larger role in maintaining the senescent state [30,31].

Additionally, the CDKIs p15^{INK4b} and p27^{KIP1} and the mitogen-activated protein kinase p38 can also be used as markers although their involvement in the senescent program is not as defined nor as global [2]. However, some of these markers, such as RB and p53, are also present in other forms of cell cycle arrest, such as quiescence. During DDR, the upregulation and activation of p53 allow for a transient cell cycle arrest to occur so the cells are able to repair damaged DNA [32]. In differentiated cells, a decrease in pRB is commonly seen to permanently arrest the cell cycle [33]. In addition, p16 expression has been

observed in various cancer cell lines [34,35]. For this reason, multiple markers, typically p16, p21, p53, p-p53 and a decrease in pRB, should be explored in order to get more conclusive information on the senescent cell cycle arrest.

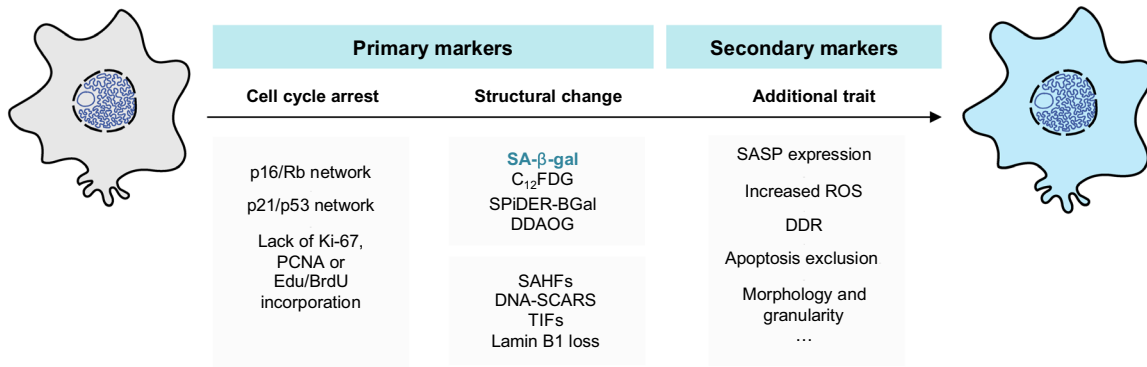
In addition to the above protein markers, the cell cycle arrest should be further assessed through cellular proliferation and/or DNA replication assays. Live and automated cell imaging and counting or spectrophotometry at different time-points over time can be used to generate and analyse the growth curve of cells *in vitro*. The assessment of DNA synthesis through the incorporation of modified nucleotides, such as 5-ethynyl-2'-deoxyuridine (EdU) or 5-bromo-2-deoxyuridine (BrdU), into the DNA of a replicating cell can also be performed. As cell cycle arrest is characterized by loss of replicative ability, senescent cells show a reduction in this incorporation of these nucleotides. After these assays, the incorporation of these nucleotides or lack thereof can be detected by fluorescence imaging or flow cytometry analysis [36]. In addition, immunostaining for other proliferation markers, such as the nuclear protein Ki-67, can also be performed [37]. Although ki-67 is not a direct measure of active proliferation like the incorporation of EdU or BrdU, this protein is present during all active phases of the cell cycle. A list of commonly used antibodies for the detection of these molecular markers is included in Table 2.

Besides protein markers themselves, quantification of the relevant mRNAs through quantitative real-time PCR (qPCR) can be employed. However, mRNA levels do not always correlate with the expressed protein level, as one study exemplified by reporting that p21 and p16 mRNA levels did not reach significant cut-offs to identify senescence [38]. Another study found that most reports using this technique only utilize one reference housekeeping gene for fold-change analysis, which hampered the correct normalization of the data [39]. It has recently been reported that the typical housekeeping genes used in normalization present an altered expression in senescence, and thus, careful design of this analysis is important to rely on data generated from this technique [39–41]. Commonly used primers for the transcriptional assessment of senescence, including relevant housekeeping genes, can be found in Table 3.

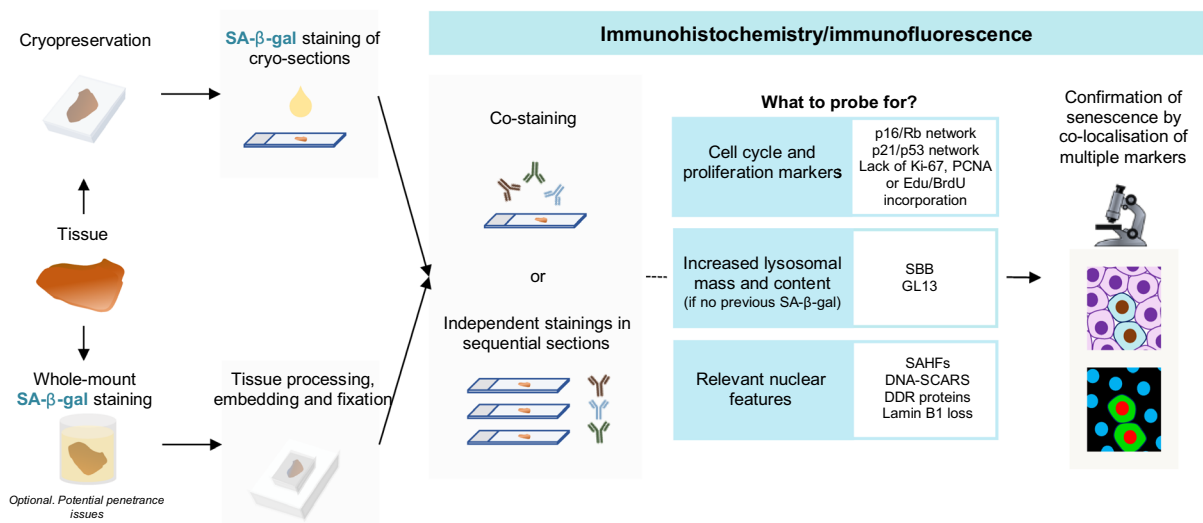
Increased lysosomal content and senescence-associated β -galactosidase (SA- β -Gal)

One of the most widely used markers of senescence is the increased levels of senescence-associated β -

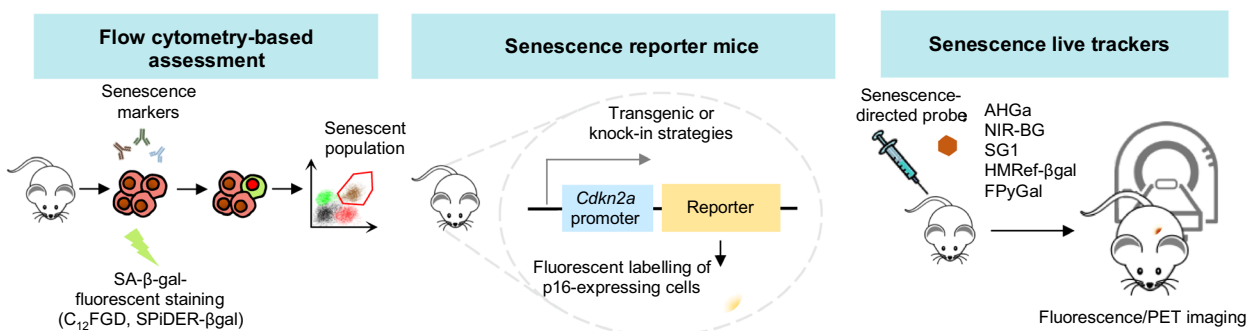
A Assessment of senescence *in vitro*



B Assessment of senescence *ex vivo*



C Identification of senescence *in vivo*



galactosidase (SA- β -Gal) activity [42]. SA- β -Gal is a hydrolase enzyme that catalyses the hydrolysis of β -galactosides into monosaccharides. For example, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), the most common substrate for SA- β -Gal activity, is catalysed by SA- β -Gal into galactose and 5-bromo-4-

chloro-3-hydroxyindole-1, which then dimerizes and forms the blue coloured precipitate indigo. Using X-Gal as a chromogenic substrate, this enzymatic assay monitors the increased expression and activity of this lysosomal protein in senescent cells and also gives indication of increased lysosomal mass [43]. Because most cells

Fig. 2. Combinatorial approaches to detect and assess cellular senescence *in vitro* and *ex vivo*. (A) Workflow for the assessment of cellular senescence *in vitro*. Cells should be induced to senesce, and sufficient time allotted for the cells to become fully senescent: chemotherapy (7–10 days), irradiation (7–10 days) and oncogene overexpression (3–7 days). Then, the primary characteristics of senescence should be assessed, starting first with cell cycle arrest, verified by cell cycle markers and the absence of proliferation. This should be followed by the verification of an additional hallmark, focusing on structural changes associated with senescence, such as increased lysosomal mass (SA- β -gal), changes in organelle structures (Lamin B1 downregulation) or markers of epigenetic changes (SAHFs). This can then be followed up with a final marker of a specific type of senescence such as DDR-related markers, increased ROS levels or SASP expression to finally verify the senescent phenotype. (B) Workflow for the assessment of cellular senescence in tissues *ex vivo*. Tissues can be processed and prepared by different methods depending on the desired immuno-detection read-out. If the sample is to be analysed by histochemistry, it is processed, preserved and fixed in a way that is incompatible with subsequent SA- β -gal staining. For this reason, samples can be whole-mount stained for such SA- β -gal activity before processing. However, this presents drawbacks, including the difficulty in assuring optimal pH (6.0) within the tissue and the poor penetrance of the staining solution, covering the entirety of the sample. Alternatively, if the tissue is cryo-preserved and cryo-sectioned, fresh sections can be stained for this enzymatic activity, overcoming the drawbacks associated with whole-mount staining. For the subsequent multiparametric staining, we recommend probing for a minimum of two additional markers (if prior SA- β -gal staining has been performed) or three markers (if no SA- β -gal staining was performed) that represent the cell cycle arrest and absence of proliferation, the increased lysosomal compartment and other relevant nuclear or structural features associated with cellular senescence. Such stainings should be performed in combination on the same tissue section or as independent stainings in strict sequential sections, so that cellular senescence can be verified with high levels of confidence by the co-localization of multiple parameters within the same cell. (C) Approaches currently used for the identification of senescence in living animals, by means of flow cytometry-based approaches, senescence reporter mice or the administration of probes directed at detecting cellular senescence *in vivo*.

Table 1. Biomarkers of cellular senescence and detection methods. IF, immunofluorescence; IHC, immunohistochemistry; WB, western blotting.

Senescent cell hallmark or trait	Marker	Expected change	Specific senescent subtype	Detection method
Cell cycle arrest				
Lack of DNA synthesis	BrdU, EdU	↓	General	Staining incorporation, IF
Lack of proliferation	Ki67	↓	General	IHC, IF
Activation of p16-pRB axis	p16INK4a	↑	General	WB, IHC, IF
	pRB	↑	General	WB, IHC, IF
	phospho-pRb	↓	General	WB, IHC, IF
Activation of p53-p21 axis	p21	↑	General, damage-induced	WB, IHC, IF
	p53	↑	General, damage-induced	WB, IHC, IF
	phospho-p53	↑	General, damage-induced	WB, IHC, IF
	DEC1 (BHLHB2)	↑	General, oncogene-induced	IHC, IF, reporter assays
	PPP1A	↑	General, oncogene-induced	IHC, reporter assays
Structural changes				
Morphology, cell size	Morphology, cell size	Enlarged, flattened	General	Light microscopy, flow cytometry
Increased lysosomal compartment and activity	SA- β -galactosidase	↑	General	Enzymatic staining
	SA- α -Fucosidase	↑	General	Enzymatic staining
DNA damage	Lipofuscin	↑	General	Dye incorporation (SSB, GL13)
	γ H2AX	↑	General, damage-induced	IF
	53BP1	↑	General, damage-induced	IF
	Rad17	↑	General, damage-induced	IF
	ATR	↑	General, damage-induced	IF
	ATM	↑	General, damage-induced	IF
	MDC1	↑	General, damage-induced	IF
ROS	TIF	↑	General, damage-induced	IF
	ROS	↑	Oxidative stress-induced, general	

Table 1. (Continued).

Senescent cell hallmark or trait	Marker	Expected change	Specific senescent subtype	Detection method
Telomere shortening SAHFs formation	Telomere	↓	Replicative-induced	Chemiluminescent oxygen detection reagents, fluorometry and flow cytometry
	DAPI/Hoechst 33342	↑	General, damage-induced	qPCR, FISH
	HIRA	↑	General, damage-induced	IF
	H3K9-methylation	↑	General, damage-induced	IF
	PML bodies	↑	General, damage-induced	IF
	HP1-gamma	↑	General, damage-induced	IHC, IF
Nuclear membrane	Lamin B1	↓	General	qPCR, IF, WB
Pro-survival				
Apoptosis exclusion	Annexin V	Absent	General	IF
	Cleaved PARP	Absent	General	IF
	Cleaved caspase 2/3/9	Absent	General	IF, IHC
	TUNEL staining	Absent	General	IF, IHC
SASP				
Cytokine secretion	IL-6, IL-8	↑	Damage-induced, OIS	ELISA, WB, SASP-responsive alkaline phosphatase assay
	CXCR2	↑	Damage-induced, OIS	ELISA, WB, SASP-responsive alkaline phosphatase assay
	IGF2	↑	Damage-induced, OIS	ELISA, WB, SASP-responsive alkaline phosphatase assay
	IGFBP3, IGFBP5, IGFBP7	↑	Damage-induced, OIS	ELISA, WB, SASP-responsive alkaline phosphatase assay
	STC1	↑	Damage-induced, OIS	ELISA, WB, SASP-responsive alkaline phosphatase assay
	GDF15	↑	Damage-induced, OIS	ELISA, WB, SASP-responsive alkaline phosphatase assay
	SERPINS	↑	Damage-induced, OIS	ELISA, WB, SASP-responsive alkaline phosphatase assay
Others				
Plasma membrane protein expression	ICAM-1	↑	Replicative-induced	IHC, IF, WB, flow cytometry
	DEP1	↑	General, OIS	IHC, IF, WB, flow cytometry
	B2MG	↑	General, OIS	IHC, IF, WB, flow cytometry
	NOTCH3	↑	General, OIS	IHC, IF, WB, flow cytometry
	DcR2	↑	General, OIS	IHC, IF, WB, flow cytometry

express endogenous β -galactosidase enzymatically active at pH 4.0, SA- β -gal is assayed by staining at nonoptimal pH (pH = 6.0) to better differentiate normal and senescence cells, and for this reason, prolonged incubation times can result in unspecific positive staining [44–46]. This staining is usually assayed with incubation periods lasting from 6 to 12 h, but we recommend optimizing the incubation time to each cell line tested, and probing nonsenescent cells in parallel to avoid false positives due to excess incubation time.

This assay is typically colorimetric, and observed with a light microscope. However, a number of probes

utilizing the same SA- β -gal cleavable chemistry have been recently developed, ranging from visible to near-infrared (NIR) and two-photon fluorescence, and even probes adapted to positron emission tomography (PET) imaging, thus demonstrating the adaptability of this chemistry [47–52]. The fluorescent probes C_{12} FDG, SPiDER-BGal and DDAO galactoside, are all modified with an incorporated galactose. This modification results in OFF-ON fluorophores that are quenched on their own, but turn on when the sugar moiety is cleaved off in the presence of SA- β -gal. As a result, these probes are amenable to new techniques,

such as fluorescence microscopy and flow cytometry-based analysis.

Finally, additional methods have been established to identify senescent cells through similar enzymatic phenotypes. SA- α -Fucosidase staining operates by similar chemistry to SA- β -galactosidase, but with α -L-Fucose containing compound as the substrate (X-Fuc) [53], which has interestingly been suggested to be more specific than SA- β -gal. However, it is currently not commonly used, probably because X-Gal has been historically more available and widespread used due to its popularity in microbiology.

Detecting nuclear alterations

Major macromolecular alterations occur not only in lysosomes, but in the nucleus as well. Senescent cells

change heterochromatin organization and associated structural proteins. In nonsenescent dividing cells, the heterochromatin is located at the nuclear periphery and is structurally less dense. However, in senescence, in an effort to silence proliferation-promoting genes, the chromatin rearranges and forms dense structures known as Senescence-Associated Heterochromatin Foci or SAHFs. SAHFs are commonly used to identify OIS of human cells, which involves staining with 4',6-diamidino-2-phenylindole (DAPI) and confocal microscopy imaging [54]. These dense structures also contain known heterochromatin-forming proteins, such as heterochromatin protein 1 (HP1), H3K9me3 and the histone H2A variant macroH2A, all of which can be immunostained for and subsequently imaged [55]. While they can serve as a marker of senescence in human cells, these structures have not been identified in murine cells so far.

Table 2. List of antibodies commonly used for the assessment of cellular senescence. IF, immunofluorescence; IHC, immunohistochemistry; RRID, research resource identification; WB, western blotting.

Senescent cell hallmark or trait	Antigen	Technique	Species	Antibody RRID
Cell cycle arrest				
Lack of proliferation	Ki-67	IF, IHC	Human	RRID:AB_398282
	Ki-67	IF, IHC	Mouse	RRID:AB_2142374
	BrdU	IF, IHC	Human, Mouse	RRID:AB_1157913
Activation of p16-pRB axis	p16 ^{INK4A}	IF, IHC, WB	Human	RRID:AB_10858268
	Rb	IF, IHC, WB	Human	RRID:AB_10860537
	phospho-Rb	IF, IHC, WB	Human	RRID:AB_1142540
	p16 ^{INK4A}	IF, IHC, WB	Mouse	RRID:AB_1126944
	p19 ^{ARF}	IF, IHC	Mouse	RRID:AB_776947
	Rb	WB	Mouse	RRID:AB_2195465
	phospho-Rb	IF, IHC, WB	Mouse	RRID:AB_2818502
Activation of p53-p21 axis	p21 ^{CIP}	IF, IHC, WB	Human	RRID:AB_10860537
	p53	IF, IHC, WB	Human	RRID:AB_2713958
	phospho-p53	IF, IHC, WB	Human	RRID:AB_2210689
	p21 ^{CIP}	IF, IHC, WB	Mouse	RRID:AB_306174
	p53	IF, IHC, WB	Mouse	RRID:AB_445445
	phospho-p53	IF, IHC, WB	Mouse	RRID:AB_2210834
Structural changes				
SAHFs formation	macroH2A	IF	Human	RRID:AB_10011253
	HP-1 γ	IF	Human, Mouse	RRID:AB_2259704
	H3K9Me3	IF	Human, Mouse	RRID:AB_11180319
	H3K9Me2	IF	Human, Mouse	RRID:AB_10986203
	γ H2AX	IF	Human, Mouse	RRID:AB_2755003
	HIRA	IF	Human, Mouse	RRID:AB_2117868
	PML bodies	IF	Mouse	RRID:AB_309932
	macroH2A	IF	Mouse	RRID:AB_514505
Nuclear membrane	Lamin B1	IF, WB	Human, Mouse	RRID:AB_648156
Others				
Plasma membrane protein expression	Dcr2	IHC	Human	RRID:AB_10863265
	Dep1	IHC	Human	RRID:AB_297361
	STX4	IHC	Human, Mouse	RRID:AB_1566783
	DEP1 (PTPRJ)	IHC	Mouse	RRID:AB_10856051
	B2M	IHC	Human	RRID:AB_305978

Table 3. List of primer sequences used for the detection of transcriptomic changes associated with cellular senescence.

Senescent cell hallmark or trait	Target gene	Protein	Forward primer (5' → 3')	Reverse primer (5' → 3')	Species
Cell cycle arrest					
Activation of p16-pRB axis	<i>CDKN2A</i>	p16 ^{INK4a}	ATCATCAGTCACCGAAGGTC	CTCAAGAGAAGCCAGTAACC	Human
	<i>CDKN2A</i>	p14 ^{ARF}	CAGCACCGGAGGAAGAAAG	AAAACCTACGAAAGCGGG	Human
	<i>Cdkn2A</i>	p16 ^{INK4a}	GGGTTTCGCCCAACGCCCCGA	TGCAGCACACCAGCGTGTCC	Mouse
	<i>Cdkn2A</i>	p19 ^{ARF}	GGTTCGCAGGTTCTTGGTC	AATCTGCACCGTAGTTGAGCA	Mouse
Activation of p53-p21 axis	<i>CDKN1A</i>	p21 ^{CIP1}	CCGAAGTCAGTTCCTTGTGGAG	CCGAAGTCAGTTCCTTGTGGAG	Human
	<i>TP53</i>	p53	GGACAGCCACGTCTGTGACTTG	CCAGTGGTTTCTTCTTTGGCTG	Human
	<i>Cdkn1a</i>	p21 ^{CIP1}	TCCCAGACTCTTGACATTGCT	TGCAGAAGGGGAAGTATGGG	Mouse
	<i>TP53</i>	p53	GGGAGGAGCCAGGCCATCA	CAGCCCCACCTCTTCAATTC	Mouse
SASP					
Cytokine & other factors secretion	<i>IL1A</i>	IL-1A	AGAGGAAGAAATCATCAAGC	TTTACTTTTGATTGAGGGCG	Human
	<i>IL6</i>	IL-6	GCAGAAAAGGCAAAGAATC	CTACATTTGCCGAAGAGC	Human
	<i>IL8</i>	IL-8	GTTTTTGAAGAGGGCTGAG	TTTGCTTGAAGTTTCACTGG	Human
	<i>SERPINE1</i>	SERPINE1	ATCCACAGCTGTCTATAGTC	CACCTGGCCCATGAAAAG	Human
	<i>CCL2</i>	CCL2	GATCTCAGTGCAGAGGCTCG	TGCTTGTCCAGGTGGTCCAT	Human
	<i>CXCL1</i>	CXCL1	GAAAGCTTGCCTCAATCCTG	CTTCCTCCTCCTTCTGGTC	Human
	<i>CXCL2</i>	CXCL2	GGCAGAAAGCTTGTCTCAA	GCTTCCTCCTTCTTCTGGT	Human
	<i>CXCL10</i>	CXCL10	GTGGCATTCAAGGAGTACCTC	GCCTTCGATTCTGGATTGAGACA	Human
	<i>IL15</i>	IL15	TTTCAGTGCAGGGCTTCTCTAA	GGGTGAACATCACTTCCGTAT	Human
	<i>LIF</i>	LIF	CCCATCACCCCTGTCAACG	GGGCCACATAGCTTGTCCA	Human
	<i>VEGFA</i>	VEGFA	ATCACGAAGTGGTGAAGTTC	TGCTGTAGGAAGCTCATCTC	Human
	<i>Il1a</i>	IL-1A	CGCTTGAGTCGGCAAAGAAAT	TGGCAGAAGTGTAGTCTTCGT	Mouse
	<i>Il1b</i>	IL-1B	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT	Mouse
	<i>Il6</i>	IL-6	TGATTGTATGAACAACGATGATGC	GGACTCTGGCTTTGTCTTTCTTGT	Mouse
<i>Cxcl1</i>	CXCL1	CTGGGATTACCTCAAGAACATC	CAGGGTCAAGGCAAGCCTC	Mouse	
Nuclear changes					
Lamin B1 loss	<i>LMNB1</i>	Lamin B1	GTATGAAGAGGAGATTAACGAGAC	TACTCAATTTGACGCCAG	Human
	<i>Lmnb1</i>	Lamin b1	ATGAAGAGGAGATCAATGAGAC	CATACTCAATCTGACGCC	Human
Housekeeping genes	<i>ACTB</i>	Beta-Actin	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG	Human
	<i>GAPDH</i>	GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	Human
	<i>TUBA1A</i>	Alpha-Tubulin	CTTCGTCTCCGCCATCAG	CGTGTTCAGGCAGTAGAGC	Human
	<i>PUM1</i>	Pumilio Homolog 1	CGGTCGTCTGAGGATAAAA	CGTACGTGAGGCGTGAGTAA	Human
	<i>Actb</i>	Beta-Actin	CCTTCTTGGGTATGGAATCCTGT	CACCTGTGTTGGCATAGAGGCTTTTAC	Mouse
	<i>Gapdh</i>	GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTT	Mouse
	<i>Tuba</i>	Alpha-Tubulin	TGTCCTGGACAGGATTCGC	CTCCATCAGCAGGGAGGTG	Mouse
	<i>Hprt</i>	HPRT	TGACACTGGCAAACAATGCA	GGTCCTTTTACCAGCAAGCT	Mouse

DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) are a possibly more global marker for senescence, because they seem to occur in a larger variety of subtypes. These are nuclear structures containing a collection of proteins associated with DDR, such as ATM, ATR and CHK2, and associated with PML nuclear bodies. DNA-SCARS help maintain the senescence-associated growth arrest and the SASP through DDR signalling and chronic p53 activation [56]. Interestingly, senescent cells can

also exhibit phosphorylated H2A histone family member X (γ -H2AX) foci, a marker of DNA damage, even without the presence of a DNA-damaging agent [57]. γ -H2AX is formally considered a type of DNA-SCARS associated histone, and it can be identified by immunoblotting or imaging techniques.

Telomeres are protective structures at the ends of linear DNA that have been observed to shorten with age in organisms, or during replication in cells, resulting in genetic instability and what is known as replicative

senescence [58]. Telomere length can be measured by qPCR and quantitative fluorescent *in situ* hybridization (Q-FISH), and dysfunctional telomeres represent another hallmark of senescence and have been used as a robust biomarker [59–61]. In addition to telomere shortening, telomere dysfunction-induced foci (TIFs) are another nuclear marker of senescence. These are characterized by telomeric regions associated with markers of DNA damage, such as γ -H2AX, ATM and 53Bp1, or described as DNA-SCARS located in the telomeres [62]. Interestingly, TIFs are still found in cells without the shortening of their telomeres, and therefore, these structures are present in more types of senescence than just replicative [58], [63]. They can be assessed in the same ways as explained above for DNA-SCARS, as many components are the same.

Another hallmark related to the nucleus is the downregulation of the protein lamin B1, a major component of the nuclear lamina. Lamin B1 is crucial to maintain the structural integrity of the nucleus. However, this integrity is lost in senescent cells, which allows the release of cytoplasmic chromatin fragments outside the nucleus, which is believed to fuel the SASP through the cGAS/STING pathway [64]. The loss of lamin b1 can be detected immunoassayed and detected by imaging or immunoblotting.

The detection of more than one of the markers that we describe as *primary*, representing and verifying the cell cycle arrest, senescent-specific nuclear changes and lysosomal alterations, can be a good indication of the senescent phenotype *in vitro*. However, given phenotype heterogeneity, we recommend improving the assessment through the detection of additional features that can give additional information on the phenotype as described below.

Detection of secondary senescence markers

The SASP

The SASP, comprises a number of factors, such as cytokines, chemokines, growth factors, metalloproteases and extracellular vesicles (EVs) that are secreted from senescent cells. The SASP is thought to be a major reason senescent cells contribute to either tissue homeostasis and dysfunction, as these factors play many roles in immune signalling, cell-to-cell communication and in the creation of pro-tumorigenic environments [65–67]. While many components of the SASP can be identified by real-time quantitative PCR (RT-qPCR) (Table 3) and proteomic techniques (such as LC MS/MS), other assays such as cytokine arrays and ELISAs can also be used. Some of the factors most commonly found upregulated in senescent cells include the interleukins IL-1a, IL-6 and IL-15, the chemokines

IL-8, GRO-a and MIP-1a, and others such as IFN- γ , VEGF, ICAM-1 and GM-CSE, among many others [65]. Regardless, their expression varies widely, changes over time and depends on the duration or induction of senescence as well as the cell type [66].

Currently, the SASP is unable to verify the senescent state outright, as many of these factors are secreted by other cell types, including immune, endothelial and cancer cells, and their secretion profiles are complicated. However, significant efforts are currently being made towards the definition of SASP signatures, and a comprehensive database known as the SASP Atlas has recently been made public [68]. This study collects information on the soluble proteins as well as exosome cargoes from oncogene-, irradiation- and therapy-induced senescent fibroblasts and epithelial cells, providing valuable information for the definition of SASP signatures in particular senescent subtypes. As shown by the authors, each type of senescence resulted in a significantly different SASP profile, and only a limited number of factors overlapped between the different conditions, making a universal SASP signature difficult to establish. While this is promising research, further work is needed to be able to use these compounds as direct biomarkers of the senescent phenotype.

Mitochondria, reactive oxygen species (ROS) and pro-survival pathways

Despite mitochondrial changes are not commonly used as a tool for the detection of senescent cells, mitochondrial dysfunction plays an important role in maintaining pro-inflammatory senescent phenotypes [69,70]. In addition, it represents a functional aim for treatment, as targeting mitochondria seems to abrogate the pro-inflammatory phenotype [71]. Interestingly, the number of mitochondria is also increased due to a reduction in their breakdown (mitophagy) upon senescence, and they grow larger showing distinct changes in their crista [69,70]. Further, changes in membrane potential have also been described, although not following a predictable pattern, with studies showing both increased and decreased potentials in senescent fibroblasts [72,73].

As introduced above, mitochondria have been used as a druggable target in senescence, with drugs and nanoparticles directed at specific mitochondrial moieties. For example, Tri-phenyl Phosphine (TPP), a lipophilic cation moiety that targets mitochondria with increased membrane potential [74], has been re-engineered as MitoTam (combined with a modified version of Tamoxifen) and as part of polymer-based nanoparticles named PLGA to efficiently target senescent cells

[75,76]. Further, a gold nanoparticle system, in which TPP was used in conjunction with the surface marker B2MG, has been reported to specifically target senescence as well [73].

With regard to the targeting for the assessment of senescence, a library of small fluorescent molecules was recently used to identify potential tools for detection in senescence, and a cyanine dye (CyBC9) was reported to efficiently label senescent mesenchymal stromal cells by targeting mitochondria [77]. Despite mitochondrial probes not being used for senescence assessment widely at the moment, more developments in the field are expected as the number of targeting species increases and our understanding of mitochondrial biology in cellular senescence improves.

It should be noted that the structural changes in mitochondria also lead to alterations such as the increase in ROS, which is considered another hallmark of senescence [78]. Interestingly, mitochondrial-derived ROS can trigger senescence both in an autocrine and paracrine manner when released by senescent cells [72,79]. Numerous dyes can be used to detect these events and are amenable to a number of common laboratory techniques such as flow cytometry-assisted analysis and plate-based spectrophotometry. Dihydroethidium (DHE) is a blue dye that oxidizes in the presence of intracellular superoxides, turning red and targeting the cell DNA. Dihydrorhodamine 123 (DHR123) is a compound that becomes fluorescent upon oxidation by general ROS levels in cells. Another commonly used dye is MitoSOX™ Red, a cation derivative of ethidium bromide that specifically measures the superoxide levels of the mitochondria. Finally, Amplex Red produces a fluorescent product in the presence of hydrogen peroxide and is used to measure extracellular ROS by spectrophotometry. Protocols and more information for all the dyes mentioned above have been reviewed in Ref [78].

Interestingly, an additional hallmark of senescence related to mitochondria is their resistance to apoptosis, resulting from the upregulation of pro-survival pathways. This event is mediated by the increased expression of antiapoptotic Bcl-2 family members located on the membranes of mitochondria as well as the endoplasmic reticulum [80,81]. Increased expression of these proteins prevents the release of cytochrome C to assemble the apoptosome, a complex structure that activates caspase signalling, which, in turn, triggers intrinsic apoptosis. Consequently, senescence can be assessed by analysing Bcl-2 expression and other antiapoptotic homologs such as Bcl-xL, Bcl-W and Mcl-1 in senescent cells. Although these proteins represent a very attractive hallmark that is commonly targeted for

effective senolysis, their expression patterns are seldom used for the assessment of this cellular state [82]. On the other hand, excluding the induction of apoptosis by confirming the lack of Annexin V, cleaved PARP or cleaved caspase-3 (Table 1) can also be very informative in terms of the senescent phenotype and is commonly performed to rule out the induction of apoptosis as a stress response.

Morphology and granularity of senescent cells

Abnormally large and flat morphology defines senescent cells *in vitro* [83]. Interestingly, researchers have recently reported that this increase in size, which translates into an increased cytoplasm to DNA ratio, can contribute to the growth arrest observed in senescent cells. As reported by the authors, protein diffusion and binding required for translation is delayed due to this cytoplasmic dilution, contributing to the cell cycle arrest [83].

Granularity also increases in senescent cells, which can be easily detected by light microscopy and flow cytometry-based analysis [28,84,85]. This seems to be due to the increase in size and number of lysosomes. However, this increase in granularity is not observed in all types of senescence, even when SA- β -gal activity is detected [86]. Multinucleation can also occur in senescent cells and is typically identified by light microscopy or confocal imaging after DAPI staining [87–89].

Of note, the aforementioned changes in the morphology and granularity of senescent cells are typically qualitative observations. We therefore recommend using these alterations for the monitoring of the induction of the senescence response *in vitro*, but also performing an accurate assessment of the senescent phenotype through the combination of different detected markers as described above (Fig. 2A).

Assessing cellular senescence in tissues and *in vivo*

Despite significant advances in the study of cellular senescence, detecting and assessing these cells *in vivo* with high levels of confidence remain challenging. Unlike in cell cultures, the vast majority of cells within the body are quiescent or terminally differentiated, meaning that some of the markers used to identify senescent cells *in vitro*, such as the lack of DNA synthesis or proliferation, are generally not valid. Additional features readily observed in cultures, such as morphological changes or increased cell size, are rarely preserved *in vivo*, owing to structural and architectural restrictions. Other markers, including cell cycle-

associated proteins, can also be expressed in specific cell types and processes like inflammation, which further restricts their reliability. Moreover, senescent cells are generally low in number within an organism, even in aged animals [67,90]. These factors, together with the heterogeneity of the senescent programme and the intrinsic complexity of a living organism, hinder the assessment of cellular senescence *in vivo*. It is for this reason that substantial efforts are currently being made in order to provide more robust and reliable strategies for the detection and assessment of senescence in fixed tissues and living animals.

Immunohistochemical detection of senescence *ex vivo*

Pinpointing cellular senescence upon histological analysis has been pivotal for the interpretation and understanding of this mechanism within preserved tissue structures and architecture. Despite obvious drawbacks owing to the unspecificity or unreliability of the markers, this conventional method has been historically instrumental for the examination of senescence *in situ*, and the International Cellular Senescence Association recently suggested a simplified workflow for its recognition within tissues [28]. In line with these guidelines, we recommend confirming the presence of cellular senescence *in situ* confidently by the concomitant immunochemical detection of at least three different markers within the same cell, representing different hallmarks of the senescent phenotype. A valid assessment could include, for instance, the presence of SA- β -gal activity together with a p16^{INK4a} and HP1-positive co-staining. A representative workflow for this detection *ex vivo* is exemplified in Fig. 2B.

Increased lysosomal mass and content

SA- β -gal activity is not only a useful *in vitro* marker as described above, but also largely used for the *ex vivo* assessment of senescence as well. In 1995, SA- β -gal activity was first reported to detect senescence in human skin samples from aged individuals [42], and it has since then been regarded as the gold standard for the detection of putative senescent cells in tissues. However, it is important to consider that, as with any other senescence biomarker, this enzymatic activity is not exclusive of senescent cells in a living organism. β -galactosidase is particularly enriched in some highly secretory cell types, such as tissue-residing macrophages and osteoclasts, cells undergoing increased lysosomal activity during autophagy, and in some cancer cells [91–93]. The staining also presents further

limitations, as it can only be performed in fresh tissue, conveying potential incompatibilities with other fixatives and sample preparation methods.

Of note, the increased lysosomal content of senescent cells also renders them positive for the histochemical staining of Sudan Black-B (SBB), which accumulates in lipofuscin due to its nonpolar nature, a pigment granule composed of lipid residues that is also considered a hallmark of ageing [94]. A biotinylated version of SBB, known as GL13, was subsequently developed and has demonstrated higher immunohistochemical specificity and decreased background signal. Importantly, its performance is not limited to fresh tissue processing as is the case with SA- β -gal, and it can be used to stain archived samples, which presents a noteworthy advantage over the classic staining [46,95].

Despite these limitations, SA- β -gal remains to be one of the most extensively used biomarkers for the cytological or histological detection of cellular senescence in tissues. A number of novel strategies have been developed exploiting this enzymatic activity, including probes for the *in vivo* tracking of senescence and fluorescence-based tools for microscopy and flow cytometry-based methodologies, which will be discussed later.

Cell cycle and proliferation molecular markers

As introduced earlier, research *in vitro* has defined the cell cycle arrest as one of the most defining features of cellular senescence. The expression of the CDK inhibitor p16^{INK4a}, encoded by the *CDKN2A* gene, is probably the most widely used genetic marker of cellular senescence *in vivo* [90,96]. As opposed to SA- β -gal, p16^{INK4a} plays crucial mechanistic roles in the implementation of the senescent programme, by halting the cell cycle through the p16^{INK4a}/Rb pathway. However, its staining in tissues should be interpreted with caution, as it can also be expressed in aged lymphocytes, cells with inactivated RB, such as tumour cells, and during certain physiological conditions such as inflammation, clastogen exposure or wound healing [8,97–99]. Moreover, current available antibodies for the histochemical detection of murine p16^{INK4a} are poor and unreliable. In order to bypass this, several mouse models have been developed in which a reporter gene is expressed under the control of p16^{INK4a} promoter, which have also provided further evidence of the expression of this CDK inhibitor in physiological contexts other than cellular senescence.

Of note, the *CDKN2A* gene also encodes for the human CDK inhibitor p14^{ARF} and its mouse homologue p19^{ARF} through an alternative reading frame

(ARF). Despite being expressed only in specific types of senescent cells, p14^{ARF} and p19^{ARF} have also been used for the histochemical detection of senescence in tissues [100]. In addition, p15^{INK4B}, encoded by *CDKN2B*, can also detect cellular senescence in tissues in the absence of p16^{INK4A} [101].

As described previously, the p53/p21 network is a second pivotal cell cycle regulator and inducer of the proliferative arrest. It is however worth noting that a transient damaging insult can activate p53 to activate DNA repair processes during quiescent states *in vivo* [67], as well as it is also a main driver of apoptosis. The role of the CDK inhibitor p21^{CIP1}, induced upon the activation of p53, is believed to be important during the onset of senescence and can also be useful in the detection of senescent cells in tissues. However, the expression of p21^{CIP1} is not usually maintained once the senescent programme has been established [102], and, thus molecular markers involved in the p16/p15/Rb axis are believed to be more reliable in the detection of senescence *ex vivo*.

Despite the vast majority of cells within tissues are terminally differentiated, analysing the absence of proliferation in some contexts such as in tumour samples, where p16 or other molecular markers might be aberrantly expressed, can be relevant for the identification of cellular senescence. In the case of mouse specimens, animals can be administered, prior to tissue collection, with nucleoside analogues that are incorporated during active DNA synthesis and therefore label dividing cells, namely EdU or BrdU. Alternatively, the use of monoclonal antibodies against antigens such as PCNA and Ki-67 are widely used as indicators of proliferation and can detect cells in the G1, S, G2 and M phases, reliably distinguishing proliferating from non-dividing cells in histological samples without the need of prior *in vivo* preparation.

Relevant nuclear features *ex vivo*

Epigenetic marks and other DNA structural alterations and rearrangements are generally considered some of the most prominent cellular senescence biomarkers. As previously introduced, SAHFs, which are directly linked to the silencing of proliferation-related genes [103], have a key causal role and are normally assayed in tissues by immunocytochemistry against HP1 speckles, as well as, albeit less frequently, against histone H2A variant mH2A, H3K9Me2 and H3K9Me3 epigenetic marks. However, these heterochromatin foci are most commonly found in OIS and can be absent depending on the cell type and other senescence drivers, and thus are not generally

considered a reliable marker in tissues [54]. Of note, SAHFs have not been identified in mouse cells so far and are therefore not used for the detection of senescence in murine tissue.

Additional nuclear marks include DNA double strand breaks, also known as DNA-SCARS, which are a common feature of damage-induced senescence [56]. These are usually detected in tissues by γ H2AX immunoreactivity or, less frequently, by accumulated DDR-related proteins such as ATR, ATM or p53 immunoreactivity. Nevertheless, DNA damage markers also present poor specificity for the senescent phenotype in tissues, not only because they can be limited to DNA damage subtypes of cellular senescence, but also because they can also be detected in quiescent and apoptotic cells. As a marker of the disrupted nuclear envelope integrity, the lack of lamin B1 can also be used to detect and quantify cellular senescence in tissues [104,105], despite this is not a largely established method.

Overall, the staining of nuclear or DNA damage markers in combination with other cell cycle-related proteins and SA- β -gal provide a reliable strategy for confirming the phenotype in fixed tissues.

Detecting senescence in living animals and live cells

The development of novel methods for the detection of senescence in live tissues and organisms has become increasingly attractive due to the possibility of screening senescence within relevant physiological and pathological contexts without damaging the cells, as well as tracking them over time to obtain greater insights into the roles and effects within the tissue (Fig. 2C). However, these strategies can sometimes be more restrictive given the difficulty in implementing a multimarker approach, and the interpretation of some of them should thus be taken with caution.

Flow cytometry

Flow cytometry-based assays have the potential to reshape the assessment of cellular senescence, as they allow for the rapid analysis and quantification of the physical traits and the immunophenotype of heterogeneous populations simultaneously. By virtue of its versatility, a number of assays based on this technique have been developed to examine cellular senescence in homogenized tissue sample suspensions, employing different hallmarks and parameters of the senescent phenotype.

As previously discussed, SA- β -gal has allowed the development of fluorogenic compounds functionalized

as substrates that allow the labelling of senescent cells when cleaved by this enzymatic activity. These include C₁₂FGD [45] and SPiDER-βgal [106], which have been successfully used to detect β-gal^{high}-expressing cells in live tissues [107–109]. However, for greater confidence, these are recommended to be assayed in conjunction with other markers that can be detected simultaneously using additional filters. As introduced above, the increased production of ROS derived from mitochondrial dysfunction can also be analysed by flow cytometry with the use of fluorescent dyes [78]. In live tissues, however, detecting ROS can be challenging and the results can be nonspecific, and it is of limited utility for measuring senescence confidently. Flow cytometry also allows the analysis of relative cell size and granularity via forward and side scatters, which has proven useful for cell cultures given the general increased cell volume, vacuolization and lysosomal compartment that characterize senescence *in vitro*. Nevertheless, there is limited evidence of senescent cells presenting an increased size *in vivo*, probably due to tissue architecture restrictions, which means this is not extensively used in flow cytometry. Exclusion of proliferation with the use of Ki-67 antibodies or cell cycle dyes also renders limited value in most contexts, given the fact that the majority of cells in tissues are quiescent or terminally differentiated.

The use of intracellular antibodies against relevant nuclear biomarkers in combination with SA-β-gal analysis can be an effective strategy. A recently developed method based on this combination, named ImageStreamX, has been used to simultaneously detect SA-β-gal, tissue-specific receptors and the loss of HMGB1 as an additional marker of senescence in murine fibrotic lungs, doxorubicin-treated xenografts and several organs harvested from aged animals [36]. However, intracellular markers are harder to measure in live tissue and usually require the alteration of the plasma membrane to penetrate into the cell, potentially impairing cell viability. In this matter, membrane antigens are of greater utility as they can allow the viable isolation of specific cell types, following what is known as combinatorial codes.

Despite a universal or more specific membrane markers of cellular senescence remain to be defined, recent studies have provided significant insights into the so-called surfaceome of senescent cells [110–115]. In fact, protocols for the detection of cellular senescence using a combination of extracellular markers have been made available, albeit only with the use of cell cultures [112]. However, some of these reported surface receptors, namely ICAM-1, NOTCH1, NOTCH3, DEP1, B2M, DPP4 STX4, NTAL and more recently uPAR, have proven to serve as reliable markers of senescence in

different murine tissues via conventional combinatorial histological staining [110–112,116–118] and therefore present the potential to be employed for flow cytometry analysis. It is worth noting that the expression of these receptors is not always specific but predominantly overexpressed in the senescent phenotype relative to the non-senescent counterparts, as is the case with other markers, and they will require further validation in additional types of cellular senescence, tissues and models.

***In vivo* mouse models**

The use of the so-called *senescence reporter mice* has been instrumental for the real-time monitoring of this mechanism in intact tissues. Being p16^{Ink4a} the most widely used genetic marker of cellular senescence, the models developed to date have engineered the expression of a reporter in a p16-dependant fashion through transgenic or knock-in approaches.

The first of these models was generated in 2011, when Baker and colleagues developed the INK-ATTAC (*Ink4a* apoptosis through targeted activation of caspase) transgenic line, in which p16^{INK4a}-expressing cells activate the expression of EGFP together with an engineered fusion protein (FKBP-Casp8), which results in the selective ablation of the cells upon alkaline phosphatase treatment [98]. This strategy allowed not only the detection and collection of such cells, but also their specific removal, which remarkably delayed the onset and attenuated the progression of age-related disorders, demonstrating for the first time the causal role of senescent cells during ageing in a living organism [98]. In 2013, the first knock-in model with the expression of luciferase under the p16^{INK4a} promoter was generated, whereby authors observed increased luminescence with age and during early neoplastic events [99]. A year later, the p16-3MR mouse strain was engineered by driving the expression of tri-modal fusion protein under the same promoter, which allowed the detection and analysis of putative senescent cells in aged tissues and during wound healing [8]. This fusion protein contains three domains, two of which enable the tracking and detection of p16-expressing cells thanks to the expression of luciferase and a red fluorescent protein monomer, while the third one drives the elimination of such cells through the activity of a truncated HSV-1 kinase, upon ganciclovir administration [8]. Lastly, a recent strategy has resulted in the generation of a new p16^{INK4a} reporter model named p16-Cre/R26-mTmG. To generate this, authors introduced a Cre-recombinase cassette within the exon 3 of the endogenous *Cdkn2a* gene and crossed the resulting knock-in line with a Rosa26-

mTmG strain. This elegant approach renders all cells within the animal positive for tdTomato and allows the continuous labelling of putative senescent cells upon p16-driven Cre-recombinase expression, which switches the red fluorescent signal to EGFP [119].

While these models have been extremely important to analyse p16^{Ink4a} increased expression in ageing, inflammation and tumorigenesis, studies at the single-cell level have remained more challenging. Through a very recent approach, however, Lui *et al.* were able to isolate, enumerate and characterize individual p16-expressing cells via a fluorescence-based reporter allele with tandem-dimer Tomato knocked into the *Cdkn2a* locus [120]. While the sole expression of p16^{Ink4a} is not always a faithful indication of cellular senescence, the isolation of such cells and further characterization through flow cytometry strategies can add robustness to these reporter strategies, rendering them very valuable tools for the study of cellular senescence in living organisms.

Probes for live tracking

A large number of chromogenic and fluorescent molecules and tracers have been developed by means of exploiting the generally increased SA- β -gal levels of senescent cells. However, the short wavelengths of most of these probes result in the dispersion of the fluorescence emission, leading to limited tissue penetrance. In addition, tissues present high autofluorescent background signal, which further hampers their detection by bioimaging techniques in living organisms. In order to overcome this, recent studies have utilized NIR strategies for novel probes, which allows deeper penetration thanks to longer emission wavelengths [121]. Two-photon probes, which require the simultaneous excitation by two photons with longer wavelengths, have also surged as an alternative that, in addition, minimizes potential photodamage to the tissue. Examples of two-photon and NIR probes include AHGa and NIR-BG, which have been successfully used to detect chemotherapy-induced senescence *in vivo* [47,49]. Other relevant ones, such as SG1 and HMRef- β gal, have been developed and demonstrated to detect β -gal^{high}-expressing cells *in vivo*, despite not in the context of cellular senescence [48,122,123]. Additional probes have been developed for the β -galactosidase enzyme translated from the *lacZ* gene, which is encoded by the bacterial *Escherichia coli* lactose operon. However, this enzyme and the endogenous lysosomal eukaryotic β -galactosidase only share 33% of similarities at the amino acid level [124] and we therefore discourage the use of those for the detection of senescence.

Imaging techniques routinely used in the clinic, such as PET imaging, are gaining attention in the development of senescence-directed tools. Because PET allows the detection of biochemical changes in tissues through radioactive tracers, the increased SA- β -gal of senescent cells is of particular use. Notably, a recently developed a PET tracer named FPyGal has showed a correlation between β -gal activity and tracer uptake in chemotherapy-treated tumours *in vivo* [125,126]. These strategies are of particular interest thanks to their potential translatability to human settings.

Substantial efforts are currently being made to generate new generation probes to decrease leakage and photodamage, as well as improve their retention within senescent cells. Despite these tools are not commercially available yet, it is certain they will become extremely popular in the coming years. They not only enable the long-term longitudinal monitoring of presumed senescent cells within relevant physiological conditions without terminating experiments, but also have the potential to be translated as diagnostic tools in the clinic.

Novel potential markers and detection methods

The senescent surfaceome

Given the complexity of the senescent phenotype, significant efforts are currently aimed at identifying novel biological signatures that enable a more strategic and quantifiable targeting and characterization of senescence. In this context, the surfaceome, or the study of the plasma membrane content of a cell, represents an exciting avenue.

As previously introduced, a number of protein receptors that distinguish senescent cells from normal cells have been identified *in vitro*, such as NOTCH1, DPP4, B2MG, SCAMP4 and uPAR [111–118]. Despite they remain to be tested in additional models and types of senescence, some of them have served as reliable histological senescent markers in tissues [110–115]. Of note, CD9 and B2MG protein receptors have also successfully been used for the engineering of nanoparticle systems aimed at targeting senescence [73,127,128], and uPAR has recently been utilized for the development of the first senolytic CAR T cell therapy in mice [118], which highlights the potential clinical relevance of surface-centred strategies.

Post-translational modification (PTM) changes of proteins have also been proposed as a hallmark of ageing [129]. Glycosylation is a type of PTM that has been strongly associated with ageing and cellular

senescence. In fact, specific cell surface glycan profiles associated with replicative senescence *in vitro* and in tissues from aged individuals have recently been described [130,131]. Of note, such changes could also be effectively quantified through lectin microarray analyses [131]. Oxidation, a different type of PTM, is a feature that has recently been described in the protein vimentin in senescent cells. As evidenced by its presence in sera from mice transplanted with bleomycin- and irradiation-induced senescent cells, this oxidized version of vimentin is not only present in the surface of senescent cells, but also secreted to the environment [132], which can be of great clinical value for monitoring purposes.

The alteration of the plasma membrane lipid composition, which results in increased membrane rigidity, has also been associated with age and the senescent phenotype [133,134]. Despite these changes are not well established in the field, a multitude of emerging technologies able to detect cells based on biomechanical properties, such as tangential flow filtration, which has been used to separate pluripotent cells from differentiated ones [135], could potentially be applied for their validation, opening up interesting novel detection strategies.

Newer techniques able to analyse and detect other biochemical changes, such as the formation and breakage of chemical bonds, are also gaining attention in the field. For instance, vibrational spectroscopy techniques, such as Raman and infrared (IR) spectroscopy [136], have been applied to effectively differentiate senescent cells from their nonsenescent counterparts [137,138]. By scanning through a wide range of wavelengths and analysing the scattered light profiles, the biochemical composition of a cell can be mapped, which can be used to differentiate cell types and cellular states. Although these strategies have not yet been used extensively in senescence, they represent promising candidates for *in vivo* settings, given the deep penetration potential of IR and Raman-based technologies in tissues.

The potential of the lysosomal compartment

We have briefly described how the versatility of the SA- β -gal has allowed the development of novel fluorescent and two-photon probes for the detection and monitoring of senescence in living animals [25]. Of note, this enzymatic activity also presents the potential to be applied to techniques that, to our knowledge, have not been explored in the field so far. A number of cancer diagnostics probes directed at intrinsic β -galactosidase have been developed with applications

ranging from fluorescence and photoacoustic tomography to magnetic resonance imaging (MRI) [122,139–142], which can be particularly relevant for *in vivo* and clinical applications of senescence. Alternatively, as the targeting SA- α -Fucosidase has exemplified, there are other enzymes upregulated in senescence, such as N-acetyl- β -hexosaminidase or mannosidase, whose targeting could prove more specific than the classic SA- β -gal assays [53,143].

The lysosomal accumulation of other materials such as pigments also confers significant autofluorescence to the cells, which has recently been proposed as a reliable marker of cellular senescence [144]. However, this is unlikely to serve as a useful indicator *in vivo*, given that tissues generally present high levels of autofluorescence.

Possibly owing to the increased lysosomal compartment as well, despite this has yet to be fully addressed, the accumulation of iron has emerged as a possible feature of senescent cells [145,146]. It remains unknown to what extent this accumulation is causative or a simple by-product of implemented metabolic changes but, given the plethora of chelators and iron detection kits readily available, it represents an easily assayed and attractive trait if proven universal for the senescent phenotype. In addition, because of this accumulation, many proteins involved in the iron metabolism could potentially be used as targets. Determining the kind of structures and forms it is stored as in senescent cells can also be informative for detection purposes. For instance, if it is bound entirely within ferritin, MRI could serve as a potential detection technique, as it has been used in Alzheimer's research already [147–149]. Other strategies employed to explore the iron content, including novel designs based on chelating nanomaterials, could possibly be used in the near future to both expand our knowledge on the role of iron in senescence and provide us with novel senescent cell assays.

Transcriptional signatures

With the implementation of the senescent programme, substantial gene expression changes take place in a tightly regulated manner through the activation and downregulation of a wide variety of signalling pathways [2]. Thanks to next-generation sequencing strategies, significant efforts have been made aimed at describing the senescent transcriptome and establishing a so-called transcriptional signature for cellular senescence [35,38,150–152]. However, it is unlikely that a *global* cellular senescence gene expression profile is ever determined. These recent studies have demonstrated that the transcriptome of senescent cells

in vitro, which are inherently more homogeneous than complex tissues, can be very variable and even change over time within the same cell type and upon the same senescence driver [35]. Actually, a study showed that the main contributor on the resulting gene expression profile of a senescent cell is the cell of origin, rather than the inducer or type of cellular senescence [38]. In addition, a transcriptional single-cell analysis of bleomycin-induced senescent fibroblasts *in vitro* showed a marked variability at the individual cell level [151], demonstrating the heterogeneity in the different transcriptional signatures reported thus far.

Taking the above into consideration, establishing a signature of senescence in living tissues is likely to be even more complex than *in vitro*. In a recent study, researchers compared the transcriptome of radiation- and replicative-induced senescent fibroblasts in cell culture to that of fibroblasts harvested from young and aged individuals and demonstrated very little overlapping between the different gene expression changes observed *in vitro* and *ex vivo* [153]. Moreover, if senescent cells are low in number, the transcriptional analysis of bulk tissue in search of a signature of senescence can be of limited value as such transcriptional changes might stand undetected. Of note, a potential strategy to bypass this confounding factor could be the isolation of putative senescent cells by flow cytometry following a multimarker combinatorial approach as previously described, prior to transcriptional analysis.

Despite this still remains a big challenge *in vivo*, gene signatures for cellular senescence will likely be redefined in the near future, and they will probably be characterized as tissue or organ-specific signatures within particular physiological or pathological contexts. Transcriptional analyses are not only important to allow the identification and the potential burden of senescence in tissues, but can also be instrumental for the discovery of novel, more targetable biomarkers and druggable targets [152].

The secreting phenotype

As with the senescent transcriptome, many efforts have been made towards the definition of SASP signatures that allow the detection and characterization of cellular senescence. The secretory profile of senescent cells can be very variable, depending on the stimulus and the cell of origin, and is highly dynamic over time [67]. In addition, single-cell RNA-seq analyses have recently revealed considerable cell-to-cell variability of SASP expression within the same senescent population [151]. While many researchers in the field have certainly contributed to identifying 'core' SASP factors (as reviewed

in Ref. [65]), we believe that the definition of the secretome in more specific biological contexts will be instrumental for senescence-based molecular signatures. A recently published database named *SASP Atlas* gathers a large collection of soluble and exosome-embedded factors detected through an unbiased proteomic analysis of different types of cells induced to senesce upon varying stimuli (irradiation, oncogenic overexpression and genotoxic stress) [68]. Of note, the authors also revealed a significant overlap with some markers detected in human plasma samples. In this context, a recent study found a significant correlation between circulating SASP factors and the chronological and biological age of individuals [154], highlighting the potential value of these signatures for *in vivo* and clinical purposes.

Extracellular vesicles have recently emerged as key communication players within the senescent secretome [155]. The release of EVs has been reported to be significantly higher in senescent cells following a wide variety of stressors, most likely due to the strong vesicle trafficking and dysfunctional lysosomal compartment in senescent cells. Intriguingly, vesicle cargoes appear to be distinct to the SASP and contain molecules other than the most widely investigated soluble factors [155,156], which can be very valuable for the development of EV-based senescent signatures. In addition, EVs can be effectively isolated and analysed from peripheral blood, potentially allowing their use as screening and monitoring tools of cellular senescence *in vivo*.

Finally, the secretion of nonprotein biomolecules, either within EVs or as free-circulators, represents an additional detection strategy. Interestingly, a recent publication reported traces of mRNA SASP factors in blood samples correlating with astrocyte senescent burden in patients suffering from cognitive decline [157]. With increasing evidence demonstrating an extranuclear DNA accumulation in the cytoplasm of senescent cells [158–160], it is likely that DNA molecules are also secreted to the extracellular environment. Despite a recent study showing that senescent cells have a decreased secretion of cell-free DNA (cfDNA) compared with apoptotic cells [161], others have demonstrated the excretion of DNA within exosomes as a mechanism to maintain homeostasis during senescence [162]. In this scenario, because senescent cells undergo extensive epigenetic modifications as previously described, high-throughput approaches able to detect epigenetic-specific signatures of cfDNA in samples represent a potential attractive and intriguing avenue. While, to our knowledge, this remains unaddressed in the field of senescence, we believe that this possibility

poses exciting opportunities for less invasive detection methods and the monitoring of cellular senescence in living organisms.

Conclusion

In this review, we present a detailed description of the biomarkers and techniques most commonly used for the assessment of cellular senescence *in vitro* and *in vivo*. Some of the major limitations in this process include the lack of universal biomarkers and the heterogeneity of the senescent phenotype, compounded by the fact that most of the changes used for detection are only relative in nature. Despite a strong correlation between most of the markers and the senescent programme, clear thresholds of expression that allow a solid discrimination between senescent and nonsenescent cells are generally needed. In addition, we believe that the senescent community should aim towards the use of comprehensive biomarker strategies that not only enable the identification of senescence, but also its quantification and monitoring.

In all probability, a universal marker of cellular senescence may never be found. We believe that this is likely because the senescence community applies a single term to define what actually embraces a plethora of varying phenotypes. It is for this reason that, as we convey in this guide, a multimarker combinatorial approach is required for the accurate assessment of cellular senescence. This has been laid out and exemplified in the field [36,163], but given the number and heterogeneity of the senescent traits that could be assessed, determining the most optimal strategy can be complicated. Thus, we propose the concurrent detection of at least three different markers that confirm (a) the cell cycle arrest, (b) the increased lysosomal compartment or another global senescent-related structural change and (c) an additional trait specific for the particular type of senescence being assessed (such as SASP or DNA damage features).

In conclusion, it is obvious that more robust markers are needed in order to better understand the biology and implications of cellular senescence. As exemplified above, significant efforts are being made to discover novel markers and detection technologies. However, in our view, some already well-established hallmarks, such as SA- β -gal, still remain to be leveraged to their full potential. In addition, we believe that the elucidation of signatures specific to subtypes of cellular senescence and particular tissues of origin will allow more vigorous characterizations. Likewise, we are confident that the development of artificial intelligence and deep learning models will allow the automated and unbiased detection

of cellular senescence in the future, potentially transforming the histological assessment of senescence within tissues in particular. Altogether, these advances will not only be key for the development of diagnostic and senolytic tools aimed at targeting senescent cells in research and the clinic, but they will also redefine how we characterize and understand cellular senescence in the future, allowing for a more comprehensive picture of how this process interacts with ageing populations and lends itself to human disease.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

EGG and AGB conceptualized and wrote the manuscript. LF and DME reviewed the manuscript and provided expert opinion and critical insights.

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