

# Monitoring senescence-mediated growth arrest of murine and human cancer cells *in vitro*

Barbara Schoerg<sup>1</sup>, Francisco José Reche Pérez<sup>1</sup>, Yvonne Heneka<sup>1</sup>, Lars Strohben<sup>1</sup>, Martin Ziller<sup>2</sup>, Ulrich Thomas<sup>2</sup>, Max Engelstädter<sup>2</sup>, Elena Dragicevic<sup>2</sup>, Michael George<sup>2</sup>, Giovanna De Filippi<sup>2</sup>, Niels Fertig<sup>2</sup>, Sonja Stoelzle-Feix<sup>2</sup>

<sup>1</sup>Eberhard Karls University of Tübingen, Tübingen, Germany.

<sup>2</sup>Nanon Technologies GmbH, Munich, Germany.



## Significance

- Cellular senescence is a key factor in ageing.
- Because of its intricate role in cancer, the investigation, detection and quantification of cellular senescence has gained major interest.
- Electric Cell-Substrate Impedance Sensing (0.1 kHz – 100 kHz) allows for the analysis of the dynamics of cell proliferation, e.g. analysis of growth arrest during senescence induction.

## Introduction

Ageing, cellular growth and cancer development appear to be different processes, but they may have common origins. The accumulation of cellular damage over time is widely regarded as a general cause of ageing and also of cancer. In addition, uncontrolled cellular growth or hyperactivity is not only involved in cancer, but also in aging-related pathologies such as atherosclerosis and inflammation<sup>1,2</sup>.

Cellular senescence is a key factor in ageing and cancer. It is characterized by a permanent and generally irreversible cell cycle arrest<sup>3</sup> with a senescence-associated secretory phenotype (SASP)<sup>4</sup>, macromolecular damage (DNA) and altered metabolism<sup>5,6</sup>.

In cancer, oncogene expression, cancerous mutations as well as radiation, chemotherapy and other cytotoxic drugs<sup>7</sup> and even inflammation-related cytokines like interferon- $\gamma$  and TNF $\alpha$ <sup>7,9,10</sup> can drive tumor cells into senescence. While therapy-induced cellular senescence can lead to permanent cessation of tumor growth, recent findings had contradicted the idea that senescence only has a positive effect on suppressing tumor growth<sup>11,12</sup>. Senescent malignant and non-malignant cells can drive tumor growth by secreting a plethora of pro-inflammatory cytokines, chemokines, growth factors and the SASP<sup>13,14</sup>. Furthermore, the SASP has a double-edged nature as it can activate the

immune system and promote tumor clearance, but can also be immunosuppressive and contribute to the growth and increased stemness of neighboring cells<sup>15</sup>. Cancer cells can override the senescence-associated cell-cycle arrest and escape from the non-proliferative compartment due to their genomic instability and potential to acquire additional mutations<sup>16,17</sup>. Because of its intricate role in cancer, the investigation, detection and quantification of cellular senescence has gained major interest.

The platform used here, AtlaZ, is a quantitative live-cell analysis system allowing real-time and label-free monitoring of cell adhesion and proliferation, cytotoxicity, GPCR, morphology changes and barrier function. Recordings can be performed in up to six 96-well plates simultaneously or independently. To test and implement the AtlaZ system for the investigation of cellular senescence, we analyzed the strength of growth arrest induced by established treatments in murine and human cancer cell lines. Cancer cells were seeded to the 96-well plates and allowed to settle and attach to the bottom (Figure 1) before the senescence-inducing treatments were added to the wells. Here, the impedance was measured over a period of up to 5 days. In our assay, the murine as well as the human cancer cells showed an arrest in cell proliferation upon treatment. The cells increased in size but stopped proliferating which was

detected by a stable arrest of the Cell Signal. We observed a time- and treatment-dependent decrease of the Cell Signal, representing proliferation arrest of murine and human cancer cells.

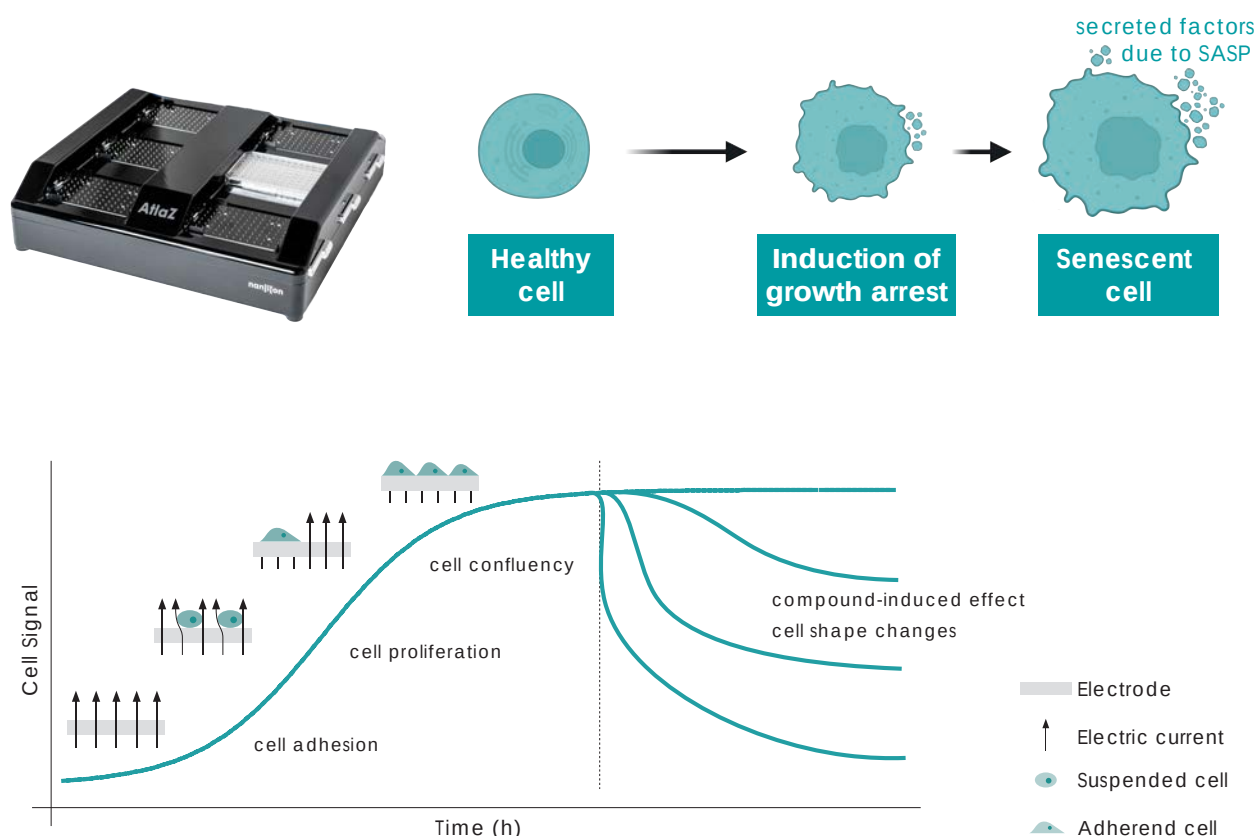
The impedance of planar gold-film electrodes that are used as growth substrate for adherent cells reveals changes in electrode coverage or cell behavior, e.g. upon treatment with senescence-inducing drugs and/or cytokines. Real-time impedance data provide insights in the temporal dynamics of senescence induction, such as changes of cell morphology and proliferation, even over prolonged periods of time.

A crucial advantage over standard assays, e.g. using cell labels and either an optical or radiometric detection technique, is the continuity of cell monitoring. Endpoint assays using dyes or labels are labor-intensive and lack kinetic information of the recorded effects. Unique culture

plates with integrated electrodes as used in the AtlaZ system enable long-term measurements over several weeks. Thus, real-time data on cell adhesion, proliferation and compound effects can be acquired.

Changes in cell morphology can be qualitatively characterized by size, shape, and structural alterations. Such physical rearrangements in cells result in corresponding alterations in their electrical properties as the cell acts as both, a resistor and a capacitor. Electrochemical impedance spectroscopy (EIS) is an analytical method to measure such electrical properties<sup>18,19</sup> and this methodology is employed in Nanion's cell monitoring technology implemented in the AtlaZ platform.

The power of Electric Cell-Substrate Impedance Sensing (ECIS) resides in its ability to detect changes in cell layer integrity in a label-free, real-time manner ranging from seconds to days.



**Figure 1** Impedance measurements offer information on e.g. cell adherence, proliferation, membrane integrity or cell death. Cell-specific profiles of different growth and cell behavior patterns such as cell size changes in late senescent cells can be detected and are for example relevant in cancer research.

## Materials and methods for cell culture

We explored the feasibility of detecting the induction and dynamics of senescent cells through impedance measurements. To achieve this, we induced senescence in two different cell lines, a primary murine  $\beta$  cell cancer line (RT2  $\beta$ -cc) and a human colon carcinoma cell line (HCT116) using established senescence-inducing protocols. Over the whole experiments, the cells were cultured on 96-well plates with planar gold film electrodes (NSP-Z, Nanion Technologies) and analyzed with the AtlaZ measurement unit (Nanion Technologies) which was positioned in a humidified incubator (Binder) at 37°C with 5% CO<sub>2</sub>.

Murine cancer cells were derived from an endogenous murine mouse model of  $\beta$ -cell cancer (RIP1-Tag2, RT2) and cultured in growth medium (Dulbecco's Modified Eagle's Medium; DMEM supplemented with 10% fetal bovine serum, FBS, Sigma-Aldrich; MEM Amino Acids, 0.5x, Sigma-Aldrich; 1 mM Sodium pyruvate, Sigma-Aldrich; 10 mM HEPES buffer, Bio&Sell; 1 mM L-Glutamine, Sigma-Aldrich; 100 U ml<sup>-1</sup> and 0.1 mg/ml Penicillin-Streptomycin, Sigma-Aldrich; 50  $\mu$ M 2-mercaptoethanol, Sigma-Aldrich). Senescence induction was achieved as described previously (Braumüller, *et al.*, 2013, Müller-Hermelink *et al.*, 2008). Briefly, adherent RT2  $\beta$ -cancer cells were washed in DPBS (Invitrogen of Thermo Fisher, USA), trypsinized with 0.05% trypsin/EDTA solution (gibco), and counted. Cells were seeded in growth medium in a 96-well NSP-Z plate (5.000 cells per well) and placed into the AtlaZ – measurement unit for monitoring of the impedance signal overnight. After 12 h, media was replaced with fresh growth medium which was either supplemented with a combination of 100 ng/ml murine IFN- $\gamma$  and 10 ng/ml murine TNF (IFN- $\gamma$ +TNF, n = 11) (R&D Systems), with 5  $\mu$ M palbociclib isethionate (palbo, n=12) (Selleckchem), or with a combination of IFN- $\gamma$ , TNF and palbo (PIT, n = 12). Control cells remained untreated (non-supplemented growth media, media control, n = 11). The recording plate with the RT2  $\beta$ -cancer cells was placed back into the AtlaZ system and the impedance spectrum (1 kHz - 100 kHz, every 4 minutes) was recorded for a period of 5 days. For final analysis, the Cell Signal at 70 kHz was used, being the most sensitive recording frequency (see Figure 2 for the concept).

The human colon carcinoma cell line (HCT116 cells)

was originally acquired from the American Type Culture Collection (ATCC, Manassas, Virginia). Cells were maintained in McCoy's 5A medium (Sigma of Merck, USA) supplemented with penicillin-streptomycin (1% v/v) and inactivated FBS (10% v/v) (Sigma of Merck). Before seeding for experiments, cells were freshly thawed and passaged at least once maintaining the same passages in every single experiment. Adherent HCT116 cells were washed in DPBS, trypsinized with trypsin/EDTA solution, and counted. In the experimental setup, cells were seeded in replicates (8x) to the 96-well NSP-Z plate (5.000 cells per well) and were allowed to settle for 24 h before being incubated in the presence of 250 nM doxorubicin (BB Pharma) for 24 h. This protocol is correlated with a notable *in vitro* expression of  $\beta$ -galactosidase, a commonly used marker for cellular senescence. In line with the murine cells, HCT116 control cells were treated with media only (Ctrl.). After 24 h, culture media was replaced, and both control and doxorubicin treated HCT116 cells were further cultured for an additional four days in the absence of the drug. Throughout this entire process, HCT116 cells were monitored by measuring the impedance (here at 50 kHz, every 20 seconds) using the AtlaZ system.

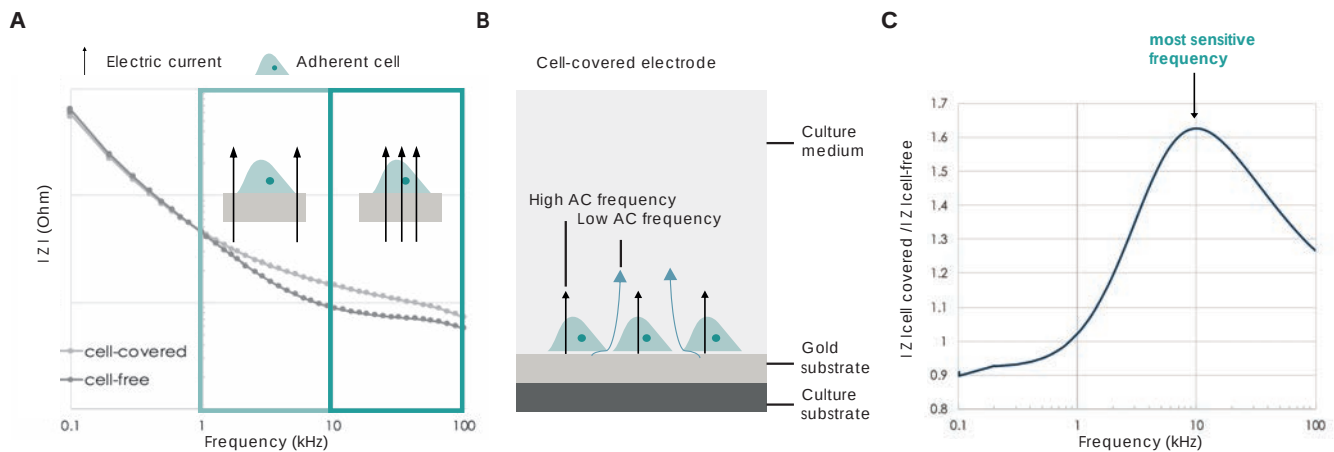
## Technological background

Impedance ( $|Z|$ ) describes the electrical resistance in response to an AC signal. It takes into account that capacitors become more conductive with increasing AC frequency. Thus, impedance spectroscopy that records the impedance at a set of discrete AC frequencies along a certain spectral window (here between 0.1 kHz and 100 kHz) enables a more diversified study of the passive electric properties of the cell layer.

The optimal frequency is cell-type specific and has to be determined empirically, but for most electrode types it ranges between 1 kHz and 50 kHz<sup>20</sup>. As a general guideline, the sensitive frequency for detection of changes in cell layer properties can be determined by dividing the impedance magnitude  $|Z|$  of a cell-covered electrode by the impedance of a cell-free electrode (Figure 2). When these ratios are plotted for each individual frequency one obtains a curve from which the maximum ratio reflects the frequency with the broadest range of relative change in impedance (Figure 2C, the most sensitive frequency).

**"The AtlaZ platform facilitates rapid and straightforward early screenings in search of senescence-inducing drugs, particularly for anticancer therapies."**

**Barbara Schoerg**, Postdoctoral Researcher, Eberhard Karls University Tübingen



**Figure 2** **A** The frequency dependent behavior of a cell-free and cell-covered electrode is presented in a Bode diagram, reflecting the impedance increase due to the cell layer between  $\sim 1 - 100$  kHz. For the specific experimental setup here, the signal is dominated by the paracellular cell layer resistance between about  $1 - 10$  kHz (range indicated by light green frame) and is increasingly influenced by capacitive currents across the cell membranes between  $10 - 100$  kHz (range indicated by darker green frame). **B** Schematic side view of a cell-covered electrode with current flow at low and high AC frequencies. **C** The ratio of the normalized impedance - calculated by dividing  $|Z|$  for a cell-covered electrode by that of the same cell-free electrode (as in Figure 2A) - is used to estimate the monitoring frequency with maximum sensitivity. Different experimental setups (e.g. various cell types within particular research areas) own specific optimal frequencies and the available frequency spectrum ( $0.1 - 100$  kHz) allows to exploit a multitude of experimental conditions.

At lower AC frequencies ( $100 \text{ Hz} - 5\,000 \text{ Hz}$ , dependent on electrode surface area), the current is forced to flow around the cell bodies, between and beneath neighboring cells (Figure 2B green arrows). At high frequencies ( $> 10\,000 \text{ Hz}$ ), the current couples transcellularly (Figure 2B, black arrows) and the impedance is dominated by the capacitance of the membrane. At intermediate frequencies the current takes a mixed combination of these paracellular and transcellular routes.

Depending on the selected recording mode, the displayed “Cell Signal” represents the impedance (Ohm) as acquired at a recording frequency of  $10 \text{ kHz}$  (Cell Monitoring mode) or  $50 \text{ kHz}$  (Cytolysis mode), or the Resistance (Transepithelial Electrical Resistance, TEER) as detected at  $1 \text{ kHz}$  in the TEER mode. This means that the “Cell Signal” is not a calculated value but represents raw data.

NSP-Z transparent sensor plates (Figure 3) allow for observation of the cells under a microscope or for making use of the plates in a complementary imaging system.

## Results

The induction of senescence in murine and human cancer cells by IFN- $\gamma$ +TNF and Palbociclib ( $\beta$  cancer cells) as well as doxorubicin (human HTC116 cancer cells) was shown previously *in vitro* as well as *in vivo*<sup>8,10</sup>. Our aim was to use

the impedance-based assay to dynamically measure the growth arrest in murine and human cancer cells, monitoring in real time the effects induced after treating the cells with the established protocols mentioned above. As control, we used equal amounts of tumor cells which are allowed to grow constantly due to normal media conditions. After experimental senescence was induced by the respective treatments, we detected a stabilization of the Cell Signal in the treated wells of both cell types (human and murine cancer cells), while the Cell Signal of the control cells was increasing constantly until a plateau was reached.

Figure 3 shows the proliferation curves of the murine RT2  $\beta$  cancer cells measured at  $70 \text{ kHz}$  over a time period of 5 days post-plating. The impedance signal of the cell monolayer was recorded every 4 minutes and is plotted over time (Figure 3A). Cells adhered overnight within less than  $24 \text{ h}$ . Next, the media was replaced and cells were treated with the senescence-inducing agents (IFN- $\gamma$ +TNF, Palbo or PIT). The senescence inducing agents resulted in a treatment-dependent growth arrest which remained stable over the recorded period of 5 days. This induction and maintenance of senescence is represented by a nearly stable Cell Signal which was for every treatment and after 3 days of senescence induction ( $t = 96 \text{ h}$ ) drastically reduced compared to the signal of the proliferating cells (media control,  $p < 0.0001$ ) (Figure 3B). The strongest effect was observed as a consequence of the cytokine treatment (IFN- $\gamma$ +TNF +/- Palbo). Here, the dynamics of the proliferation was calculated as the slope of a regression curve (Palbo:  $0.45x$ ;

IFN- $\gamma$ +TNF: -0.45x; PIT:-0.46x; media control: 2.79x) (Figure 3C). In sharp contrast to the Cell Signal of the treated cells, we detected an increasing signal over a period of 3 days deriving from the physiologically normal cells (media control) which represents stable cell proliferation (Figure 3A, B). The induction of senescence was verified using enzymatic  $\beta$ -gal staining (Figure 3D).

HCT116 cells were seeded on NSP-Z plates (5000 cells/well) and adhesion and proliferation was monitored with AtlaZ over the next 24 hours. At  $t = 24$  h, the cells were treated with doxorubicin, a chemotherapeutic drug which is known to induce senescence at certain concentrations. (Figure 4). Control wells were kept under normal conditions. After another 24 h, the media of the treated wells was replaced with culture media (without doxorubicin). Cells were cultured for another period of 3 days. The Cell Signal of the cell monolayer was recorded (measurement interval: every 2 minutes) over the whole experiment (Figure 4A). The data shows a nearly steady signal in consequence of the doxorubicin treatment. The Cell Signal was not increasing over time which most likely represents cellular growth arrest over the recorded period of 3 days. This growth arrest and induction of senescence could be verified using enzymatic  $\beta$ -gal staining (Figure 4D). In contrast to the doxorubicin treated cells, normal cell proliferation represented by an increasing Cell Signal was observed in wells which were treated with media control (values,  $p < 0.0001$ ) (Figure 4A, B).

Finally, we assessed the strength of growth arrest, which is represented by the slope of the regression curve (doxo: 0.02; media control: 1.23) (Figure 4C).

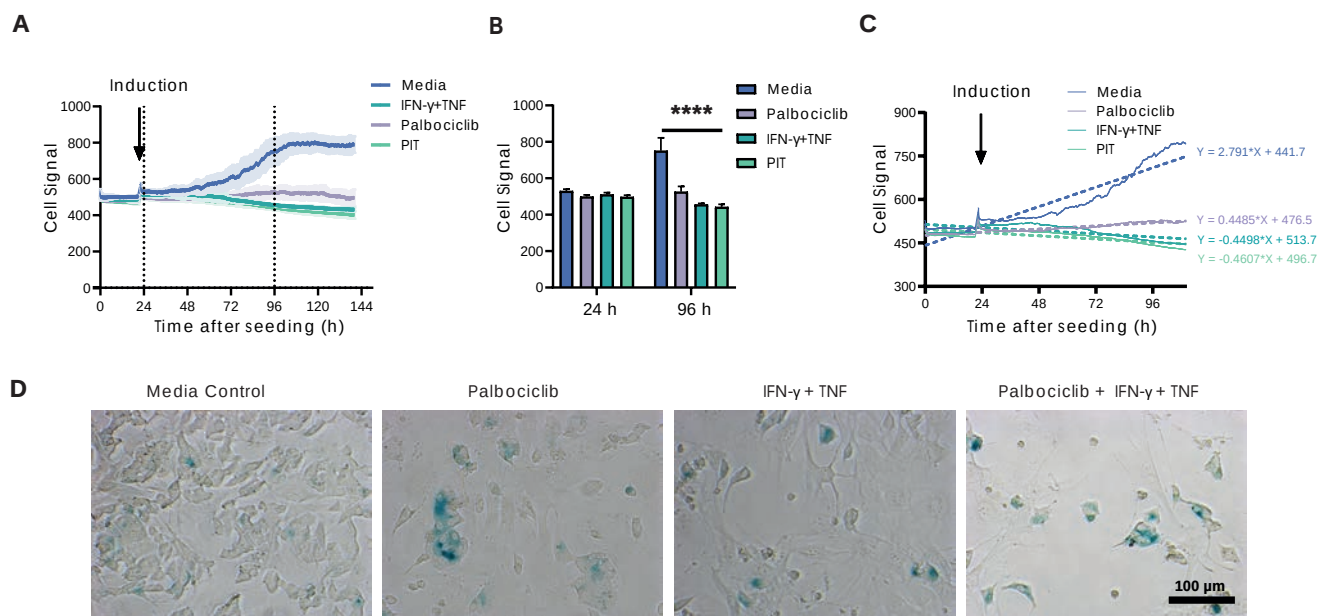
Overall, the results for both the murine RT2  $\beta$  cancer cells as well as the HCT116 cells demonstrate senescence-mediated growth arrest which was validated by the well established protocols as reported in the literature.

In conclusion, these data show the applicability of the impedance technique as implemented by AtlaZ system to monitor the dynamics of cellular senescence. This enables an easy comparison of the power of newly discovered or even clinically approved senescence inducing drugs.

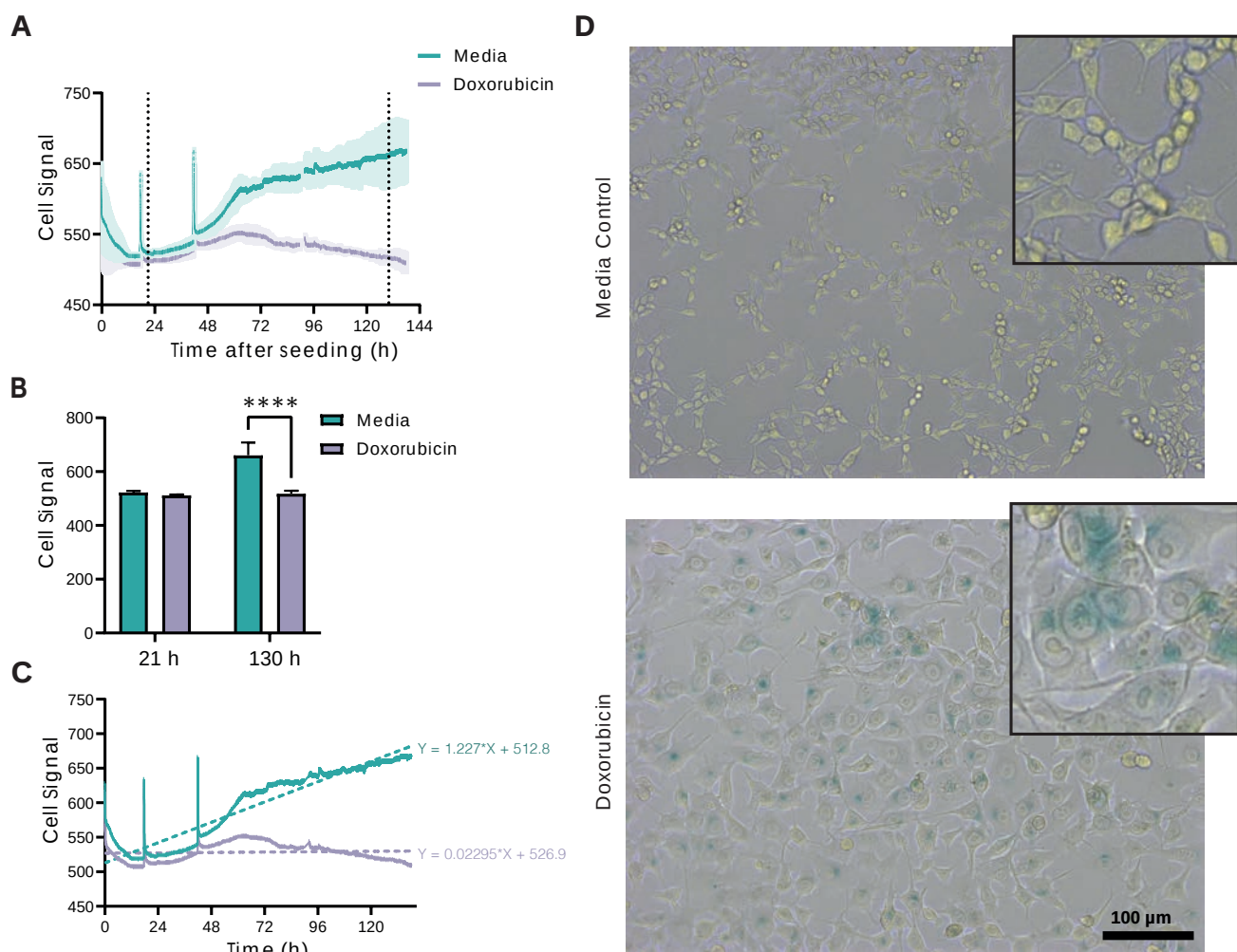
## Summary

The interconnection between ageing, cancer development, and cellular growth suggests common origins despite distinct outward processes. The accumulation of cellular damage is widely acknowledged as a primary cause of ageing, potentially leading to abnormal cellular advantages (aberrant properties of cells, enabling them to bypass normal growth controls) and cancer. Uncontrolled cellular overgrowth is implicated in age-related pathologies like atherosclerosis and inflammation.

Impedance analysis of mammalian cells grown on planar



**Figure 3** **A** Proliferation curves of the murine RT2  $\beta$  cancer cells measured at 70 kHz over a time period of 5 days post-plating. At  $t=24$  h cells were treated with the senescence-inducing agents IFN- $\gamma$ +TNF, Palbo or PIT. **B** Bar graph plots of Cell Signal amplitudes at  $t=24$  and  $t=96$  h. **C** Strength of the senescence induction calculated as the slope of a regression curve (Palbo: 0.45x; IFN- $\gamma$ +TNF: -0.45x; PIT:-0.46x; media control: 2.79x). **D** Induction of senescence as verified using enzymatic  $\beta$ -gal staining.



**Figure 4** **A** Proliferation curves of the HCT116 cells measured at 50 kHz over a time period of 5 days post-plating. At  $t=24$  h cells were treated with the senescence-inducing doxorubicin. At  $t=48$  h, the media was replaced with culture media without doxorubicin. Cells were cultured for another period of 3 days. **B** Bar graph plots of Cell Signal amplitudes at  $t=21$  h and  $t=130$  h. **C** Strength of the senescence induction calculated as the slope of a regression curve (Doxo: 0.02; Media Control: 1.23). **D** Induction of senescence as verified using enzymatic  $\beta$ -gal staining.

film electrodes provides a label-free, non-invasive and unbiased observation of cellular properties addressing the biological response to putative senescence inducers, drugs, toxins or stressors in general<sup>21</sup>. Being label-free, automation and continuous monitoring of barrier function are among the most significant advantages offered by electrical techniques in comparison to macromolecular solute permeability studies<sup>20</sup>.

The label-free AtlaZ impedance recording system enables acute and chronic assessment of cellular toxicity as well as senescence in a continuous fashion from living cells under physiological temperatures and without the confounding effects of dyes that may affect cell function. The system uses 96-well plates with 96 parallel sensors offering a time resolution of down to 1 s for impedance measurements, thus allowing investigation of fast effects like GPCR related morphology changes. A frequency spectrum can be

recorded ranging from 100 Hz – 100 kHz.

In general, cell adhesion and proliferation assays such as immune-cell-mediated killing of cancer cells can be successfully performed.

We exemplarily showed that:

- senescence-mediated growth arrest can be detected using impedance measurements of cancer cells;
- dynamics of senescence-mediated growth arrest, induced by compound treatments, can be investigated using AtlaZ. A persistency of the effect during chronic exposure proves that not only acute but also chronic effects can be analyzed;
- frequency spectrum function offers wide application of

impedance measurements for cell structural changes, such as cell size changes.

Multi-frequency impedance data allow for a non-invasive continuous monitoring of cells with the option to zoom in on certain cell aspects or to tailor the readout to individual cell types for improved data interpretation.

We conclude that the AtlaZ system provides a versatile tool for *in vitro* cell monitoring addressing the demands for versatility, physiological relevance and throughput.

## References

1. López-Otín, C., et al., 2013. The hallmarks of aging. *Cell* 153(6):1194-217.
2. Vijg, J., & J. Campisi, 2008. Puzzles, promises and a cure for ageing. *Nature* 454(7208):1065-71.
3. Wang, E., et al., 1995. Senescent Human Fibroblasts Resist Programmed Cell Death, and Failure to Suppress bcl2 Is Involved. *Cancer Research* 55(11):2284-2292.
4. Campisi, J., et al., 2011. Cellular senescence: a link between cancer and age-related degenerative disease? *Semin Cancer Biol* 21(6):354-9.
5. Passos, João F, et al., 2010. Feedback between p21 and reactive oxygen production is necessary for cell senescence. *Mol Syst Biol* 6(1) 6(1):347.
6. Rodier, F., et al., 2011. DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *Journal of Cell Science* 124(1):68-81.
7. Kirkland, J. L., 2023. Tumor dormancy and disease recurrence. *Cancer and Metastasis Reviews* 42(1):9-12.
8. Braumüller, H., et al., 2013. T-helper-1-cell cytokines drive cancer into senescence. *Nature* 494(7437):361-5.
9. Müller-Hermelink, N., et al., 2008. TNFR1 signaling and IFN-gamma signaling determine whether T cells induce tumor dormancy or promote multistage carcinogenesis. *Cancer Cell* 13(6):507-18.
10. Brenner, E., et al., 2020. Cancer immune control needs senescence induction by interferon-dependent cell cycle regulator pathways in tumours. *Nature Communications* 11(1):1335.
11. Camphausen, K., et al. Radiation therapy to a primary tumor accelerates metastatic growth in mice. *Cancer Res.* 2001 Mar 1;61(5):2207-11. PMID: 11280788.
12. Guccini, I., et al., 2021. Senescence Reprogramming by TIMP1 Deficiency Promotes Prostate Cancer Metastasis. *Cancer Cell.* 11;39(1):68-82.e9. doi: 10.1016/j.ccell.2020.10.012. Epub 2020 Nov 12. PMID: 33186519.
13. Demaria M., et al., 2017. Cellular Senescence Promotes Adverse Effects of Chemotherapy and Cancer Relapse. *Cancer Discov.* 7(2):165-176. doi: 10.1158/2159-8290.CD-16-0241. Epub 2016 Dec 15. PMID: 27979832; PMCID: PMC5296251.
14. Faget, D.V., et al., 2019. Unmasking senescence: context-dependent effects of SASP in cancer. *Nat Rev Cancer*; 19(8):439-453. doi: 10.1038/s41568-019-0156-2. Epub 2019 Jun 24. PMID: 31235879.
15. Carpenter, V.J., et al., 2021. Senolytics for Cancer Therapy: Is All That Glitters Really Gold? *Cancers (Basel).* 10;13(4):723. doi: 10.3390/cancers13040723. PMID: 33578753; PMCID: PMC7916462.
16. Milanovic, M., et al., 2018. Senescence-associated reprogramming promotes cancer stemness. *Nature.* 4;553(7686):96-100. doi: 10.1038/nature25167. Epub 2017 Dec 20. PMID: 29258294.
17. Saleh, T., et al. 2019. Tumor cell escape from therapy-induced senescence. *Biochem Pharmacol.* 162:202-212. doi: 10.1016/j.bcp.2018.12.013. Epub 2018 Dec 19. PMID: 30576620.
18. Reiß, B. & Wegener, J., 2015. Impedance analysis of different cell monolayers grown on gold-film electrodes. *IEEE Eng. Med. Biol.* 7079-7082.
19. Lukic, S. & Wegener, J., 2015. Impedimetric monitoring of cell-based assays. *Encyclopedia of Life Sciences*, Wiley & Co. <https://doi.org/10.1002/9780470015902.a0025710>
20. Stolwijk, J.A., et al. 2015. Impedance analysis of GPCR-mediated changes in endothelial barrier function: overview, and fundamental considerations for stable and reproducible measurements. *Pflügers Arch.* 467(10): 2193–2218. doi:10.1007/s00424-014-1674-0.
21. Zinkl, M., & Wegener, J., 2019. Using animal cells as sensors for xenobiotics: monitoring phenotypic changes by multimodal impedance assays, *Current Opinion in Environmental Science & Health*, Volume 10, Pages 30-37, ISSN 2468-5844, <https://doi.org/10.1016/j.coesh.2019.08.007>.

## Contact Information

### Dr. Barbara Schoerg

Postdoctoral Researcher  
Eberhard Karls University of Tübingen, Germany  
[barbara.schoerg@med.uni-tuebingen.de](mailto:barbara.schoerg@med.uni-tuebingen.de)

### Dr. Sonja Stoezel-Feix

Director, Scientific Affairs  
Nanion Technologies GmbH  
[Sonja.Stoezel-Feix@nanion.de](mailto:Sonja.Stoezel-Feix@nanion.de)  
[www.nanion.de](http://www.nanion.de)

## Acknowledgments

Figures have been partially designed using  
[BioRender.com](https://www.biorender.com/).

