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"How to detect senescent cells and intercellular junctions by in vivo confocal microscopy. An algorithm to approach new in vivo longevity parameters using Artificial Intelligence applied to stratum granulosum and stratum spinosum images"

Pedro Pinto [1], Joana Pereira [2] , Ruben Cunha[2] , Sara Faro[2] , Ana Severo[2] , Maria Elias[2] , Nuno Galego[1], Manuel Fitas[1],

[1]R&D; [2]Technical Department, PhD Trials, Lisboa, Portugal

1. Introduction

Cell morphology, which includes factors such as the shape and structure of the cytoskeleton, reveals the biochemical and mechanical states of epidermal cells. In the aging process, there is an increase in senescent cells and changes in the mechanics of the cytoskeleton, resulting in morphological changes that can be quantified. These characteristics can be used as biomarkers of age [1].

Cytoskeletons are composed of well-structured components such as actin filaments and microtubules. These maintain their structure and internal tension thanks to efficient proteostasis and remodelling mechanisms [2]. When this system is maintained, the cytoskeletal structures are thinner, more defined and functional, preserving the characteristics of a young cell. Over time, these structures become disorganized, proteins aggregate and internal tension decreases [3].

This disorganization is observed in various model organisms. Recent studies have demonstrated, for example, that in species such as Caenorhabditis elegans, ageing leads to filaments becoming disorganized and weak. Similarly, the neuronal cytoskeleton appears to be susceptible to the effects of ageing, undergoing structural changes like those found in epidermal tissues. These results prove how cellular ageing affects the organization of the cytoskeleton in various biological situations [4-5].

As we get older, cells suffer morphological changes which modify their shape, becoming more irregular, less circular and, if their circularity drops to half its normal state, it is a sign of cellular senescence. Thus, cell circularity is a morphological measurement system often used to assess structural changes associated with senescence, where values close to 1 designate a perfect circular shape and lower values reflect elongated or irregular shapes. Previous studies have indicated that senescent cells show a significant reduction in circularity compared to

young cells, due to changes in the organization of the cytoskeleton and an increase in cell area [4,5,7].

To evaluate cell morphology in the epidermis, it is possible to use Confocal Scanning Laser Microscopy (CSLM), which is a modern imaging technology that allows non-invasive characterization of skin strata from the epidermis to the superficial dermis, with high resolution and in real time. This technique stands out for its speed, reproducibility and ability to produce images in vivo, which makes this technology very relevant in various fields, such as dermatological research or clinical diagnosis [6]. This method allows visualization of the epidermal layers, such as the stratum granulosum and stratum spinosum.

In this context, we developed an algorithm, programmed in Python, capable of distinguishing and quantifying cell morphological changes. This methodology was based on the analysis of cell circularity and cytoskeleton thickness from images obtained with CSLM. The aim of this analysis was to accurately characterize age-dependent structural changes in different age groups, specifically in the layers of the stratum granulosum and stratum spinosum.

2. Materials and Methods

After informed written consent, two hundred female subjects were included in this study. The subjects were stratified into four distinct age groups to allow a comparative assessment of the mechanical behaviour of the epidermis along the spectrum of adult aging. The groups were defined as follows: young (18-35 years; mean age: 28.1), middle-aged (36-45 years; mean age: 41.8), older (46-65 years; mean age: 54.9) and elderly (66 years or older; mean age: 70.0). The selection and grouping of participants were structured to ensure a representative cross-section of adult life.

The morphological analysis was obtained from CLSM images, specifically of the stratum granulosum and stratum spinosum of each subject. To guarantee the uniformity and quality of the data, all the images were named and cropped to a standardized size and converted to 8-bit TIF format, without compression. This was done to eliminate noise and normalize the analysis conditions.

An algorithm was developed in Python to process these analyses. The algorithm starts by detecting the nuclei of each cell, expanding the detection to the cell boundaries, which allows it to calculate the circularity, the thickness of the cytoskeleton and distinguish senescent cells from normal cells. The cells were then analysed based on their morphological appearance and classified into two groups: senescent cells and normal cells. For this study, cells with a circularity between 0 and 0.439 were considered morphologically altered and therefore subsenescent cells. Cells with a circularity between 0.440 and 1.0 were considered normal cells. This approach was supported by previous studies that used automatic image analysis to characterize cell morphology associated with ageing [7]. A complementary Ridge Detection algorithm was also used to analyse intercellular distance.

All statistical analysis was performed using SPSS 23 (IBM) and adopting a confidence level of 95%.

3. Results

A total of 400 epidermal images were analysed, encompassing samples from two hundred female participants distributed across four age groups: young (18–35 years), middle-aged (36–45 years), older (46–65 years), and elderly (66 years and older). Image quality was consistent across samples, allowing for standardized segmentation and analysis.

In the stratum granulosum, as can be seen in Table1, there was a statistically significant increase in the number of senescent cells occurred along side with the increase of age. In the young group the mean count was 64 whilst the elderly group had a count of 76 senescent cells. This is paired with a decreased of circularity as age increases. The young group had a mean circularity of 0.440 whilst the mean for the elderly group went down to 0.432.

Table 1. Analysis of senescent cells in the stratum granulosum, across four age groups. Count is expressed in number (n), and circularity values are reported in arbitrary displacement units.

Senescent cells	Count (n)	Circularity (AU)
Young	64	0,440
Middle-aged	72	0,434
Old	73	0,434
Elderly	76	0,432

Normal cells, in the stratum granulosum, show the same behaviour as senescent cells. In Table 2, we can see that the number of normal cells decreases with age, as so it does the circularity. In the young group the mean count of normal cells was 401 whilst the elderly group had a count of 380. As for the circularity of normal cells, there was a slight decrease with increasing of age. The young group had a mean value of 0.673 for circularity and the elderly group 0.671.

Table 2. Analysis of normal cells in the stratum granulosum, across four age groups. Count is expressed in number (n), and circularity values are reported in arbitrary displacement units.

Normal cells	Count (n)	Circularity (AU)
Young	401	0,673
Middle-aged	395	0,673
Old	392	0,672
Elderly	380	0,671

In table3, analysing all the cells, regardless of their circularity, we can observe that both the total cell count and the mean circularity slightly decrease with age. Young subjects displayed 466 cells and 0.641 of mean circularity. The elderly group showed a total of 458 normal cells and a mean circularity of 0.639 (Table 3).

Table 3. Analysis of all cells in the stratum granulosum, across four age groups. Count is expressed in number (n), and circularity values are reported in arbitrary displacement units.

Total cells	Count (n)	Circularity (AU)
Young	466	0,641
Middle-aged	465	0,640
Old	461	0,639
Elderly	458	0,639

These results show a clear tendency of increase in the number of senescent cells over age and a decrease in the circularity at the same time. Consequently, the number of normal cells with age decreases and so does the mean circularity.

As previously mentioned, a Ridge Detection analysis was performed to determine the thickness of the cells in the stratum granulosum. Figure 1 presents a graph showing the median cell thickness for each age group.

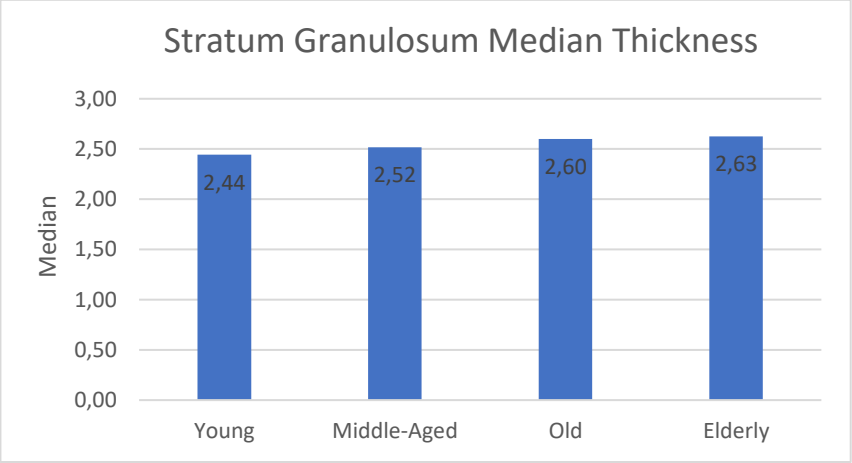

Figure 1. Representative graphics of the cellular thickness in the stratum granulosum, from each age group: young, middle-aged, old and elderly.

Cytoskeletal thickness analysis revealed progressive changes associated with aging. In the stratum granulosum, we can observe thickness increasing gradually over the groups. The young group started with a mean of thickness of 2.44 and the elderly group presented a 2.63 of mean thickness. These results show a consistent profile with our previous discoveries. Younger subjects are showing a well-organized and tension-maintained cytoskeletal network. On the contrary, the elderly group is showing filament thickening and aggregation.

Figure 2 shows representative images from each age group, resulting from the analysis performed on the stratum granulosum.

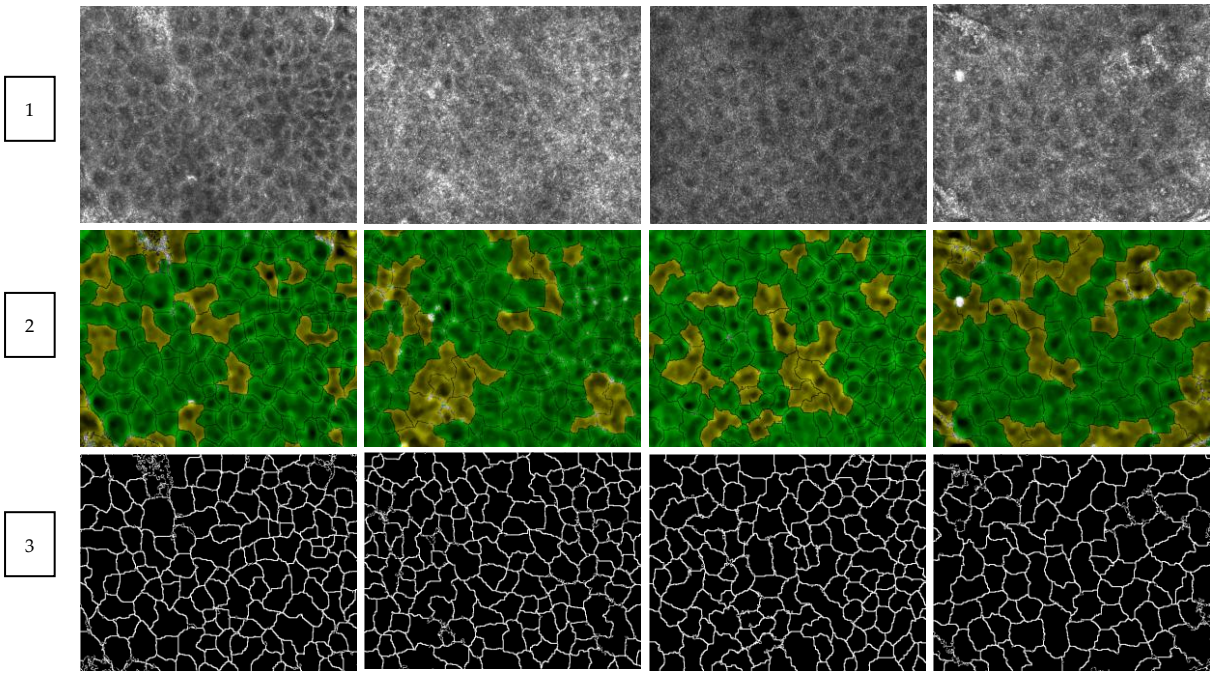

Figure 2. Representative images, obtained by the analysis of the cellular morphology in the stratum granulosum, from each age group: (a) young, (b) middle-aged, (c) old and (d) elderly. In line 1 we can see original images taken by confocal in the stratum spinosum. On line 2, yellow cells represent the senescent cells while green cells represent normal cells and in black, we have the nuclei of each cell. Finally, on line 3 we can observe cellular thickness. These are the images used to run Ridge Detection.

Regarding Stratum spinosum, all epidermal cells from the stratum spinosum were analysed from the same four age groups: young (18–35 years), middle-aged (36–45 years), older (46–65 years), and elderly (66 years and older).

The stratum spinosum shows in Table 4 a slight increase in the number of senescent cells and a slight decrease in the circularity, as the age goes up. In the young group the mean count was 17 whilst the elderly group had a count of 23 senescent cells. This is paired with a decreased of circularity as age goes up. The young group had a mean circularity of 0.397 whilst the mean for the elderly group went down to 0.390.

Table 4. Analysis of senescent cells in the stratum spinosum, across four age groups. Count is expressed in number (n), and circularity values are reported in arbitrary displacement units.

Senescent cells	Count (n)	Circularity (AU)
Young	17	0,397
Middle-aged	19	0,393
Old	23	0,391
Elderly	23	0,390

The number of normal cells, in the stratum spinosum showed a significant decrease across age groups, starting at 524 cells in the younger group and ending with 485 in the elderly group. As shown in Table 5, the younger group presents a circularity of 0.670 and the elderly group a circularity of 0.662. Although mean circularity with age also showed a decrease, it wasn't as significant as the count of cells

Table 5. Analysis of normal cells in the stratum spinosum, across four age groups. Count is expressed in number (n), and circularity values are reported in arbitrary displacement units.

Normal cells	Count (n)	Circularity (AU)
Young	524	0,670
Middle-aged	492	0,664
Old	491	0,663
Elderly	485	0,662

When analysing all images of the stratum spinosum, the total count of cells had a significant decrease between age groups and so did the mean circularity. As presented in Table 6, the count of cells for the younger group was 376 and for the elderly group was 340. As for the mean circularity, the younger group showed values of 0.635 and the elderly group 0.615.

Table 6. Analysis of all cells in the stratum spinosum, across four age groups. Count is expressed in number (n), and circularity values are reported in arbitrary displacement units.

Total cells	Count (n)	Circularity (AU)
Young	376	0,635
Middle-aged	347	0,623
Old	340	0,615
Old plus	340	0,615

These results show the same tendency as within the stratum spinosum. As we get older, our cells suffer morphological alterations, in shape and number, they become more irregular, and the number of cells affected by this becomes higher. These results validate this method to identify and quantify aging biomarkers.

The Ridge Detection analysis was applied to determine the thickness of the cells in the stratum spinosum. Figure 3 displays a graph illustrating the median cell thickness for each age group.

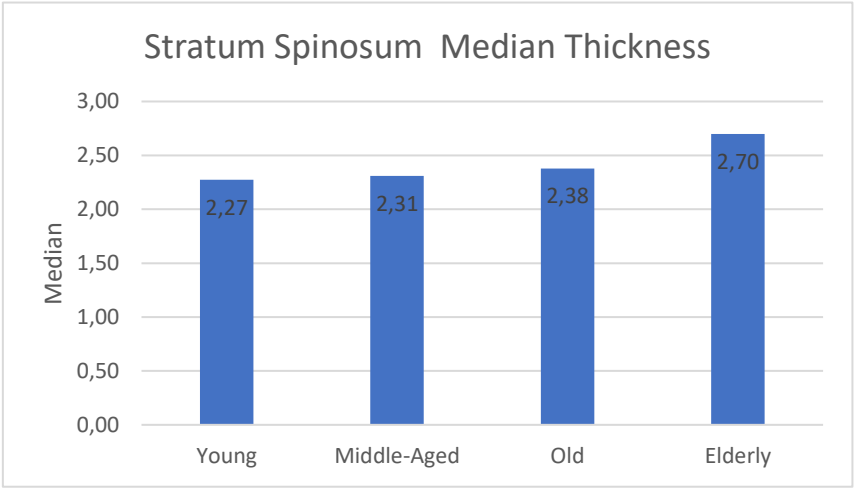

Figure 3. Representative graphic of the cellular thickness in the stratum spinosum, from each age group: young, middle-aged, old and elderly.

In both stratums, granulosum and spinosum, cytoskeletal thickness analysis revealed progressive alterations associated with aging. However, in the stratum spinosum, we can observe thickness increasing gradually over the missile-aged and old groups. The elderly group showed a much-aggravated profile of thickness growth. The young group started with a mean of thickness of 2.27 and the elderly group presented a 2.70 of mean thickness.

These results, within the stratum granulosum and spinosum, align with the hypothesis that aging induces cytoskeletal reorganization, characterized by filament bundling, increased apparent thickness, and loss of fine structural integrity in epidermal cells.

Figure 4 presents representative images from each age group, derived from the analysis performed on the stratum spinosum.

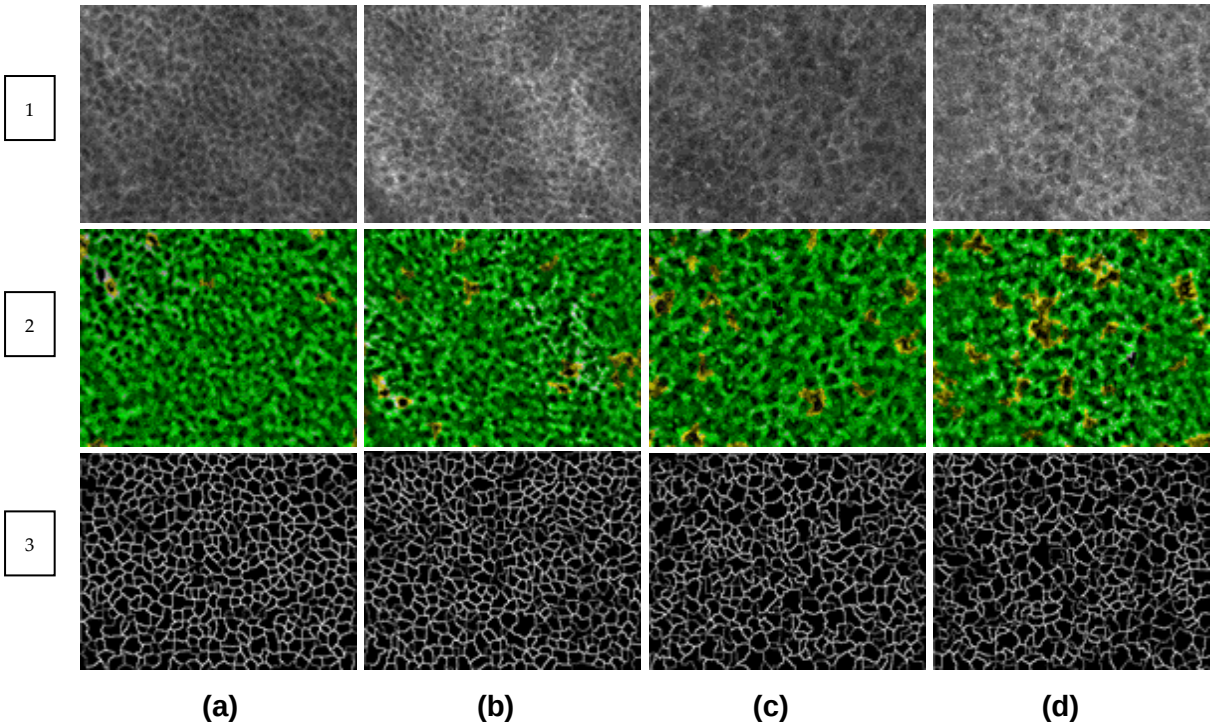

Figure 4. Representative images, obtained by the analysis of the cellular morphology in the stratum spinosum, from each age group: (a) young, (b) middle-aged, (c) old and (d) elderly. In line 1 we can see original images taken by confocal in the stratum spinosum. On line 2, yellow cells represent the senescent cells while green cells represent normal cells and in black, we have the nuclei of each cell. Finally, on line 3 we can observe cellular thickness. These are the images used to run Ridge Detection.

4. Discussion

The aim of the study was to quantify cellular morphology, its organization and the thickness of the cytoskeleton, in the stratum granulosum and stratum spinosum, and correlate these changes with skin aging. Analysing the results obtained, the aim of the study was a success, as we can clearly see a tendency between the different age groups.

In both strata, we can observe a significant decline in circularity as we go up in age, proposing structural remodelling of the epidermis and changes in cellular morphology as this is aligned

with cellular senescence. Younger subjects presented a mean value in circularity higher than the older subjects, which is an indicator of a more regulated, cohesive cell structure. Lower values of circularity, as observed in older subjects, indicate a loss of structural integrity and higher deformation of the cells. This finding is consistent with existing research. Previous studies show a disruption in cytoskeletal organization, notorious of irregular cell shapes and impaired tissue homeostasis.

The count of senescent cells increased as of age increased. However, for normal cells, the count decreased as age increased as well. This implies that with scaling of the age, normal cells tend to be reduce, because they are being morphologically altered and by consequence the number of senescent cells increases. This pattern aligns with the known biological hallmark of aging characterized by the accumulation of dysfunctional cells within tissues.

This became even more confirmed as we analysed cell thickness, and the results demonstrated what we expected would happen. In both strata, younger subjects presented thinner cytoskeletal profiles, while older subjects exhibited progressively thicker and more bundled structures.

This can be explained by the age-associated disorganization of the actin cytoskeleton and increased protein aggregation, resulting in less defined and more robust filamentary networks. Comparing both strata, we can see a difference in the distribution of the results. Such as in the stratum granulosum the values are mostly concentrated towards higher values, which indicates that superficial epidermal layers might be more susceptible to age-driven cytoskeletal remodelling.

In conclusion, this discovery corroborates the effectiveness of this algorithm to detect morphological differences of aging at cellular level. This also proves the importance of morphological biomarkers, such as circularity and cellular thickness to study and quantify skin aging.

5. Conclusion

The aim of the study was to quantify cellular morphology, its organization and the thickness of the cytoskeleton. The study was carried out using a Python-based algorithm designed to analyze cells in the stratum granulosum and stratum spinosum, providing quantitative data on cytoskeleton thickness and cellular circularity. This analysis allows us to distinguish normal cells from senescent ones based on their cytoskeleton thickness and circularity.

Supported by physiology knowledge and when we analyze our results, we can confidently say that skin aging is directly linked to a decrease in cellular circularity and an increase in cytoskeleton thickness. When these two parameters are combined, we see a change in the balance of normal cells and morphologically altered cells, called senescent cells.

These findings could aid future studies on the efficacy of anti-aging products and improve our understanding of age-related pathologies. In the future, we could enhance this discovery by incorporating biomarkers, creating 3D visualizations, or utilizing artificial intelligence to further explore the mechanisms of skin aging.

6. References List

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