Chapter 13

Assessing Collagen Deposition During Aging in Mammalian Tissue and in *Caenorhabditis elegans*

Alina C. Teuscher, Cyril Statzer, Sophia Pantasis, Mattia R. Bordoli, and Collin Y. Ewald

Abstract

Proper collagen homeostasis is essential for development and aging of any multicellular organism. During aging, two extreme scenarios are commonly occurring: a local excess in collagen deposition, for instance during fibrosis, or a gradual overall reduction of collagen mass. Here, we describe a histological and a colorimetric method to assess collagen levels in mammalian tissues and in the nematode *Caenorhabditis elegans*. The first method is the polychrome Herovici staining to distinguish between young and mature collagen ratios. The second method is based on hydroxyproline measurements to estimate collagen protein levels. In addition, we show how to decellularize the multicellular organism *C. elegans* in order to harvest its cuticle, one of the two major extracellular matrices, mainly composed of collagen. These methods allow assessing collagen deposition during aging either in tissues or in whole organisms.

Key words Collagen, Aging, Tissue, Herovici staining, Hydroxyproline, Cuticle, Isolation, Freeze-cracking, Age-synchronizing, Extracellular matrix, *C. elegans*

1 Introduction

Cells and tissues are embedded within extracellular matrices (ECM), which are important for tissue geometry, integrity, and function [1]. In mammals, collagens constitute about 30% of the total protein mass in the body and are the major component of the ECM [2]. In humans, collagen turnover can be extremely slow as observed in the lens of the eye and in cartilage structures (with a half-life time of 114 years [3–5]) or extremely fast, within 72 h after physical exercise, in Achilles tendons [6]. Hence, depending on the tissue or organ, some collagens are synthesized, secreted, and integrated in the ECM once early in life and are not replaced over

Alina C. Teuscher and Cyril Statzer contributed equally to this chapter.

Irit Sagi and Nikolaos A. Afratis (eds.), *Collagen: Methods and Protocols*, Methods in Molecular Biology, vol. 1944, https://doi.org/10.1007/978-1-4939-9095-5_13,

the entire lifetime, whereas other collagens have a higher turnover rate.

During aging, the connective tissue or ECM integrity declines due to accumulation of damage from collagen fragmentation, oxidation, and glycation [2, 7–12] leading to a continuous reduction of collagen mass, as best illustrated by wrinkles and sagging skin [13, 14], but also observed in other connective tissues that support organ function. This deterioration in ECM integrity has been implicated in many age-dependent diseases, such as diabetes, cancer, chronic liver diseases, and cardiovascular diseases [2, 7–12]. In parallel, the accumulation of molecular damage [15], chronic inflammation (inflammaging) [16], or injury during aging might locally drive abnormal collagen deposition resulting in fibrosis [17]. These observations suggest that in general the overall collagen mass tends to decline as a function of age, but due to the increasing incidence of damage or injury, atypical collagen accumulation might occur more locally in certain parts of the body. Therefore, proper assessment of the extent of collagen deposition might reveal novel insights in maintenance and remodelling of the ECM during aging.

A direct role for proper collagen deposition and remodelling during aging has become evident by studying model organisms, such as mice and *C. elegans*. For instance, deficits in the ECM remodelling enzyme (MMP14) lead to premature aging, short lifespan, and cell senescence in mice [18]. Interestingly, long-lived mice preserve connective tissue elasticity and integrity during aging [19, 20]. Consistent with these findings, in the nematode *C. elegans*, collagen synthesis declines during aging. Moreover, most if not all long-lived *C. elegans* mutants delay this progressive decrease in collagen mass [21]. Delaying this progressive reduction in collagen mass by actively prolonging the biosynthesis of collagens during lifetime is required and sufficient for healthy aging and longevity in *C. elegans* [21].

In addition to studying the progressive age-dependent decrease in ECM integrity in mice, *C. elegans* provides additional unique opportunities to explore this phenomenon during aging, since (1) *C. elegans* is transparent, thereby allowing ECM components to be tagged by fluorescent proteins to directly monitor ECM homeostasis and integrity noninvasively in vivo [21], and (2) *C. elegans* is a well-established aging model because of its short lifespan (about 3 weeks) and powerful established genetics. However, histological methods are not as commonly used or even developed in *C. elegans* compared to mice. Below, we describe a histological method (Herovici staining) to be used for both *C. elegans* and mammalian tissue, a colorimetric method (measurement of hydroxyproline levels) to estimate collagen levels in both *C. elegans* and mammalian tissue, and a technique to isolate the

C. elegans cuticle (extracellular matrix) to be used for further assessment of collagen deposition or other analysis.

The Herovici staining protocol [22] is one of the several histological methods used to assess the extent of collagen deposition in a tissue. Compared to other widely used collagen staining techniques (e.g., picrosirius red staining or Masson's trichrome staining), the Herovici protocol allows to distinguish between young (e.g., collagen III) and mature collagen (e.g., collagen I) without cross-polarized filters, providing an obvious advantage in the visualization and analysis of processes, where changes in collagen ratios are expected or known to occur. The sequential use of several dyes results in a polychromatic staining, with young collagen appearing blue, mature collagen colored pink to red, and cytoplasm counterstained in yellow (Fig. 1). Moreover, cell nuclei are stained dark blue to black by hematoxylin. The Herovici method is mostly used for histological studies in skin; however, the staining protocol works well in other tissues or organs too (e.g., liver or lung).

Collagen levels can also be determined by using hydroxyproline measurements. Collagens have characteristic [Gly-X-Y] repeats, whereby glycine is at every third position, X is frequently proline, and Y is frequently 4-hydroxyproline. These prolonged stretches of [Gly-X-Y] repeats in the collagen protein are important to form a stable triple helix from the three constitutive collagen chains [2]. Since collagens are enriched in hydroxyproline and this post-translational modification is not as common as in other proteins, measuring hydroxyproline abundancy results in an estimate of total collagen levels [23, 24]. For a more accurate estimation of collagen amounts by hydroxyproline measurements, one could decellularize tissue samples of interest [25] in order to decrease the levels of other hydroxyproline-containing proteins found in the cytoplasm.

Here we show techniques to isolate the *C. elegans* cuticle, a procedure to further enrich for collagen content during sample harvesting for instance for hydroxyproline measurements. The cuticle and basement membranes are the two major extracellular matrices of *C. elegans* [26]. The *C. elegans* cuticle is mainly composed of collagens [27]. Two methods are described to isolate this cuticle. The first and simpler method was modified from Leushner et al. [28], and the second method was adapted from Cox et al. [29]. The advantages and differences of each method are explained in Subheading 3 below.

In summary, collagen deposition and remodelling become faulty during aging resulting either in a generalized decline in collagen biosynthesis or, locally, in excessive and amorphous collagen deposition (fibrosis). The methods described here are tailored for mammalian tissue samples and the organism *C. elegans* to enable the quantification of total collagen protein levels (hydroxyproline measurement), to distinguish between young and mature collagen ratios, and to enrich for collagen-containing extracellular

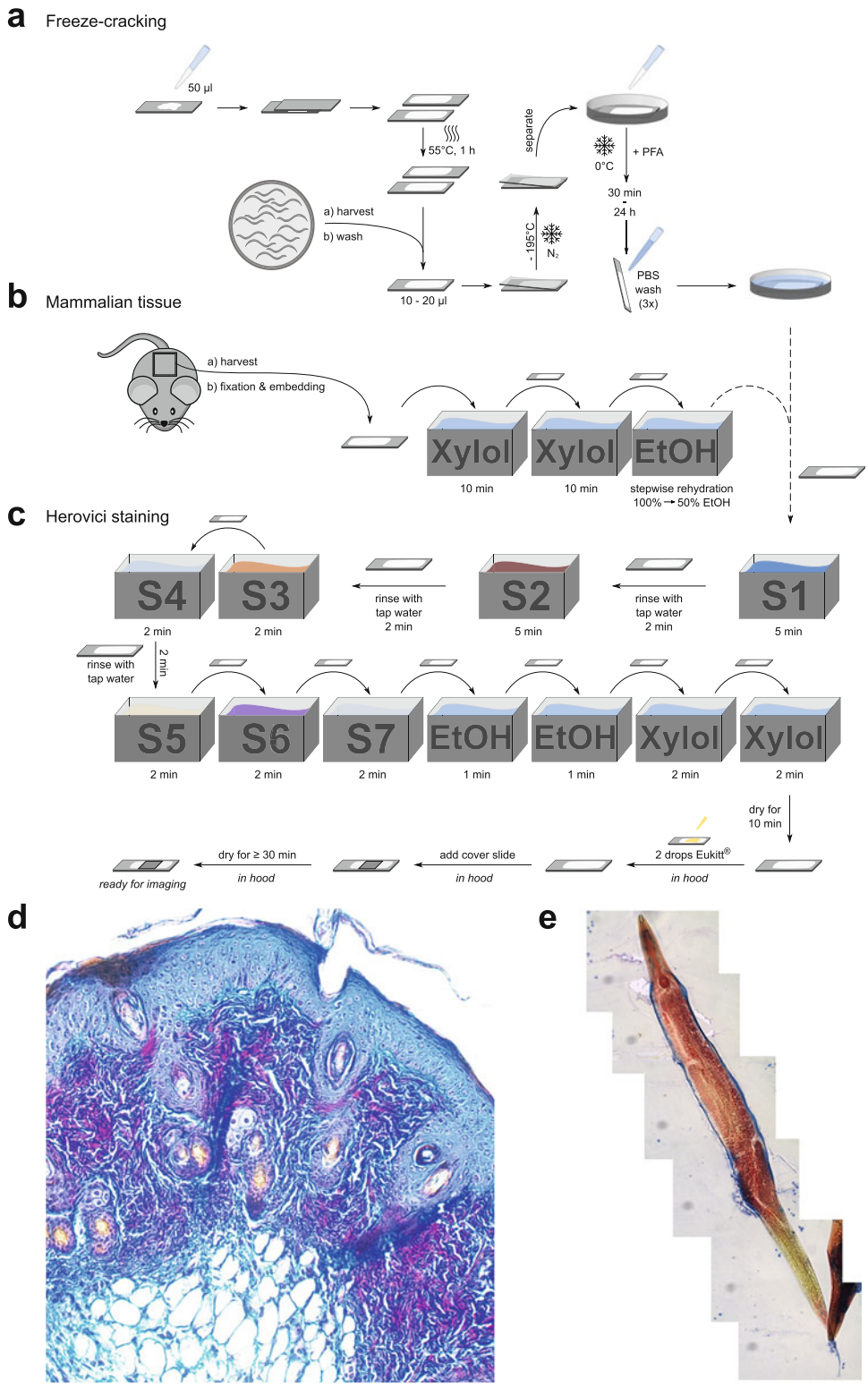


Fig. 1 Herovici staining for mammalian tissue and *C. elegans*. (**a**–**c**) A schematic of the individual steps of the Herovici staining described in Subheading 3 (*see* Subheading 3.1). (**a**) Freeze-cracking of *C. elegans*

matrices in *C. elegans*. In addition, we describe in detail how to synchronize *C. elegans* in order to start a time course for aging studies. With these methods in hand, the investigator should be able to assess collagen deposition during aging and in age-related pathologies.

2 Materials

General mouse necropsy and mammalian tissue harvesting or *C. elegans* maintenance are described elsewhere [30, 31]. *C. elegans* strains can be acquired from the *Caenorhabditis* Genetics Center (CGC, https://cgc.umn.edu, [31]). For the preparation of all solution, distilled water and analytical grade reagents are recommended. All Herovici staining solutions are stored in glass bottles at room temperature protected from light. Disposal of waste materials should be carried out according to specific local regulations. Suitable protection (lab coat, safety glasses, and gloves) and the use of a fume hood are recommended for the handling of acids and other hazardous substances.

2.1 Herovici Staining for Mammalian Tissue and C. elegans

1. 70–100% EtOH.
2. Microscope slides with cut edges and frosted end, 1 mm thick, 76 × 26 mm.
3. Poly-L-Solution: 400 mg Sigma P1524 poly-L-Lysine; 0.2 g sodium azide (0.1%) in 200 ml ddH_2O. Can be reused and stored at 4 °C.
4. PBS: 8 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, 0.24 g KH_2PO_4, pH adjusted with HCl to pH 7.4.
5. PFA: 4% paraformaldehyde solution in PBS.
6. Herovici's Solution 1: Add 270 ml of deionized water to a glass beaker. Weigh 1.25 g celestine blue powder, and transfer it to the glass beaker. Weigh 15 g of aluminum potassium sulfate dodecahydrate (CAS: 7784-24-9; 31242, Merck) and add it to the glass beaker. Heat and boil the solution until the powders are dissolved (approximately 3–5 min). Let the solution cool down to room temperature, and transfer to a 500 ml graduated cylinder, and add distilled water to a final volume of 300 ml.

Fig. 1 (continued) (*see* Subheading 3.1.1). (**b**) Mammalian tissue harvesting and deparaffinization (*see* Subheading 3.1.2). (**c**) Shared part of the Herovici staining protocol for both *C. elegans* and mammalian tissue (*see* Subheading 3.1.3). (**d**) Herovici-stained mouse skin specimen. The young collagen (collagen type III) is stained in blue, whereas the mature collagen (collagen type I) is stained in red. Cytoplasm is counterstained in yellow and cell nuclei dark blue to black. Three layers forming the skin (epidermis, dermis, hypodermis) can be identified. (**e**) Herovici-stained *C. elegans* at day 1 of adulthood

Add 60 ml of glycerol once the solution has cooled down to room temperature (*see* **Note 1**).

7. Herovici's Solution 2: Weigh 1 g of hematoxylin powder, transfer it to a glass beaker, and dissolve it in 100 ml of 95% ethanol in distilled water (alcoholic hematoxylin). Weigh 4.5 g of ferrous II sulfate ($FeSO_4$) powder and 2.5 g ferric III chloride ($FeCl_3$) powder, and transfer them to a separate glass beaker containing 298 ml distilled water. Add 2 ml of 36% (concentrated) hydrochloric acid (toxic, *see* **Note 2**). Stir the solution on a magnetic stirrer till powders dissolve (*see* **Note 3**). Mix this solution with the alcoholic hematoxylin from **step 1** (*see* **Notes 4**–**6**).
8. Herovici's Solution 3: Add 300 ml of distilled water to a glass beaker. Weigh 1.25 g of metanil yellow, and transfer it to the glass beaker. Add 25 drops (use a 1 ml pipette tip) of glacial acetic acid (*see* **Notes 7** and **8**).
9. Herovici's Solution 4: Add 1800 ml of distilled water to a glass beaker. Add 9 ml of glacial acetic acid (toxic, *see* **Notes 7** and **9**).
10. Herovici's Solution 5: Weigh >1 g of lithium carbonate (Li_2CO_3) powder, and dissolve it in 78 ml distilled water (aqueous Li_2CO_3). The solution will be saturated (*see* **Note 10**). Add 1 ml of saturated aqueous Li_2CO_3 to 500 ml distilled water to a glass beaker (*see* **Notes 11** and **12**).
11. Herovici's Solution 6: Weigh 0.15 g of methyl blue powder, and dissolve it in 150 ml distilled water in a glass beaker. Weigh 0.2 g of acid fuchsin powder, and dissolve it in 150 ml of saturated aqueous picric acid in a separate glass beaker. Mix the two solutions together. Add 30 ml of glycerol and 0.15 ml of saturated aqueous Li_2CO_3 (*see* **Notes 11** and **13**).
12. Herovici's Solution 7: Add 10 ml of glacial acetic acid to 1 L of distilled water (*see* **Notes 7** and **14**).

2.2 Total Collagen Quantification for Mammalian Tissue and C. elegans

1. 5 N NaOH.
2. 5% sodium hypochlorite solution (or household bleach).
3. Sterile ddH_2O.
4. M9 buffer: 3 g KH_2PO_4, 7.52 g Na_2HPO_4, 5 g NaCl, and 0.05 g $MgSO_4$ in 1 L ddH_2O, autoclaved [31].

2.2.1 Age Synchronization of C. elegans

2.2.2 Total Protein Quantification

1. QuickZyme Protein Assay Kit (QuickZyme Biosciences) or equivalent kit from other manufactures suitable for protein quantification of acid hydrolyzed samples.
2. Pierce™ BCA Protein Assay Kit (Thermo Fisher) or equivalent kit from other manufactures.

2.2.3 Total Collagen Quantification

1. QuickZyme Total Collagen Kit (QuickZyme Biosciences) or equivalent kit from other manufactures.
2. Aqueous hydrogen chloride solution: 4 M, 6 M, and 12 M.

2.3 Materials for Cuticle (Extracellular Matrix) Isolation in C. elegans

1. Sodium dodecyl sulfate (SDS) buffer: 1% SDS in ddH_2O.
2. Triton X-100 buffer: 0.5% Triton X-100 in ddH_2O.
3. 15 ml glass conical centrifuge tubes with screw gaps.

2.3.1 Cuticle Isolation (Freeze-Thaw Protocol)

2.3.2 Cuticle Isolation (ST Buffer Protocol)

1. M9 buffer: 3 g KH_2PO_4, 7.52 g Na_2HPO_4, 5 g NaCl, 0.0493 g $MgSO_4$ in 1 L ddH_2O, autoclaved.
2. Sonication buffer: 10 mM Tris–HCl, pH 7.4; 1 mM EDTA; in ddH_2O.
3. 0.1 M PMSF buffer: 1 mM phenylmethanesulfonylfluoride in isopropanol.
4. ST buffer: 1% SDS; 0.125 M Tris–HCl, pH 6.8; in ddH_2O.

3 Methods

3.1 Herovici Staining for Mammalian Tissue and C. elegans

All procedures are carried out at room temperature, if not indicated otherwise. Glass slides with tissue sections fixed in 4% paraformaldehyde (PFA in PBS) are used for the Herovici staining protocol. Reusable Herovici solutions can be poured back in their original container. At the end of this procedure, tissue sections will be ready to be imaged under the microscope. In this section, we first describe the harvesting and preparation for mammalian tissue (*see* Subheading 3.1.1) and then for *C. elegans* (*see* Subheading 3.1.2) followed by the shared methodical part (*see* Subheading 3.1.4).

3.1.1 Harvesting and Preparing Mammalian Tissue Sections (Fig. 1a)

1. Organs or tissues of interest are harvested as described elsewhere [30], fixed in 4% paraformaldehyde for 24 h at 4 °C, submersed in increasing concentrations of ethanol, embedded into paraffin [32], and sectioned with a microtome (3.5 μm sections).
2. Deparaffinize tissue sections by immerging the glass slides in xylol for 10 min. Repeat this step once for a total of two.
3. Rehydrate the tissue sections stepwise from 100% to 50% (100%, 96%, 90%, 80%, 70%, 50%) ethanol by immerging the glass slides for 1 min in each solution.
4. Continue the protocol; *see* Subheading 3.1.4.

3.1.2 Preparation of Poly-L-Slides for Freeze-Cracking of C. elegans

1. Clean microscope slides with Kimwipes and 70%–100% EtOH.
2. Pipette 50 µl Poly-L-Solution on the non-frosted part of the microscope slide. Place another microscope slide on top, so that the transparent parts completely overlay and the frosted parts are hanging over on the opposite sides.
3. Slide the two slides apart: one side per slide should now be covered with a thin layer of Poly-L-Solution.
4. Turn the slides around, so that the Poly-L-Solution sides are facing upward, and place the slides in a 55 °C incubator for 1 h to dry. Slides can now be used directly or stored for several months in a clean slide box at 4 °C.

3.1.3 Harvesting and Preparing C. elegans Samples Using a Freeze-Cracking Method (Fig. 1b)

The described *C. elegans* freeze-cracking method was adopted from Duerr 2006 [33]. For freeze-cracking use a minimum of 4000 *C. elegans* or better about 8000–12,000 *C. elegans* (*see* **Note 15**). For a protocol to age-synchronize and culture *C. elegans* (*see* Subheading 3.2.2):

1. Place the fixation solution (4% paraformaldehyde (PFA in PBS)) and several empty 10 cm petri dishes on ice in order to pre-chill for later use.
2. Wash *C. elegans* off culturing plates using M9 buffer, and collect them in a 15 ml conical centrifuge tube.
3. Centrifuge for 1 min at 1000 rpm (standard table centrifuge is ca. 200 × *g*; *see* **Note 16**), and remove supernatant. Wash the *C. elegans* until the supernatant is clean and no bacteria are visible (*see* **Note 17**).
4. Rinse *C. elegans* by filling up the 15 ml conical centrifuge tube with 14 ml ddH_2O, centrifuge for 1 min at 1000 rpm (200 × *g*), remove supernatant, and add ddH_2O to the pelleted *C. elegans* to a total volume of 100 µl.
5. Using a glass Pasteur pipette, place a small drop of animals (ca.10–20 µl) on a Poly-L-Solution covered slide. Wait shortly to let the *C. elegans* settle on the slide. Use the pipette to spread the *C. elegans* out so that they do not overlap.
6. Carefully place another Poly-L-Solution covered slide on top of the slide with the *C. elegans* so that the Poly-L-covered sides face each other, while the frosted parts are not overlapping.
7. Put on safety goggles and gloves. Hold the two slides slightly pressed together into liquid nitrogen until the bubbling stops. Take the slides out. Wait for ca. 20 s. Then start to snip with your finger carefully against one of the slides until they rip apart.

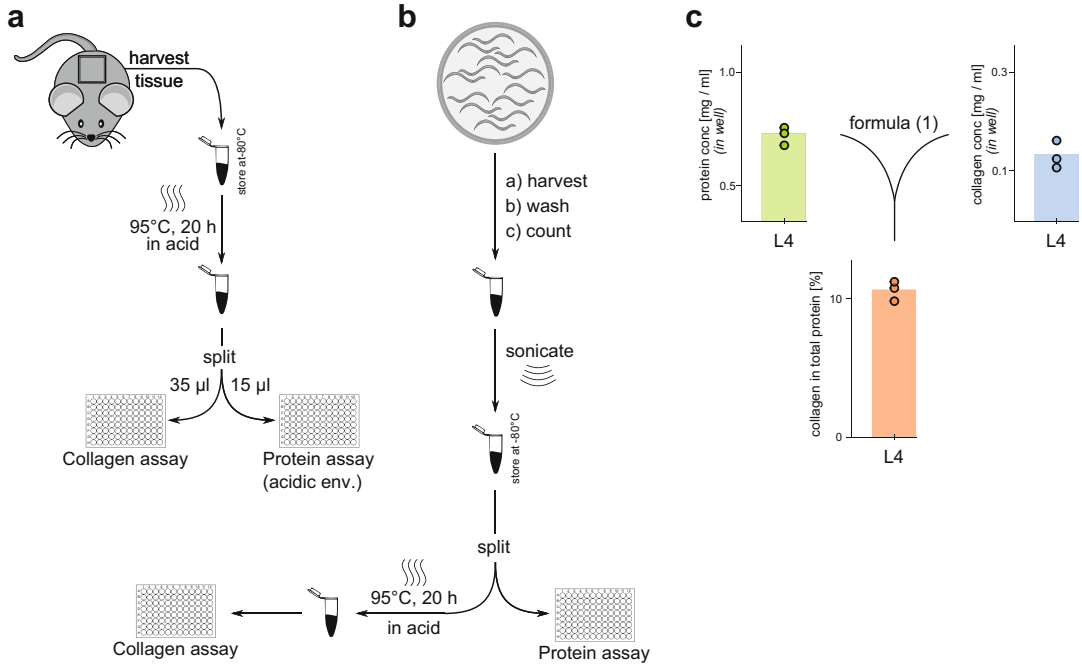
8. Check which slide has most of the *C. elegans* attached to it, and place either only this slide or both slides separately into individual empty 10 cm petri dishes, which are standing on ice.
9. Pipette 100–200 µl 4% PFA pre-chilled onto the *C. elegans* covered part of the slides. Fix the *C. elegans* by incubating the slides at 4 °C for at least 30 min or up to 24 h.
10. After fixation, rinse the slides gently at least three times with PBS. Proceed immediately with staining, or store the slides in a Coplin jar or petri dish containing PBS at 4 °C for several days.

3.1.4 The Herovici Staining for Both Mammalian Tissue and C. elegans (Fig. 1c)

1. Immerse slides in Solution 1 for 5 min (*see* **Note 1**).
2. Rinse slides under running tap water for 2 min (*see* **Note 18**).
3. Immerse slides in Solution 2 for 5–6 min (*see* **Notes 5**, **6**, and **19**).
4. Rinse slides under running tap water for 2 min (*see* **Note 18**).
5. Immerse slides in Solution 3 for 2 min (*see* **Note 8**).
6. Transfer slides directly to Solution 4 for 2 min (*see* **Note 9**).
7. Rinse slides under running tap water for 2 min (*see* **Note 18**).
8. Immerse slides in Solution 5 for 2 min (*see* **Note 12**).
9. Transfer slides directly to Solution 6 for 2 min (*see* **Note 13**).
10. Transfer slides directly to Solution 7 for 2 min (*see* **Note 14**).
11. Immerse the slides in 100% ethanol for 1 min. Repeat this dehydration step once for a total of two.
12. Immerse the slides in xylol for 2 min. Repeat this step once for a total of two.
13. Let the slides dry for a minimum of 10 min (*see* **Note 20**).
14. Mount the slides with a mounting media (e.g., Eukitt®), and let them dry under a fume hood (*see* **Note 21**).
15. After adding the cover slip, the specimen is ready for imaging. Examples of a Herovici-stained mammalian tissue section and a *C. elegans* are shown in Fig. 1d, e, respectively.

3.2 Total Collagen Quantification for Mammalian Tissue and C. elegans

The collagen content in a tissue or whole-organism sample can be determined in multiple ways (Fig. 2). It is generally advised to select the normalization entity to be as conclusive as possible. Here, total collagen quantification is combined with either absolute animal number (*C. elegans*) or with total protein determination for both mammalian tissue and *C. elegans*. Here, we focus mainly on how to establish aged *C. elegans* cultures and on the assessment of collagen levels in *C. elegans*, since the use of hydroxyproline measurement in mammalian tissue is well-established.



d

$$\frac{collagen}{protein} = \frac{[Coll_{Well}] \cdot dil_{assay}^{coll} \cdot dil_{digestion}}{[Protein_{Well}] \cdot dil_{assay}^{prot}} \quad (1)$$

$$\frac{collagen}{individual} = \frac{[Coll_{Well}] \cdot dil_{assay}^{coll} \cdot dil_{digestion}}{\frac{animals_{counted}}{Vol_{counted}}} \quad (2)$$

$[Coll_{Well}]$ = collagen concentration per well as obtained by the assay.

dil_{assay} = dilution in assay well (e.g. to avoid signal saturation).

$dil_{digestion}$ = dilution through HCl and then water addition.

$\frac{animals_{counted}}{Vol_{counted}}$ = average of counted animals divided by the volume used for counting (e.g. 20 µl).

$\frac{collagen}{protein}$ = ratio of collagen to total protein, can be expressed as collagen in total protein [%]

Fig. 2 Total collagen quantification using hydroxyproline assay for mammalian tissue and *C. elegans*. (**a**, **b**) Schematic of the collagen assay that can be performed using mammalian tissue (**a**) as well as *C. elegans* samples (**b**). Please *see* Subheading 3 (*see* Subheading 3.2) for detailed protocol steps. (**c**) An example of how to quantify the collagen-to-protein ratio for larval L4 stage *spe-9(hc88) C. elegans* samples is shown. (**d**) Formula (1) shows how to calculate the ratio of collagen content to total protein levels. The dilution term referring to the digestion is omitted when the total protein content is determined using the hydrolyzed sample. Formula (2) shows how to normalize collagen content per individual *C. elegans*

3.2.1 Mammalian Tissue (Fig. 2a)

There are several ways to assess hydroxyproline levels in mammalian tissues. Here, we briefly outline the use of a colorimetric method using the QuickZyme Kit for both hydroxyproline and total protein measurements. The advantage of this protocol is that each HCl hydrolyzed tissue sample can be split to measure (1) hydroxyproline levels to estimate collagen protein content and (2) to measure total protein levels from the same sample in order to normalize the collagen content to total protein levels. Equivalent kits or protocols

from other manufacturers can be used. For more details, please follow the manufacturer's instruction.

1. Organs or tissues of interest are harvested as described elsewhere [30]. Frozen organs/tissues and formalin-fixed or paraffin-embedded tissues can also be used.
2. Hydrolyze the tissue in 6 M HCl in a safety centrifuge tube for 16 h at 95 °C in a one 1:10 ratio of wet tissue weight to acid volume (*see* **Note 22**). Split the hydrolysate: 35 μl is used for collagen quantification and 15 μl to assess the total protein content (*see* **Note 23**).
3. To generate the standard curve for the colorimetric quantification of collagen and total protein levels, please follow the manufacturer's instructions.

3.2.2 Synchronizing C. elegans for an Age-Dependent Time Course Analysis (Fig. 2b)

C. elegans samples are prepared in one batch and harvested in user-defined intervals over the animal's lifespan. To age-synchronize the *C. elegans* population, we describe a bleach preparation protocol to harvest eggs from gravid *C. elegans* adults. For each time point and condition, 2000–5000 animals are required; therefore ca. 10,000–100,000 eggs should be harvested in the initial lysis step.

1. Use multiple culturing plates containing a large amount of gravid *C. elegans* adults to perform a population lysis. Wash the culturing plates with sterile H_2O. Pipette the H_2O across the plate several times to loosen *C. elegans* that are stuck onto the bacterial lawn.
2. Collect the liquid containing the *C. elegans* in a sterile 15 ml conical centrifuge tube. Centrifuge for 1 min at 1000 rpm (standard table centrifuge is ca. 200 × *g*; *see* **Note 24**). Discard supernatant. Wash residual bacteria away from *C. elegans* by filling up the 15 ml conical centrifuge tube with sterile H_2O, spin down, and discard the supernatant. Repeat this step until the supernatant is clear. Spin down to pellet gravid *C. elegans*, and add H_2O to a total volume of 3.5 ml.
3. Put on gloves and eye protection. Add 1 ml of 5% sodium hypochlorite solution (or household bleach), and add 0.5 ml of 5 N NaOH. Close 15 ml conical centrifuge tube, and shake well. In 1-min intervals, hold the 15 ml conical centrifuge tube under a dissecting scope to observe lysis of gravid *C. elegans*, and shake well after observation to optimize lysis. Once almost all *C. elegans* are lysed, add M9 buffer to a total volume of 15 ml, and centrifuge at 3000 rpm (ca. 1800 × *g*) for 1 min to pellet the eggs (*see* **Note 25**). Discard supernatant, and wash the eggs at least three times by filling up to 15 ml with M9 buffer and re-pelleting the eggs by centrifugation at 3000 rpm (ca. 1800 × *g*) for 1 min. Discard the supernatant (*see* **Note 26**).

4. After lysis and subsequent washing steps, the samples are incubated while rotating for 12 h in M9 buffer supplemented with 5 μg/ml cholesterol.
5. 500–1000 larval L1 *C. elegans* larvae are placed on each 10 cm culturing plate. If a temperature-sensitive sterile strain is used, the sterility mechanism has to be activated at the needed time point (*see* **Note 27**). If floxuridine (FUDR) is used to avoid offsprings, the animals are transferred at the larval L4 stage to 50 μg/ml FUDR culturing plates (*see* **Notes 28** and **29**).
6. 2000–5000 *C. elegans* (2–10 plates) are harvested for each condition and time point. Transfer all animals from the plates to a 15 ml conical centrifuge tube. The animals are centrifuged briefly at low speed (200 rpm, ca. 8 × *g*) to pellet aged *C. elegans* adults. The suspension containing unhatched eggs or larvae (if FUDR is used) is aspirated and discarded. Repeat this washing step three times or until only the desired *C. elegans* adults are present and supernatant is clear (*see* **Note 30**).
7. From the last wash that was filled up with M9 buffer to a total volume of 14 ml, take five times 20 μl aliquots, and place these aliquots on a petri dish lid to count the number of animals in each 20 μl drop using a dissecting scope (*see* **Notes 31** and **32**). To estimate total number of *C. elegans*, average the number of *C. elegans* from each 20 μl drop and multiply by 700 to get an estimate of the total number of *C. elegans* present in 14 ml sample.
8. Centrifuge for 1 min at 1000 rpm (ca. 200 × *g*) to softly pellet *C. elegans*. Discard supernatant. Use a glass Pasteur pipette to transfer the *C. elegans* pellet to a labelled 1.5 ml Eppendorf collection tube, fill up with sterile H_2O to a total volume of 200 μl, and store at −80 °C (*see* **Notes 16**, **33**, and **34**).
9. At the end of the time course, once all samples have been collected, proceed by thawing all tubes. At least 3–5 freeze-thaw cycles should be performed by thawing at room temperature and freezing in liquid nitrogen (or dry ice with EtOH or by placing back into the −80 °C).
10. Then sonicate all samples on ice until all animals are disrupted. The *C. elegans* cuticle is the hardest to break. With a standard sonication device, it will take several repeats to break down all *C. elegans* (*see* **Note 35**).

3.2.3 Total Protein Quantification for C. elegans (Fig. 2)

To determine the total collagen content as a fraction of total protein abundance, the total protein content has to be determined separately.

1. Pipette 25 μl of each sonicated sample as well as of the provided standard solutions into a 96-well plate, and quantify the total protein content following the manufacturer's instructions (Pierce™ BCA Protein Assay Kit, Thermo Fisher, or equivalent kit from other manufacturers). If needed, dilute the sample with M9.
2. Use a plate reader to quantify the color change at the specified wavelength.

3.2.4 Total Collagen Quantification for C. elegans (Fig. 2)

Here, we determine the total collagen abundance by digesting the sample and directly measuring the concentration of the amino acid hydroxyproline in each sample.

1. The sonicated samples are transferred into heat-stable tubes provided by the kit manufacturer (QuickZyme Total Collagen Kit, QuickZyme Biosciences, or equivalent kit from other manufacturers). The provided collagen standard does not require to be sonicated but is otherwise treated identically as the samples.
2. According to the manufacturer's instructions, the sample and standard are mixed with 12 M HCl solution and incubated for 20 h at 95 °C.
3. In brief, after the digestion the supernatant is isolated, the acid content lowered, assay buffer added, and the plate incubated at room temperature for 20 min followed by the addition of the detection reagent and subsequent incubation at 60 °C for 1 h. The color change is quantified using a plate reader (*see* **Note 36**).
4. The total collagen content per sample in a well can be directly determined through the measured color change. In a first step, the collagen reference sample (1.2 mg/ml of rat tail collagen) is used to generate a standard curve relating the magnitude of the color change to the collagen content of the well. Over the used concentration range, this relationship is approximately linear. With the standard curve available, the collagen content of the samples can be quantified. It must be noted that the collagen standard supplied in the QuickZyme Biosciences Kit contains rat tail collagen. To validate the use of rat tail collagen as a reference for hydroxyproline levels for *C. elegans* collagens, the potential hydroxyproline occurrences on the Y position in the *C. elegans* [Gly-X-Y] triple repeats in collagens were estimated in silico. The relative proline abundance on the Y position is similar between *C. elegans* and rat tail collagens (personal communication Jan M. Gebauer). Example results for *C. elegans* protein and collagen concentration are shown in Fig. 2b.
5. The total collagen-to-total protein ratio can be determined by combining the measured collagen and protein concentrations according to formula (1). In the case of *C. elegans*, the total collagen abundance per animal can be calculated by subjecting

the measured collagen concentration to formula (2). All formulas to determine the relative collagen contents are depicted in Fig. 2c.

3.3 Methods for Cuticle (Extracellular Matrix) Isolation in C. elegans

3.3.1 Cuticle Isolation (Freeze-Thaw Protocol)

This method was modified from Leushner et al. [28], for the use in *C. elegans*. The two advantages of using this protocol compared to the ST protocol is (1) its simplicity and (2) the SDS-cleaned cuticles might still contain Schiff base products or other adducts that might be of importance of the desired analysis [34]. The major problem with this protocol is that the *C. elegans* cuticle tends to stick on plastic; this can be avoided by using glass materials instead. All steps after the freeze-thawing should be carried out on ice, except steps involving SDS. We recommend to use about 12,000 *C. elegans* per sample to isolate a sufficient number of cuticles. Please *see* Subheading 3.2.2 for a protocol to age-synchronize the *C. elegans* samples. An example of an aged *C. elegans* (7-day adult) is shown in Fig. 3a for comparison with the isolated cuticles Fig. 3b, c.

1. Use 12,000 adult *C. elegans* (about 8–12 full culturing plates). Pipette around 3 ml M9 buffer on each plate. Gently tilt plates to collect the liquid on one side, and carefully pipette the M9 buffer several times across the plate to loosen up the *C. elegans* stuck on the bacterial food lawn. Transfer the animals from the plates into a 15 ml conical centrifuge tube.
2. Wash the *C. elegans* three times with 15 ml ddH_2O by centrifugation at around 1000 rpm (ca. 200 × *g*) for 1 min, and discard the supernatant (*see* **Note 33**).
3. After the last washing step, discard the supernatant again, and use a glass Pasteur pipette to transfer the *C. elegans* pellet into a 1.5 ml Eppendorf microfuge tube (*see* **Note 37**). Centrifuge 1.5 ml tube at around 100 × *g* for 30 s, discard supernatant, and fill up with ddH_2O to 100 µl.
4. Freeze the tubes in liquid nitrogen for 1 min (or until frozen), followed by a thawing step at room temperature. Repeat this freeze-thaw cycle at least three times.
5. Sonicate the samples five times in intervals of 20 s each at an amplitude of 80% with 20 s breaks on ice in between (Sonoplus mini20 from Bandelin) (*see* **Note 38**).
6. Use a glass Pasteur pipette to transfer the ruptured *C. elegans* to 15 ml glass tubes (with screw gaps) (*see* **Note 39**), and suspend them in 10 ml 1% SDS solution, followed by overnight incubation onto a rotor at 37 °C. The 1% SDS should wash out all the internal cells and organs from the disrupted cuticles (Fig. 3b).

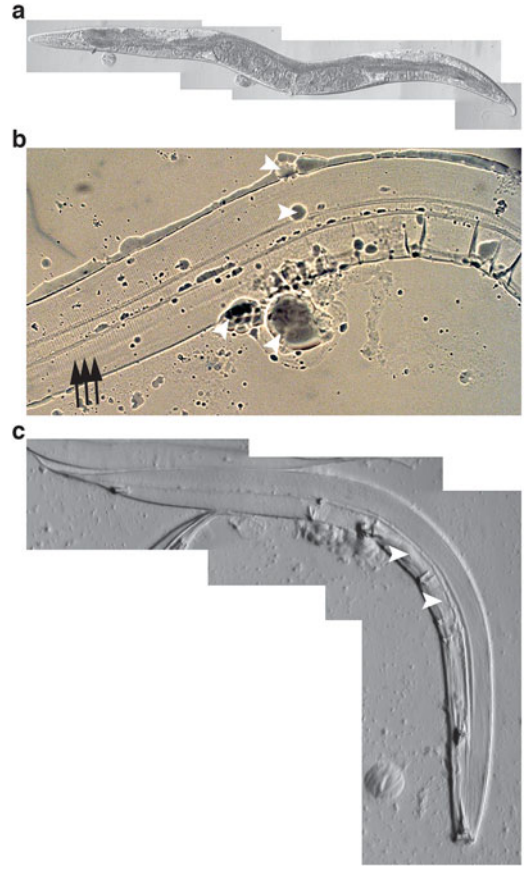


Fig. 3 Isolated *C. elegans* cuticles. (**a**) Differential contrast image of a day 7 adult *C. elegans*. Anterior to the left and ventral side down. (**b**) Intact *C. elegans* cuticle after 1% SDS before washing step. Black arrows point to the annuli structure of the cuticle. White arrowheads point to debris. (**c**) Intact *C. elegans* cuticle after washing. Sometimes other extracellular matrices, such as the basement membranes of the gonad (white arrowheads), are still attached

7. On the following day, wash the cuticles with sterile ddH_2O containing 0.5% Triton-X by centrifuging at 3000 rpm (ca. 1800 × *g*) for 1 min; discard supernatant (*see* **Note 40**).
8. The cleaned cuticles (Fig. 3c) can now be further processed, analyzed, or stored at −20 °C.

3.3.2 Cuticle Isolation (ST Buffer Protocol)

This method was adapted from Cox et al. [29]. Please *see* Subheading 3.3.1 for the comparison with the other cuticle isolation freeze-thaw method, and *see* Subheading 3.2.2 for a protocol to age-synchronize the *C. elegans* samples. All steps after the sonication should be carried out on ice, except steps involving SDS.

1. Collect 12,000 (adult) *C. elegans* with M9 buffer in a 15 ml tube as described in the first step of Subheading 3.3.1.

2. Wash the *C. elegans* pellet three times by centrifugation at around 1000 rpm (ca. 200 × *g*) for 1 min, and discard the supernatant (*see* **Note 33**).
3. After the last washing step, discard the supernatant, and resuspend the *C. elegans* pellet in 5 ml sonication buffer. Incubate on ice for 10 min.
4. Add 30 µl 0.1 M PMSF to the solution, and sonicate five times for 20 s with an amplitude of 80% with 20 s breaks on ice in between (Sonoplus mini20 from Bandelin).
5. Wash the ruptured *C. elegans* cuticles three times with the sonication buffer by centrifugation at 1500 rpm (around 450 × *g*) for 1 min, and discard the supernatant.
6. Discard the supernatant, and transfer the cuticles with a glass Pasteur pipette to a 1.5 ml microfuge tube (*see* **Note 37**). Centrifuge the 1.5 ml tube at around 100 × *g* for 30 s, discard supernatant, and fill up with sonication buffer to 100 µl.
7. Add 1 ml ST buffer, and heat the tube for 2.5 min at 95 °C.
8. Incubate the tubes on a rotator overnight at room temperature.
9. Wash cuticles three times with 0.5% Triton-X in sterile ddH_2O by centrifuging at 3000 rpm (ca. 1800 × *g*) for 1 min; discard supernatant.
10. The cleaned cuticles can now be further processed, analyzed, or stored at −20 °C.

4 Notes

4.1 Herovici Staining for Mammalian Tissue and C. elegans

1. Solution 1 can be reused.
2. Caution. Hydrochloric acid should be handled with care and added to the solution under a fume hood.
3. If required filter the solution to eliminate visible impurities.
4. Alcoholic hematoxylin and the ferrous solution are prepared separately and mixed at the end.
5. Solution 2 has a shelf life of approximately 2–3 months due to oxidation.
6. Solution 2 can be reused.
7. Caution. Glacial acetic acid should be handled with care and added to the solution under a fume hood.
8. Solution 3 can be reused.
9. Do not reuse Solution 4.

10. By adding more than 1 g of Li_2CO_3 to 78 ml distilled water, the solution will be saturated. Do not shake to try to dissolve the precipitate. Let the solution stand.
11. Pipette the aqueous Li_2CO_3 from the top without disturbing the precipitate accumulated on the bottom of the container.
12. Solution 5 can be reused.
13. Solution 6 can be reused.
14. Do not reuse Solution 7.
15. Per plate you can make 2–3 freeze-cracked *C. elegans* slides.
16. To prevent adhesion of animals to plastic pipette tips, one can either add 0.5% Triton-X as a detergent or switch to glass pipettes.
17. Bacteria prevent the animals to properly stick to the slides.
18. Regular water. This step can be performed directly in the sink.
19. A longer incubation may be needed if the solution has not been freshly prepared.
20. A longer drying time (30 min–1 h) is perfectly fine.
21. Eukitt® will need approximately 30 min to dry.

4.2 Total Collagen Quantification for Mammalian Tissue and C. elegans

22. Ideally, a 1:10 ratio of wet tissue weight to acid volume is recommended, with a minimal volume of 200 µl 6 M HCl per tube. For example, 50 mg of tissue mixed with 500 µl of 6 M HCl can be used.
23. To be able to relate the total collagen content of the sample as robustly as possible to the total protein abundance, we recommend to measure the later directly in the hydrolyzed sample. To be able to quantify the free amino acid level present in the acidic hydrolyzed sample, a suitable kit has to be chosen. We recommend the Total Protein Assay, QuickZyme Biosciences.
24. When centrifuging *C. elegans*, either turn off the breaks or turn down the deceleration to a minimum.
25. In general, complete lysis can take about 4–8 min. Hence, it is important to regularly check if all *C. elegans* are lysed. If lysis is performed for too long, the eggs will suffer from increasing damage.
26. Sometimes three washes are not enough to wash out all bleach solution. Hence, check by smelling the 15 ml conical centrifuge tube if bleach odor is still present.
27. We recommend the *C. elegans* mutant *spe-9(hc88)*. Due to a temperature-sensitive defect in spermatogenesis, the animals are not able to lay fertilized eggs when shifted to 25 °C during development. To ensure all animals are sterile, L1 animals can be cultured at 25 °C until day 1 or 2 of adulthood.

28. To avoid food scarcity, check the grow-up plates often, and if needed, transfer to fresh plates.
29. To check the effectiveness of the intervention, a selected number of plates can be used for validation by, for example, placing a fluorescent reporter strain on them.
30. Since the collagen content is changing over the lifespan of *C. elegans*, it is crucial to work with age-synchronous populations. Thus, all eggs and larvae must be removed as best as possible. This can be achieved by transferring animals often to new plates and rigorous washing or through filtration.
31. Because adult *C. elegans* will sink to bottom of the conical centrifuge tube, shake the 15 ml conical centrifuge tube well between taking the aliquots for counting. Try to pipette from the middle of the 15 ml conical centrifuge tube for consistency.
32. Use a pipette with a large orifice to sample *C. elegans* during the counting procedure. Since adults grow in size, a too small pipette tip can lead to an underestimation of older populations.
33. At this step of the protocol is a good time point for a break or to wait until all time points from before are collected, for instance, if you collect daily samples over the 2–3 weeks lifespan of *C. elegans*.
34. It is recommended to fill up all sample *C. elegans* pellets to 200 μl to simplify the downstream steps.
35. Example sonication configuration using a Bandelin Sonoplus, UW mini20 device: Amplitude 80%, 30s intervals, pulse 1.0 s on, 1.0 s off. However, using a more powerful sonicator is recommended to accelerate *C. elegans* fragmentation.
36. It is advised to perform a few test reactions prior to processing the entire batch for both quantification methods to be able to adjust sample dilution should it be too concentrated.

4.3 Cuticle (Extracellular Matrix) Isolation in C. elegans

37. *C. elegans* tend to stick on the plastic tip or on the plastic tubes. Hence, make sure to use a glass Pasteur pipette and pipette the *C. elegans* on the bottom of the plastic tube to avoid sticking on the side of the tube.
38. If the cuticles in the freezing protocol are ruptured too much, you can consider performing only the freezing and thawing cycles or only sonication.
39. One of the major problems is losing too many cuticles or *C. elegans*. Therefore, use always glass pipettes to transfer worms and cuticles.
40. Triton-X prevents the cuticles from sticking to the tubes during the washing steps.

Acknowledgments

We thank Marjolein Wildwater for sharing her improved freeze-cracking protocol, Eline Jongsma for her assistance in adapting the freeze-cracking protocol, Salome Brütsch and Hayley Hiebert for their help to develop the *C. elegans* Herovici staining protocol, Anna Bircher for contributing to the early stages of the cuticle isolation protocols and imaging cuticles, Max Hess for providing the *C. elegans* example image, and Jan M. Gebauer for bioinformatic prediction of potential prolines in collagens that might become hydroxylated in *C. elegans*. Some *C. elegans* strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). This work was supported by the Swiss National Science Foundation [PZ00P3 161512] to S.P. and M.R.B. and [PP00P3 163898] to A.C.T., C. S., and C.Y.E. Alina C. Teuscher and Cyril Statzer contributed equally to this work.

References

1. Hynes RO (2009) The extracellular matrix: not just pretty fibrils. Science 326:1216–1219
2. Ricard-Blum S (2011) The collagen family. Cold Spring Harb Perspect Biol 3: a004978–a004978
3. Sivan S-S, Wachtel E, Tsitron E et al (2008) Collagen turnover in normal and degenerate human intervertebral discs as determined by the racemization of aspartic acid. J Biol Chem 283:8796–8801
4. Heinemeier KM, Schjerling P, Heinemeier J et al (2016) Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage. Sci Transl Med 8:346ra90–346ra90
5. Toyama BH, Hetzer MW (2013) Protein homeostasis: live long, won't prosper. Nat Rev Mol Cell Biol 14:55–61
6. Kjaer M, Langberg H, Miller BF et al (2005) Metabolic activity and collagen turnover in human tendon in response to physical activity. J Musculoskelet Neuronal Interact 5:41–52
7. Myllyharju J, Kivirikko KI (2001) Collagens and collagen-related diseases. Ann Med 33:7–21
8. Fisher GJ, Quan T, Purohit T et al (2009) Collagen fragmentation promotes oxidative stress and elevates matrix metalloproteinase-1 in fibroblasts in aged human skin. Am J Pathol 174:101–114
9. Shoulders MD, Raines RT (2009) Collagen structure and stability. Annu Rev Biochem 78:929–958
10. Sell DR, Monnier VM (2012) Molecular basis of arterial stiffening: role of glycation–a mini-review. Gerontology 58:227–237
11. Snedeker JG, Snedeker JG, Gautieri A et al (2014) The role of collagen crosslinks in ageing and diabetes–the good, the bad, and the ugly. Muscles Ligaments Tendons J 4:303–308
12. Myllyharju J (2004) Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet 20:33–43
13. Fenske NA, Lober CW (1986) Structural and functional changes of normal aging skin. J Am Acad Dermatol 15:571–585
14. Shuster S, Black MM, McVitie E (1975) The influence of age and sex on skin thickness, skin collagen and density. Br J Dermatol 93:639–643
15. López-Otín C, Blasco MA, Partridge L et al (2013) The hallmarks of aging. Cell 153:1194–1217
16. Franceschi C, Campisi J (2014) Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci 69(Suppl 1):S4–S9
17. Wynn TA (2007) Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. J Clin Investig 117:524–529
18. Gutiérrez-Fernández A, Soria-Valles C, Osorio FG et al (2015) Loss of MT1-MMP causes cell senescence and nuclear defects which can be reversed by retinoic acid. EMBO J 34:1875–1888

19. Flurkey K, Papaconstantinou J, Miller RA et al (2001) Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. Proc Natl Acad Sci U S A 98:6736–6741
20. Wilkinson JE, Burmeister L, Brooks SV et al (2012) Rapamycin slows aging in mice. Aging Cell 11:675–682
21. Ewald CY, Landis JN, Porter Abate J et al (2015) Dauer-independent insulin/IGF-1-signalling implicates collagen remodelling in longevity. Nature 519:97–101
22. Herovici C (1963) A polychrome stain for differentiating precollagen from collagen. Stain Technol 38:204–205
23. McAnulty RJ (2005) Methods for measuring hydroxyproline and estimating in vivo rates of collagen synthesis and degradation. Methods Mol Med 117:189–207
24. Qiu B, Wei F, Sun X et al (2014) Measurement of hydroxyproline in collagen with three different methods. Mol Med Rep 10:1157–1163
25. Naba A, Clauser KR, Hynes RO (2015) Enrichment of extracellular matrix proteins from tissues and digestion into peptides for mass spectrometry analysis. J Vis Exp 101: e53057
26. Kramer JM (2005) Basement membranes, WormBook : the online review of C elegans biology. pp 1–15
27. Page AP and Johnstone IL (2007) The cuticle, WormBook : the online review of C elegans biology. pp 1–15
28. Leushner JR, Semple NL, Pasternak J (1979) Isolation and characterization of the cuticle from the free-living nematode *Panagrellus silusiae*. Biochim Biophys Acta 580:166–174
29. Cox GN, Kusch M, Edgar RS (1981) Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. J Cell Biol 90:7–17
30. Treuting PM, Snyder JM (2015) Mouse necropsy. Curr Protoc Mouse Biol 5:223–233
31. Stiernagle T (2006) Maintenance of *C. elegans*, WormBook : the online review of C elegans biology. pp 1–11
32. Fischer AH, Jacobson KA, Rose J et al (2008) Paraffin embedding tissue samples for sectioning. CSH Protoc 2008:pdb.prot4989
33. Duerr JS (2006) Immunohistochemistry, WormBook : the online review of C elegans biology. pp 1–61
34. Davis BO, Anderson GL, Dusenbery DB (1982) Total luminescence spectroscopy of fluorescence changes during aging in *Caenorhabditis elegans*. Biochemistry 21:4089–4095