Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen® and Hoechst 33258

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Summary

Objective: To compare two fluorometric assays, utilizing (1) the bisbenzimidazole Hoechst 33258 and (2) PicoGreen®, for determining DNA content in human cartilage.

Methods: Human articular and nasal septal cartilage explants were digested using proteinase K. Portions of sample digest were analysed for intrinsic and dye-enhanced fluorescence with either Hoechst 33258 or PicoGreen®.

Results: Intrinsic tissue fluorescence in both articular and septal cartilage increased with age and was prominent at wavelengths used for Hoechst 33258 but relatively low at wavelengths used for PicoGreen®. The relative contribution of intrinsic fluorescence to total dye-enhanced fluorescence of human cartilage was markedly greater for Hoechst 33258 (19–57%) than for PicoGreen® (2–7%). Thus, in many situations, DNA in human cartilage can be assayed using PicoGreen® without the need to correct for intrinsic cartilage fluorescence.

Conclusion: PicoGreen® can be used in a rapid and sensitive assay to quantify DNA in small samples of human cartilage. © 2002 Published by Elsevier Science Ltd on behalf of OsteoArthritis Research Society International.

Key words: Human cartilage, DNA, Fluorescence, Hoechst 33258, PicoGreen®.
or if such properties also exist in other types of human cartilage, such as that from the nasal septum. A number of fluorescent dyes other than Hoechst 33258, including TOTO, YOYO-1, YO-PRO-1, and PicoGreen®, have been developed recently and interact sensitively and specifically with DNA. Of these dyes and Hoechst 33258, PicoGreen® has the highest sensitivity for DNA. It is unclear if PicoGreen® would be useful for determining the DNA content of human cartilage, especially with small tissue samples and in a multi-well assay format.

The objective of this study was to compare the use of Hoechst 33258 and PicoGreen® for quantifying DNA content in human articular cartilage and human nasal septal cartilage. To accomplish this, human cartilage samples were solubilized and analyzed for intrinsic fluorescence over a range of excitation and emission wavelengths, for fluorescence enhancement by addition of Hoechst 33258 or PicoGreen®, and for the specificity and sensitivity of fluorescence enhancement by the DNA in the tissue digests. As an application of these DNA assays, samples of adult human articular cartilage and septal cartilage from donors of various ages were analyzed for intrinsic fluorescence enhancement by the DNA in the tissue samples and in a multi-well assay format.

Materials and methods

Materials

Hoechst 33258, DNA from calf thymus, Deoxyribonuclease I (DNase; 2300 Kunitz units/mg), trizma hydrochloride (Tris–HCl), phenylmethylsulfonyl fluoride, Benzamidine–HCl, N-ethylmaleimide, magnesium chloride (MgCl2), and DNA from human placenta were purchased from Sigma Chemicals Co (St Louis, MO). Disodium ethylenediamine tetraacetate (Na2EDTA), sodium chloride (NaCl), and sodium phosphate were from Fisher Scientific (Tustin, CA). PicoGreen® dsDNA quantification reagent and 20X TE (200 mM Tris–HCl, 20 mM Na2EDTA, pH 7.4) were from Molecular Probes (Eugene, OR). Ribonuclease A (RNase; 50–75 Kunitz units/mg) isolated from bovine pancreas was from Worthington Biochemicals (Freehold, NJ). Proteinase K was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Dulbecco’s phosphate-buffered saline (PBS) without calcium and magnesium, pH 7.1 was from Life Technologies (Grand Island, NY).

Stock solutions

Hoechst 33258 was stored at 2 μM (1 mg/ml) in distilled water in a light-tight bottle at 4°C. Just before use, a working solution was made by dilution of the stock solution to 0.2 nM (0.1 μg/ml) in TEN (10 mM Tris, 1 mM Na2EDTA, 0.1 M NaCl, pH 7.4). The working solution was held in and dispensed from a light-tight container.

PicoGreen® was stored at 50× in DMSO in a light-tight container at −20°C. Just before use, a working solution was made by dilution of the stock solution to 1× in TE. The working solution was held in and dispensed from a light-tight tube.

Calf thymus DNA as well as human placenta DNA were dissolved in TE, made to 5 μg/ml in TE assuming OD260 nm=1.0 (DU 480 Spectrophotometer, Beckman Coulter, Fullerton CA) corresponds to 50 μg/ml (based on the proportion of A-T to G-C base pairs in human cartilage, the exact OD260 would be 1.02, and stored at −20°C.

Sample preparation

Human articular cartilage was obtained from the femoral condyle or patellofemoral groove of young adult (age 20–40 years, N=5) or old adult (age >60 years, N=5) tissue donors post mortem. The young joints were macroscopically normal, while the old joints were moderately degenerate (grade 3). Osteochondral fragments were rinsed with PBS with proteinase inhibitors (2 mM Na2EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM Benzamidine–HCl, 10 mM N-ethylmaleimide), stored at −70°C, subsequently thawed in PBS, weighed to determine wet weight (WW), and digested overnight at 60°C in a solution of proteinase K (0.5 mg/ml) in PBE (100 mM sodium phosphate, 5 mM Na2EDTA, pH 7.1) to obtain tissue samples of 15–35 mg WW/ml. The inferior region of the nasal septum was selected from human cartilage specimens removed during routine septoplasty procedures in young adult (34±3 years, N=5) and old adult (71±9 years, N=5) donors according to a protocol approved by the UCSD Institutional Review Board, with informed consent by the patients. Samples were stored in PBS at 4°C for up to 24 h, weighed wet, and digested overnight at 60°C by addition of proteinase K in PBE to obtain tissue samples of 15–45 mg WW/ml. Following digestion, all samples were diluted with PBE to 12 mg WW/ml and stored at −20°C until further use.

Intrinsic and dye-enhanced fluorescence spectra of human cartilage digests

Spectral scans at excitation (EX) wavelengths of 250–580 nm and emission (EM) wavelengths of 270–600 nm were performed on samples of (a) DNA (200 ng/ml), (b) digested articular cartilage (0.6 mg WW/ml) from young adults (30±5 years, N=2), (c) digested articular cartilage (0.6 mg WW/ml) from old adults (84±0 years, N=2), (d) digested septal cartilage (0.6 mg WW/ml) from young adults (27±8 years, N=2), and (e) digested septal cartilage (0.6 mg/ml) from old adults (77±5 years, N=2). To obtain the indicated concentration of cartilage digests, the cartilage digests at 12 mg WW/ml were diluted with TE (intrinsic and PicoGreen®) or TEN (Hoechst 33258). Samples (1 ml) were analyzed either (1) alone for intrinsic properties, (2) with added Hoechst 33258, or (3) with added PicoGreen® in disposable acrylic cuvettes (Sarstedt Inc., Newton, NC) using a fluorometer (F-2000 Fluorescence Spectrophotometer, Hitachi, San Jose CA), with excitation and emission bandpass each at 5 nm, step size at 5 nm, PMT voltage of 700 V, and a scan rate of 1200 nm/min. During the scan, photobleaching was relatively mild, with an ~12% reduction in fluorescence at EX/EM wavelengths corresponding to local excitation maxima (i.e., EX 270 nm/EM 290–600 nm, EX 300 nm/EM 320–600 nm) upon repeated analysis. The fluorescence spectral data was corrected for the spectral absorbance properties of the acrylic cuvette by correcting for the absorbance of light through one wall of the cuvette at the fluorescence excitation wavelength and also for the absorbance through one wall at the emission wavelength.
DYE-ENHANCED FLUORESCENCE OF MIXTURES OF DNA AND CARTILAGE DIGESTS

Fluorometric assays were performed on mixtures of calf thymus DNA and proteinase K digested old adult human articular cartilage. Samples consisted of (a) 0–200 ng DNA ± 1.2 mg cartilage/ml and (b) 0–1.2 mg cartilage + 100 ng DNA/ml, both in buffers of TEN±Hoechst 33258 and TE±PicoGreen®. With TEN±Hoechst 33258, fluorescence was measured at EX 365/EM 458 nm in the fluorometer using disposable cuvettes with excitation and emission bandpass each at 5 nm, and step size at 5 nm, PMT voltage of 700 V, and averaging fluorescence over 3 s per sample. For each measurement, 100 µl of sample was mixed with 1 ml of TEN±Hoechst 33258 solution. With TE±PicoGreen®, fluorescence was measured at EX 480/EM 520 nm in a fluorometric platereader (Spectramax Gemini, Molecular Devices, Menlo Park, CA) on automatic sensitivity using black 96-well plates with clear polystyrene bottoms (Corning Costar, Corning, NY) and averaging over six measurements per sample with an auto emission cut-off at 515 nm. For each measurement, 100 µl of sample was mixed with 100 µl of TE±PicoGreen® -5 min prior to fluorescence detection in the platereader. Fluorescence data from the platereader were converted to the same relative fluorescence units obtained from the cuvette reader by measuring values for a 500 ng/ml DNA standard using PicoGreen® in both fluorometers and using the ratio of the values as a conversion factor.

SENSITIVITY OF FLUORESCENCE ENHANCEMENT TO NUCLEASE DIGESTION

To further assess the specificity of fluorescence enhancement for DNA-dye interactions as well as to assess the contribution of RNA-dye interactions, samples were analysed for fluorescence enhancement both with and without treatment with DNase, RNase, or both in combination. Once nucleotides are cleaved to small fragments, their interaction with fluorescent DNA binding dyes is diminished greatly (98%,16). Samples were prepared by mixing volumes of (a) calf thymus DNA (3 µg/ml), (b) old adult human articular cartilage (6 mg/ml), (c) old adult human sepal cartilage, or (d) PBS (as a blank) with (1) PBS (sham digestion), (2) DNase (100 µg/ml, final concentration) in PBS with 125 mM MgCl₂, (3) RNase (50 µg/ml) in PBS, or (4) a combination of DNase (100 µg/ml) and RNase (50 µg/ml) in PBS with 125 mM MgCl₂. Sample mixtures (N=3 each) were incubated at 37°C for 30 min. Aliquots from each solution were assayed for fluorescence with TEN±Hoechst 33258 and TE±PicoGreen® as described above. Each fluorescence value was corrected for background fluorescence by subtracting the appropriate value for blanks for each nuclease. These values were then divided by the values for the corresponding sham digested samples (also corrected for blank).

CALIBRATION OF FLUORESCENCE ENHANCEMENT TO DNA STANDARDS

Dilutions of calf thymus DNA and human placental DNA were assayed with Hoechst 33258 in the cuvette fluorometer at final concentrations of 0–500 ng DNA/ml. Dilutions of calf thymus DNA and human placental DNA were assayed with PicoGreen® in the fluorescence platereader at final concentrations of 0–500 ng DNA/ml.

CONTRIBUTIONS OF INTRINSIC AND DYE-ENHANCED FLUORESCENCE IN HUMAN ARTICULAR AND NASAL SEPTAL CARTILAGE DURING ADULT AGING

Solutions of human articular cartilage and human nasal septal cartilage digests were assayed by addition of TEN±Hoechst 33258 and TE±PicoGreen® to a final assay concentration of 0.6 mg WW/ml, as described above. DNA content was determined by comparing the fluorescence enhancement with Hoechst 33258 or PicoGreen® to the enhancement with DNA standards (0–500 ng/ml), fit by linear regression. DNA values obtained for PicoGreen® were corrected for damping of the fluorescence enhancement by the presence of PBE in the sample by determining the change in slope (~15% decrease) of DNA standards with varying concentrations of PBE present.

STATISTICAL ANALYSIS

For samples that were mixtures of cartilage digest and DNA, linear regression was used to analyse the dependence of fluorescence values on cartilage and on DNA. Differences between slopes (±S.E.) were assessed by a two-tailed t-test. Linear regression was also used to assess the relationship between DNA estimates using Hoechst 33258 and PicoGreen®. For fluorescence enhancement of DNA standards, linear regression was used to analyse the dependence of fluorescence values on type of DNA. Differences between slopes were assessed by a two-tailed t-test. Two-way ANOVAs were used to assess the effect of age (young, old) and cartilage type (articular, sepal) on intrinsic fluorescence, dye-enhanced fluorescence, and DNA content. In addition, a three-way ANOVA was used to determine the effect of age, cartilage type, and nuclease treatment on fluorescence enhancement relative to untreated samples (expressed as percentage and arcsin transformed before statistical analysis), with different dyes as a repeated measure. Data of specific experimental groups are presented as mean±S.D.

Results

INTRINSIC AND DYE-ENHANCED FLUORESCENCE SPECTRA OF HUMAN CARTILAGE DIGESTS

The fluorescence spectra of DNA and cartilage digests exhibited a variety of local maxima that depended on the presence or absence of Hoechst 33258 or PicoGreen®, and one representative plot of the two samples analysed are shown in Fig. 1. Contour spectral plots of DNA samples alone showed little background fluorescence [Fig. 1(A)] and intense fluorescence enhancement with the addition of DNA binding dyes [Fig. 1(F), (K)]. With addition of Hoechst 33258 to DNA, maximal fluorescence was at EX 365 nm/EM 455 nm [Fig. 1(F)]. With addition of PicoGreen® to DNA, the maximum in fluorescence was at EX 480 nm/EM 505 nm [Fig. 1(K)].

In contrast, both young adult [Fig. 1(B), (D)] and old adult [Fig. 1(C), (E)] articular [Fig. 1(B), (C)] and sepal [Fig. 1(D), (E)] human cartilage exhibited significant background fluorescence, with intense peaks at EX 280 nm/EM 350 nm and
EX 330 nm/EM 390 nm. With addition of Hoechst 33258, both young and old cartilage showed marked fluorescence enhancement (i.e. fluorescence with the intrinsic fluorescence subtracted) with a maximum at EX 365 nm/EM 460 nm [Fig. 1(G)–(J)]. With addition of PicoGreen®, both young and old cartilage showed fluorescence enhancement that was maximal at EX 480 nm/EM 510 nm [Fig. 1(L)–(O)].

DYE-ENHANCED FLUORESCENCE OF MIXTURES OF DNA AND CARTILAGE DIGESTS

It was possible that the components of the cartilage digest interfere with the interaction of DNA with fluorescent dye. To test this possibility, the fluorescence enhancement of dye with purified DNA was compared both alone and in the presence of cartilage digest. The enhancement of dye fluorescence due to mixtures of DNA and cartilage digest could be attributed primarily to an additive effect of fluorescence enhancement by DNA alone and fluorescence enhancement by cartilage digest alone. Fluorescence increased linearly with increasing concentration of DNA with either Hoechst 33258 [Fig. 2(A)] or PicoGreen® [Fig. 2(C)] in the cases of DNA alone, DNA with an added 1.2 mg/ml cartilage, and DNA with an added 1.2 mg/ml cartilage with intrinsic cartilage fluorescence subtracted. Regression of the fluorescence vs DNA data yielded similar slopes in the absence or presence of cartilage digest (1.96–2.08 {F}/ng DNA/ml for Hoechst 33258, \( P = 0.68 \); and 8.54–8.67 {F}/ng DNA/ml for PicoGreen®, \( P = 0.53 \)). Fluorescence also increased linearly with increasing concentration of cartilage with either Hoechst 33258 [Fig. 2(B)] or PicoGreen® [Fig. 2(D)] in the cases of cartilage alone, cartilage alone with intrinsic cartilage fluorescence subtracted, cartilage with an added 100 ng/ml DNA, and cartilage with an added 100 ng/ml DNA with intrinsic cartilage fluorescence subtracted. Regression of the fluorescence vs cartilage concentration data yielded slopes that were diminished only slightly (5–10%) with the addition of exogenous DNA (555 to 522 {F}/(mg WW/ml) without subtracting intrinsic fluorescence and 169 to 151 subtracting intrinsic cartilage fluorescence for Hoechst 33258, \( P = 0.03 \); 686 to 638 without subtracting intrinsic fluorescence and 675 to 628 subtracting intrinsic cartilage fluorescence for PicoGreen®, \( P = 0.04 \)). Thus, for the same concentration of cartilage digest or DNA in solution assayed, the relative enhancement in fluorescence (over background levels) was much greater with PicoGreen® than with Hoechst 33258, and components of digested cartilage had little effect on DNA determination.

SENSITIVITY OF FLUORESCENCE ENHANCEMENT TO NUCLEASE DIGESTION

The enhancement in fluorescence due to addition of either Hoechst 33258 or PicoGreen® to purified DNA or cartilage digest was susceptible to nuclease digestion (Table I; \( P < 0.001 \)). In particular, pre-treatment of both articular and septal cartilage samples with DNase led to an elimination of most (94–98%) of the fluorescence enhancement. In contrast, pre-treatment with RNase had no statistically detectable effect, reducing the fluorescence enhancement a maximum of 10% in the case of articular and septal cartilage with Hoechst 33258. Digestion with both RNase and DNase showed reductions of fluorescence enhancement to the same degree as DNase digestion alone (\( P = 0.75 \) for Hoechst 33258, \( P = 0.96 \) for PicoGreen®).

CALIBRATION OF FLUORESCENCE ENHANCEMENT TO DNA STANDARDS

Fluorescence enhancement of human placental and calf thymus DNA standards with both Hoechst 33258 and PicoGreen® were compared using a linear regression of data points. The fluorescence enhancement with Hoechst 33258 was 22% greater for human placental DNA samples (1.63 vs 1.37 {F}/(ng DNA/ml), \( P < 0.001 \)), but was similar with PicoGreen® (4.45 vs 4.56 {F}/(ng DNA/ml), \( P = 0.20 \)).

CONTRIBUTIONS OF INTRINSIC AND DYE-ENHANCED FLUORESCENCE IN HUMAN ARTICULAR AND NASAL SEPTAL CARTILAGE DURING ADULT AGING

The relative amount of intrinsic and dye-enhanced fluorescence (i.e., attributable to DNA) in cartilage samples...
depended both on the dye used as well as donor age. Since the normalized intrinsic fluorescence at Hoechst 33258 wavelengths was much higher than that at PicoGreen® wavelengths (Table II), these data were analysed separately. The intrinsic fluorescence at the wavelengths for the Hoechst 33258 assay, normalized to the total dye-enhanced fluorescence of young articular cartilage, was higher for old samples (50 relative units for articular and septal combined) than young adult cartilage samples (29 relative units, \(P<0.01\)). There was a trend toward a lower fluorescence in septal cartilage (33 relative units for young and old combined) than in articular cartilage (46 relative units, \(P=0.06\)), but with no interactive effect (\(P=0.30\)). At wavelengths used for the PicoGreen® assay, intrinsic fluorescence was low (<5 for all ages and types of cartilage samples) and also not significantly affected by age (\(P=0.13\)) or tissue source (\(P=0.13\)) with no interactive effect (\(P=0.94\)). With Hoechst 33258, the portion of overall fluorescence that was intrinsic tissue fluorescence was much higher for cartilage from old donors (46%) than young (25%, \(P<0.001\)). With PicoGreen®, intrinsic fluorescence was slightly higher for old than young (6% vs 3%, \(P<0.01\)). The relative intrinsic fluorescence was higher for articular cartilage than septal cartilage with Hoechst 33258 (44% vs 27%, \(P<0.001\)) but not PicoGreen® (5% vs 4%, \(P=0.46\)).

The estimated DNA contents, using human DNA standards and subtracting background tissue fluorescence [Fig. 3(a)], were higher for young adult cartilage than old adult cartilage (140 vs 81 ng DNA/mg WW, \(P<0.05\)), higher for septal cartilage than articular cartilage (141 vs 80 ng DNA/mg WW, \(P<0.05\)), without an interactive effect between age and cartilage type (\(P=0.72\)). The estimates of DNA content using Hoechst 33258 and using PicoGreen® were similar (\(P=0.25\)) and highly correlated [slope=1.01, \(r^2=0.95\), Fig. 3(B)].

**Discussion**

This study investigated the use of the Hoechst 33258 and PicoGreen® fluorescent dyes in the analysis of DNA content in proteinase K digested samples of human articular and nasal septal cartilage. Particular attention was paid to the extent of intrinsic fluorescence and its changes with aging in the adult. The specificity for DNA by both Hoechst 33258 and PicoGreen® was examined by (i) comparing fluorescence spectra of calf thymus DNA, articular cartilage, and septal cartilage in the presence or absence of DNA binding dyes, (ii) measuring the effect of interaction between DNA and cartilage digest solution on fluorescence enhancement, and (iii) measuring the sensitivity of fluorescence enhancement to DNase and RNase. The results
indicate that when intrinsic fluorescence is accounted for, both Hoechst 33258 and PicoGreen® allow analysis of DNA with the contribution of intrinsic fluorescence of human cartilage on DNA content determination.

(i) Three-dimensional scans of dye-enhanced fluorescence for DNA and articular and nasal septal cartilages show similar peaks, caused by the addition of DNA binding dyes for both Hoechst 33258 and PicoGreen® which are unchanged by the age of tissue present. Fluorescent maxima for the dye-enhanced fluorescence are similar to those previously published for Hoechst 33258 and PicoGreen®. This suggests that both Hoechst 33258 and PicoGreen® are interacting with DNA in the proteinase K digested cartilage to produce fluorescence enhancement.

(ii) The possible interaction between the dye and non-DNA components in cartilage was also assessed. For both DNA binding dyes, changes in slope of fluorescence with and without constant concentrations of cartilage [Fig. 2(b), (d)] show a change in slope of less than 5%, showing cartilage tissue digest has a minimal interference with fluorescence enhancement caused by addition of DNA binding dyes.

(iii) The contribution of DNA and RNA enhancement in proteinase K digested cartilage was examined by nuclease digestion (Table I). DNase digestion of cartilage digests resulted in a decrease in fluorescence, using both Hoechst 33258 and PicoGreen®, to less than 7%, a residual percentage similar to that of a sample of pure DNA. In addition, treatment with RNase produced little decrease in the overall fluorescence using both dyes. The existence of a slight residual fluorescence enhancement in samples of digested cartilage as well as purified DNA is in agreement with a previous study and this strongly suggests that residual DNA fragments interact slightly with these dyes. Indeed, DNase usually leaves fragments that average four base pairs in length. If these fragments are of particular sequences, (e.g., A-T bases with Hoechst 33258), they may bind to the DNA binding dye and emit fluorescence. In addition, RNase treatment of both cartilage digests and purified DNA caused a slight decrease in fluorescence enhancement in both cases, suggesting that this decrease was not due to RNA in the cartilage samples.

A direct comparison indicates that PicoGreen® is more sensitive to DNA than Hoechst 33258, generating a higher amount of fluorescence enhancement for the same concentration of 0.6 mg WW/ml. Fluorescence data was normalized so that the total (intrinsic+enhanced) averaged 100 for the young articular samples. Data presented is mean±S.D., N=4–5.

Table II

<table>
<thead>
<tr>
<th>Age</th>
<th>Cartilage type</th>
<th>Intrinsic fluorescence (relative units)</th>
<th>Dye-enhanced (DNA) fluorescence (relative units)</th>
<th>Total fluorescence (relative units)</th>
<th>Intrinsic fluorescence/overall fluorescence (%)</th>
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<td>Hoechst 33258</td>
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<td>Young</td>
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<td>32±9</td>
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</table>

Cartilage samples are from young (20–40 years old) and old (>60 years old) donors and assayed at a final concentration of 0.6 mg WW/ml. Fluorescence data was normalized so that the total (intrinsic+enhanced) averaged 100 for the young articular samples. Data presented is mean±S.D., N=4–5.

Fig. 3. Comparison of DNA content measured using Hoechst 33258 and PicoGreen®. (A) DNA reactive dye is (■) Hoechst 33258 and (□) PicoGreen®. Variation in DNA content by type (articular, septal) and age (young, 20–40 years old; old, >60 years old) of cartilage (N=4–5). (B) Correlation of apparent DNA content of human articular and septal cartilage using Hoechst 33258 and PicoGreen®. Slope=1.01, r²=0.95.
cartilage for human articular cartilage. This is much more sensitive than Hoechst 33258 (6 ng/ml dye solution in a cuvette fluorometer)\(^{10}\). For assaying cartilage DNA content with a cuvette fluorometer rather than a fluorescence plate reader, the volumes of sample test solution and PicoGreen\(^{\circledR}\) dye solution may be scaled up to generate a larger total sample volume appropriate for the particular fluorometer. This in itself would achieve a five-fold higher sensitivity with PicoGreen\(^{\circledR}\) than Hoechst 33258. In addition, the intrinsic fluorescence of cartilage at wavelengths used for Hoechst 33258 is much higher than that at wavelengths used for PicoGreen\(^{\circledR}\).

There are a number of technical details that should be addressed when using either Hoechst 33258 and PicoGreen\(^{\circledR}\) to assay DNA content. The intrinsic fluorescence of human cartilage should be taken into account when using Hoechst 33258, and checked when using PicoGreen\(^{\circledR}\) where it usually contributes slightly. Also, it is important to use DNA standards appropriate to the sample being analysed. As shown by comparison of bovine and human DNA standards, the dye-enhanced fluorescence can be somewhat different for equal DNA concentrations from different mammalian species. In addition, data from DNA standards may need to be corrected for the buffer constituents used in the cartilage digest samples, since different buffer solutions can affect the fluorescence signal. This method may also be useful for other types of cartilage, such as auricular cartilage, that are classified as elastic; the presence of elastin\(^{19}\) and other types of matrix components that may contain fluorophores\(^{20}\) could complicate the use of this assay.

The intrinsic tissue fluorescence of human cartilage must be considered in simple one-step assays of cartilage DNA using crude tissue digests, especially for Hoechst 33258 and less so for PicoGreen\(^{\circledR}\). Previous work has shown that intrinsic fluorescence can cause a false determination of DNA content using Hoechst 33258 in human cartilage, and this artefact was removed by subtracting fluorescence caused by DNase digestion of samples\(^{6}\). However, DNase digestion can still leave residual fluorescence even when background fluorescence is subtracted (Table I) due to incomplete nuclease digestion or residual fluorescence, leading to an overestimate of intrinsic fluorescence and thus an underestimate of DNA content. Simple subtraction of intrinsic fluorescence has the advantage of a less time consuming procedure than DNase digestion, but may overestimate DNA content. At wavelengths used for PicoGreen\(^{\circledR}\), however, human cartilage has very low intrinsic fluorescence (Table II), making reliable DNA content determination possible through a single measurement with PicoGreen\(^{\circledR}\).

The contributors to the intrinsic fluorescence of cartilage digest remain to be determined. Intrinsic fluorescence of human cartilage was detected at wavelengths used for both Hoechst 33258 and PicoGreen\(^{\circledR}\) DNA assays. Three-dimensional scans over a range of excitation and emission wavelengths revealed higher intrinsic fluorescence of old adult than young adult cartilage digests. Three-dimensional fluorescence scans of articular and sepal cartilage digest samples show intrinsic peaks at wavelengths similar to those seen in purified articular cartilage\(^{5}\). In particular, the EX 330/EM 390 nm peak is consistent with the published excitation and emission maximum for pentosidine\(^{6,8}\), while the EX 280/EM 350 nm peak is consistent with the published excitation and emission maximum for pyridoxal phosphate\(^{6,8}\). An increase in fluorescence at EX 330/EM 390 nm in old samples over adult samples is consistent with fluorescence obtained from purified pentosidine from human cartilage\(^{6,8,9}\), although sepal cartilage exhibited a smaller increase than articular cartilage. At wavelengths relevant to DNA measurements with Hoechst 33258, old cartilage exhibited greater intrinsic fluorescence than young cartilage, as shown by the increased fluorescence in old cartilage due to trail-off from a higher pentosidine peak [Fig. 1(B), (C)]. The EX 280/EM 350 nm peak remained relatively constant throughout aging, consistent with previous studies showing constant pyridinoline content with aging following the completion of skeletal development\(^{6,8}\). To determine conclusively whether pyridinoline and pentosidine contribute primarily to the intrinsic fluorescence of cartilage digests, it would be necessary to purify these molecules and compare the extent of fluorescence and spectra of the fractions obtained during purification. It would be of interest to compare more generally the variation in intrinsic cartilage fluorescence within an individual donor (or patient) between various cartilage tissues at different sites to distinguish between site-specific and donor-associated variability. However, it was not feasible for us in the present study to obtain both nasal septal and articular cartilage from the same donor.

Determination of cellularity in human cartilage is valuable for quantitative studies with cartilage explants. Values obtained in this study of DNA content per wet weight are consistent with previous results for articular cartilage\(^{2,3,21,22}\). While the apparent decrease in cellularity with age in articular cartilage may be affected by the degree of tissue degeneration and swelling after tissue isolation\(^{23}\), the similar change of DNA content with age in septal cartilage is consistent with a process associated with aging. DNA assays using both Hoechst 33258 and PicoGreen\(^{\circledR}\) can be very useful in determining cellular content in various systems, such as metabolic experiments and determination of native cartilage composition. Both assays rely on proteinase K digests of tissue, which provides simple, one-step solubilization of tissue components. These digests can be used for a variety of biochemical quantification procedures, such as glycosaminoglycan\(^{24}\) and collagen\(^{25}\) content. In particular, the PicoGreen\(^{\circledR}\) assay allows for quick data collection in a 96-well fluorometric plate reader with high sensitivity for small sample size and minimal effects of tissue intrinsic fluorescence.

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