Near Infrared Spectroscopy for Rapid Determination of Mankin Score Components: A Potential Tool for Quantitative Characterization of Articular Cartilage at Surgery

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Purpose: The purpose of this study was to demonstrate the potential of near infrared (NIR) spectroscopy for characterizing the health and degenerative state of articular cartilage based on the components of the Mankin score. Methods: Three models of osteoarthritic degeneration induced in laboratory rats by anterior cruciate ligament (ACL) transection, meniscectomy (MSX), and intra-articular injection of moniodoacetate (1 mg) (MIA) were used in this study. Degeneration was induced in the right knee joint; each model group consisted of 12 rats (N = 36). After 8 weeks, the animals were euthanized and knee joints were collected. A custom-made diffuse reflectance NIR probe of 5-mm diameter was placed on the tibial and femoral surfaces, and spectral data were acquired from each specimen in the wave number range of 4,000 to 12,500 cm⁻¹. After spectral data acquisition, the specimens were fixed and safranin O staining (SOS) was performed to assess disease severity based on the Mankin scoring system. Using multivariate statistical analysis, with spectral preprocessing and wavelength selection technique, the spectral data were then correlated to the structural integrity (SI), cellularity (CEL), and matrix staining (SOS) components of the Mankin score for all the samples tested. Results: ACL models showed mild cartilage degeneration, MSX models had moderate degeneration, and MIA models showed severe cartilage degenerative changes both morphologically and histologically. Our results reveal significant linear correlations between the NIR absorption spectra and SI (R² = 94.78%), CEL (R² = 88.03%), and SOS (R² = 96.39%) parameters of all samples in the models. In addition, clustering of the samples according to their level of degeneration, with respect to the Mankin components, was also observed. Conclusions: NIR spectroscopic probing of articular cartilage can potentially provide critical information about the health of articular cartilage matrix in early and advanced stages of osteoarthritis (OA). Clinical Relevance: This rapid nondestructive method can facilitate clinical appraisal of articular cartilage integrity during arthroscopic surgery.

At the histologic level, osteoarthritis (OA) severity is often assessed visually using the 14-point grading scale devised by Mankin et al.¹ This method is effective for overall matrix grading; however, it is commonly used as a total score to broadly determine cartilage health. We have recently shown the importance of the extra information obtained by retaining the individual scores of the matrix health.² This has been shown to identify types of cartilage degeneration associated with different surgically induced joint damage, thereby acknowledging the asymmetric nature of OA and related damages. Although histologic examination provides the accurate information and insight into specific manifestations of damage, it requires destructive excision (biopsy) of the diseased tissue for histologic evaluation. Furthermore, histologic protocols generally take days or weeks and are therefore not suitable for surgical applications, in which time, cost, and extent of tissue removal are critical parameters. Initial cartilage evaluation is currently achieved through techniques such as radiologic (radiography) examination and magnetic resonance imaging. However, cartilage is not visible in radiographic images, and...
the poor resolution, availability, and cost of magnetic resonance imaging limits its use. In addition, they cannot be adapted for use in real time during surgery. This dearth of sensitive and viable options for improved diagnostics for the various degrees of OA cartilage changes has led to extensive research into methods that can be applied intra-articularly and in real time for cartilage evaluation at time of surgery. Promising methods include optical techniques such as fiberoptic Raman spectroscopy,\textsuperscript{3} arthroscopic optical coherence tomography probing,\textsuperscript{4} and near infrared (NIR) spectroscopy. The current study is an extension of our recent research\textsuperscript{5} in which we revealed the capacity of NIR to accurately characterize the extent of cartilage degeneration in artificially induced OA models in laboratory rat specimens by correlating NIR spectral data with total Mankin score. The purpose of this study was to demonstrate the potential of NIR spectroscopy for characterizing the health and degenerative states of articular cartilage by correlating NIR data with components of the Mankin score. Hence, we hypothesized that there is a relationship between the NIR absorption spectrum and component histologic scores that constitute the Mankin scoring system and that such a relationship could facilitate cartilage assessment at surgery.

**Methods**

**Animals**

Animal ethics approval was obtained from the relevant authorities before commencement of this project. Male Wistar Kyoto rats (11 to 12 weeks old) were purchased from the Medical Engineering Research Facility (MERF) (Brisbane, Australia). Each animal weighed about 300 to 350 g. Rats were housed under conditions that included a controlled light cycle (light/dark: 12 hours each) and a controlled temperature (23°C ± 1°C). They were allowed to habituate themselves to the housing facilities for at least 7 days before surgery.

**Rat OA Models**

Three types of OA models were used in this study; 2 were surgically induced\textsuperscript{6,7} and the third was chemically induced. The surgical methods included (1) removing the medial compartment meniscus disk (MSX) and (2) transecting the anterior cruciate ligament (ACL). The chemically induced method involved a single intra-articular injection of monooiodoacetate (MIA).\textsuperscript{8} Briefly, for the MSX model, after giving anesthesia with Zoetil (tiletamine 15 mg/kg, zolazepam 15 mg/kg; Virbac Philippines, Taguig City, Philippines) and xylazine 10 mg/kg, the medial collateral ligament was transected just below its attachment to the meniscus so that when the joint space opened, the meniscus was reflected toward the femur. The meniscus was cut at its narrowest point without damaging the tibial surface, resulting in complete transection of the medial meniscus. The surgical wounds were closed by suturing in 2 layers. A sham group underwent the same surgical procedure on the left knee but without excision of the ligament or meniscus manipulation. For the ACL model, the right knee was exposed through a medial parapatellar approach. The patella was dislocated laterally and the knee was placed in full flexion followed by ACL transection with microscissors. The joint capsule and subcutaneous layer were sutured separately, and the skin was closed with No. 3-0 Vicryl suture (Ethicon, Somerville, NJ). A sham group underwent the same surgical procedure with the omission of transection of the ACL. After the surgery, both the MSX and ACL animals received pain killer (buprenorphine 0.05 mg/kg) and antibiotic (gentamicin 5 mg/kg). For the MIA model, the rats were anesthetized and MIA was injected (1 mg in 50 μL volume in 0.9% saline) into the right joint cavity through the patellar ligament; control animals were injected with 0.9% saline only. A total of 36 rats were tested, with 12 rats in each group; no animal was excluded during the experiments.

**Sample Preparation and NIR Spectroscopy Data Acquisition**

Whole knee tibial joints were removed by dissection at 8 weeks after surgery, and NIR spectral data were acquired from the joints as described in the following sections.

**NIR Spectroscopy: Instrumentation and Data Acquisition.** Diffuse reflectance NIR spectroscopy was performed using a Bruker MPA FT-NIR (Fourier Transform NIR) spectroscope (Bruker Optics, Billerica, MA), with a detector spanning the full NIR spectral range. The spectroscopy was equipped with a custom-made fiberoptic probe of 5-mm outer diameter and 2-mm-diameter window. It consisted of a centrally placed 60-μm fiber for transmitting the NIR light, and 6 peripherally positioned 600-μm fibers for collecting the diffusely reflected light from the tissue. The spectroscopy was connected to a computer running OPUS software, version 6.5 (Bruker Optics, Billerica, MA) for equipment triggering and spectral data acquisition.

Before sample scanning, a reference spectrum was taken from a 99% Spectralon reflectance standard—SRS-99 (Labsphere, North Sutton, NH) for converting raw data into reflectance spectra. Spectral data (Fig 1) were obtained over the full NIR wavelength range at 16 cm\textsuperscript{-1} resolution, with each spectrum consisting of 64 co-added scans. This scanning condition was established as adequate for NIR probing of cartilage from preliminary experiments. A single scan (which is the average of 64 individual scans) was taken of each joint. It was also ensured that any further analyses were conducted on tissue extracted from the same region as that exposed to
NIR spectroscopy. Precautions to avoid experimental errors from probe vibration and offset were observed to ensure the accuracy and repeatability of acquired spectral data. Each spectrum consisted of 1,102 data points.

Morphologic and Histologic Characterization of OA Samples: Mankin Score. Subsequent to NIR scanning, the samples were fixed in 4% paraformaldehyde and decalcified in 10% ethylenediaminetetraacetic acid over a period of several weeks. After dehydration and paraffin embedding, serial 5-μm sagittal sections were cut from the lateral and medial compartments of the joint. Two sections obtained at 100-μm intervals from the non-weight-bearing region and weight-bearing region of each knee joint were stained with safranin O fast green. For safranin O fast green staining, 5-μm paraffin-embedded sections of tibia from the rat samples were counterstained with hematoxylin before being stained with 0.02% aqueous fast green for 4 minutes (followed by 3 dips in 1% acetic acid) and then 0.1% safranin O for 6 minutes. The slides were then dehydrated and mounted with crystal mount medium. OA severity in the tibial plateau was evaluated according to the modified Mankin histologic grading system, which assesses structure (0 to 6 points), cellularity (0 to 3 points), matrix staining (0 to 4 points), and tidemark integrity (0 to 1 point), up to a maximum score of 14 points. The scores for each histologic section are based on the most severe changes observed in multiple sections from each specimen. The final scores for each section were calculated as the averaged score from 3 independent assessors. A total of 12 rats were assessed in each group. Only the structural integrity (SI), cellularity (CEL), and matrix staining (SOS) components of the Mankin score were considered for correlation with NIR spectral data in this study.

Statistical Evaluation and Overview of Multivariate Approaches

Statistical analyses for the Mankin parameters were performed on GraphPad Prism statistics software, version 5.0 (GraphPad, La Jolla, CA). The data were expressed as mean ± quartiles (upper and lower quartiles) and were compared using one-way analysis of variance; P < .05 was considered to be statistically significant. The normal distribution assumption was tested using D’Agostino and Pearson omnibus normality test and passed for all groups before analysis. Because of the relatively small sample size (N = 36; 12 samples/group), power analysis (using G*Power statistical power analysis software) was used to compute sample size effect for all parameters, testing whether or not the quantity used is sufficient for analysis of variance. An average effect size $f = 0.9819$ was obtained, which is adequate relative to the effect size convention proposed by Cohen.

To classify the Mankin score parameters of the different OA models based on their NIR spectral data, a method is required to reduce the large amount of data (high dimensionality) that constitutes the total NIR spectra of all the samples into fewer variables (low dimensionality). We used principal component analysis (PCA) as a form of multidimensional scaling methodology. It is a linear transformation of the original variables into a lower dimensional space that retains a maximal amount of information about the variable. For example, we could look at how the spectrum changes with the different OA models. The principal components (PCs) are combinations of the original variables after linear transformation. The first PC reflects the greatest variance in the original variables (spectral data), followed by the second, third, and so on. Here, we consider only the first 2 PCs. The lower
dimensional data are represented by the score plots of these PCs. These scores were then classified using discriminant analysis (DA). In general, DA establishes relations between members in a data set or group\textsuperscript{15}; hence it was used to distinguish/discriminate between our experimental groups.

To investigate the capacity of NIR spectroscopy to predict the individual components of the Mankin score, a single y-variable partial least squares algorithm (PLS)\textsuperscript{16} was adopted. Regression (calibration) models were developed and validated for the 3 Mankin components. Specific regions of the spectrum were considered and separately correlated (Table 1). The leave-one-out cross-validation method was used to determine the optimal number of PLS factors and estimate the performance of the regression models. The left-out sample was predicted with the model and the procedure was repeated, with each sample being left out of the calibration set. Finally, the correlation coefficients between the predicted and measured values were calculated during calibration and validation. This validation method was adopted because it is effective in analyzing small sample sizes. Optimal model selection was based on the highest $R^2$ and lowest root mean square error of cross-validation.

The multivariate techniques used here are best suited to modeling linear phenomena and hence may not adequately model the raw spectral data because of the possibilities of multicollinearity between spectral data points. As a result, preprocessing algorithms including multiplicative scatter correction, straight line subtraction, and derivative pretreatment were used to correct nonlinearity such as that resulting from light scattering variations in reflectance spectroscopy.\textsuperscript{17} PLS analyses were performed with and without preprocessing. PCA and PLS spectral analyses were performed using OPUS Quant2 software (Bruker Optics, Billerica, MA), whereas DA analysis was performed using available functions in the statistics toolbox in MATLAB (Mathworks, Natick, MA).

### Results

#### Analysis of Animal Model Degeneration

In this article, it should be realized that the score for the sham is zero, leading to a zero ranking for control samples that can be considered to be in intact normal condition. Consequently, the following analyses and evaluation are relative to the normal zero-rank condition. The Mankin component scores for the 3 different OA models are presented in Table 1. ACL models show mild cartilage degeneration (mean Mankin score = 5) exhibited by surface irregularities and clefts to the transitional zone, normal cellular behavior through diffuse hypercellularity, and slight to moderate staining loss. MSX models present moderate cartilage damage (mean Mankin score = 8.56), including clefts to the radial and calcified zones—cellular behavior similar to that of the ACL models—however, with a higher prevalence of diffuse hypercellularity, and moderate through severe staining loss. MIA models display more severe cartilage degenerative changes (mean Mankin score = 13.17) both morphologically and histologically, including complete structural disorganization, cell cloning and hypocellularity, and complete absence of staining for proteoglycans (Fig 2).

#### Classification of Mankin Score Components

The effect of OA degeneration on the NIR spectra of each group, relative to the Mankin components, can be observed from the PCA plot presented in Fig 3. For SOS, the samples group according to their level of staining intensity along the second PC, whereas samples within a group are distributed along the first PC (Fig 3A). The

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**Table 1. Components of the Mankin Score for All Rat Samples Tested**

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<thead>
<tr>
<th>Parameter</th>
<th>Rat 1</th>
<th>Rat 2</th>
<th>Rat 3</th>
<th>Rat 4</th>
<th>Rat 5</th>
<th>Rat 6</th>
<th>Rat 7</th>
<th>Rat 8</th>
<th>Rat 9</th>
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samples can also be observed to group into 3 main classes along the second PC axis: “class 1” consists of the ACL samples with relatively high staining intensity, “class 2” consists of the MSX samples with medium staining intensity, and “class 3” encompasses the MIA-treated models with practically no staining. DA applied to these PCA scores shows the region in space where the scores for each OA group would lie statistically (Fig 3A). High discrimination, with no overlap between models, can be observed here.

Classification based on SI (Fig 3B) shows that the samples group according to their structural viability along the first PC, whereas samples within a group are distributed along the second PC. DA results show slight overlap between the ACL and MSX groups (8.3%) and the ACL and MIA groups (16.7%). Nevertheless, relatively high discrimination can be observed with this component. Classification based on CEL is presented in Fig 3C. It shows that the CEL influences both the first and second PCs. DA also shows slight overlap between the ACL and MIA groups (16.7%) and between the MIA and MSX groups (8.3%).

NIR Spectral Preprocessing and Single Y-Variable Partial Least Squares Algorithm Calibration and Validation

After spectral data preprocessing, correlations were performed between the NIR spectral data and Mankin parameters of all the samples tested (Table 2). SI was optimized using straight line subtraction, whereas first and second derivatives were optimal for SOS and CEL, respectively. The models developed using the NIR spectral regions A, B, and D (Table 2) present significantly high correlations and low errors (Figs 4-6) with the Mankin components investigated in this study. Improvement in the correlation coefficient and error of the calibration models, and their validation, was also observed with the application of preprocessing (Table 2). On the contrary, spectral data in region C, which covers the section characterized by the OH peak, have weaker relationships with all the parameters. The calibration and validation plot showing the correlation between NIR spectral data and SOS after first derivative preprocessing in region A is presented in Fig 4. After straight line subtraction preprocessing of the data in region D, Fig 5 presents the calibration and validation plots of the NIR spectral data and SI correlation. Slightly lower R² values were obtained for the NIR absorption spectra and CEL correlation, as presented in Fig 6.

Discussion

In this study, significant correlations between NIR absorption spectra and components of the Mankin score were observed (Figs 3-6). These findings are consistent with our previous results in which we showed the potential of NIR spectroscopy as a tool for determining the total Mankin score of articular cartilage during degeneration. Relative to earlier studies in the literature in which NIR spectroscopy was applied for evaluating cartilage defects in sheep and human patients, the results of this study constitute a...
significant improvement in the adaptation of this method for assessing cartilage integrity. The improvement is arguably caused in part by the multivariate analytic approach to data analysis adopted in this study, as opposed to the univariate approach applied in the former studies.

The ACL model is indicative of mild early-stage osteoarthritic degeneration in the joint and presents scores that are higher than seen in the shams (Table 1), despite the relatively smooth articular surface of the samples in this group. Further and dramatic increase in the Mankin components score is also evident from the histologic sections of the MSX and MIA samples (Fig 2A), which represent moderate and severe osteoarthritis degeneration, respectively (Table 1). These results are consistent with our previous study5 because the differences can be observed from the overall Mankin score of the sample. Nevertheless, the importance of monitoring variations in the components that make up the overall Mankin score for each sample was pointed out by Moody et al.,² because samples with different Mankin component scores can yield the same overall score. This is significant because degenerative changes in OA are complex and nonlinear, hence knowledge of these individual scores is important for identifying the degenerating components of the matrix, facilitating patient-specific treatment, and indicating whether or not total replacement, partial replacement, or regenerative medicine (localized cell-based treatment) is adopted, with consequence for cost and rehabilitation of patients.

NIR absorption characteristics of articular cartilage, like other biological materials, arise predominantly from C-H, N-H, O-H, and S-H bonds [8, 10] and also reflect both microscopic and macroscopic properties of the tissue. Therefore, the spectral data of cartilage matrix embed latent information on its physical, structural, and morphologic characteristics. Furthermore, the penetrating property of NIR spectroscopy into biological tissues,²¹ beyond the thickness of articular cartilage,⁹,²² makes it suitable for assessing the tissue’s material integrity. In addition, its capacity to monitor key chemical, physical, and morphologic properties of organic materials²³ presents the potential to facilitate detection and quantification of changes in the tissue based on the Mankin components.

Spectral data in region D, in which SI is optimized, is characterized by first overtone bond vibrations caused
by CH\textsubscript{n} and SH absorptions, which are arguably representative of the collagen network of articular cartilage. Light in the wavelength encompassed by region B, characterized by second overtone CH\textsubscript{n} vibrations and also representative of collagen network, penetrates deeper, and the data in this region equally presents a significantly high correlation with SI. This region can be used as a reliable substitute for region D in the prediction of SI. Third overtone vibrations caused by CH\textsubscript{n} and ROH, which characterizes cartilage proteoglycans, are responsible for absorptions in region A, where SOS is optimized. NIR light in this wave number region penetrates beyond the cartilage matrix into the subchondral bone, enabling full-thickness probing. NIR correlation with CEL in region B is characterized by a combination of second and third overtone vibrations mainly resulting from CH\textsubscript{n} and RNH\textsubscript{2} bond vibrations. This is likely caused by the multicomponent and complex structure of the matrix chondrocytes. Preprocessing improved both calibration and validation results in all parameters. Straight line subtraction that was used for optimizing SI eliminates offsets or different linear baselines, and derivative preprocessing used for pretreating SOS and CEL.

Table 2. Assessment Statistics of Articular Cartilage Mankin Component Parameters To NIR Correlation Based on Distinct Regions of the Spectrum With and Without Preprocessing

<table>
<thead>
<tr>
<th>Regions</th>
<th>Range (cm\textsuperscript{-1})</th>
<th>Structural Integrity</th>
<th>No Preprocessing</th>
<th>Staining Score (SOS)</th>
<th>No Preprocessing</th>
<th>Cellularity</th>
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<tr>
<td></td>
<td></td>
<td>Calibration R\textsuperscript{2} (%)</td>
<td>Validation R\textsuperscript{2} [R (%)</td>
<td>RMSEP</td>
<td>Calibration R\textsuperscript{2} (%)</td>
<td>Validation R\textsuperscript{2} [R (%)</td>
<td>RMSEP</td>
</tr>
<tr>
<td>A</td>
<td>12,436 to 9,967</td>
<td>96.39</td>
<td>93.43 [96.66]</td>
<td>0.235</td>
<td>93.22</td>
<td>87.22 [93.39]</td>
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</tr>
<tr>
<td>B</td>
<td>10,500 to 7,500</td>
<td>91.33</td>
<td>76.78 [87.62]</td>
<td>0.443</td>
<td>83.82</td>
<td>60.99 [78.1]</td>
<td>0.574</td>
</tr>
<tr>
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<td>79.04</td>
<td>45.96 [67.79]</td>
<td>0.675</td>
<td>86.57</td>
<td>48.21 [69.43]</td>
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</tr>
<tr>
<td>D</td>
<td>6,113 to 5,446</td>
<td>87.78</td>
<td>80.62 [89.79]</td>
<td>0.404</td>
<td>64.26</td>
<td>54.63 [73.91]</td>
<td>0.619</td>
</tr>
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NIR, near infrared; RMSEP, root mean square error of prediction; SOS, safranin O staining.

\textsuperscript{1}The region composing the water peak of articular cartilage.

Fig 4. Relationship between near infrared (NIR) spectral data and safranin O staining (SOS) component of the Mankin score of the osteoarthritic cartilage models. (A) Calibration and (B) validation.
corrects for baseline variations because derivatized spectra are generally free of baseline variations because the derivative of any function eliminates constant variables.  

DA classification and demarcation of the PCA scores (Fig 3) indicate the region of the plot space where the PCA scores of each group (of degeneration) are likely to be located, although more data are required to clearly distinguish between ACL and MIA groups for SI and CEL components of the Mankin score. These analyses reveal significant clustering of the samples according to their level of degeneration with respect to the individual Mankin components (Fig 3). The source of the overlap observed between the ACL and MIA groups in the PCA score plots with respect to SI and CEL parameters (Fig 3, B and C) is unclear because degeneration in ACL is mild, whereas that in MIA is severe. However, this may be caused by the inconsistent/nonlinear nature of cartilage matrix deterioration in OA. In other words, the mild degenerative state of ACL does not necessarily imply mild changes in its SI or CEL score.

The poor correlation observed in region C caused by severe absorption of the NIR light by OH bonds in water is experienced in this region. The higher error in this region is likely to negatively influence the accuracy of any model developed using the whole spectrum for analysis. Validation of the suitability of the regions chosen for analyses (Table 1, column 4) beyond the calibration $R^2$ values (Table 1, column 3) shows that the choices are adequate for the prediction of the Mankin parameters. In addition, the region-specific analyses adopted present a computationally faster option to using the whole spectrum because the amount of spectral data involved in the analyses for each component is reduced considerably. This translates to lower latency in converting data to useful information necessary for diagnosis and decision-making in surgery.

The multivariate analytic approach adopted in this study, as in our previous studies, presents more accurate and robust means of adapting NIR for prediction of the Mankin components. Using multivariate analysis with spectral preprocessing and wavelength selection based on distinct regions, correlations between NIR spectra and component-specific properties of articular cartilage have been shown by the distinction between different osteoarthritic models tested (Fig 3) and accurate estimation of their Mankin parameters.
(Figs 4-6). This is significant in contributing to knowledge that could potentially advance nondestructive and rapid evaluation of cartilage integrity with possible benefit to decision-making and treatment optimization for degenerated joints at surgery. Combined with robust multivariate statistical and spectral analyses and fiberoptic technologies, NIR spectroscopy has the potential for in vivo quantitative and histologic evaluation of cartilage condition in defective joints, allowing for monitoring of early to advanced progression of OA during arthroscopic surgery, and thus enabling selective and optimized treatment options and outcomes for each patient.

Limitations

This study is based on characterization and results obtained from rat articular cartilage, which is approximately 4 to 8 times thinner than human articular cartilage, thus posing a limitation. Consequently, the outcome would have to be extended by adapting the experimental protocol and statistical analyses to human cartilage samples to optimize the method for clinical applicability. It is worth noting, however, that this limitation does not affect or negate the hypothesis behind the study. Another possible limitation is the issue of probe vibration during data acquisition; however, this can easily be corrected (or compensated for) by software and hardware stabilization techniques.

Conclusions

NIR spectroscopic probing of articular cartilage can potentially provide critical information on the health of articular cartilage matrix in early and advanced stages of OA.

References


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