Comparison of Near-Infrared Spectroscopy with Needle Indentation and Histology for the Determination of Cartilage Thickness in the Large Animal Model Sheep

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Abstract

The suitability of near-infrared spectroscopy (NIRS) for non-destructive measurement of cartilage thickness was compared with the gold standard needle indentation. A combination of NIRS and biomechanical indentation (NIRS-B) was used to address the influence of varying loads routinely applied for hand-guided NIRS during real-life surgery on the accuracy of NIRS-based thickness prediction. NIRS-B was performed under three different loading conditions in 40 osteochondral cylinders from the load-bearing area of the medial and lateral femur condyle of 20 cadaver joints (left stifle joints; female Merino sheep; 6.1 ± 0.6 years, mean ± standard error of the mean). The cartilage thickness measured by needle indentation within the region analyzed by NIRS-B was then compared with cartilage thickness prediction based on NIRS spectral data using partial least squares regression. NIRS-B repeat measurements yielded highly reproducible values concerning force and absorbance. Separate or combined models for the three loading conditions (the latter simulating load-independent measurements) resulted in models with optimized quality parameters (e.g., coefficients of determination R^2 between 92.3 and 94.7) and a prediction accuracy of < 0.1 mm. NIRS appears well suited to determine cartilage thickness (possibly in a hand-guided, load-independent fashion), as shown by high reproducibility in repeat measurements and excellent reliability compared with tissue-destructive needle indentation. This may provide the basis for non-destructive, intra-operative assessment of cartilage status quo and fine-tuning of repair procedures.

Keywords

near-infrared spectroscopy (NIRS), needle indentation, histology, articular cartilage thickness, large animal model

Traumatic and osteoarthritis (OA) cartilage defects show limited regeneration capacity, aggravated by secondary loss of cartilage substance in the adjacent tissue. A number of surgical techniques are currently used to repair such cartilage defects.^{1,2} However, as they are mostly not curative, there is a continuing need to optimize the approaches, for example, by using large animal models to verify specific aspects of the interventions. Intra-operative mapping of cartilage thickness with high regional resolution by non-destructive near-infrared spectroscopy (NIRS) may be superior to previously applied, non-invasive, high-quality magnetic resonance imaging (MRI) and may thus allow fine-tuning of repair procedures for articular cartilage injury, for example, a more precise definition of the defect borders.

In animal models, the gold standard for the determination of cartilage thickness is the method of invasive, tissue-destructive needle indentation,³ and only a limited number

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of studies report on non-invasive procedures such as MRI,⁴ ultrasound,⁵ or NIRS.⁶⁻¹¹ Thus, there is a high need for non-destructive and convenient techniques to analyze the thickness of healthy or diseased articular cartilage in preclinical and human set-ups.

NIRS has been previously applied for the non-destructive assessment of normal or degenerated cartilage in porcine, ovine, equine, rat, and bovine animal models,⁶⁻⁹ in tissue-engineered cartilage constructs,¹² and for the quantification of cartilage alterations in degenerative human OA.^{10,13} In these studies, NIRS showed good correlations with clinical injury scores, biomechanical properties,¹³ histological grading,¹³ and biochemical features. NIRS may thus be suitable as a non-destructive, clinically applicable method for cartilage analysis, also considering that Afara *et al*¹¹ have already used NIRS for the determination of the articular cartilage thickness under static conditions.

The present study aimed at determining the accuracy of NIRS-based cartilage thickness prediction and the influence of varying loads and/or data preprocessing. A specific and novel approach of this study was to perform the measurements under non-static conditions. For this purpose, a combination of NIRS and biomechanical indentation (NIRS-B) was used, as previously established in our group.^{9,14} This combined approach was chosen to address the question of whether *ex vivo* non-destructive NIRS is suitable for the determination of cartilage thickness in a force-independent fashion, which takes into account the varying loads during hand-guided NIRS. This may then eventually provide the basis for intra-operative assessment of cartilage status quo in humans and experimental models. In the present study, NIRS-B was thus successfully applied for the first time to predict the cartilage thickness on the femur condyles in the large animal model sheep, and to validate it by comparison with the gold standard indentation and the values obtained by histology.

Materials and Methods

Sample Preparation Method

Twenty left cadaver stifle joints (adult female Merino sheep; age 6.1 ± 0.6 years (mean $\pm$ standard error of the mean [SEM]; body weight 78.5 ± 2.7 kg; frozen at -20°C) without cartilage alterations were used. The joint samples were derived from either unpublished studies of experimental chondral repair (permission from the governmental commission for animal protection, Free State of Thuringia, Germany; registration number 02-007/11) or published studies on the injection of calcium phosphate cement into bone defects of lumbar vertebral bodies.^{15,16}

After thawing (24 hours), the joints were opened, the femur was fixed in a clamp, and the cartilage kept humid with isotonic saline solution. One osteochondral cylinder

Figure 1. Sample preparation (left knee). One osteochondral cylinder each (diameter 10 mm; depth 20 mm) was extracted with a trepan drill from the main load-bearing area of the medial and lateral femur condyle (A) for subsequent near-infrared spectroscopy (NIRS) (B; x = center of fiber probe), needle indentation at 5 defined locations covering the region analyzed by NIRS (C), and conventional histology at 3 different levels representing the locations analyzed by NIRS and needle indentation (D).

each (diameter 10 mm; depth ~20 mm; original orientation on the condyle marked by an incision) was extracted with a trepan drill from the main load-bearing area of the medial and lateral femur condyle (**Fig. 1A-D**), visually examined for approximately equal cartilage thickness along its circumference, transferred to isotonic saline solution, and stored at 20°C until further analysis (within 30 minutes).

Combination of NIRS and Biomechanical Indentation (NIRS-B)

NIRS-B was performed on the cartilage surface of the osteochondral cylinders (**Fig. 1B**) in a climatized room (20°C) using a NIRS system (Arthrospec; Jena, Germany). As shown in Supplementary Figure 1, this system was synchronized with a linear translation stage (Thorlabs, Dachau, Germany) driving a 2-mm diameter fiber probe and a force sensor (ME Systeme, Hennigsdorf, Germany) to simultaneously measure indentation force (force range 0-10 N; sampling rate 32 Hz) and corresponding tissue NIRS absorbance spectra (range of 947-1649 nm; spectral resolution 3 nm; dynamic range 16 bit; sampling rate of 9 Hz).⁹

Thirty minutes after warm-up, the NIRS-B system was calibrated using a polytetrafluoroethylene (PTFE) optical reference standard. Subsequently, the osteochondral cylinders were placed in the sample holder and immersed in isotonic saline until the cartilage surface was covered.

NIR spectra of 40 samples were acquired for unloaded cartilage (interval 1, minimal indentation), loaded cartilage (interval 2, indentation 0.1 mm) and loaded cartilage after relaxation (interval 3, indentation 0.1 mm) as shown in **Figure 2**. These 3 load conditions were chosen to represent the forces occurring during hand-guided, intraoperative NIRS assessment when: (1) just lightly touching the

Figure 2. Movement of the fiber probe during the NIRS-B (near-infrared spectroscopy in combination with biomechanical indentation) measurements. The position of the fiber probe during the NIRS measurements is shown in relation to the surface of the osteochondral samples (position 0); the intervals 1 (minimal indentation), 2 (maximal indentation), and 3 (relaxation), in which NIRS spectra were recorded, are also indicated.

surface; (2) applying variable forces during locus screening (“navigation force”) or actual quantitative measurement (“procedure force”^{8,17}); or (3) assessing cartilage relaxation during more extended application of the fiber probe.

Each measurement sequence started by lowering the fiber probe onto the surface of the sample with a speed of 0.2 mm/s to reach the feed position $s = 0$ mm, as defined by $F_0 = 0.5$ N. After recording 10 NIR spectra in this minimal load position (interval 1; **Fig. 2**), the fiber probe was removed for a 30-second sample resting period and then advanced toward the sample surface with a speed of 0.2 mm/s until maximal indentation of 0.1 mm was reached. At this point, 10 NIR spectra and the indentation force $F_{\max}$ were recorded (interval 2; **Fig. 2**). The indentation depth was chosen in the present set-up, since the application of a specified strain as a percentage of the cartilage thickness was impossible in undamaged cartilage of unknown thickness (i.e., without prior needle indentation).

After a relaxation time of $t = 180$ seconds with no probe movement, further 10 NIR spectra and the indentation force F_{Relax} were recorded (interval 3; **Fig. 2**). The 10 spectra of each interval were averaged and the absorbance was calculated as $A = -\log_{10}(\text{Sample Signal} - \text{Dark Signal}/\text{Reference Signal} - \text{Dark Signal})$.

To determine the reproducibility of NIRS-B measurements, 3 repeat measurements were conducted on one sample, using a cartilage relaxation period of approximately 1 minute between individual measurements to reduce re-swelling artifacts.

The spectral data set acquired under all 3 loading conditions (complete range of wavelengths from 947 to 1649 nm; exemplary depiction of the loadings and factor weights of several principal components based on the factor analysis of one NIR spectra shown in Supplementary Fig. 2) and the reference thickness set determined by needle indentation or histology (location 5 on the respective condyle; **Fig. 1C** and **D**) of all 40 cartilage samples were then used to develop calibrations. In the case of the spectra from interval 1 (virtually unloaded cartilage), calibration was performed using the

original reference thickness set, in the case of spectra from intervals 2 and 3 (constant indentation depth 0.1 mm), 0.1 mm were subtracted from the reference thickness values.

The Bruker OPUS 7.0 software was then used for data preprocessing, partial least squares (PLS) regression, and leave-one-out cross validation. PLS was preferred in the present study because: (1) it is the standard method for NIRS analysis; (2) due to its widespread use, it favors comparability of the data with other publications in the field; and (3) it prepares the ground for future applications with a higher number of parameters such as varying tissue features (including unhealthy tissue), temperatures, feeds, and so on.

The resulting PLS models were evaluated and optimized on the basis of the parameters coefficient of determination (R^2 ; maximum of 100),¹⁸ root mean square error of cross validation (RMSECV),¹⁸ residual prediction deviation (RPD; with values <2.5 classified as poor and not recommended for screening), and number of principal components (rank; desirable rank between 3 and 9 to avoid underfitting or overfitting).¹⁸ The RPD is the ratio of the standard deviation to the standard error of prediction, and is a common quality parameter routinely used in NIR spectroscopy.

To investigate the influence of varying loads, either models based on separate spectra from intervals 1, 2, and 3 (models 1-3, respectively; 40 spectra each), or based on combined spectra from all 3 intervals (model 4; 120 spectra), were developed.

To investigate the influence of noise reduction, modeling was fully repeated with spectral data previously smoothed using a pixel weighted mean filter, resulting in 8 models each for the calibration algorithms using either needle indentation or histology as reference (**Tables 3** and **4**).

Needle Indentation

Cartilage thickness was validated at 5 different locations on each specimen (**Fig. 1C**), covering the region analyzed by NIRS.⁵ A hypodermic needle (25 G; 0.5×16 mm; Sterican; B. Braun Melsungen AG; Melsungen; Germany) was driven through the cartilage by a linear servomotor at a constant speed (0.3 mm/s) while continuously recording force and position. Cartilage thickness was determined as the distance from the initial surface contact to the tidemark, as defined by a characteristic change in the curve slope due to the different material properties of uncalcified and calcified cartilage (Supplementary Fig. 3).⁵

Histology

Immediately after performing NIRS-B and needle indentation, the osteochondral cylinders were fixed in 4% paraformaldehyde, decalcified in Osteodec solution for 3 weeks (Bio-Optica, Milan, Italy; weekly exchange of the solution),

dehydrated, and embedded in paraffin. The samples were then cut into 8- μ m sections and stained with hematoxylin and eosin.

The thickness of the articular cartilage (tidemark to surface) at the locations of all NIRS/needle indentation measurement points (see **Fig. 1B-D**) was measured in the respective location of one paraffin section each using an Axiophot microscope, a 4 $\times$ EC Plan-Neofluar objective (both Carl Zeiss, Jena, Germany) and the Axiovision 4.2. software (Carl Zeiss Vision GmbH, Jena, Germany).

Statistical Analyses

Results (means $\pm$ SEM) were statistically analyzed using the Wilcoxon signed rank test and SPSS 22.0 (IBM SPSS, Armonk, NY, USA). Correlations among parameters were assessed using the Spearman rank correlation test. Statistical significances were accepted at $P \leq 0.05$.

Results

This section presents representative full NIRS spectra and describes the data from the NIRS-B assessment and its reproducibility. It then shows the results from the gold standard determination of the cartilage thickness by needle indentation (or histology), and, finally, it reports on the development of models for the prediction of cartilage thickness by NIRS on the basis of values derived from either needle indentation or histology.

Full NIRS Spectrum Depiction

Full NIRS spectra show the characteristic wavelength pattern of three repeat measurements for one cartilage sample in interval 1 (minimal indentation; $F_0 = 0.5$ N) with maxima at $\lambda = 950$ nm (second overtone of O-H bonds), $\lambda = 1170$ nm (second overtone of C-H, C-H₂, and C-H₃ bonds), and $\lambda = 1450$ nm (first overtone of O-H bonds; **Fig. 3**; for the depiction of the spectra of either the same location under the influence of the different loads at the different intervals or in cartilage samples of varying thickness see Supplementary Figs. 4 and 5, respectively). Low variability of the data, exemplified for the low-noise wavelength of 950 nm (inset in **Fig. 3**), was observed throughout the NIRS absorbance spectrum. In subsequent **Figures 4B** and **5B**, the variability of the absorbance data is exemplarily depicted for the characteristic low-noise wavelength of 950 nm (see inset).

Reproducibility of NIRS-B Measurements

Repeated measurements of the cartilage in one sample (sheep 3) yielded highly reproducible values for force ($F_{\max}$, and F_{Relax} ; SEM maximum 4.4% of the mean; **Fig. 4A**; **Table 1**) and absorbance in intervals 1 to 3 at the positions

Figure 3. Near-infrared spectroscopy (NIRS) absorbance spectrum in interval 1 (minimal indentation; $F_0 = 0.5$ N). The complete NIRS absorbance spectrum of 3 repeat measurements for one cartilage sample in interval 1 displays the characteristic wavelength pattern of cartilage with maxima at $\lambda = 950$ nm, $\lambda = 1170$ nm, and $\lambda = 1450$ nm. In subsequent **Figures 4B** and **5B**, the variability of the absorbance data is exemplarily depicted for the characteristic low-noise wavelength of 950 nm (see inset).

minimal indentation ($s = 0$), maximal indentation, and relaxation, respectively (SEM maximum 0.4% of the mean; **Fig. 4B**; **Table 1**).

The unloaded tissue in interval 1 showed a numerically higher absorbance than the loaded tissue in intervals 2 and 3. The absorbance slightly increased between intervals 2 and 3, whereas the force decreased from $F_{\max}$ to F_{Relax} (**Fig. 4A** and **B**; **Table 1**).

NIRS-B assessment. Indentation force and complete NIRS spectra (947–1649 nm) of 40 samples were then measured in one defined location on the load-bearing area of the medial and lateral femur condyle (see **Fig. 1**) in intervals 1, 2, and 3 (**Fig. 2**). During cartilage surface compression in interval 2, the force significantly increased (2.19 ± 0.23 N) and then significantly decreased again (0.59 ± 0.06 N) toward the end of the relaxation period (interval 3; **Fig. 5A**; **Table 2**).

Interval 1 (minimal indentation) showed variable NIRS absorbance (exemplified for $\lambda = 950$ nm; mean of 0.3459 ± 0.0119 AU; **Table 2**). In contrast to the force pattern, the absorbance significantly decreased during the initial compression (interval 2), and then significantly increased until the end of the relaxation period (**Fig. 5B**; **Table 2**).

Needle indentation. Cartilage thickness showed regional heterogeneity for the 5 measurement points on the medial and lateral femur condyles ($n = 20$ each; **Fig. 6A** and **B**; for details see figure legends).

Figure 4. Accuracy of NIRS-B (near-infrared spectroscopy in combination with biomechanical indentation) repeat measurements. **(A)** Three-dimensional force-feed-time graph of triple repeat measurements in one sample; **(B)** change of absorbance at the wavelength of $\lambda = 950$ nm in triple repeat measurements in one sample; after numerically higher absorbance values in interval 1, the values slightly increased in the period between intervals 2 and 3, in parallel with a numerically decreased F_{Relax} in comparison with the respective F_{max} .

Figure 5. NIRS-B (near-infrared spectroscopy in combination with biomechanical indentation) measurements of all samples. **(A)** Three-dimensional force-feed-time graph of all samples ($n = 40$); **(B)** change of absorbance at the wavelength of $\lambda = 950$ nm in all samples; with variable values for the absorbance of the 40 individual samples, the force increased during compression of the cartilage surface in interval 2, and then decreased again at the end of the relaxation period (interval 3). In contrast, the absorbance significantly decreased during initial compression, and significantly increased until the end of the relaxation period.

Table 1. Reproducibility of NIRS-B: Three Repeat Measurements of Force and Absorbance on One Sample in Intervals 1, 2, and 3, Exemplified for $\lambda = 950$ nm.

Repeat	Interval 1, F_0 (N)	Interval 2, F_{max} (N)	Interval 3, F_{Relax} (N)	Interval 1, A_1 (AU) at 950 nm	Interval 2, A_2 (AU) at 950 nm	Interval 3, A_3 (AU) at 950 nm
1	0.50	3.05	0.75	0.4345	0.4186	0.4221
2	0.50	3.16	0.79	0.4327	0.4180	0.4198
3	0.50	3.36	0.87	0.4283	0.4147	0.4184
Mean		3.19	0.80	0.4319	0.4171	0.4201
SEM		0.09	0.04	0.0018	0.0012	0.0011
SEM/mean (%)		2.8	4.4	0.4	0.3	0.3

Table 2. NIRS-B Measurement: Force and Absorbance (Exemplified for $\lambda = 950$ nm) of 40 Cartilage Samples Measured in Intervals 1, 2 and 3.

Repeat	Interval 1, F_0 (N)	Interval 2, $F_{\max}$ (N)	Interval 3, F_{Relax} (N)	Interval 1, A_1 (AU) at 950 nm	Interval 2, A_2 (AU) at 950 nm	Interval 3, A_3 (AU) at 950 nm
Mean	0.5	2.19 [†]	0.59*	0.3459	0.3276 [†]	0.3331*
SEM		0.23	0.06	0.0119	0.0117	0.0119
(min; max)		(0.88; 6.68)	(0.24; 2.40)	(0.2169; 0.5448)	(0.2137; 0.5251)	(0.1976; 0.5308)
SEM/mean (%)		10.5	10.2	3.4	3.6	3.6

[†] $P \leq 0.05$ in comparison with F_0 or $A_{\text{Interval 1}}$.

* $P \leq 0.05$ in comparison with $F_{\max}$ or $A_{\text{Interval 2}}$.

Figure 6. Determination of cartilage thickness by needle indentation ($n = 40$). Cartilage thickness at the 5 measurement locations on the medial femur condyle (**A**) and the lateral femur condyle (**B**). Values shown are means $\pm$ standard error (SEM) of the mean for each location, as well as those for all locations). The symbols indicate $P \leq 0.05$ in comparison with: * measurement point 1 (lateral-distal), # measurement point 2 (medial-distal), + measurement point 3 (medial-proximal), & measurement point 4 (lateral-proximal), and § medial femur condyle.

The cartilage thickness of all 5 measurement points on each condyle was averaged for comparison with subsequent NIRS-B; the overall mean for the medial condyle (1.14 ± 0.07 mm) was significantly higher than that for the lateral condyle (0.78 ± 0.05 mm; **Fig. 6A and B**).

On the medial condyle, the values for measurement point 3 were significantly lower than those for measurement points 1, 2, 4, and 5 ($P \leq 0.05$); in addition, measurement points 2, 4, and 5 showed significantly lower values than measurement point 1 (A).

On the lateral condyle, the values for measurement point 4 were significantly lower than those for measurement points 2, 3, and 5 (B); in addition, measurement points 2, 3, and 5 showed significantly higher values than measurement point 1, and measurement point 5 (central) showed significantly lower values than measurement point 2 (B).

NIRS-B Thickness Prediction Based on Needle Indentation

For all 8 models, a prediction accuracy of < 0.1 mm and models with highly optimized quality parameters were

obtained (R^2 from 92.43 to 94.74; RMSECV < 0.1 ; optimized values for RPD between 3.8 and 4.4; acceptable number of principal components between 4 and 10). Performance of models with raw or smoothed data was comparable (i.e., noise did not cause substantial deterioration of the models; **Table 3**), indicating that non-modified data can be used for future analyses. Correlations between NIRS and needle indentation values for the different models were also highly significant (rho between 0.953 and 0.967; all P s = 0.000; **Fig. 7**).

Regarding differences among the thickness predictions for individual cartilage samples derived from the 3 models for the different loading conditions, and from model 4 based on all 3 conditions (total of $n = 6$ prediction values for each sample), the variability was very limited (SEM between 0.9% and 5.7% of the mean; data not shown).

Independently performed linear regression analysis for repeated measures confirmed a highly significant, positive correlation between needle indentation and NIRS-B in model 4 ($n = 120$; $P \leq 2.2 \times 10^{-16}$; $R^2 = 0.958$; Supplementary Figure 6B), confidence intervals close to the prediction accuracy of

Table 3. NIRS-B Thickness Prediction Based on Needle Indentation (Minus 0.1 mm Offset for Intervals 2 and 3; Measurement Point 5): Evaluation of ($n = 40$) Samples.^a

	Preprocessing	R^2	RMSECV	RPD	No. of Principal Components (Rank)	No. of Spectra (Data Source)
Model 1r	Raw	94.04	8.61	4.1	5	40 (A_1)
Model 1s	Smoothed	94.12	8.57	4.1	4	40 (A_1)
Model 2r	Raw	94.09	8.59	4.12	4	40 (A_2)
Model 2s	Smoothed	94.74	8.10	4.36	8	40 (A_2)
Model 3r	Raw	92.43	9.72	3.64	4	40 (A_3)
Model 3s	Smoothed	92.94	9.39	3.76	10	40 (A_3)
Model 4r	Raw	93.63	9.00	3.96	9	120 (A_1, A_2, A_3)
Model 4s	Smoothed	93.91	8.80	4.05	8	120 (A_1, A_2, A_3)

^aCoefficient of determination (R^2), root mean square error of cross validation (RMSECV), residual prediction deviation (RPD), and number of principal components (rank); calibrations were either based on the separate use of 40 spectra each from intervals 1, 2, or 3 (models 1, 2, and 3, respectively), or of 120 spectra from all intervals (model 4).

NIRS-B (approximately 0.17 mm; Supplementary Fig. 6A), and showed no significant indications for an offset of the values for the 2 methods from zero (Supplementary Fig. 6B), a significant interobserver variability (Supplementary Fig. 6B), or a deviation from a normal distribution (Supplementary Fig. 6C).

Histology

As observed by needle indentation, the histologically determined cartilage thickness for the 5 measurement points on the medial and lateral femur condyles ($n = 20$ each) showed regional heterogeneity (see legend to Fig. 8A and B).

When the cartilage thickness of all 5 measurement points on each condyle was averaged, the overall mean value for the medial condyle (0.91 ± 0.04 mm) was significantly higher than that for the lateral condyle (0.63 ± 0.03 mm; Fig. 8A and B).

The histology values resulted significantly lower than the needle indentation values for measurement points 1, 4, and 5 on the medial condyle and for measurement points 2, 3, and 5 on the lateral condyle (Fig. 8A and B). However, there was a weak, but significant correlation between the thickness values derived from histology and needle indentation on both condyles ($\rho = 0.544$ and 0.367 , respectively; Fig. 8C and D).

On the medial condyle, the values for measurement point 3 were significantly lower than those for measurement point 2 ($P \leq 0.05$); in addition, measurement point 4 showed significantly lower values than measurement points 1, 2, and 5, and measurement point 2 showed significantly higher values than measurement points 1 and 5 (A).

On the lateral condyle, the values for measurement points 3 and 4 were significantly lower than those for measurement point 1; in addition, the values for measurement points 4 and 5 were significantly higher than those for measurement point 3 (B).

NIRS-B Thickness Prediction Based on Histology

When using histology as a reference set, a high prediction accuracy (< 0.1 mm), good-quality parameters (R^2 from 86.44 to 90.84; RMSECV ≤ 0.1 ; acceptable number of principal components; Table 4), and significant correlations were obtained for models 1, 2, and 4 (ρ between 0.900 and 0.936; all P s = 0.000; Supplementary Fig. 7). Lack of improvement after data smoothing again indicated the principal suitability of non-modified data (Table 4). Instead, model 3 based on NIRS absorbance during relaxation showed deteriorated quality parameters (Supplementary Fig. 7).

Discussion

In the present study, aimed at determining the accuracy of NIRS-based thickness prediction under the influence of varying loads using a combination of NIRS and biomechanical indentation (NIRS-B), NIRS-B proved highly reproducible in measuring the absorbance of normal articular cartilage in different states of compression or relaxation. Following development of optimized data analysis models in comparison with the articular cartilage thickness determined by needle indentation (and, to a lesser degree, also in comparison with histology), NIRS-B was capable of predicting the articular cartilage thickness with a high accuracy (< 0.1 mm). Thus, non-destructive, force-independent NIRS-B appears highly suitable for the assessment of cartilage thickness in experimental or human cartilage pathophysiology.

NIRS-B Reproducibility

Repeated measurements of the cartilage yielded highly reproducible values with very limited variation for force and absorbance levels throughout the NIRS-B assessment protocol. This result shows that NIRS-B yields stable and reliable experimental results, and further confirms the

Figure 7. Correlations between the values obtained by near-infrared spectroscopy (NIRS) and needle indentation. Visual depiction of the correlations between the values obtained by NIRS and needle indentation for the different prediction models (including ρ , P , and n for each correlation; compare with Table 3).

methodological validity of combined NIRS analysis and biomechanical indentation, as previously suggested by our group and others.^{9,14,19}

Time Course of Force and Absorbance

When compressing the cartilage on the femur condyle during indentation with the NIRS fiber probe, the force increased to a maximum ($F_{\max}$; 2.19 N) and subsequently decreased again toward the end of the relaxation period (F_{relax} ; 0.59 N). This well-known cartilage stress-relaxation phenomenon,²⁰

previously observed using force measurements,⁹ NIRS,⁹ and ultrasound,^{21,22} is also reflected in an initial decrease of the absorbance during maximal indentation of $s = 0.1$ mm and a subsequent increase between intervals 2 and 3. The initial decrease of NIRS absorption during maximal indentation likely results from the extrusion of water (the main absorber of NIR radiation) from the cartilage extracellular matrix.^{9,23} However, the increasing NIRS absorption during subsequent cartilage relaxation is partially unexpected, since the cartilage should continue to lose water under continuous compression, as confirmed by a progressive decrease of the compression forces.

However, a compressed extracellular matrix and/or a partial loss of chemically bound, fixed water from collagens or glycosaminoglycan side chains (GAGs) of cartilage proteoglycans (see Padalkar *et al.*²³ and references therein) may also underlie the increasing NIRS absorption in this phase. Finally, such “water-reduced” GAGs may partially revert the extrusion of water from the cartilage and cause an osmotically driven influx of water.^{9,23}

The existence of free and bound water in the cartilage matrix, as well as their differential detection by bands of different wavelengths in static NIRS, has been demonstrated previously.²³⁻²⁵ It remains to be shown whether such differential detection of free and bound water is also applicable to the dynamic combined NIRS-B protocol performed in the present study, and whether further biochemical analysis can identify the specific molecules undergoing an alteration of their NIRS signal under these conditions.

NIRS-B Thickness Prediction

In the present study, suitable calibration algorithms were developed for the comparison of the dynamic NIRS-B and needle indentation data using PLS regression. This resulted in robust models with a high accuracy for the prediction of the articular cartilage thickness (< 0.1 mm).

These results confirm previous high-accuracy findings of Afara *et al.*^{6,11} with static NIRS. However, these authors mounted fiber probe and specimen in a rig to keep both components stable and avoid vibrations during NIRS, a setup difficult to achieve during manual application in arthroscopic surgery. In the latter case, a standard hook probe is pressed on the tissue surface with forces between 0.5 and 4 N,^{8,17} a range well covered in the present study. Thus, the current study demonstrates for the first time that, at an experimental level, prediction of cartilage thickness can not only be performed by static NIRS but also by dynamic combined NIRS-B.

The models based on separate data from intervals 1 to 3 (constant load, 40 spectra each) and the model 4 based on all 3 intervals (varying load, 120 spectra) all showed high-quality parameters. Notably, model 4 combining data with largely varying forces (range 0.5–6.68 N) also showed high

Figure 8. Comparison between the cartilage thickness values obtained by histology and needle indentation ($n = 40$ for all measurement points). Cartilage thickness at the various locations on the medial femur condyle (**A**) and the lateral femur condyle (**B**; data shown as means $\pm$ standard error (SEM) of the mean for each location, as well as those for all locations); Spearman rank correlations between the values obtained by needle indentation and histology on the medial (**C**) and lateral condyle (**D**); $P \leq 0.05$ in comparison with: * measurement point 1; # measurement point 2; + measurement point 3; & measurement point 4; \$ medial femur condyle; and \$ for the comparison between needle indentation and histology. For reasons of clarity, significant differences among the needle indentation values for the individual measurement points are not shown in this figure, but only in **Figure 6** (see above).

performance, indicating that exact prediction of cartilage thickness by NIRS-B may also be feasible under intraoperative, hand-guided conditions. This may more closely reflect real-life surgery, during which different surgeons apply a fiber probe with variable forces during locus screening (“navigation force”) or actual quantitative measurement (“procedure force”^{8,17}). Also, model performance was largely unchanged by data smoothing, indicating that more easily available raw data without any loss of information can be used for future analyses.

One particular example for NIRS-B thickness prediction based on needle indentation is now depicted in Supplementary Figure 2, in which 5 individual factors with their respective factor weights contribute to the prediction

model (model 2s, smoothed; coefficient of determination: 48.32%, 82.42%, 87.62%, 89.88%, 91.64% for 1, 2, 3, 4, and 5 factors, respectively). Since this contribution is mainly situated in the wavelength range around 1170 nm (second overtone of C-H, C-H₂, and C-H₃ bonds), it can be speculated that NIRS signal changes reflect alterations of the main organic components of cartilage, that is, large aggregating proteoglycans and collagens. In this case, changes in water content appear to be less important, since the 2 main water peaks at 950 and 1450 nm are not included. Thus, NIRS-B thickness prediction may be based on differences in matrix content and/or distribution between the NIRS probe on the cartilage surface and the NIR-reflecting border to the subchondral lamella in cartilage of varying

Table 4. NIRS-B Thickness Prediction Based on Histology (Minus 0.1 mm Offset for Intervals 2 and 3; Measurement Point 5): Evaluation of ($n = 40$) Samples.^a

	Preprocessing	R^2	RMSECV (%)	RPD	No. of Principal Components (Rank)	No. of Spectra (Data Source)
Model 1r	Raw	90.37	8.57	3.23	7	40 (A_{11})
Model 1s	Smoothed	90.57	8.48	3.3	9	40 (A_{11})
Model 2r	Raw	86.76	10.00	2.77	8	40 (A_{12})
Model 2s	Smoothed	86.44	10.20	2.72	6	40 (A_{12})
Model 3r	Raw	89.39	8.99	3.07	9	40 (A_{13})
Model 3s	Smoothed	89.76	8.83	3.13	10	40 (A_{13})
Model 4r	Raw	91.36	8.23	3.4	8	120 (A_1, A_2, A_3)
Model 4s	Smoothed	90.84	8.47	3.31	9	120 (A_1, A_2, A_3)

^aCoefficient of determination (R^2), root mean square error of cross validation (RMSECV), residual prediction deviation (RPD), and number of principal components (rank); calibrations were either based on the separate use of 40 spectra each from intervals 1, 2, or 3 (models 1, 2, and 3, respectively), or of 120 spectra from all intervals (model 4).

thickness. However, it is has to be clearly pointed out that (1) NIR spectra reflect numerous, very broad, and strongly superimposing overtone and combination bands; (2) cartilage contains a multitude of organic and inorganic components, including up to 70% of water; (3) the NIR bands in the cartilage cannot be directly interpreted, but must be analyzed using complex statistical approaches. The conversion of such speculations to scientific insight into the molecular basis of the NIRS signal changes will thus require much more detailed NIR spectra of aqueous solutions with defined concentrations of one or few organic components and their respective NIRS profiles.²⁶ As in the case of the needle indentation-based models, the histology-based NIRS model 4 showed high performance (RPD >3.6), further underlining that dynamic NIRS is in principle able to predict cartilage thickness under intra-operative, hand-guided conditions with varying loads.

Needle Indentation

Marked regional heterogeneity of the cartilage thickness was observed on both medial and lateral femur condyles, with individual values for the medial condyle from 0.51 mm (medial-proximal) to 1.89 mm (lateral-distal; overall mean 1.14 ± 0.07 mm) and values for the lateral condyle from 0.35 mm to 1.29 mm (both lateral-proximal; overall mean 0.78 ± 0.05 mm).

To our knowledge, measurements of cartilage thickness on the medial and lateral femur condyle in sheep by needle indentation have not been published before; however, the present values were well in the range of those obtained by conventional formalin/paraffin histology.^{27,28} This confirms that, apart from its invasiveness, invasive needle indentation is a gold standard for the localized determination of cartilage thickness, giving more accurate measures than A-mode ultrasound or manual/stereomicroscopic measurement.^{5,11}

Histology

To our knowledge, a direct comparison between histology and needle indentation has also not been reported. Other studies have either determined the cartilage thickness by 1 of these 2 methods (sometimes in comparison to third acoustical or optical procedures),^{5,14,29,30} or applied other modalities such as high-resolution MRI or laser displacement sensing.^{31,32} The discrepancies between needle indentation and histology found in the present study are likely due to alcoholic dehydration for histology. However, significant, positive correlations between needle indentation and histology or between NIRS-B and histology indicate that technically simple, inexpensive, and broadly applicable histology may still be valuable for the (comparative) estimation of cartilage thickness in experimental (large) animal models and humans. Alternatively, more sophisticated histological techniques only available in specialized laboratories, such as undecalcified plastic embedding or the cryostat cutting of undecalcified samples^{33,34} may provide information regarding the observed discrepancies.

Limitations

The present investigation was performed with normal sheep cartilage in order to first establish the system for unaltered, healthy cartilage. It has to be expected that the situation in unhealthy cartilage will be different and possibly more complex and will likely require new analyses and calibrations.^{10,13}

In addition, all data in the present study were obtained with osteochondral plugs extracted with a drill from the medial and lateral femur condyle, in order to allow a positioning of needle indenter and NIRS fiber probe as close as possible to an exactly perpendicular orientation. Future studies will have to address the question, whether this exact positioning of the sensors can be reproduced in vivo on intact condyles under the tightly restricted spatial access

during minimally invasive surgery.³⁵ In the current study, measurement point 5 of the needle indentation has been used as a reference for the calibration of the NIR spectra for cartilage thickness prediction, resulting in robust models with high quality parameters and a high accuracy for the prediction of the articular cartilage thickness. In order to consider not only the NIRS signal from the central cartilage area directly below the fiber probe but also a possible contribution from a wider conus extending to the measurement points 1 to 4 of the needle indentation, the average of the measurement points 1 to 5 may be used (data not shown). The fact that this approach also yielded high-quality parameters (R^2 between 88.66 and 93.53) and accuracy (RMSECV between 7.93 and 10.5%), can be interpreted as a sign of the high stability and reproducibility of the present calibration algorithms.

In the present experimental setup, the application of a specified strain as a percentage of the cartilage thickness (as performed in Maenz *et al.*^{36,37}) was impossible, since NIRS was executed in undamaged cartilage with unknown thickness (i.e., without prior needle indentation). The choice of a defined, fixed 0.1 mm indentation depth, aimed at generating highly reproducible and highly accurate experimental conditions for all samples and measurements, completely eliminated any influence of creep, since a time-dependent motion of the 2 mm diameter fiber probe into the cartilage under constant load was impossible. This assumption is further supported by the fact that the force showed a rise to a peak, followed by a slow stress-relaxation process until a substantially lower force value above 0 N was reached.

In contrast, it may be very difficult to define an appropriate force suitable to generate such reproducible experimental conditions, because of the variable viscosity of the tissue with time and among different samples (due to, e.g., creep and relaxation). Furthermore, single measurements at full cartilage relaxation may require time periods in the range of hours, with the serious risk of substantial cartilage alterations (e.g., progressive water loss or tissue break-down). Finally, an unequivocal determination of the time point of full cartilage relaxation may further complicate or extend the measurements. In our opinion, the choice of the indentation depth therefore does not question, but rather strengthens the validity of the present results.

The range of the measurement error in the present study extends from approximately 8% to 10% and thus compares well with the relative measurement errors reported in previous studies comparing either needle probe and ultrasonic measurements (range between 8.7% and 20.5%, depending on cartilage thickness and indenter radius⁵) or needle probe and static NIRS measurements (range between 5.5% and 7.1%¹¹). The accuracy of cartilage prediction in the present study is thus regarded as highly satisfactory for a non-destructive technique potentially applicable for hand-guided NIRS during real life surgery.

Conclusion

NIRS-B allows the prediction of articular cartilage thickness with a high accuracy (< 0.1 mm) and high-quality parameters after development of an optimized data analysis model in comparison with the gold standard needle indentation. Prediction of cartilage thickness can therefore not only be performed by static NIRS but also by dynamic combined NIRS-B, at least at an experimental level. Non-destructive, force-independent NIRS may thus also be suitable for intra-operative determination of the cartilage thickness in human disease or experimental models.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

Animal Welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

Ethical Approval

Ethical approval was not sought for the present study because this was an ex vivo study with pre-existing animal material (see also the paragraph ‘Sample Preparation Method’ in the Materials and Methods section).

Supplementary Material

Supplementary figures for this article are available online.

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