Nanostructure surveys on macroscopic specimens by small-angle scattering tensor tomography

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Many materials show distinct mechanical properties based on the macroscopic arrangement and orientation of their nanostructure. Their ordering can span a wide range of length scales. In biology, this principle of hierarchical ordering is often used in order to maximize functionality, such as strength and robustness of the material, while minimizing weight and cost. Methods for nanoscale imaging provide direct visual access to the ultrastructure, but the field of view is limited and does not easily allow a full correlative study of changes in the ultrastructure over a macroscopic sample. Other methods to probe ultrastructure ordering, such as small-angle scattering of X-rays or neutrons, can be applied to macroscopic samples. However, these scattering methods remain constrained to two-dimensional specimens¹⁻⁴ or to isotropically oriented ultrastructures⁵⁻⁷. This limits their use for studying nanostructures with more complex orientation patterns, abundant in nature and materials science. Here, we introduce an approach that combines small-angle scattering (SAS) with tensor tomography to probe nanoscale structures in three-dimensional macroscopic samples in a non-destructive way. We demonstrate experimentally the method by measuring the main orientation and degree of orientation of nanoscale mineralized collagen fibrils in a human trabecula with 25 micron spatial resolution. The computational effort can be relaxed by explicit constraints of known symmetries of the sample, such as cylindrical symmetry in the case of bone mineralized collagen fibrils (References). With the correct adjustment of these constraints, SAS tensor tomography is widely applicable to both biological and materials science specimens, and it opens new avenues for understanding and characterizing smart or bio-inspired materials. Moreover, due to the non-destructiveness of the method, it is amenable for *in situ* measurements and allows, for example, studying the

33 **role of the ultrastructure for the mechanical response of the biological tissue or**
34 **manufactured material under investigation.**

35 The arrangement of the nano- and microscale building blocks over extended regions is of
36 high importance for the functional properties of many materials. This is particularly the case
37 for many biological structures, where hierarchically ordered organization ensures the required
38 properties, *e.g.* the high mechanical stability of bone necessary for structural support of the
39 body, force transmission for locomotion and mechanical protection of vital organs^{8,9}. High-
40 resolution techniques exist which can directly image nanoscale structures^{10,11}. Besides the
41 importance of such information, the overview of how this structure is changing over
42 macroscopic length scales is pivotal to understanding the function of hierarchically organized
43 tissues and materials. In the case of human bone, the structural element of interest is the
44 mineralized collagen fibril, and their orientation is among the factors determining bone
45 strength in different structural levels¹²⁻¹⁴. Experimental techniques exist to probe structural
46 orientations by using light, X-rays and electrons, making use of direction-dependent
47 polarization¹⁵⁻¹⁷, small- or wide-angle scattering^{1,15} or diffraction^{18,19}, rather than directly
48 imaging nanostructures. These techniques can cover a large field of view of thin sections or
49 of the sample surface. However, studying 3D samples without the need of sectioning can be
50 of high benefit. Cutting can influence the structures at the surface, and for some samples
51 cutting is not feasible or applicable at all.

52 X-ray techniques are highly adept for probing the inside of macroscopic objects due to their
53 high penetration depth, even in dense materials such as mineralized bone tissue. X-ray
54 photons scatter where changes of electron density occur. The scattering angle, or momentum
55 transfer q , is inversely related to the characteristic sizes of the structure under investigation.
56 Thus, at high scattering angles atomic length scales can be resolved (wide-angle X-ray
57 scattering, WAXS, or X-ray diffraction, XRD), whereas at small angles scattering from
58 structures at the nanometre scale can be detected (small-angle X-ray scattering, SAXS).
59 Oriented ultrastructure results in an anisotropic scattering pattern, which can be captured with
60 a 2D detector, and the orientation can be analysed in each q -range separately³. Spatially
61 resolved SAXS, where a specimen is scanned through an X-ray pencil beam is a valuable tool
62 for mapping ultrastructural features in macroscopic samples^{1-3,20}. However, the samples
63 should be thin or at least of uniform structure in direction of the incoming beam to avoid
64 losing the spatial information by averaging along the beam path. There have been efforts to
65 combine SAXS or WAXS with computed tomography (CT) by rotating the sample with

66 respect to the X-ray beam and scanning at different angular steps^{5-7,21,22}. In these cases the
67 reconstruction was carried out by integrating the scattered intensity in a q -range of interest
68 and applying standard CT reconstruction algorithms, i.e. filtered backprojection or algebraic
69 reconstruction techniques. This is only applicable if the scattering is invariant with respect to
70 sample rotation, which is the case for isotropic scatterers or tissues and materials with
71 structural symmetry around the rotation axis. For the more general case of anisotropically
72 distributed ultrastructures, a full 3D reciprocal space map of the 3D SAXS distribution is
73 needed. Thus in each sub-volume, i.e. voxel, not merely a scalar, such as X-ray absorption,
74 but a tensor needs to be reconstructed. Tensor tomography is known in magnetic resonance
75 imaging (MRI), where the orientation-dependent magnetic relaxation is used to measure the
76 diffusion of water in tissue (diffusion tensor imaging, DTI)²³. Recently, directional dark-field
77 imaging (DDFI) was successfully combined with tensor tomography to investigate structures
78 and orientations at the micrometre scale²⁴. We have developed a new algorithm, allowing the
79 combination of small-angle scattering with tensor tomography, which allows structures in the
80 nanoscale to be probed and offers the possibility for a reconstruction of the 3D SAXS
81 distribution for each q -range individually. Figure 1 schematically shows the experimental
82 setup and the principle of the method, which we refer to as SAS tensor tomography. It allows
83 tissues and materials with anisotropically oriented nanostructure to be studied at 3D spatial
84 resolutions limited by the X-ray beam size, which is typically at the micrometre scale.

85 To demonstrate the method, we present a study of a trabecular bone specimen with a size of
86 about $1 \times 1 \times 2.5$ mm³ extracted from a human vertebra. The scattering angles covered by the
87 detector correspond to structures in the range of 5.4 nm up to 300 nm. In the hierarchy of
88 multi-scale structural characteristics of bone tissue, the arrangement of the hydroxyapatite
89 mineral crystal platelets with sizes of approximately $3 \times 25 \times 50$ nm³, their intra-fibrillar
90 spacings (~ 65 nm), as well as the diameter of collagen fibrils (50-200 nm) lie within this size
91 range^{9,25,26}. Most of the SAXS signal originates from the electron density contrast between
92 the mineral crystal platelets and surrounding lower electron density materials such as
93 collagen fibrils and water¹. It has been shown that the orientation of crystals, collagen fibrils
94 and fibril bundles and fibres are closely related^{2,9,14,20}. Following this assumption, any of
95 these structures can be probed to estimate the orientation of mineralized collagen fibrils in
96 bone.

Figure 1: Schematic illustration of the experimental setup for SAS tensor tomography.

The sample is scanned through an X-ray pencil beam ($25 \times 25 \mu\text{m}^2$) at different sample orientations while a two-dimensional detector is recording the small-angle scattering pattern for each scanning point. In addition to rotation around the tomographic axis (y), different tilt angles around the x-axis are measured. For the tomographic reconstruction the sample volume is subdivided into small sub-volumes, i.e. voxels, defined by the dimension of the X-ray pencil beam. The detector frame probes ultrastructural length scales from a few nanometres up to a few hundred nanometres. This allows for instance to probe mineralized collagen fibrils, as schematically illustrated. The distinct Bragg reflection highlighted by an arrow originates from the regular arrangement of the $\sim 65 \text{ nm}$ gaps of collagen fibrils containing mineral crystals. The scattering of the fan-shaped profile perpendicular to it originates from the shape, size and lateral arrangement of the mineral crystals, and is also affected in the investigated range (indicated with white circles) by the lateral packing of the collagen fibrils²⁵.

The sample is scanned through a pencil X-ray beam, which had a dimension of $25 \text{ by } 25 \mu\text{m}^2$ for this experiment. In each scanning point a 2D scattering pattern is recorded, which is a sum of the scattering of all sub-volumes in the beam path. Similar to 1st-generation tomography this scan is repeated for each orientation around the tomographic (y) axis. If the step size of the scanning and the angular spacing are chosen appropriately, the spatial resolution of the tomogram is limited only by the X-ray beam size. Accordingly the scanning step size was $25 \mu\text{m}$ and the angular spacing was 4.5° between 0 and 180° . In contrast to the X-ray absorption probed in conventional tomography, the 2D scattering pattern emerging from each probed

voxel depends on the relative orientation of the underlying nanostructure with respect to the X-ray beam, thus changes with sample orientation. Hence, for the reconstruction of the 3D SAXS distribution in each voxel additional data need to be collected requiring multiple tilt angles of the tomographic axis. Here data were collected at six different tilt angles of the tomographic axis, resulting in a total of 240 measured projections, each of which was measured by scanning 122×55 positions, resulting in 1.6 million scattering patterns with a total exposure time of 22.5 h. For each scattering pattern we reduced the data by azimuthal integration over 16 sectors and radial integration over the q -range corresponding to distances from 85 to 165 nm as indicated by the white circles in the detector frame in Figure 1.

For reconstruction of the orientation we model the 3D reciprocal-space map with a series of spherical harmonics Y_l^m , which provide a complete basis for the intensity distribution on the sphere corresponding to a fixed q -range. Spherical harmonics have been used in texture analysis to reconstruct orientation distribution functions based on pole-figure analysis from XRD measurements^{27,28}. One advantage of using spherical harmonics is that known symmetries can be enforced by choosing the appropriate degree l and order m . For the trabecular bone studied, we assume that the mineral plate surface normal is randomly oriented in the plane perpendicular to the fibrils, thus a point symmetry around $q = 0$ and rotational symmetry around one axis in the 3D reciprocal space map are expected in the q -range investigated. Therefore we use here only the spherical harmonic functions with even degree l and zero order m . An optimization algorithm is used to minimize the error between the modelled intensity of sub-volumes in the beam path projected to the detector plane and the measured intensity for each scanning point under all rotation angles. Figure 2 visualises how all measured scattering patterns are used to reconstruct the reciprocal space map from which the orientation of the nanostructure can be retrieved.

Figure 2: Data processing. More than a million SAXS pattern (left) are used to reconstruct the 3D reciprocal space map for each voxel, which is modelled using spherical harmonics (middle) and provides a representation of the nanoscale structure distribution. From the reconstruction the main ultrastructure orientation as well as the degree of orientation can be retrieved, which is visually represented by a 3D vector (right). The degree of orientation is illustrated both by the colour and the length of the cylinders.

The main orientation in the 3D reciprocal space map is defined by the relative orientation of the zenith of the spherical harmonic functions with respect to the coordinate system of the sample. Thus the main orientation in each voxel is given by polar and azimuthal spherical coordinate angles, which are optimization parameters of the reconstruction. The degree of orientation can be calculated as the ratio between the anisotropic scattering (described by spherical harmonic functions with degree $l > 0$) and the total scattering. Assuming co-alignment between collagen fibrils and mineral crystals^{2,9,14,20}, the main orientation of the scattering in the investigated q -range reveals the spatial arrangement of the mineralized collagen fibrils. The anisotropic scattering of bone is related to the fraction of aligned mineralized fibrils on average over a certain voxel. Hence, the higher the degree of orientation retrieved, the higher is the ratio of aligned fibrils compared to randomly oriented fibrils²⁹ (see also illustration in Figure 3). The degree of orientation is calculated directly from the coefficients of the spherical harmonic functions, which are additional optimization parameters of the reconstruction. For details see the online methods. In the visualisation of the reconstructed ultrastructure the degree of orientation is represented both by the colour and the length of the cylinders (Figure 2, right).

Simultaneous to the SAXS patterns the X-ray absorption was measured with a photo diode mounted on the beam stop blocking the direct beam in front of the detector. The corresponding CT reconstruction is shown in Figure 3a. The result of SAS tensor tomography on the human trabecular bone is shown in Figure 3b as a 3D visualization (see also supporting information movie). One tomographic slice of the reconstruction is shown in

Figure 3c. Domains of low degree of orientation (blue) and high degree of orientation (red) can be identified.

Figure 3: Orientation of collagen fibrils within a human trabecular bone sample. **a**, CT reconstruction retrieved from the transmitted intensity using standard filtered backprojection. **b**, Orientation of bone ultrastructure as retrieved from SAS tensor tomography and **c**, one tomographic slice of the reconstruction. The cylinder orientation represents the main orientation of collagen fibrils in the corresponding voxel as schematically depicted in **d** and **e**. The degree of orientation is represented by both colour and length of the cylinders, where a low degree of orientation (blue) means low ordering of the collagen fibrils (**d**), while in regions with a high degree of orientation (red) the collagen fibrils are well aligned with respect to each other (**e**). Scale bars in **a** and **b** correspond to 0.5 mm.

The presence of domains with a size of several tens of micrometres in size is in agreement with previous findings on trabecular bone as well as the observation that high degrees of orientation are found in places with higher curvature in the trabecula⁴. As expected, in these curved regions the collagen fibrils follow closely the trabecular bone microstructure.

The technique presented here can be tuned to specific characteristics of the samples under investigation. For example, the range of sizes of the ultrastructure probed can be varied by changing the analysed scattering angles, *i.e.* the q -range. One advantage of the method is that the angular scattering range of interest is digitally extracted from the scattering frames such that different length scales can be probed simultaneously and reconstructed from the same dataset. Independent of the q -range, the real-space resolution of the spatial mapping can be adapted by the size of the X-ray beam and the scanning step size. The analysis algorithm would also allow the reconstruction of the full q -range, similar to what has been shown for

SAXS tomography for isotropic scatterers³⁰. This opens the possibility to retrieve size and form of the scattering objects, as done for standard small-angle scattering analysis. The method is not limited to such symmetries as could be applied in the example of trabecular bone. Spherical harmonics provide a complete basis for modelling the 3D reciprocal space, and azimuthal structure can be captured by adding azimuthal orders m into the optimization. Yet, prior knowledge on symmetry of the scattering object can be used for reducing the complexity of the optimization problem and, potentially, the number of projections needed to reconstruct the structure. While limits on the complexity of a 3D reciprocal space map that can be recovered with the method presented here as well as rational sampling requirements remain to be explored, the flexibility of resolution both in real and reciprocal space and the non-destructiveness of SAS tensor tomography already make this imaging technique widely applicable in biological and materials science, providing information complementary to nanoscale imaging, which will aid the adequate modelling and understanding of biological tissues and complex materials.

Supplementary Information is available in the online version of the paper.

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Author contributions M.L., M.G., A.M., P.S., O.B., and M.G.-S. conceived the research project. M.G. prepared the sample. M.L, M.G., and M.G.-S. carried out the X-ray experiments. M.L. developed the data analysis framework with support from O.B. and M.G.-S. Results were interpreted by M.L, M.G., P.S., and J.K. M.L. wrote the manuscript with contributions from all authors.

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296 **Methods** (online only)

297 **Sample preparation.** A trabecula was extracted from a T12 human vertebra from a 73 year
298 old man and cleaned from soft tissue. The trabecula ($\sim 1 \times 1 \times 2.5$ mm³) was isolated using a
299 scalpel and embedded into poly-methyl methacrylate (PMMA). The human vertebra had been
300 obtained from the Department of Anatomy, Histology, and Embryology at the Innsbruck
301 Medical University, Innsbruck, Austria, with the written consent of the donor according to
302 Austrian law. All subsequent procedures were in accordance to Swiss law, the Guideline on
303 Bio-Banking of the Swiss Academy of the Medical Sciences (2006) and the Swiss ordinance
304 814.912 on the contained use of organisms.

305 **Experiment.** Experiments were carried out at the cSAXS beamline (X12SA) of the Swiss
306 Light Source, Paul Scherrer Institut, Switzerland. The 12.4 keV X-ray beam was defined by a
307 fixed-exit double-crystal Si(111) monochromator to an energy bandwidth of about 2×10^{-4} and
308 focused to a beam size of approximately 25 by 25 μm^2 . Horizontal (sagittal) focusing was
309 achieved by bending the 2nd monochromator crystal and vertical (meridional) focusing by
310 bending a Rh coated mirror. The sample was mounted on a goniometer on two perpendicular
311 rotation stages on top of a two-axis scanning stage. A 7 m long flight tube under vacuum was
312 inserted between sample and detector to minimize air scattering and X-ray absorption.
313 Scattering patterns were collected with a PILATUS 2M detector³¹ placed 7.15 m downstream
314 of the sample. Simultaneously, the transmitted beam was measured with a diode mounted on
315 a beam stop blocking the direct beam prior to the exit window of the flight tube. 40
316 projections at tilt angles between 0 and 180 degrees around the tomographic rotation axis
317 were collected for 6 different tilt angles of the rotation axis between -30 and 45 degrees, with
318 6710 scan points each. This resulted in a total of 1,610,400 scattering patterns and a total

exposure time of 22.5 h, with an individual frame exposure time of 50 milliseconds. Each voxel was exposed for 12 seconds in total. The dose d was estimated using $d = \mu N_0 \epsilon / \rho$,³² where μ/ρ is the mass attenuation coefficient (37.5 cm²/g for bone at 12.4 keV³³), N_0 is the number of incident photons per unit area, and ϵ is the photon energy. Thus the dose imparted on the bone specimen is about 2.9×10^7 Gy. In order to assure that no structural changes were induced by radiation damage the projection at 0 rotation around both axes was repeated at the beginning, in the middle and the end of the experiment. In future experiments with increased resolution or on specimens prone to radiation damage the dose could be reduced by using fewer tilt angles, lower photon counting statistics and a higher X-ray energy than in this proof-of-concept experiment.

The total time for the experiment was 35.5 h, which includes an overhead of 13 hours due to motor movements. This will be significantly reduced by faster sample stages with precise positioning which will also enable higher spatial resolution. Since the exposure time was limited by detector data transfer and not by photon flux, we expect that the availability of faster detectors³⁴ soon will allow for significantly reduced acquisition times or increased number of resolved voxels at existing facilities.

Reconstruction. The SAXS data were reduced by azimuthal integration within 16 segments. For further analysis we integrated in a q -range of 0.0379 to 0.0758 nm⁻¹, which corresponds to length scales of 165 to 85 nm. Projections were aligned with subpixel accuracy based on the absorption measurements on the beamstop diode using an efficient image registration approach based on cross-correlation³⁵. A gradient-based optimization algorithm was used to minimize the error metric $s = 2 \cdot \sum (\sqrt{I} - \sqrt{\hat{I}})^2$, where I is the measured and $\hat{I}$ is the modelled intensity from the current solution. This error metric was chosen since minimizing it is a first-order approximation to a maximum likelihood estimation for photon-counting Poisson noise³⁶. Due to the high number of parameters to optimize, the gradient of the error metric with respect to such parameters is calculated using an analytical expression rather than finite differences.

The modelled intensity $\hat{I}$ is obtained by calculating the intensity pattern of each voxel for each orientation and by adding them up along the corresponding beam path, also termed a projection. To reduce artefacts due to projections in a rectangular 3D grid along arbitrary orientations, the volume was upsampled by a factor of 2 before the projection. For the

projection bilinear interpolation was used followed by a triangular two-dimensional filter and by a final downsampling. The intensity in each voxel was calculated using a series of spherical harmonic functions Y_l^m of degree l and order m as $I = |\sum a_l^m Y_l^m(\theta, \phi)|^4$ where θ and ϕ are the polar and azimuthal angle, respectively. To model the scattering from the collagen fibrils, we used the spherical harmonic functions Y_l^m of degree $l = 0, 2, 4$ and 6 and of order $m = 0$. The square of the sum was used in order to avoid negative intensities. To guide the solution towards the *a priori* known rotational symmetry of the collagen fibrils smooth penalty terms were introduced for the coefficients a_l^m . For the gradient calculation a backprojection operator is needed, which was implemented using bilinear interpolation. In order to accelerate convergence of the reconstruction, the gradient of the error metric with respect to the coefficients a_l^m was spatially convolved with a 3D Hamming window of $3 \times 3 \times 3$ voxels, which can be understood as an application of Grenader's method of sieves³⁷. The parameters of the optimization in each voxel were the polar angle θ and azimuthal angle ϕ , representing the orientation of the probed ultrastructure in 3D as well as the coefficients a_l^m of the spherical harmonics, from which the degree of orientation ρ is calculated as

$$\rho = \frac{\int (a_2 Y_2^0 + a_4 Y_4^0 + a_6 Y_6^0)^2 \sin\theta d\theta d\phi}{\int (a_2 Y_2^0 + a_4 Y_4^0 + a_6 Y_6^0)^2 \sin\theta d\theta d\phi},$$

which corresponds to the ratio between the anisotropic scattering and the total scattering. In order to accelerate convergence the optimization was performed following a two-step approach. First, the angles that define the spherical harmonics were optimized while the coefficients were kept fixed, which helps optimizing initially a subset of the parameters. This was followed by optimizing the coefficients of the spherical harmonics while the angles obtained in the first step were kept constant. Finally, all parameters were optimized simultaneously, with this last step reaching convergence after only 2 more iterations. Typically 20 iterations were performed for the first two steps. Using a development prototype implementation, the reconstruction took 3 days running on a standard compute server with 12 CPU cores and up to 24 threads processed in parallel. The algorithm lends itself well towards further parallelization, and an optimized code running on a computer cluster would speed up the processing significantly.

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