

Organ-to-Cell-Scale Health Assessment Using Geographical Information System Approaches with Multibeam Scanning Electron Microscopy

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The linking of local and global events is as critical for medicine as it is for environmental science. Yet a means to image and analyze relationships between organs and their inhabitant cells, akin to zooming in on a satellite view of a rainforest ecosystem and its inhabitant trees,^[1,2] has remained elusive. Here, we combine a novel imaging technique, developed originally for rapid throughput quality assessment and detection of nanometer-sized defects in silicon wafers,^[3] with a geographical information system approach, to create navigable anatomic maps of hip joints from patients undergoing hip joint replacement.^[4] Analysis of spatial information acquired by localizing relevant map landmarks, including both viable and pyknotic cells as well as blood vessel edges, enabled an understanding of organ health in the context of its cellular inhabitants. Applying this approach for the first time to human tissues, it was shown that viable, pyknotic, and interstitial osteocytes show significant differences in their relative proximities to nearest neighboring osteocytes. In contrast, no significant relationship was observed between viable or pyknotic cells and their proximity to the blood supply. While current imaging modalities allow for organ-centric and cell-centric imaging of global and local regions of the organs and tissues comprising our bodies, this study demonstrates the feasibility of seamless multiscale imaging of human organ systems to elucidate relationships between organ health and the viability of organs' individual cellular inhabitants. This

step forward can potentially pave the way for retrospective elucidation of disease etiologies and the development of rapid throughput diagnostic imaging modalities in the future.

Cells are the living inhabitants of our organs and tissues. From bone to brain, cells' viability, spatiotemporal arrangement, and local extracellular matrix milieu reflects their life history and future health.^[5–11] Environmental, e.g., rainforest, health offers an analogous albeit scaled up, systems biology paradigm. Satellite imagery and geographical information system approaches provide a perfect means to separate the “trees from the forest and the forest from the trees” while further allowing assessment of ecosystem health measures, such as tree viability and watersheds, over time.^[1,2] No such diagnostic medical imaging modality is currently available.

High resolution and throughput imaging techniques do exist, e.g., for electron microscopy of ultrastructural pathological changes in diagnosis of malignant schwannoma,^[12] but regions of interest are limited. There is currently an acute need for such a technology for assessment of kidney biopsies which, e.g., are currently sent physically from Dubai to the Cleveland Clinic core laboratories via daily express shipments, for electron microscopy and pathology readouts that are entered online in Cleveland for reading by doctors in Dubai. Furthermore, quantitative approaches, such as calculation of fractal dimension in confocal imaging datasets, demonstrate the feasibility of automated, rapid throughput diagnostic measures in small fields of view (Figure 1, Table 1). However, the potential for associated sampling errors have limited the clinical use of such approaches.^[12,13] Hence, the approach described in this manuscript may have transformative potential for the field.

An understanding of emergent structure-function relationships during tissue genesis by cells (e.g., during development, tissue engineering) and degeneration and failure of tissues as a consequence of inefficient or insufficient adaptation (in context of aging, pathophysiology), necessitates a means to image these processes seamlessly across length scales.^[1,2] Yet the paucity of rapid throughput imaging technologies that allow for seamless bridging of structure-function relationships across length scales (10^{-2} – 10^{-9} m) stymies the elucidation of tissues' emergent properties. This area of unmet need, identified by both the U.S. National Science Foundation as well as the National Institutes of Health,^[14,15] provided the impetus for the current study.

Here, we aimed to develop and utilize, for the first time for human life sciences applications, multibeam scanning electron microscopy (mSEM) together with big data visualization methods for massive image data sets. Multibeam scanning

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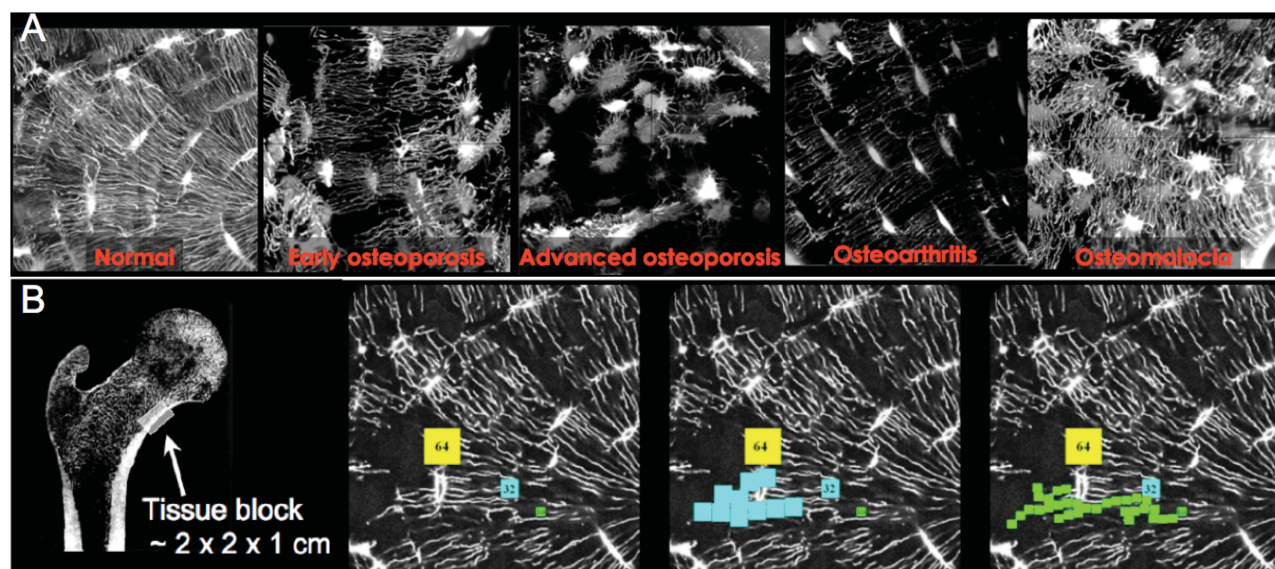


Figure 1. Use of fractal dimension as a measure of connectivity and disease diagnostic in bone cell networks. A) Laser scanning confocal images of cortical bone from patients with normal, osteoporotic, osteoarthritic, and osteomalacic cortical bone from the femoral neck of human patients (IRB approved, Cleveland Clinic, acquired during the course of hip replacement surgery, B) Images reproduced with permission.^[10] Copyright 2004, Elsevier. Fractal dimension is calculated using the box count method on the bone cell (osteocytic) network from femoral neck cortical bone (tissue block). Quadrants or boxes made up of 64 (yellow), 32 (turquoise), and 16 (green) pixels are assessed and compared from healthy, osteoporotic, osteoarthritic, and osteomalacic specimens Table 1).

electron microscopy uses a single electron optical column for multiple electron beam sources, allowing for a scale up of imaging speed, and thus specimen size. Multibeam scanning electron microscopy has the potential to increase image acquisition rates by orders of magnitude, allowing, for the first time to our knowledge, rapid throughput imaging of anatomical human tissue blocks, such as whole joints, with electron microscopic resolution.^[3]

As a first step toward elucidating structure-function relationships in the cortical bone of the femoral neck, we imaged human hip joint tissue resected during the routine course of hip replacement surgery (Cleveland Clinic Institutional Review Board approved study of stem cell niches and organ to cell structure-function relationships,^[4] Figure 2).

A major challenge in the implementation mSEM imaging of human musculoskeletal tissues was the tissue preparation, as the quality of electron microscopy images depends strongly not only on the microscope but also on sample fixation, embedding, and contrast. Indeed, this process took several iterations. Coronal sections of the femoral head and transverse sections

of the femoral neck were sectioned by an orthopaedic pathologist and prepared for mSEM. To maximize infiltration and to minimize artifacts on specimen surfaces, block specimens were embedded in polymethylmethacrylate rather than typical EM epoxy resins such as Epon. Additionally, atomic force and electron microscopy methods, routinely used to prepare and process samples no larger than a millimeter on edge, were developed to prepare three orders of magnitude larger femoral head and neck sections for mSEM.^[3,12,13] Selective etching of the samples revealed further details of intrinsic organic and inorganic tissue structure, similar to characteristics found in previous studies using atomic force microscopy,^[16,17] albeit expanding from the mesoscopic to nanoscopic length scales (Figure 2, Figure 3, and Figure 4).

Using the mSEM prototype, we successfully imaged joint tissue blocks containing complex tissue composites across length scales (10^{-2} – 10^{-9} m) and in a rapid throughput manner (Figure 2, Figure 3, Table 2). The mSEM technology enabled imaging of several cm^2 of surface area, at nanometer resolution, over the course of hours, and resulted in terabyte-sized image datasets comprising tens of thousands of high-resolution images.

To maximize accessibility of data for scientists and the public alike, further steps were taken, including automated, registered image stitching (using Fiji^[18] and TrakEM2^[19] libraries), tile pyramid rendering (TrakEM2,^[20]), and visualization using the Google Maps API^[21] to enable seamless zooming in and out and rapid navigation of tiled image maps.^[22,23] Global mapping paradigms are particularly useful to differentiate the proverbial “forest from the trees and the trees from the forest”.^[1,2] Yet, stitching of so many ultrahigh resolution images was not trivial. Furthermore, anomalies noticed during stitching

Table 1. Fractal dimension (FD) calculation for healthy and diseased femoral neck cortical bone (white shaded area in Figure 1B, arrow).

Diagnosis	Normal	Osteoarthritic	Osteoporotic	Osteomalacic
FD range	1.49 – 1.60	1.28 – 1.36	1.25 – 1.42	1.55 – 1.65
FD mean	1.54	1.30	1.35	1.60
Standard deviation	0.028	0.019	0.057	0.038
Standard error	0.008	0.005	0.016	0.010
Sample size	13	13	13	13

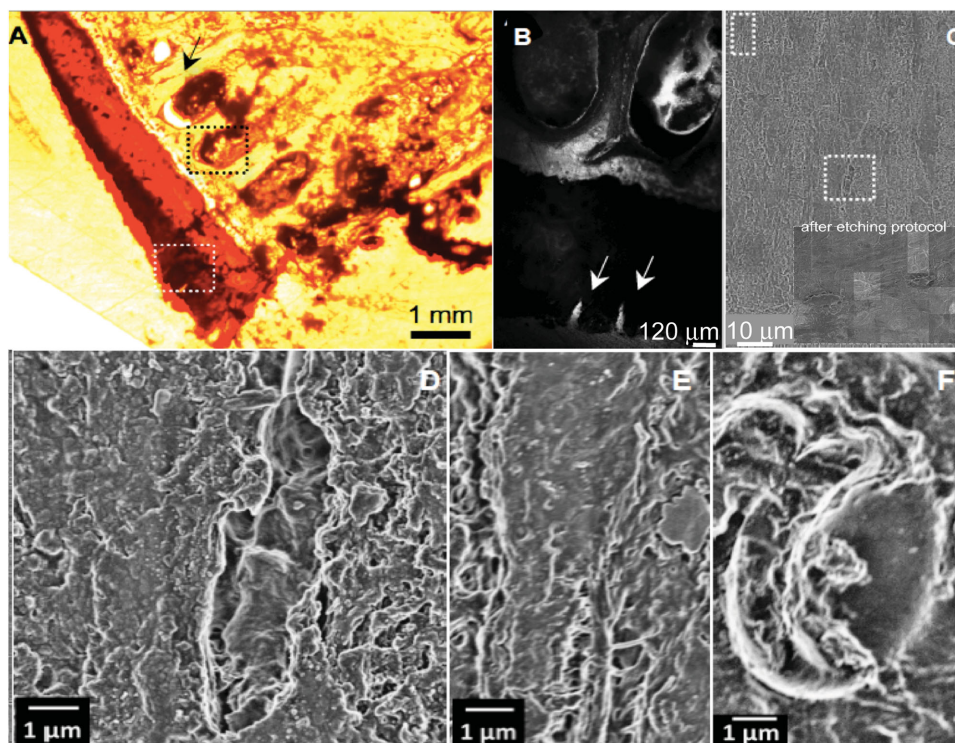


Figure 2. Seamless multiscale imaging of hip joint tissue, prior to and after (inset in (C) etching step. A) Transmitted light image of OsO₄ stained femoral head section, with greatest penetration of contrast agent (darkest red) in articular cartilage (white dotted square) and marrow spaces of the subchondral bone (black dotted square). B) Laser scanning confocal image of specimen shows autofluorescence (light areas) of subchondral bone and marrow spaces (ellipsoids at top) as well as cartilage (dark), with two visible cartilage defects (bright cracks, arrows). C) Large scale, tiled mSEM image of same area with single field of view showing (sub-)cellular resolution of D,E) bone and F) blood cells without additional etching.

required development of custom code to correct for tracking errors intrinsic to precision micromachined systems measuring in the nanometer range and the physics of the interaction between the multiple electron beams and the specimen.

TrakEM2 is capable of managing extremely large image data sets; using TrakEM2,^[24] the volumes of interest do not need to be read wholly into the computer memory. This overcomes the typical limitations related to memory consumption. Thereafter, we used the Javascript API of Google Maps to visualize the resulting tiled pyramid structure, allowing for interactive browsing by the user (Figure 3 and Figure 4). To create a first map of the human hip, 55 000 mSEM images were stitched together using Fiji and TrakEM2 libraries and exported as two million 256 × 256 prerendered tiles. Google Maps API and associated interactive navigation capacities allowed for visualization and interaction with the image datasets for scientists and the general public alike (Figure 3, <http://www.mechbio.org>).

As a first case study to elucidate the relationship between bone health and cell health in the femoral neck of the human hip, the Google JavaScript API was used to enable marking of physiologically relevant landmarks on the navigable map of the femoral neck (Figure 4). For proof-of-concept, a blinded observer marked blood vessel edges, viable osteocytes, and pyknotic osteocytes using standard blue, green, and red pins, respectively. Healthy osteocytes were defined as having a minimum of three visibly distinct cell processes while pyknotic osteocytes were defined as having less than three processes

in the plane of view.^[9] Osteocytes within osteons were identified along radii extending from the edges of Haversian blood canal vessels (inner boundary), outward toward cement lines delimiting the outer edges of osteons. Interstitial osteocytes, defined as those cells located in interstitial bone (by definition outside of osteons) were also identified. The respective position coordinates of the pins within the map were exported and distances were calculated using the MATLAB script. Statistical distributions and non/parametric tests were run between the dataset distributions of viable, pyknotic, and interstitial osteocytes (JMP, SAS Institute), as related to neighboring blood vessels and osteocytes. This enabled testing of specific hypotheses related to bone health as well as osteocyte viability, connectivity, and transport path distances.

Subtle but statistically significant differences ($p < 0.0001$) were observed in distances between viable, pyknotic, and interstitial osteocytes (Figure 4), where healthy osteocytes exhibited the shortest distance (less than 8 μm with smallest variance and exponential distribution) to other viable cells. While pyknotic cells also exhibited an exponential distribution, these cells had a longer path distance and higher variance compared to viable cells. In contrast, interstitial cells exhibited a normal distribution with mean path distance to the next viable cell more than five times that of viable cells and four times that of pyknotic cells. No statistically significant differences in distance to nearest blood supply were observed between viable, pyknotic, and interstitial cells ($p > 0.05$).

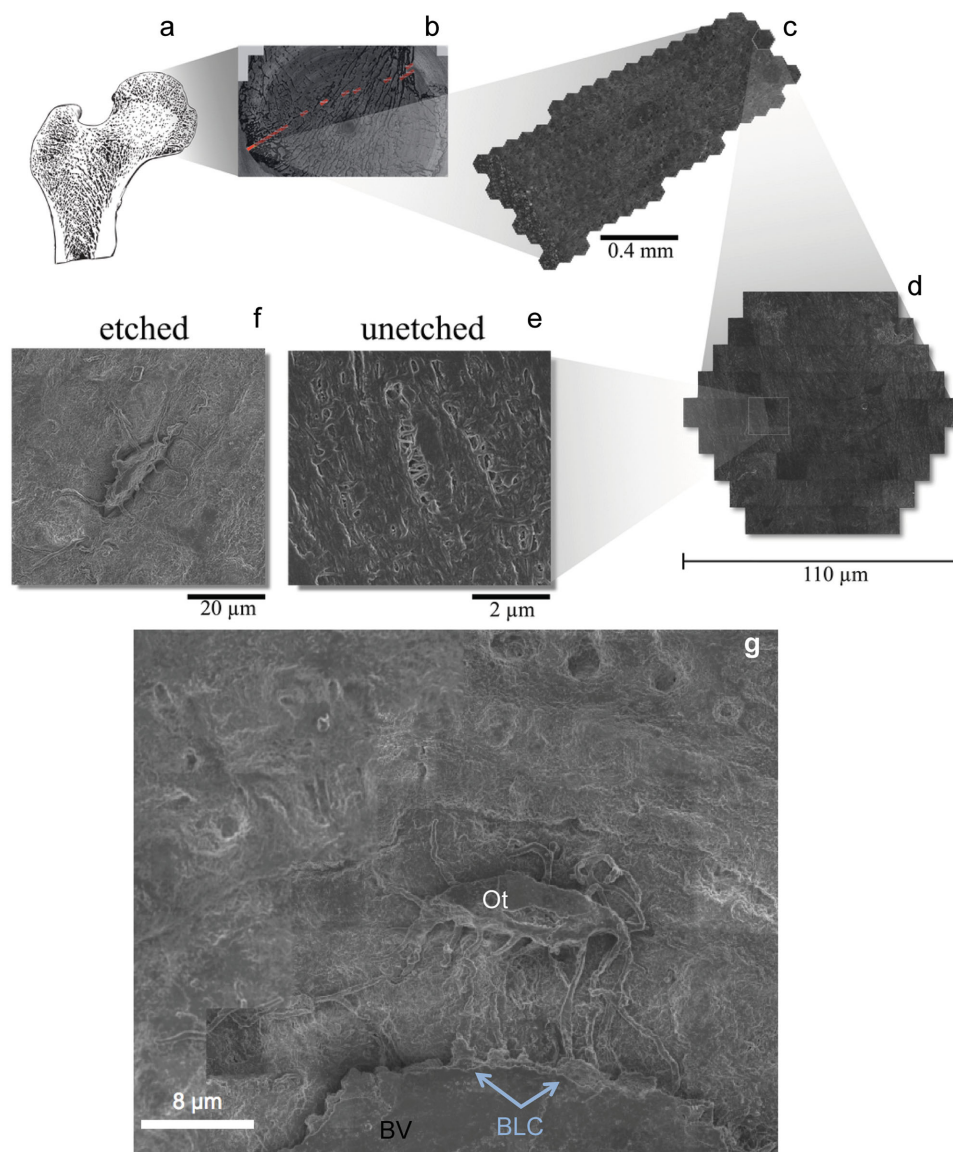


Figure 3. Creation of a first navigable map of the human hip using a full imaging data set of the femoral neck across length scales. A) The femoral neck (circa 4.8 cm in diameter) was imaged as a series of B) polygonal regions. C) One polygonal region (circa 2.3 cm), comprised over fifty-five thousand SEM images, which were then stitched together using Fiji and TrakEM2 libraries, and exported as two million 256×256 prerendered tiles, to visualize using Google Maps API (Figure 2). D) One hexagonal region comprised 61 single images. E) One such image from an unetched specimen, with a resolution of ≈ 4 nm per pixel, resulting in circa 112×10^9 pixels. F) A corresponding etched specimen. G) Rendering of pyramidal data storage using Google Maps API to achieve “zoomable” image, akin to a “Google Maps” for tissues and organs of the body. Representative zoomed in screen shot from the first data set of a patient’s hip. Osteocyte (Ot) interacts with the tightly coupled cells (Bone Lining Cells, BLC) lining the blood vessel (BV) via numerous osteocytic processes. Full, navigable and “zoomable” dataset and an animation demonstrating navigation of the maps can be accessed at <http://www.mechbio.org>.

Hence, osteocyte viability in femoral necks from aging patients undergoing arthroplasty appears to relate more to cell network connectivity than mean path length to blood supply. While no statistically significant differences in mean path length to the endosteum, periosteum or nearest blood vessel were observed in viable, pyknotic or interstitial osteocytes, a small but significant increase in distance to the next viable osteocyte was observed in pyknotic compared to viable cells. This may implicate connectivity to other viable cells as a factor in osteocyte survival and tissue, respectively, organ health with

age. Automated segmentation routines are in development to facilitate automated and rapid throughput diagnostic assessment of imaging datasets.

Combined with geospatial approaches, this novel imaging technology opens the door for a range of network modeling applications which will enable disease epidemiology studies in populations of cells that inhabit tissues and organs. For example, to further explore the role of cellular connectivity in osteocyte survival, network analysis tools such as clustering methods can be used to identify spatial relationships between

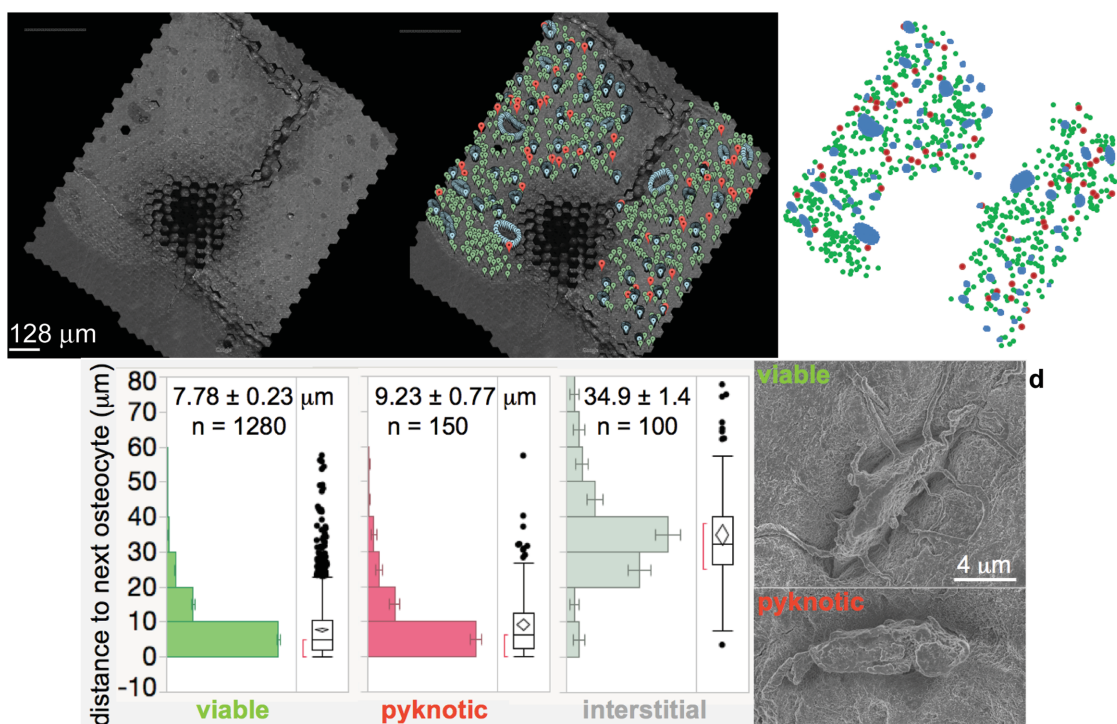


Figure 4. The first navigable map of the human hip was used to investigate relationships between path distances between cells and the blood supply. The dark area in the center of the image represents an artifact of sample preparation and for this reason, areas including the artifacts were not included in quantitative studies. A,B) Applying navigation features typical for Google Maps (through use of the Google JavaScript API), pins were dropped on landmarks including viable (green) and pyknotic (red) osteocytes as well as blood vessel edges (blue). C) Landmarks were imported and rendered graphically in a statistics program to test hypotheses. While no statistically significant differences were observed between nearest path distance to the blood supply and pyknotic or viable osteocytes (data not shown), statistically significant differences were observed between D) viable, pyknotic, or interstitial cells and the distance to the next viable osteocyte ($p < 0.0001$). This may implicate connectivity to other viable cells as a factor in osteocyte survival with age.

and among groups of viable and/or pyknotic cells.^[25–28] Network connectivity, shortest path and search algorithms can be implemented to further investigate distance-based relationships between pairs of osteocytes and/or osteocytes and blood vessels. Given additional samples from individuals in varying disease states, the temporal progression of bone disease can also be modeled at the network level, wherein the structural evolution of the cellular network can be explored. Bringing this ensemble approach to the next level, i.e., to develop rapid throughput diagnostics, requires both development of new image processing pipelines and expansion of computational power. Through automated segmentation of landmarks, including cells and blood vessels, as well as epidemiological approaches as noted above, these methods may have great potential for early diagnosis or to identify high-risk patients well in advance of debilitating disease progression.

Table 2. Technical specifications for mSEM imaging protocols used in the study.

Sample dataset	Beam number	Single-beam FoV width, height [μm]	Landing energy [keV]	Pixel resolution [nm]	Dwell time [ns]
1	61	12.60, 10.96	1.5	9.78	100
2	61	12.60, 10.96	1.5	9.78	100
3	61	12.60, 11.20	1.5	3.76	100

In conclusion, joint tissue blocks containing complex tissue composites across length scales (10^{-2} – 10^{-9} m) were successfully imaged in a rapid throughput manner. Visualizing the seamless mSEM images using the Google Maps API presents a novel opportunity to tie local health events at a cell and tissue length scale to organ health and disease, providing an unprecedented means to assess the health of the hip joints' individual living inhabitants (cells) to elucidate etiology of bone disease. Use of the ubiquitous Google Maps API platform for navigation of and documentation of relevant landmarks in human tissue samples further eases access to and interaction with the data, by the scientific community as well as the general public. In addition, the new technological platform allows for unparalleled examination of emergent structure-function physiological relationships in human health and disease. Like a multiscale and multidimensional puzzle, our approach serves as a platform for mapping the human body and its cellular inhabitants.

This novel platform presents a new set of challenges and limitations, in part due to its cutting edge nature, as well as in consideration of the sheer size of the resulting datasets. The “sheer size limitation” presented itself when we enabled public access to the first map of the human hip, comprising two million prerendered images, with sample sizes in the range of hundreds of gigabytes

via a university server. Finally, dissemination of results was a major hurdle, given the technical challenge to publish results consisting of such massive datasets, or in more than two dimensions; this presents a profound barrier to transfer of information and knowledge (power) to the public as well as the field as a whole. This barrier can be overcome through a combination of expertise in handling and sharing of massive data sets as well as dedicated computing power, paving the way not only to understand and reverse natural processes of organ and tissue failure as we age, but also to ignite the imagination of the public in the power of research and pursuit of knowledge.

Experimental Section

Specimen Acquisition: Femoral necks and heads, normally discarded after hip replacement surgery, were collected after sectioning in the coronal plane of the femoral head and the transverse plane of the femoral neck (to facilitate chemical fixation) by the Cleveland Clinic Pathology Department per IRB protocol guidelines (Cleveland Clinic IRB protocol 12-335).^[4]

Specimen Preparation: Tissues were fixed, undecalcified, in glutaraldehyde (2.5%), formaldehyde (4%) in cacodylate buffer (0.2 M) at 4 °C. They were then processed for bulk embedding in poly(methyl methacrylate) (PMMA), which polymerized slowly under vacuum. Once hardened, the PMMA embedded specimens were precision Computer Numerical Control (CNC)-milled to achieve mirror-like planarity. Thereafter, specimens were prepared for carbon coating and imaging. Between imaging steps, PMMA embedded specimens were selectively etched with HCl (0.02 M) for 90 s and/or NaOCl (10%) for 11 min per our previous atomic force microscopy protocols^[16,17] to image the respective inorganic or organic phase of the extracellular matrix from respective tissues of the joint complex.

mSEM Imaging: Samples were imaged with a 61-parallel beam Zeiss prototype mSEM, using a 100 ns of dwell time per beam and a pixel size of 9.8 nm (Table 2). Sixty-one single-beam images per hexagonal multibeam field-of-view (mFoV) were generated per scanning passage, each imaging 12.60 and 10.96 μm in box width and height, respectively (1288 by 1120 pixels). Each beam simultaneously generated a rectangular subimage with a FoV on the order of circa 10 μm (Figure 3). To scan 5.85 mm² of the mapped sample (Figure 3), 898 mFoV regions were obtained at an acquisition rate of 100 TB d⁻¹, each comprised of 88 MB of image data and stored in a lossless format. Overlapping single images in the same mFoV and between adjacent mFoV had roughly 8% and 80% overlap, respectively, required for stitching procedures.

Image Stitching: Python scripts were developed in Fiji,^[18] using the libraries from the registration toolkit TrakEM2,^[19] to automatically generate individual images of complete datasets (adapted from S. Preibisch et al.).^[24]

Pixel coordinates for each single-beam image were available from the microscope metadata, providing a first approximation for relative stitching. In the resulting tiled image sets, nanometer-scale discrepancies in stitching were observable as gaps or imperfect alignment of biological structures at image boundaries. These imperfections were most likely attributable to interactions between electron beams and specimen sample. To address these, and after first verifying some consistency of this error across the entire specimen, the isolated stitching of a representative mFOV region was processed and the resultant translation offset vectors were applied to the correspondent tiles in other regions. This greatly reduced the number of image registration calculations in the stitching process.

To alleviate computational load, stitching was calculated within and between adjacent hexagonal field-of-view regions. Brightness disparities amongst image tiles were minimized using nonlinear blending.^[24]

Maps: A TrakEM2 prerendered tile exporter script (Beanshell script developed by Stephan Saalfeld, <https://github.com/axtimwalde/fiji-scripts/blob/master/TrakEM2/catmaid-export2.bsh>) was adapted

to process our datasets tiled pyramid structure. The reconstructed mosaic, with a total pixel space of 315486 $\times$ 356226, of all single-beam images was partitioned in PNG-compressed 256 $\times$ 256 pixel tiles, which comprised the highest, full-resolution zoom level of the pyramid. Pixel size was increased twofold for each unitary decrement in zoom level. Lower zoom level tiles were rendered by recursively grouping four tiles (512 $\times$ 512 pixels) and downsampled to 256 $\times$ 256 pixel tiles. The pyramid tiles were saved in a web server with unique directory paths as follows

.. / (maxzoom – zoom) / y / y_x (maxzoom – zoom).png

where maxzoom represents the maximum zoom level, and x and y are the position in the tile coordinate system, as described in the Google Maps API documentation [<https://developers.google.com/maps/documentation/javascript/maptypes> (Google Maps custom map documentation)].

Google Maps API manages the retrieval and displaying of the necessary tiles. The creation of a custom map, using the Google Maps API, requires MapType object for translation between (screen to tile) coordinate frames.

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