DEFINITELY NOT BONE IDLE

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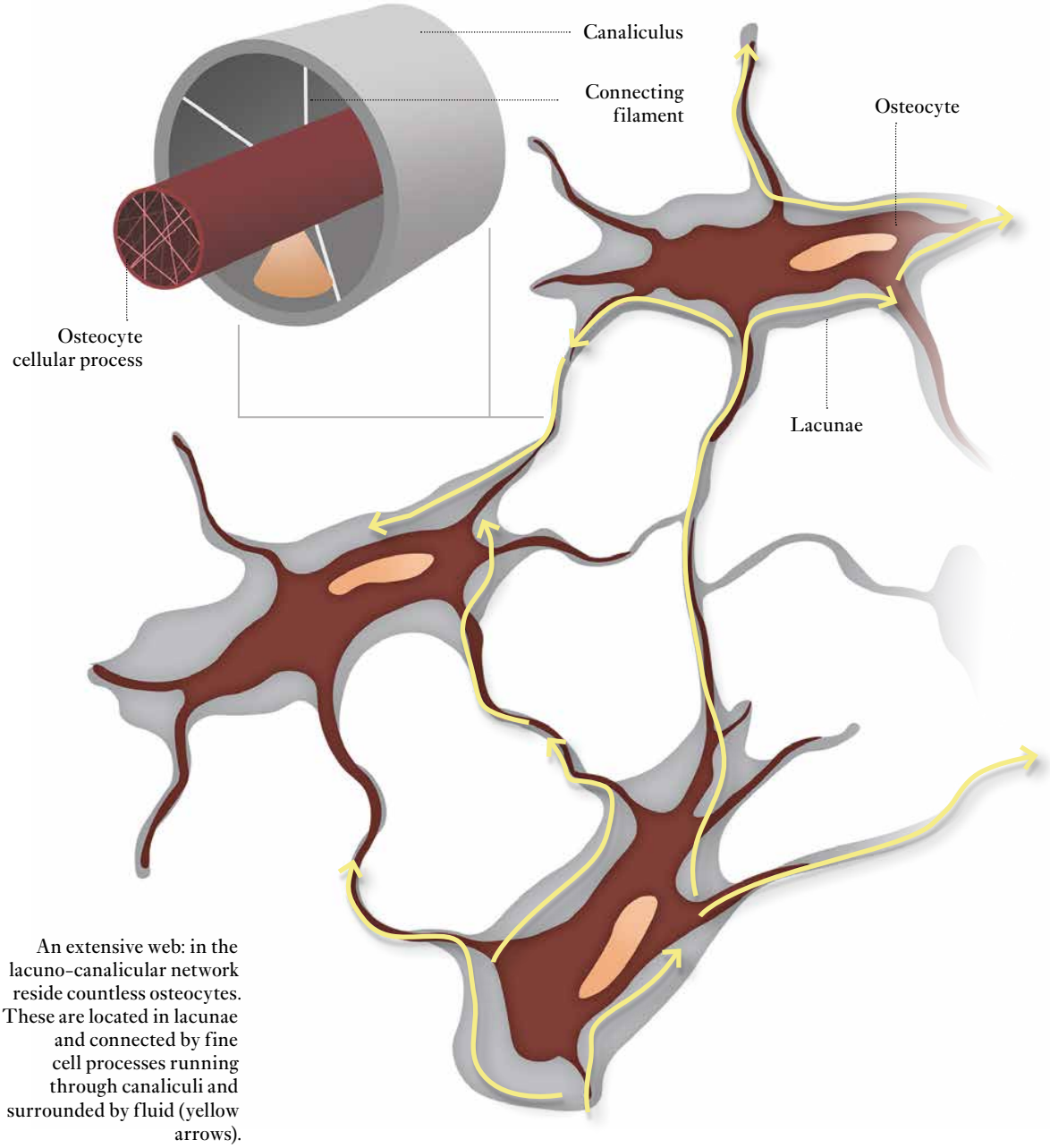
PHOTO: DAVID AUSSERHOFER

Your skeleton provides support for your body. But this framework is anything but static; mechanical stress causes your bones to constantly renew and remodel themselves. Richard Weinkamer and Wolfgang Wagermaier at the Max Planck Institute of Colloids and Interfaces are investigating precisely how this happens and what structure makes bones stiff and strong. Their findings could also prove relevant for medicine and materials science.

Transilluminated: the Potsdam team uses a variety of methods to study the structure of bone tissue – for example, using a micro-computed tomography scanner. The researchers place bone samples into the tubes of these scanners. Here, a thin section has been stained with rhodamine for further examination.

Fractures, crooked spines, worn hip joints – as a surgeon at the Charité, Julius Wolff had seen a lot. X-rays had not yet been discovered when he formulated his law of transformation of bones (otherwise known as "Wolff's law") in 1892. When he held them up to the light, thin slices of bone cut with an ivory saw revealed that the fine, sponge-like matrix of the bones' interiors could remodel themselves in response to mechanical stress. Form follows function, as we now say. And when it comes to the architecture of bone, that design concept can be taken literally. To dissipate tension and achieve maximum stability, bone reacts actively and permanently to mechanical stimuli. This means that material is added in areas that bear heavier loads and removed in areas under less load. But the precise factors triggering and governing this process are still a mystery to scientists 130 years later.

At the Department of Biomaterials of the Max Planck Institute of Colloids and Interfaces in Potsdam, two Austrian scientists have been using state-of-the-art techniques to delve deep into this question – and are



An extensive web: in the lacuno-canalicular network reside countless osteocytes. These are located in lacunae and connected by fine cell processes running through canaliculi and surrounded by fluid (yellow arrows).

ILLUSTRATION: GCO BASED ON AN MPG DESIGN.

SUMMARY

Bones are constantly being renewed and remodeled. Osteocytes and the lacuno-canalicular network play a key role in this process.

Studies conducted by Max Planck researchers corroborate the fluid flow hypothesis. According to the hypothesis, osteocytes can sense changes in the external pressure based on changes to the velocity of fluid flow through the lacuno-canalicular network. There is increasing evidence that osteocytes and the lacuno-canalicular network are also involved in recycling bone material.

Improving our knowledge of how bone renewal is controlled could enhance our understanding of diseases such as osteoporosis.

Detailed knowledge of bone structure is inspiring the development of particularly strong and rigid materials.

steadily getting closer to the answer. One of them, Richard Weinkamer, a mathematician and physicist, is chiefly interested in the mechanobiology of bone. The other, Wolfgang Wagermaier, with his background as a materials scientist, is fascinated by bone, which is a natural hybrid material. Their research focuses on fundamental insights that on the one hand will help us to better understand bone diseases, and, on the other, will also lead to the discovery of synthetic materials with interesting new properties.

Bones are composed of an inherently soft collagen matrix, in which extremely fine platelets of calcium phosphate are embedded. This construction lends the material not only strength and rigidity, but also a degree of elasticity. "Hybrid materials composed of proteins and minerals such as calcium phosphate, calcium carbonate, silicon oxide or iron oxide are widespread in nature. Mother-of-pearl, diatoms and teeth are obvious examples. As well as, of course, bone," says Wagermaier. "We wouldn't get far if our bones were merely soft masses of tissue. Not only that, bones also act as reservoirs of calcium in the body." In this way, they maintain a very sustainable and practicable materials management system. It needs to be practicable, because the more than 200 bones, which constitute about ten percent of an adult's body weight, are constantly being remodeled. It wouldn't be feasible to continually consume the amounts of new minerals needed for that job through our diet alone.

Thirty million cells per cubic centimeter

The material that makes up a compact bone like the tibia is entirely remodeled within ten years. In the case of spongy bones like the vertebrae, this occurs even faster – within five years. This task of demolition and reconstruction is performed by specialized cells: osteoblasts build up bone material, while osteoclasts break it down again. However, 95 percent of all bone cells are osteocytes. As if they were straight out of an Edgar Allan Poe novel, they live for years, sometimes even decades, walled up alive in small cavities in the bone – some 30 million cells per cubic centimeter! Because they are not directly involved in the restructuring processes and are difficult to access in their cavities, cells of this type were overlooked by researchers for a long time. That makes them all the more interesting to Richard Weinkamer and Wolfgang Wagermaier. Osteocytes perform important tasks, so the researchers in Potsdam are investigating these in a combination of diverse methods: optical techniques, characterization methods from material science, mathematical computations and simulations. Osteocytes, for example, are not only involved in maintaining mineral balance; they even secrete hormones and, as the researchers have recently discovered, serve as pressure sensors.

Osteocytes need to be enclosed in their bony prisons to perform some of their functions. And they get imprisoned by their own actions. "At some time in the past, osteoclasts have eaten small tunnels into the bone at these sites. Then osteoblasts have come along and filled the tunnels back up again, layer by layer, thereby enclosing themselves and differentiating into mature bone cells – into osteocytes," explains Richard Weinkamer.

The osteocyte network

To investigate the confined osteocytes, the researchers first saw off a sample of bone, embed it in plastics, and viewed it under an electron microscope. The first things that stand out are the large dark spots. They are channels that traverse the bone, through which blood vessels run. They are surrounded by "osteons": concentric structures similar to annual tree rings and only a shade darker than the lighter part of the bone sample. "Here, surrounding the blood channel, the bone is even younger and less mineralized," explains Wolfgang Wagermaier. "The small, dark spots in the middle are the lacunae. And in these oval cavities, some 15 micrometers in diameter, reside the osteocytes."

If you look very closely, you'll immediately see a fine structure that extends away from the blood vessels toward the exterior: a labyrinth of channels. The researchers immerse the bone sample in a solution containing the fluorescent stain rhodamine and view it under a confocal laser scanning microscope, revealing a bright, filigree structure on a black background, like an extremely fine crochet pattern: the "lacuno-canalicular network." Even though the cell bodies of the osteocytes are locked inside the lacunae, they are connected to their neighboring cells via up to 80 finger-like cellular protrusions running through this network of channels. It resembles the network of neurons in the brain. "The stain allows us to reveal areas that would otherwise be inaccessible. It covers the entire inner surface of the cavity system, and the laser light causes it to become excited and fluorescent," explains Wagermaier. Bone isn't

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transparent, but it is possible to look through thin layers with a light microscope. Confocal microscopy, in which a focused laser beam scans the sample, can be used to capture images of different layers of the bone, which are then combined into a 3D image. Doing so allows the researchers to gather detailed information on the density and connectivity of the lacuno-canalicular network, down to a depth of 40 micrometers. Drawing on his mathematical background, Weinkamer used a computer to virtually extract it from the bone material and calculate the extent of the network. "If you could string together lengthwise all the channels from just one cubic centimeter of bone, they would extend for about 74 kilometers! It's truly spectacular."

Wolff's law states that bone is reinforced exactly where it is mechanically needed. But where is the sensor that controls the process? We know that cells are sensitive to mechanical stimuli, like pressure. However, the pressure exerted from the outside on the relatively stiff bone barely deforms it and isn't sufficient to activate osteoblasts and osteoclasts. Osteocytes may play an important role in this, as was suggested back in the early 1990s in the "fluid flow hypothesis." The idea is that the entire network of channels and cavities containing osteocytes is filled with fluid. "It's essentially like a wet sponge. When the sponge is squeezed, the pressure forces the liquid away, toward other areas where it can escape. If the bone is subjected to mechanical stress, the fluid within this network is forced into the wide channels around blood vessels," explains Weinkamer. The osteocytes feel the forces of the fluid as it flows over their surface. As a simplification, one could say: the greater the load on the bone, the faster the fluid flows. "And it's this information that the osteocytes pass on through the network to the bone surface. That's where the osteoblasts and osteoclasts reside, which then take over."

Bone training for mice

Being able to image the canal network in the bone meant this hypothesis could now be tested by the Potsdam researchers. Together with his colleague Bettina Willie from McGill University in Quebec, Canada, Weinkamer studied three genetically identical mice. The rodents were anesthetized, one of their hind legs was placed in a leg press similar to one in a gym, and a small amount of mechanical force was applied along its longitudinal axis. The untrained second leg served as a control. The mice were then placed in the computed tomography scanner to scan their bones and awakened again. The mice went through this entire procedure several times. Analyzing all the data gave an accurate picture of where new bone was being formed after fitness training and where it was not.

The Potsdam team then examined in detail the lacuno-canalicular networks containing several million small channels in the bones of the three mice. Using the data gathered about the networks, the researchers then calculated the flow of fluids through them. This allowed them to simulate where in the cross-section of the mouse leg they would expect bone to be formed and where it would be resorbed. The simulation did indeed precisely predict where this bone remodeling took place. Based on the evidence from the bone training in the three Canadian mice, the researchers verified that the mechanical sensor in the bone really does functions as predicted by the fluid flow hypothesis. During the training, the bones of the three mice all responded to the training – but to different degrees. Richard Weinkamer discovered why when he compared the channel networks in great detail using samples from the crucial bone areas. His flow velocity calculations established that the architecture of mouse two's network (which gained less bone during training) only allowed fluid to flow slowly.

Bone formation from an embryo to an adult vertebrate follows a sophisticated program. Constant rebuilding is a part of this from the very beginning. "It all starts with a cartilage condensation. As soon as a small amount of bone is present, some of it is removed from the inside and added to the outside," explains Richard Weinkamer. "This is how, little by little, the radius of a bone in-

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Close-ups of bones from a horse (top left). An image obtained by scanning electron microscopy reveals the larger, circular openings for blood vessels and the smaller cavities of the lacunae in a section through the femur (top right). Under a confocal laser scanning microscope, rhodamine staining highlights the channels of the lacuno-canalicular network (lower left). Polarized light microscopy reveals that the collagen fibers are arranged in a ring around the blood vessels (bottom right).

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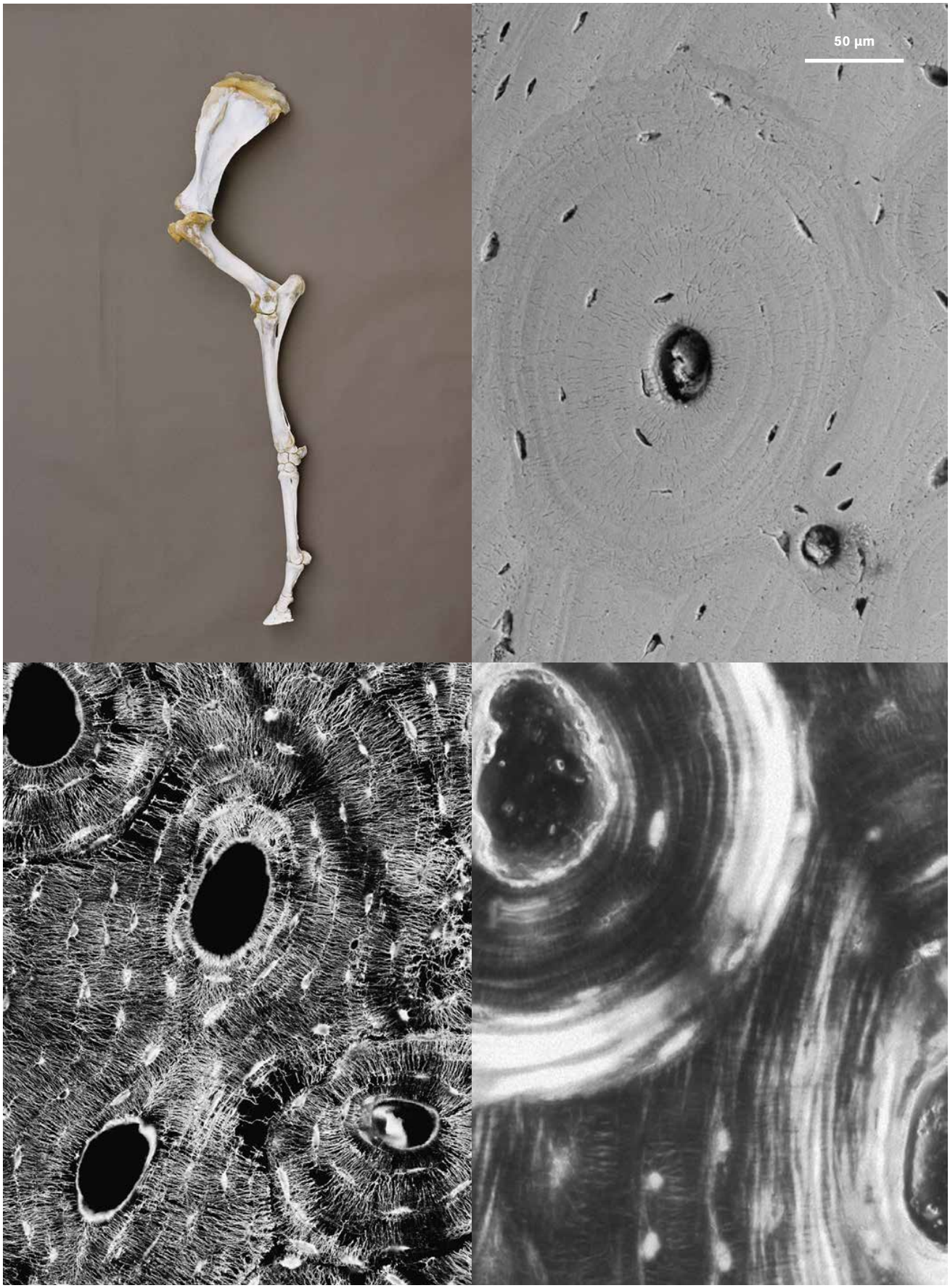
RICHARD WEINKAMER



PHOTOS: SCIENCE PHOTO LIBRARY, MPI FOR COLLOID AND INTERFACE RESEARCH (2), KERSCHNITZKI, WAGERMAIER ET AL., J STRUC BIOL 2011, CELLS TISSUES ORGANS 2011 (CLOCKWISE FROM TOP LEFT)

PHOTO: DAVID AUSSERHOFER

"Tennis players possess more bone volume in their dominant, racket arm."

WOLFGANG WAGERMAIER

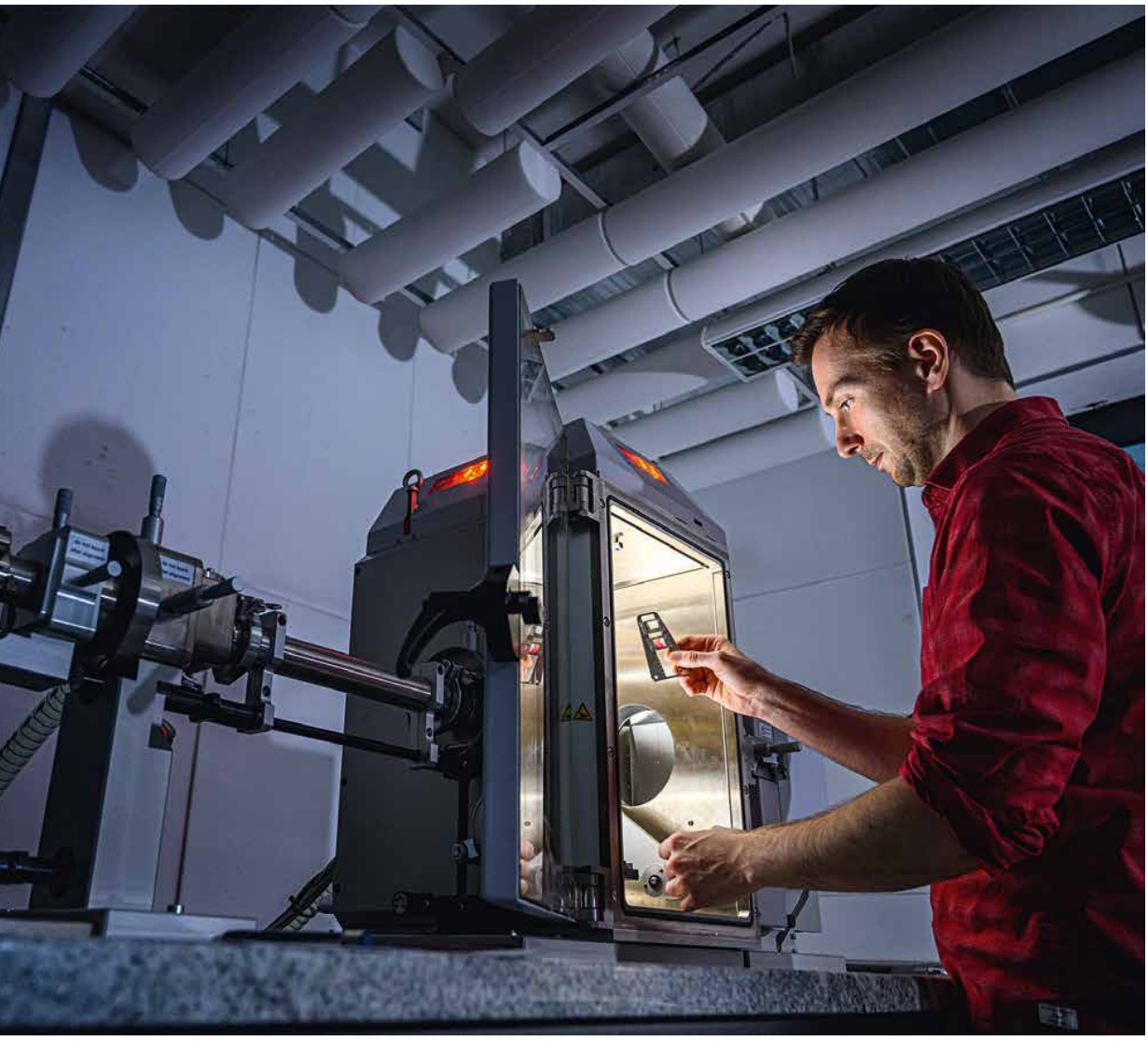
creases. Longitudinal growth is a little more complicated." In this case, the calcium phosphate is broken down in a two-step process. First, the osteoclasts secrete acid to dissolve the mineral component. They do this by developing "fingers" – tiny cell protrusions that they literally push into the material. In the second step, they use enzymes to degrade the collagen matrix. But how do the osteoblasts recover the building material? This transport route for calcium phosphate is currently being studied by Wagermaier and Weinkamer. "Obviously, some kind of recycling is taking place. Some of the mineral is packed into vesicles. We're using the electron microscope to search for these vesicles and calculating how many would have to pass through to mineralize the bone," Weinkamer explains. There is now increasing evidence, including that from the Potsdam team's research, that osteocytes are likewise involved in regulating mineral balance. There's no doubt that the breakdown and incorporation of calcium phosphate, a process known as mineral homeostasis, is also a very laborious process. "The lacuno-canalicular network may also serve to ensure that the mineral is extracted without completely destroying the collagen matrix," says Wolfgang Wagermaier.

Dysfunctions in bone mineralization and demineralization are the cause of many bone diseases. Osteoporosis and brittle bone disease (osteogenesis imperfecta) are two examples. Using animal models like mice that carry specific genetic defects with a pattern of disorders similar to human bone diseases, researchers around the world are attempting to learn about these diseases – and to identify potential therapeutic approaches.

While Richard Weinkamer is concentrating on the lacunae and the web of channels, materials scientist Wolfgang Wagermaier is primarily interested in the areas that appear black under the confocal laser scanning microscope: the compact bone material surrounding the canal network, like the rock of a mountain surrounding a cave system. To visualize it, Wagermaier's team is bombarding samples with short-wavelength X-rays at the electron storage ring BESSY II in Berlin-Adlershof. This procedure yields diffraction images, which they can use to determine the sizes of the mineral particles in the collagen matrix. Wagermaier is searching for inspiration from biology to create new materials, in this case the relationships between the material parameters – the orientations, for instance, of collagen fibers or the sizes of mineral particles – and the architecture of the lacuno-canalicular network. Polarized light reveals the ring-shaped structure of the collagen matrix. Small-angle X-ray scattering reveals deeper structures, including collagen fibrils. Wide-angle X-ray scattering provides even more detail. "Each fibril contains numerous collagen molecules that are arranged in a surprisingly regular fashion," Wagermaier says. "Each individual collagen molecule is twisted into a triple helix about 300 nanometers long. The collagen helices are separated by gaps of about 40 nanometers." In these gaps, the mineral platelets begin to grow, attaining a thickness of two to seven nanometers, depending on the type of bone.

Synthetic materials with mineral nanoparticles

The combination of flexible fibers and hard particles can also be harnessed to optimize synthetic materials. A study conducted together with Hans Börner of the Humboldt University in Berlin, for example, has shown that when mineral nanoparticles are embedded in a polymer matrix using a kind of adhesive at the interfaces, synthetic materials become significantly stronger and stiffer. The work performed by osteoblasts in building the bone structure, which provides a model for materials science, is extremely elaborate. However, its effects are often visible on a macroscopic scale. "People who play tennis frequently possess more bone volume in their dominant, racket arm," Wagermaier states. And in testing greyhounds who repeatedly race in one direction around a circular track, it was found that the bones of the outer legs are often slightly denser than those of the inner legs. In general, bones obey the following principle: absence of a mechanical stimulus causes them to lose mass. This is not just a problem that occurs due to lack of exercise in old age; it also affects astronauts during extended space missions. In a study conducted by NASA, subjects



Sample for the "nanostar": technician Daniel Werner prepares to analyze a sample of bone using small-angle X-ray scattering (SAXS) to determine the size of its mineral particles.

who were not permitted to leave their beds for four weeks lost both muscle and bone mass. The extent of this varied from person to person. Weinkamer and Wagermaier suspect that, similar to the mice, differences in the network structure of the bones play a role.

Bones can remodel and renew themselves throughout life. Nevertheless, bone density starts to decrease at around the age of 35. "We don't know why. Does the sensitivity of the bones' mechanosensing system decrease with age? Just like our other sensory organs – our eyes, and ears – it becomes less sensitive," muses Wagermaier. In his view, using drugs as a prophylactic measure would be ill-advised. It would be better to take countermeasures, like exercise and gentle strength training. This would maintain the pressure on bones to prevent their restoration from decreasing, even into advanced old age.

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GLOSSARY

LACUNO-CANALICULAR NETWORK
The micrometer-fine channel system that runs through bone. It connects the lacunae in which osteocytes reside and is filled with fluid.

OSTEOBLASTS
Cells that secrete collagen and calcium phosphate and thus build bone material.

OSTEOCLASTS
Cells that break down bone material and make this material available for rebuilding.

OSTEOCYTES
Cells that are derived from osteoblasts and are located in the lacunae of the lacuno-canalicular network.