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Multiscale imaging of bone microdamage

Atharva A. Poundarik¹ and Deepak Vashishth^{1,**}

¹Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY

Abstract

Bone is a structural and hierarchical composite that exhibits remarkable ability to sustain complex mechanical loading and resist fracture. Bone quality encompasses various attributes of bone matrix from the quality of its material components (type-I collagen, mineral and non-collagenous matrix proteins) and cancellous microarchitecture, to the nature and extent of bone microdamage. Microdamage, produced during loading, manifests in multiple forms across the scales of hierarchy in bone and functions to dissipate energy and avert fracture. Microdamage formation is a key determinant of bone quality, and through a range of biological and physical mechanisms, accumulates with age and disease. Accumulated microdamage in bone decreases bone strength and increases bone's propensity to fracture. Thus, a thorough assessment of microdamage, across the hierarchical levels of bone, is crucial to better understand bone quality and bone fracture. This review article details multiple imaging modalities that have been used to study and characterize microdamage; from bulk staining techniques originally developed by Harold Frost to assess linear microcracks, to atomic force microscopy, a modality that revealed mechanistic insights into the formation diffuse damage at the ultrastructural level in bone. New automated techniques using imaging modalities such as microcomputed tomography are also presented for a comprehensive overview.

Bone fragility due to osteoporosis and poor bone quality is a major and increasing concern [1]. In both genders, aging is associated with significant loss of bone mass [2]. Currently the gold standard technique for bone mass density (BMD) measurements is dual emission x-ray absorptiometry (DXA). However, for a given bone mass, risk of fracture increases with age suggesting the inadequacy of BMD alone, as a reliable predictor of bone fracture [3]. Recent evidence has demonstrated that bone quality is crucial to accurately predicting fracture risk. Bone quality encompasses various attributes of the bone matrix from the quality of its material components (type-I collagen, mineral and non-collagenous matrix proteins) and cancellous microarchitecture, to the nature and extent of bone microdamage [4]. Microdamage accumulates with age and disease [5–8] and its understanding is imperative to better understanding bone fracture.

Microdamage manifests in multiple forms across the scales of hierarchy in bone [9] (Fig. 1). At the ultrastructural level of a mineralized collagen fibril, consisting of type-I collagen fibril infused with mineral crystals, and non-collagenous proteins, mechanisms like sacrificial bonding [10–13], dilatational bands [14] and fibrillar sliding [15] have been

^{**}Corresponding Author Contact Information: vashid@rpi.edu, Tel: 518-276-2296, Fax: 518-276-4233.

shown to dissipate energy, preventing the formation of larger morphologies of microdamage. At the subsequent hierarchical level, multiple collagen fiber bundles (comprising collagen fibrils) are arranged to form rings called lamellae. Microdamage at the lamellar level manifests in the form of linear microcracks (60–130µm) [16–20], diffuse damage (comprising cracks <1µm) [21–23] and uncracked ligaments (>100µm) [24–26]. Lamellae can range in thickness from 2–7µm and are layered concentrically around the Haversian canal forming an osteon. Osteons run longitudinally along the length of the bone and can be up to 250µm in diameter. The angular orientation of the collagen fibrils in adjacent lamellar layers normally varies and is important in determining the mechanical properties of the resulting osteon. Cement lines are present at the boundary of osteons and interstitial bone, and promote mechanisms like crack deflection and osteon pullout [27–29] at the highest level of hierarchy in cortical bone.

Bone microdamage has been shown to affect the quality of bone matrix through a range of biological and physical mechanisms. For example, increased rates and magnitudes of loading, commonly seen in athletes or military personnel, can lead to accumulation of microdamage and eventually stress fractures [30–32]. It is also well known that microdamage formation stimulates bone remodelling by initiating bone resorption and bone formation [33–39]. Recent studies investigating this phenomenon have implicated osteocyte apoptosis in microdamage induced remodeling [40, 41]. Furthermore, the nature of matrix damage also impacts the mechanical properties of bone [42, 43]. Thus, due to the significant role of bone microdamage in bone quality and its implications in disease and bone fractures, an understanding and visualization of mechanisms the various levels of bone matrix hierarchy is critical.

Indeed, the use of multiple imaging modalities has enabled researchers to further their understanding of bone microdamage. This review explores the imaging tools and techniques that have been widely used to understand the damage phenomena in bone, from macroscopic bulk staining techniques to ultra-high resolution microscopy techniques that can probe bone's ultrastructure to its very fundamental constituents.

Bulk Staining and Light Microscopy

Use of bulk staining by basic fuchsin is the most widely known approach to characterizing bone microdamage. First developed by Frost [19], bulk staining was devised to identify in vivo damage whilst avoiding artefactual damage that may arise during the sectioning and polishing of specimen for microscopic evaluation. Bulk staining using fuchsin is a two-step process [44]. The first step involves dehydrating and staining a sample with basic fuchsin, soluble in an ethanol medium (6% fuchsin at 26 °C), to allow penetration of stain into microcracks and matrix voids. Post staining, the dehydrated basic fuchsin stained sample is rehydrated and sectioned. Because fuchsin is insoluble in water, rehydrating the already stained sample prevents any leakage of the stain. Thus, artefacts created during the process of sectioning and polishing, are not stained [19, 45]. The bulk staining approach for identifying microdamage has been criticized for using alcohol as a medium, which could cause the creation of artefactual damage through dehydration of the sample. However, this theory has been disproved by showing that the total number of microcracks in human rib

samples which were ground and stained thereafter, and those that we bulk stained, are the same [46]. Moreover, the current protocol developed by Burr and co-workers [46] involves progressive dehydration of bone by gradually increasing the ethanol concentration (70% to 100%) and decreasing the incubation time (48hrs to 12 hrs). Electron microscopy images have confirmed basic fuchsin staining at microcrack locations [47]. In another approach, Vashishth et al. [48] developed the double staining technique using toluidine blue (water soluble) and basic fuchsin (ethanol soluble) in a sequential fashion (Fig. 2). Here, the specimen is bulk stained with toluidine blue after machining, but before mechanical testing. After testing, the sample is stained with basic fuchsin. Thus, cracks that are artefactual or inherent in the specimen will show double staining (toluidine blue and basic fuchsin), and microdamage formed during mechanical testing will only exhibit the basic fuchsin stain.

Both, linear microcracks and diffuse damage have been well investigated using en block staining. Whilst linear microdamage can be seen as sharp, micron dimension cracks within bone matrix, the diffuse damage morphology is a contained volume of multiple submicron cracks. Under fatigue loading, linear microcracks form on the compressive side, and diffuse damage on the tensile side [7], and both contribute independently to bone matrix quality [22]. Linear microcracks are a predominant form of damage in bone matrix, and easily identifiable using the bulk staining method (Fig. 2). As per Burr and Stafford [46], linear microcracks are intermediate in size, larger than canaliculi but smaller than vascular channels. They have sharp borders and are surrounded by a halo of basic fuchsin. They are stained through the thickness of the section and on changing the depth of focus, crack edges appear more sharply stained than rest of the crack. They are known to form during bone fatigue and their morphology varies with the microstructure and extent of loading. Compressive loading results in the formation of long cracks that can be as long as 300 microns. Tensile regions show micro-crack toughening as opposed to singular linear cracks [20].

In addition to linear microcracks, the bulk staining method allows for identification and quantification of diffuse damage. Comprising sub-micron cracks over an area, diffuse damage appears as a region of pooled stain as viewed under an optical microscope [49] (Fig. 3). Unlike linear microcracking, diffuse damage enables bone matrix to confine microdamage to a limited region, thereby restricting crack propagation and eventual matrix failure. This is possible because the creation of a large surface area of submicron cracks dissipates energy that would otherwise result in the linear microcrack growth and propagation. Diffuse damage is thus a superior toughening mechanism over linear microcracks, that averts failure in bone by dissipating energy and stalling crack propagation [7]. It is seen in areas of tension under fatigue [14, 23] and contains nanoscale deformation zones called dilatational bands that have been characterized using other imaging modalities. It has also been demonstrated that the ability of bone to form diffuse damage is reduced with age [21].

Both, linear microcracks and diffuse damage are also present in another energy dissipating mechanism that increases the fracture resistance of bone, known as microcrack toughening [50]. Microcrack toughening (Fig. 4) occurs in two stages i) formation of the frontal process zone and ii) formation of the wake zone. The first stage involves micro-cracking around the

main crack tip that causes a reduction in stress intensity around the crack tip. A build-up of cracks in the frontal zone eventually causes softening and crack propagation [7, 14, 20, 28]. The wake zone contains microcracks that are left behind as the crack propagates into the matrix. This process is repeated as the main crack grows into the matrix.

Light microscopy has also been employed to evaluate microdamage in the context of matrix healing. Schaffler and co-workers demonstrated the role of osteocyte apoptosis in the remodeling and repair of linear microcracks [40, 41]. They showed that osteocyte apoptosis is necessary to initiate intracortical bone remodeling to repair fatigue-induced microdamage. Furthermore, they suggest that the extent of osteocyte apoptosis may be determined by the extent of microdamage. Recent work has also revealed that diffuse damage has the tendency to self-heal in vivo, without the need of intracortical remodeling [51].

It is important to note that some studies published in literature do not report the adherence to the established guidelines and include images of microdamage analyzed. This makes the data suspect and the comparison between studies difficult.

In vivo histomorphometry and sequential fluorochrome labeling

Developed over four decades ago by Frost and others [52, 53], tetracycline labeling has been widely used in the histology of bone, in vivo. Because tetracyclines appear yellow-green under transmitted light, the use of multiple dyes like alizarin red, calcein blue and xylenol orange, in addition to tetracycline, has allowed for comprehensive assessment of bone remodeling in disease. All the above dyes can chelate to the calcium ions on exposed mineral surfaces in bone and are fluorochromes, making them candidates for evaluation using LSCM [54, 55]. Recent studies have investigated the use of selective fluorochromes to detect bone microdamage [56, 57].

Whilst most studies have focused on an in vivo assessment of bone formation/resorption, Stover et al, [58] used in vivo labeling with calcein blue for microdamage assessment in the metacarpals of horses afflicted by stress fractures. The presence of microcracks was confirmed on one transversely cut specimen by bulk staining with basic fuchsin. Fluorescence microscopy confirmed that microcracks were also labeled with calcein blue. O'Brien et al. [59] used fluorescent chelating agents to sequentially label microcracks and investigate the effect of crack length on propagation. By applying the chelating agents at particular time intervals within a compressive fatigue test, O'Brien et al found that microcracks less than 100µm stopped at cement lines, those greater than 100µm continued to grow after encountering a cement line and only the ones greater than 300µm could penetrate osteons and ultimately cause failure.

Another technique that permits high resolution 3-D imaging of bone matrix is epifluorescence-based serial block face imaging [60, 61]. In this technique, multiple fluorochromes within a single specimen can be reconstructed and visualized in three dimensions (Fig. 5). Kazakia et al. [60] used this method to study fluorochrome-labelled microdamage in trabecular bone (5mm diameter) with voxel size of $3 \times 3 \times 8 \mu\text{m}^3$. Local damage volume fraction (an indicator of matrix heterogeneity) and global damage volume

fractions based on the 3-D reconstruction, were computed to be 0.1–0.28 and 0.15 respectively.

Laser confocal scanning microscopy

Laser scanning confocal microscopy (LSCM) has been widely used for the evaluation of bone microdamage. In LSCM, fluorescent dye/s or fluorochromes are used to stain bone sections, that are subsequently embedded and cover-slipped onto a glass slide prior to imaging. LSCM employs lasers with specific wavelengths to excite the fluorochromes. Upon excitation, the fluorochromes emit a characteristic spectrum of longer wavelengths that are filtered (typically using long-pass or band-pass filters), recorded, and analysed to identify areas of the embedded bone section stained by a particular fluorochrome. The use of multiple fluorescent stains, a high spatial 3-D resolution and multiple filter arrangements, makes LSCM a very versatile technique [8, 62, 63]. Fluorescein (FITC), rhodamine and basic fuchsin are all commonly used fluorochromes [44]. Basic fuchsin absorbs light of wavelength 545 nm [45, 64], that is green in color. When this particular wavelength is used to excite fuchsin, it emits light of a longer wavelength that appears orange when viewed under a red filter. In addition to organic fluorochromes, the use of antibody fluorescent stains has been used to evaluate the role of proteins in microdamage formation [14].

In LSCM, the incident laser/s can be focused to image a plane of the stained section, allowing very good spatial accuracy. Furthermore, images of multiple consecutive planes can be stacked and reconstructed into a 3-D image, allowing comprehensive qualitative assessment of damage morphology. Zioupos [65] used LSCM to identify microcracks labelled with FITC, and correlate microcrack parameters with bone matrix toughness. Reilly & Currey [66] and Boyce et al. [67] compared damage morphologies of bone loaded under tension and compression. Diab et al. [23] (Fig. 6) used the technique on basic fuchsin stained fatigued human bone, to evaluate linear microcrack and diffuse damage morphologies. O'Brien et al. [68] compared elliptical microcracks in human rib sections to reconstructions from serial histological sections and theoretical predictions [69], noticing similarities in all the observations. More recently, Poundarik et al. [14] imaged microdamage in fatigued bone specimen at higher magnification and showed the presence of multiple submicron cracks and dilatational bands (Fig. 7) within diffuse damage regions.

Other studies have also looked at microdamage formation in cortical bone tissue surrounding a screw insert. Like fatigue microdamage, both linear microcracks and diffuse damage have been visualized in bone surrounding screw implants. A recent study [70] examined the association between resorption cavities, and microdamage induced during the placement of self-tapping titanium screws into the tibial diaphysis, in a New Zealand white rabbit model. LSCM showed that resorption pits were especially in contact with linear microcracks. In another recent study [71] the authors used LSCM to compare tissue organization in bone remodeled following microdamage formation. On examining tissue types from equine, murine and bovine bone, they concluded that osteoblasts use the primary scaffold-like tissue to facilitate the deposition of bone matrix that is well organized and mechanically competent. Without a substrate that allows osteoblasts to align and deposit the

new matrix collectively, osteoblasts act in isolation and fail to form matrices with long-range order, or hierarchy, as seen in bone.

Finally, the use of multiple fluorescent antibody stains can reveal detailed information about the role of the organic matrix in microdamage formation [14]. As opposed to using band-pass or long-pass filters that permit the transmission of signals over large wavelength intervals, the use of multispectral confocal microscopy allows for acquisition of multiple and discrete spectral wavelengths allowing retention of only the wavelengths that have maximum emission signal. This approach minimizes spectral overlap between multiple stains, that may not otherwise be eliminated using conventional filters. In order to determine optimal spectral regions, each stain's emission spectrum is characterized and an effective use of controls for determining imaging parameters is critical for reliable data acquisition. Use of multispectral confocal microscopy was used by Poundarik et al [14] to demonstrate the association of non-collagenous proteins osteocalcin and osteopontin with regions of diffuse microdamage of fatigued bone specimen (Fig. 8). Sections stained with basic fuchsin were incubated in primary antibodies for osteopontin (raised in rat) and osteocalcin (raised in mouse). Following incubation, the samples were rinsed and incubated in secondary antibodies for OC and OPN including a donkey anti-rat Alexa Fluor 488 and a goat anti-mouse Alexa Fluor 405. The excitation/emission wavelengths (nm) for each stain were; basic fuchsin=543/560, OPN=495/519, OC= 402/421. The colocalization of these proteins with regions of diffuse damage containing dilatational bands is strongly suggestive of their role in bone quality [14].

MicroCT

Conventional methods used to visualize and quantify bone microdamage involve sectioning (histology) and imaging of histological sections through transmitted light or fluorescence microscopy techniques, using basic fuchsin stain or fluorochromes [19, 46, 55–58,65–69]. However, these methods are destructive, i.e. do not preserve the 3-D structure of bone [44]. The preservation of 3D architecture is particularly important when studying cancellous bone, where microdamage is the result of strong interplay between material quality and trabecular microarchitecture [43]. Recent studies have investigated non-invasive, 3D imaging techniques requiring contrast agents, to view bone microdamage, in vitro using lead sulfide [72], lead uranyl acetate [73], barium sulfate (BaSO_4) [74–76], iodinated molecules [77], and sodium fluoride (Na^{18}F) [78, 79] as contrast agents with higher X-ray attenuation than the extracellular matrix of bone.

Use of lead based staining was adapted from protocols developed on 500 micron thick sections, for 2D electron microscopic evaluation of microdamage [80]. Tang et al, [73] used lead uranyl acetate to bulk stain and image microdamage in femoral cancellous bone specimens (100–200 μm thick trabeculae), at a 10 μm voxel resolution using microCT (Fig. 9). For staining, the specimens (previously subjected to compressive mechanical tests) were soaked in 70% ethanol solution containing a lead uranyl acetate complex for 14 days, allowing proper attachment of the stain to exposed surfaces. The samples were subsequently immersed in 1% ammonium sulfide in acetone for a week to fix the stain. Thresholds were adjusted based on empirical evaluation of attenuation properties prior to imaging. Because

stains like lead–uranyl acetate selectively label bone microdamage, total microdamage accumulation can be evaluated by computing the damaged volume fraction (DV/BV). Unlike stereological measures of damaged regions that are obtained from 2-D measurements, DV/BV is a true volumetric measure of microdamage [73]. Furthermore, Tang and Vashishth [81], used the damage surface (DS) to damage volume (DV) ratio (DS/DV) to distinguish between linear microcracks and diffuse damage. Because linear microcracks are plane like, DS/DV for this damage morphology approaches infinity. In contrast, diffuse damage is more spherical and DS/DV for the diffuse damage morphology is finite. Moreover, there is a continuous spectrum of damage morphologies that span between linear microcrack and diffuse damage. DS/DV therefore provides a numerical index of damage morphology. Tang and Vashishth and Karim et al, [82] showed that this index relates to and explains the mechanical properties of bone. Recently, lead uranyl acetate staining has also been used to visualize microdamage due to fatigue and monotonic loading at the nanoscale, using transmission x-ray microscopy (TXM) [83]. Although destructive, and needing extensive sample preparation, TXM allows examination of microarchitectural features at finer resolution (~10nm) when compared to microCT (~10µm). Furthermore, partial volume effects that result in an exaggerated visualization of damage volumes, in microCT can be avoided by the use of TXM. The finer resolution also allows imaging of the canalicular network, and allows an evaluation of its role in bone microdamage.

Contrast agents like barium sulfate [74–76] are also increasingly being used for radiological evaluation of microdamage, at a resolution of 10µm. The staining is a result of the following precipitation reaction: $\text{BaCl}_2(\text{aq}) + \text{Na}_2\text{SO}_4(\text{aq}) \rightarrow \text{BaSO}_4(\text{s}) + 2\text{NaCl}(\text{aq})$. Barium and sulfate ions from two independent solutions diffuse into voids within the tissue including microdamage and vasculature. These spaces provide nucleation sites for precipitation of barium sulfate on tissue surfaces [74]. Turnbull et al [76] used stain volume (SV) to total volume (TV) as a quantitative measure of microdamage in rat femora, loaded in fatigue, to 5 and 10% degradation in secant modulus (Fig. 10). Microdamage staining using BaSO₄ has been validated using basic fuchsin, energy dispersive spectroscopy and back-scattered SEM. However, more work using this promising technique, is needed to reduce variability in microdamage assessment as compared to other more established methods [45, 46, 66, 67, 72, 73].

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) allows observation of microdamage at the sub-micron or nanoscale level, due to its superior magnification and depth of field over other light microscopies [22, 28, 50, 80]. In particular, SEM allows a thorough evaluation of fracture mechanisms that dissipate energy during fracture processes. Because SEM involves the use of an electron beam to visualize sample surfaces, it is required that sample surfaces are conductive in order to avoid charge build-up that may result in poor image quality and artefacts. Most non-conductive materials, including bone, need to be coated with a layer of conducting material (gold, platinum, etc) prior to imaging.

Contrast agents like lead uranyl acetate have been used to facilitate the visualization of microdamage in bone through back-scattered electron microscopy [84]. Akin to microCT

techniques where x-ray attenuation results in image contrast, electron microscopy leverages compositional differences between the lead based stain and bone matrix to identify microdamage. Back-scattered electron microscopy is used to distinguish areas of different elemental compositions. Regions of bone that are electron dense (such as areas rich in lead uranyl acetate) will appear brighter, due to higher electron density, as compared to areas of bone, rich in mainly calcium and phosphorus from bone mineral.

SEM is also routinely used in the qualitative analysis of fracture surfaces, otherwise known as fractography. Wise et al. [85] assessed the fracture surfaces of the bones of multiple mice strains. Femora were subjected to three point bending tests and comprehensively evaluated for the presence of bone microdamage on tensile and compressive sides of the fracture surface. Both forms of loading are known to generate different microdamage morphologies, with diffuse damage being more prominent on the tensile side and linear microcracks on the compressive [86]. Fiber bridging, interlamellar separation and microcracking were identified as the major toughening mechanisms in the mice strains examined. Interlamellar separation or delamination, ruptured collagen fiber bundles and osteon pull-out have been observed in multiple studies employing electron microscopy [28, 87, 88]. Vashishth et al. [28] suggested, through fractographic analysis, that bone fracture involves separation and pull-out of mineralized collagen (Fig. 11) [28].

Interfaces in bone have been shown to be more ductile and thereby have greater propensity to deform. At the osteonal level, crack deflection brings into play interfaces that are contained in the bone matrix like lamellar interfaces and cement lines [27, 89]. A transverse/radial crack passing through the bone matrix may deflect along the interface before reverting back to its original direction [24]. Crack deflection increases the energy dissipated during crack propagation by increasing the path the crack has to travel through the matrix. Investigations on fractured cortical and trabecular human bone has revealed significant information on the nanoscale damage [24–26]. Crack bridging mechanism (Fig. 12) between fiber bundles or lamellar interfaces is strongly evident in regions of damage and has a major role in crack growth prevention. Crack bridging mechanisms [26] inhibits crack growth through the presence of bridges that span the crack. These bridges, in the form of non-collagenous proteins, collagen fibers or uncracked ligaments, resist the ability of the crack tip to propagate by attempting to hold the crack faces together. In either case, transfer of stress through the bridges reduces the stress concentration at the crack tip and stalls its growth further into the matrix.

Atomic Force Microscopy (AFM)

The atomic force microscope is an extremely versatile imaging tool. In AFM, a cantilever traverses the material surface, and its deflections are transduced into electrical signal by the means of a laser (red beam) that deflects off the top of the cantilever onto a position sensitive detector. Measurement of the position of the beam at any point of time allows determination of the position of the cantilever tip on the sample surface. The nature of deflection, as indicated by the motion of the laser (up-down or left-right), on the detector, reflects bending or torsion of the cantilever. Two most common modes of operation for imaging sample topography are the contact mode and tapping mode. The contact mode of

operation requires constant tip-sample contact that results in cantilever deflection and thus a force. Sample topography is accurately mapped by maintaining the contact force at a constant value by the adjusting cantilever position. As biological samples are soft and easily damaged, the alternative tapping mode is preferred to scan their surfaces. In this mode, the cantilever oscillates at a resonant frequency as it scans the sample surface. The tapping mode ensures minimal tip-sample interaction.

AFM has been widely used in imaging bone microdamage and fracture surfaces of human bone. Topographical analysis of fractured bone surfaces reveal mineral particle sizes of 50–250 microns [90] that is consistent with other studies on bone [14]. Imaging studies by Hansma and coworkers strongly suggest the existence of a glue-like phase in bone that prevents the separation of individual collagen fibrils [10, 91]. Further investigations have provided evidence for a nanoscale damage mechanism called sacrificial bonding, which involves divalent cations, in particular Ca^{2+} ions, and the extrafibrillar matrix (like osteopontin, a non-collagenous protein), to form and re-form bonds during the process of mechanical loading [92]. Additionally, through AFM investigations, Nicolletta et al. [93] have proposed that the separation of mineralized collagen fibrils is governed by constituents of the organic matrix (such as non-collagenous proteins) that bridge the microcracks, and play a role in resisting crack growth.

In that regard, recent work has implicated non-collagenous proteins osteocalcin and osteopontin in the formation of bone microdamage. Through the imaging of diffuse damage regions using AFM, it was found that dilatational bands exist in diffuse damage regions of bone (Fig. 13). The authors concluded that at the nanoscale, dilatational band formation results from the tensile loading of non-collagenous matrix proteins, including osteocalcin and osteopontin, both of which are known to bind adjacent mineral aggregates through calcium ion mediated complexes [14]. In the absence of continuous tensile loading, the deformed protein-complexes may ‘close’ or completely recover the deformation to the initial unloaded state in a time dependent manner (anelastic behavior). However, under continued loading, inelastic deformation of the protein complexes results in the formation of dilatational bands. Subsequent loading of the dilatational bands will cause shearing and rupture of mineralized collagen fibrils and formation of diffuse damage.

In summary, this review describes various techniques that have been frequently used, and have the potential to further the study bone microdamage. Applicability of each technique will depend on the nature of required information and specimens availability. Whilst bulk staining is a quick and useful way to confirm and assess linear microcracks and diffuse damage, other modalities allow a more in depth evaluation. MicroCT and confocal microscopy (that can be used on bulk stained samples) allow visualization at the micron scale, and are useful tools to understand the 3D nature of bone microdamage. Other advanced techniques like TXM, SEM and AFM lack the ability to investigate large regions of microdamage, but allow users to probe in extensive details, the nanoscale mechanisms that underlie microdamage formation in bone. Ultimately, we recommend the use of multiple imaging modalities along with other mechanical evaluation of damage (for example by fracture mechanics [7] or indentation testing [94] to obtain mechanistic information about bone quality and microdamage formation, across the scales of hierarchy in bone.

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Figure 1.

Scales of hierarchy of bone from the osteonal level to the mineral aggregate level at the nanoscale. Each scale of hierarchy exhibits a predominant form of microdamage mechanism that aids in resisting crack propagation within the bone matrix. [Poundarik et al. PNAS 2012]

Figure 2.

Images showing **a)** bone microcrack stained with toluidine blue and **b)** subsequently, with basic fuchsin. **c)** an artefactual crack stained with both toluidine blue and basic fuchsin, and **d)** a loading microcrack stained with only basic fuchsin.

Figure 3. Light microscopy images of linear microcrack in **a)** cancellous and **b)** cortical bones. Diffuse damage in cancellous **c)** and cortical **d)** bone. [Vashishth, 2007]

Figure 4.

Stages of micro-crack toughening. In stage I, the extension of the main crack (da) results in the formation of a frontal process zone and is accompanied by an increase in crack growth resistance (V_p). The process zone is a collection of submicron cracks (linear microcracks and diffuse damage). Their formation increases energy dissipation and reduces the elastic modulus of the region ahead of the main crack tip. Propagation of the main crack through the frontal process zone with a reduced elastic modulus, exhibits decreased crack growth resistance (stage II). The graphs on the right of the above figure show how velocity of the

crack (vertical axis) increases as the material ahead of the crack tip softens and then decreases as the crack moves on to newer parts of the matrix. [Vashishth et al., 2000].

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Figure 5.

3-D rendering of damaged bovine trabecular bone specimen imaged using the serial milling technique. **a)** Bone volume imaged with UV excitation and DAPI emission filter and **b)** microdamage stained with xylene orange imaged using a TRITC filter set. **c)** and **d)** show magnified volumes of bone and microdamage. Finally, bone volume and microdamage are overlaid in **e)** for a more comprehensive visual evaluation. [Kazakia et al. 2007]

Figure 6. Diffuse damage in human cortical bone as seen by light microscopy (**left**) and confocal microscopy (**right**). Scale bars = 50 μm . [Diab and Vashishth, 2007].

Figure 7.
Dilatational bands present in diffuse damage regions under confocal microscopy (scale bar = 20 microns). [Poundarik et al. 2012]

Figure 8.

Immunohistochemical staining of fatigued specimen. Associations of dilatational bands (**above**) and diffuse damage (**below**) with osteocalcin (blue) and osteopontin (green). As seen in the micrographs, osteocalcin and osteopontin co-localize with the bands (highlighted red by basic fuchsin, which stains for damage), indicating their possible role in band formation. [Poundarik et al. 2012]

Figure 9. MicroCT image of lead uranyl acetate stained trabecular bone specimen. Darker regions are indicative of microdamage. [Tang and Vashishth, 2007]

Figure 10. 3-D microCT images of (**top**) control and (**middle**) 5% and (**bottom**) 10% fatigue loaded rat femora, where microdamage can be seen stained with barium sulfate. [Turnbull et al. 2011]

Figure 11.

Fractographic SEM images of fractured human bone at the osteonal level (**left**), lamellar level (**middle**) and the collagen fibril level (**right**), confirm the mechanisms at play across multiple hierarchical levels in human bone. [Vashishth et al. 2000]

Figure 12. Scanning electron microscopy image of fractured bone surface showing collagen fiber bridging. [Nalla et al. 2003]

Figure 13. Formation of dilatational bands in damaged bone studied via atomic force microscopy
 The images show the progression of dilatational band formation in bone. Mineral aggregates connected by osteocalcin and osteopontin complex separate to form dilatational bands (encircled). A single dilatational band as imaged by atomic force microscopy (**left**). Growth of dilatational bands subsequently leads to the rupture and separation of mineralized fibrils (**middle**) and formation of diffuse damage regions (**right**). [Poundarik et al. 2012]