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Title: Rupture of Osteocyte Processes Across Microcracks: the Effect of Crack Length and Stress

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Rupture of Osteocyte Processes Across Microcracks:

the Effect of Crack Length and Stress

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Abstract

Bone cells are connected to one another in a network, *via* their dendritic cellular processes. Previously, we hypothesised that these processes could be ruptured by microcracks. We proposed this as a mechanism by which osteoctyes could detect the presence of microcracks. In order for this mechanism to be effective, the number of ruptured processes would have to increase with microcrack length and also with the applied cyclic stress applied. This paper presents for the first time experimental data which shows that this is indeed the case. We examined samples of bovine, ovine and murine bone *ex vivo* and observed processes passing across crack faces: some were still intact whilst others had ruptured. The number of intact processes per unit crack length decreased significantly with increasing crack length, and also decreased in samples which had been tested *in vitro* at higher stress levels. A theoretical model which we had developed previously was able to predict the overall magnitude and general trends in the experimental data. This work has provided further support for our "scissors" model which proposes that microcracks can be detected because they disturb the osteocyte network, specifically by rupturing cellular processes where they pass across the crack faces.

Keywords
Bone: microcracks; osteocytes; processes; fracture

1. Introduction

The mechanism by which osteocytes detect microcracks in the bone matrix has not yet been established. This is a critical question in the field of mechanobiology, because it is vital to the maintenance of healthy, load-bearing bone. Bones contain large numbers of microcracks (Frost, 1960; Mori and Burr, 1993) which form due to fatigue as a result of cyclic loading. These microcracks are repaired by the actions of BMUs: orchestrated systems of osteoclasts and osteoblasts which remove old, damaged bone and replace it with new bone. If this repair process were to cease, our bones would fail by stress fractures within a matter of months (Taylor *et al.*, 2004). Microdamage has also been implicated in bone adaptation: the number and size of microcracks could act as a signal to initiate bone deposition in response to high stresses or bone resorption in the case of disuse (Prendergast and Taylor, 1994).

It has been known for some time that BMUs are targeted towards damage (Burr and Martin, 1993; Burr, 2002; Lee *et al.*, 2002), i.e. the removal of bone is not a random process but is directed to places where microcracks exist. This implies that something in the bone is able to detect the presence of these cracks, and the only likely candidate for that role is the osteocyte network. Circumstantial evidence for the role of osteoctyes in damage control includes increased hip fracture linked to reduced osteocyte numbers (Dunstan *et al.*, 1993) and increased microcrack accumulation related to areas of osteocyte reduction (Qiu *et al.*, 1997). A number of theories have been advanced to explain how this detection process might work (Klein-Nulend *et al.*, 2005). For example it is known that osteocytes, along with many other cell types, are capable of detecting deformations in their cell walls, making them sensitive to changes in substrate strain and fluid flow (You *et al.*, 2000); microcracks can affect fluid flow by disrupting the canalicular network (Tami *et al.*, 2002). The strain levels which normally occur in the bone

matrix are very small compared to the strains which have been shown to elicit responses, but magnifying effects due to the flow of fluid over the cell wall have been proposed to explain this (Han *et al.*, 2004). Another possible mechanism of strain measurement is the cell's primary cilium (Temiyasathit and Jacobs, 2010). It has also been found that cells in the vicinity of microcracks tend to undergo apoptosis (Bentolila *et al.*, 1998; Noble *et al.*, 2003; Verborgt *et al.*, 2000), suggesting a potential signalling pathway.

We have proposed a different mechanism, which we call the "scissors" model. Examining the various ways in which cellular material might be affected by the presence of a crack, we proposed that cellular processes spanning the crack might be ruptured. The concept is illustrated schematically in fig.1. Osteocytes are connected together in a network via their dendritic processes, which typically number 50-100 processes per cell (Sugawara *et al.*, 2005). If a microcrack is present, some of these processes pass across the crack, from one crack face to the other. These cracks are typically elliptical in shape and oriented parallel to the osteonal structure, at a slight angle to the bone's longitudinal axis. Under applied compressive loading the crack faces will be pressed together and will undergo relative shear motion. This scissor-like action, we proposed, would rupture the dendritic processes. However, not all processes are expected to rupture: the magnitude of the shear motion depends on location (i.e. distance from the crack tip) and also on the crack length and applied stress. Therefore, we predicted that more processes would be cut if the crack was longer and/or if the stress applied to the bone was higher. We showed that, theoretically, the number of ruptured processes could be used as a signal to indicate whether a crack needed repairing or not. In our theoretical model (Taylor *et al.*, 2003) a significant signal arose if the crack length was greater than 100μm for normal physiological loading, and this critical crack length became smaller for higher stresses corresponding to strenuous exercise.

Our previous study included some microscopy which established that cracks had unbroken processes passing across them whilst other processes appeared to be ruptured (Hazenberg *et al.*, 2006). However we did not attempt to quantify the number of broken and intact processes as a function of crack length or stress.

The aims of the present work were as follows:

1) To examine cellular processes passing across cracks in more detail.
2) To quantify the number of broken and intact processes as a function of:
    a) crack length; b) applied stress and; c) species type
3) To compare these experimental results with predictions using the theoretical model which we developed previously.

2. Methods and Materials

We obtained samples of cortical bone from cows, sheep and mice. These materials were already available from other projects being conducted in the Trinity Centre for Bioengineering. In all cases the animals concerned were skeletally mature and free from bone diseases and the bones had been subjected only to normal *in vivo* loadings. Bones were stored in a freezer at -20^{o}C. Samples 1cm thick were

obtained using transverse cuts. Some bovine samples were subjected to further loading *in vitro* by applying cyclic compressive stresses at a frequency of 2Hz. These samples were machined into cylindrical, waisted specimens and tested according to a protocol which we have used extensively in the past: see for example (O'Brien *et al.*, 2002). The stress cycle chosen was sinusoidal with a ratio between the maximum stress and minimum stress of 10 to 1, the stress range being defined as the difference between the maximum and minimum stress. Samples were subjected to one of three loading conditions: 70MPa for one million cycles, 100MPa for 30,000 cycles and 110MPa for 30,000 cycles. For comparison, the maximum stress normally experienced *in vivo* is of the order of 40MPa and the number of cycles experienced *in vivo* is of the order of one million per year.

Samples were prepared for examination in a scanning electron microscope (SEM) using a staining technique developed by Schaffler *et al* (Schaffler *et al.*, 1994). They were fixed in 4% paraformaldehyde solution for 24 hours at 4^{o}C and washed three times in phosphate buffered saline (PBS). In order to confirm that the cracks being examined had occurred *in vivo* rather than during sample preparation, a heavy metal stain was applied by placing the samples into a solution of 70% acetone containing lead-uranyl complex (20% lead acetate; 8% uranyl acetate). They were then rinsed in 70% acetone and placed into a solution of 1% ammonium sulphide in acetone for 7 days to form lead and uranyl sulphide precipitates, followed by rehydration. As a result the original *in vivo* cracks became stained in a manner which was visible in the scanning electron microscope, allowing us to distinguish them from preparation artifacts (see fig.2).

Samples were cut into transverse slices 100μm thick using a diamond saw (Struers, Minitron) and ground flat using silicon carbide paper. After fixing in paraformaldehdye (see above) they were washed in a series of alcohol solutions of increasing concentration up to 100%. They were then subjected to critical-point drying using liquid CO_2 which was vented off, giving a dry sample without the structural damage which can result from air drying.

They were examined in an SEM using a backscattered electron detector. Fifty cracks were examined from each of six groups: bovine *in vivo*; ovine *in vivo*; murine *in vivo*; bovine loaded *in vitro* at 70MPa, at 100MPa and at 110MPa.

3. Results

SEM images showed cellular processes spanning the crack: some were intact whilst others had ruptured, (see fig.3). It was rather difficult to observe ruptured processes but easy to observe intact ones, so we counted the intact ones. Fig.4 shows results for 50 cracks in bovine bone which had experienced normal *in vivo* loadings. In presenting this and subsequent data we have normalised the number of processes by dividing by the length of the crack, giving results in terms of the number of intact processes per millimetre of crack length. This is plotted as a function of the crack length. Logarithmic plots are used because the quantities measured varied by several orders of magnitude. As fig.4 shows, there was a lot of scatter in the plot (the reasons for which will be discussed below) but there was a clear trend in which the number of intact processes per unit crack length

decreased with increasing crack length. This clearly indicates that a greater proportion of processes have ruptured in the larger cracks.

Fig.4 also shows a prediction line drawn using our theoretical model, which will be discussed below. Fig.5 shows results from samples which had been subjected to *in vitro* cycling at various stress levels higher than those normally encountered *in vivo*. Again there is considerable scatter but a clear tendency towards smaller numbers of intact processes. (It should be noted that the points placed along the axis at a value of 1 process/mm were in fact cracks in which no intact processes were found: it's not possible to plot a value of zero on this logarithmic scale). To check the statistical significance of these results we divided the cracks into two groups according to whether they were greater or less than 100μm in length. Fig.6 shows the average number of intact processes per unit crack length for these two groups, for each of the four stress levels. Table 1 shows the p values obtained using an unpaired t-test. There are highly significant differences ($p<<0.05$) between the data obtained from cracks loaded *in vivo* and cracks subjected to higher stress levels. For the *in vivo* data there is a significance difference between the shorter cracks and the longer ones and this is also true for one of the elevated stress levels (110MPa). For the other two stress levels the p values are 0.1 and 0.11, indicating a likely difference. All of these p values could be reduced by carrying out a more sophisticated analysis which would take account of the fact that there is a general tendency for values to decrease with crack length: this tendency itself creates some of the apparent scatter. In this case however we felt that the results of this simple t-test were sufficient to make the point.

Figure 7 shows results from ovine and murine bone subjected only to *in vivo* loadings. The data are quite similar but there is a tendency towards smaller crack lengths and more intact processes per millimetre with decreasing animal size. T-tests conducted on the entirety of data from each group showed a highly significant difference ($p = 0.009$) between bovine and murine, and p values of 0.06 between bovine and ovine and 0.17 between ovine and murine.

4. Theoretical Model

We compared these data to predictions made using a theoretical model described in an earlier publication (Taylor *et al.*, 2003), in which we developed equations to predict the relative movements of the crack faces using fracture mechanics theory and computer simulations. The shear displacement δs of each crack face is given by:

$$\delta s(x) = \left(3.25 \left(\frac{b}{a} \right)^{0.1} \frac{\sigma}{E} (1 - \upsilon^2) \sqrt{(a^2 - x^2)} \right) \sin(\beta) \cos(\beta) \qquad (1)$$

Here σ is the applied stress: we assume that the maximum value which occurs *in vivo* under normal activities is 40MPa. E and υ are the Young's modulus and Poisson's ratio of bone respectively. The values of these elastic constants have been measured for cortical bone from many different species. The values obtained vary considerably depending on the test method used as well as species type and bone location (see for example (Yamada, 1970; Currey, 2002; Chattah *et al.*, 2009)). Taking account of results from various sources we decided to use E values

of 20GPa, 15GPa and 9GPa for bovine, ovine and murine bone respectively; we used a Poisson's ratio of 0.39 throughout. The crack was assumed to be elliptical in shape, with major and minor axes *b* and *a* respectively: we assumed that *b/a* = 4 (Taylor and Lee, 1998). The parameter x describes distance along the crack face, measured from the centre. The largest displacements occur near the centre of the crack, falling to zero at the crack tip. The angle β describes the inclination of the crack within the bone; it is the angle between the major axis and the transverse plane. We used a value of 70^o (Taylor and Lee, 1998). This equation applies only along the centre line of the crack, i.e. along its minor axis. The significance of this will be discussed below.

To predict the number of broken and intact processes, we assumed that a process passing across between the crack faces will be ruptured if the relative shear displacement of the two faces 2δs is larger than 0.2μm, this being the typical diameter of a process. For convenience in manipulating the equation we can group together a number of constants as follows:

$$C = \frac{\delta s}{3.25\left(\frac{b}{a}\right)^{0.1}\left(\frac{1}{E}\right)\left(1-\upsilon^2\right)\sin\beta\cos\beta} \tag{2}$$

Equation 1 can then be simply rewritten as:

$$\frac{C^2}{\sigma^2} = a^2 - x^2 \tag{3}$$

When δs takes the critical value of 0.1μm, x takes a critical value x_c such that all processes lying at $x<x_c$ are predicted to break whilst all processes at $x>x_c$ will remain intact. If the average distance between processes is d then the number of intact processes per unit crack length is:

$$\#_{\text{int}} = \frac{\left(a - x_c\right)}{ad} \tag{4}$$

Therefore:

$$\#_{\text{int}} = \frac{1}{d}\left(1-\frac{1}{a}\right)\sqrt{a^2 - \frac{C^2}{\sigma^2}} \tag{5}$$

The value of d depends on the number density of osteocytes and the number of processes per osteocyte. We assumed 30,000 osteocytes/mm^3 for bovine bone, 40,000 for ovine bone and 90,000 for murine bone, following the trend of experimental data reported by Mullender et al (Mullender *et al.*, 1996). The average spacing of processes was estimated by assuming 100 processes per osteocyte (Sugawara *et al.*, 2005) , assuming that the osteoctyes are regularly spaced, and noting that, on average, one sixth of the processes coming from a cell will pass across the plane of a nearby crack.

5. Discussion

The experimental data reported above have established, for the first time, that the number of cellular processes which are ruptured is a function of both crack length and applied stress. Cracks less than 100μm long subjected to normal physiological loads have almost all their processes still intact. Longer cracks have many more of their processes ruptured than small cracks, and the application of cyclic stresses above physiological levels increases this number significantly. This lends considerable weight to our "scissors" hypothesis: if these ruptures can be detected by the cell, then their number provides a very convenient way to assess the severity of the crack and the need to repair it. Evidence that ruptures to the cell network can initiate cell signalling pathways has already been provided by our recent work on cell networks cultured *in vitro* in 3D gel matrices (Mulcahy *et al.*, 2011).

Our experimental results (figs 4, 5 and 7) show considerable scatter: the number of intact processes for a given crack length varies by more than an order of magnitude. It can be shown that most of the scatter arises simply from the random locations of osteocytes and processes. The distribution of processes along the crack can be considered to be a Poisson distribution because the diameter of a process is very small compared to the length of the crack, so a process constitutes a relatively rare event. On fig.4 we have indicated the scatter band that would be expected to arise simply due to this Poisson distribution. The two dashed lines indicate the 10% and 90% limits, so if this were the only source of scatter we would expect 80% of the data points to lie within the scatter band. This analysis demonstrates that, once this source of scatter is taken into account, there is very little scatter arising from any other cause, such as specimen-to-specimen variations or experimental errors.

Scatter will also occur due to variation in a number of parameters which we have identified as affecting the shear displacement δs (equation 1). Applied stress varies with location in the bone and with exercise level for individuals; the angle of inclination of the crack to the bone axis β will vary from crack to crack and we have no data on this variation. Bone's elastic material properties also vary, as will the shape *b/a* of individual cracks. We examined cracks on the surface of cut sections: equation 1 is valid only for the centre line (i.e. minor axis) of the crack, but in practice we are looking at random transverse sections. We assume that rupture of a cellular process occurs if δs is greater than 0.1μm; this value will no doubt vary from one process to another, and we have not taken account of the fact that this displacement will occur cyclically and so may cause fatigue failure. We are counting the number of processes which we can see when looking into the crack: we assume that we would only be able to see processes which are close to the surface, within the average spacing, d. In estimating this value of d we are assuming typical values taken from the literature for the number density of osteocytes and the number of processes per osteocyte, because special techniques are required to measure these quantities accurately.

Our theoretical model predicts the general trends in the data quite well, considering the simplifying assumptions made and the potential sources of scatter and uncertainty in the chosen parameter values. The model has a large number of parameters in it whose values have to be chosen. In all cases we have used parameter values taken from the literature and not from the experiments reported

in this paper, so the predictions are independent of the data. For bovine bone subjected to *in vivo* loadings (fig.4) the prediction line follows the general trend of the data and lies within the scatter band, except for the smallest crack lengths where it tends to overestimate the number of intact processes. For crack lengths less than 100μm the theoretical model predicts that there will be no broken processes at all, hence the horizontal part of the prediction line, and this seems to coincide with a cut-off in the experimental data points. For higher stress levels (fig 5) the number of intact processes is predicted to decrease; the prediction lines mirror the general trend and tend to fall within the data, but the scatter increases considerably owing to the fact that the average number of intact processes is decreasing, to the point where some cracks had no intact processes at all. The predictions for ovine and murine bone (fig.7) were also quite accurate. The larger density of osteocytes in smaller animals such as mice may reflect the need to detect smaller cracks in these bones, due to their much smaller overall bone size.

6. Conclusions

- Osteocytes and their cellular processes can be observed in the vicinity of microcracks. Some of these processes rupture at the point where they pass across the crack faces, whilst others remain intact.
- The number of intact processes per unit crack length decreases significantly with increasing crack length in bone subjected to physiological loadings. This indicates that, in longer cracks, a larger proportion of processes are being ruptured.
- The proportion of broken processes also increases significantly when cyclic stresses above normal physiological levels are applied.
- The general trend of these results can be predicted using fracture mechanics theory based on our "scissors" model.
- These observations support the "scissors" model as a viable mechanism of microcrack detection by the osteocyte network.

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Table 1
Statistical significance for data on bovine bone: "short" and "long" cracks are cracks greater than or less than 100μm long

Comparison	p value
In vivo short cracks v long cracks	1.4×10^{-6}
70MPa short cracks v long cracks	0.11
100MPa short cracks v long cracks	0.095
110MPa short cracks v long cracks	0.007
In vivo v 70MPa (all cracks)	0.0025
In vivo v 100MPa (all cracks)	0.0020
In vivo v 110MPa (all cracks)	0.0056

Figure Captions

Figure 1: Schematic illustration of the "scissors" model. A microcrack is oriented at an angle with respect to the applied stress, which is largely compressive. Cellular processes passing across the crack can be ruptured if the crack face displacement δs is greater than the diameter of the process.

Figure 2: An example of a crack which occurred *in vivo*, stained for examination under the scanning electron microscope.

Figure 3: Scanning electron microscope photograph showing part of a crack, with an intact process (black arrow) and a broken process (white arrow) passing across between the crack faces.

Figure 4: Experimental data showing the number of intact processes per millimetre of crack length, as a function of crack length, for 50 different cracks, in bovine bone which has experienced only *in vivo* loadings. The solid line shows a theoretical prediction based on our "scissors" model. The two dashed lines indicate the expected scatter band (10% and 90% limits) assuming the data conform to a Poisson distribution.

Figure 5: Data and predictions as in fig.5 but including bovine bone which was subjected to elevated stress cycles *in vitro*: 1 million cycles at a stress level of 70MPa; 30,000 cycles at 100MPa and 30,000 cycles at 110MPa. *(Note: data points lying along the horizontal axis, at a value of 1 process/mm, indicate cracks which had no intact processes at all; they are placed here because it's not possible to record a value of zero on the logarithmic scale.)*

Figure 6: Average number of intact processes per unit crack length for bovine bone at four different stress levels. "Short" and "Long" cracks here are cracks whose lengths are less than or greater than 100μm.

Figure 7: Data and predictions for ovine bone and murine bone, subjected to *in vivo* loadings.

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Figure 1
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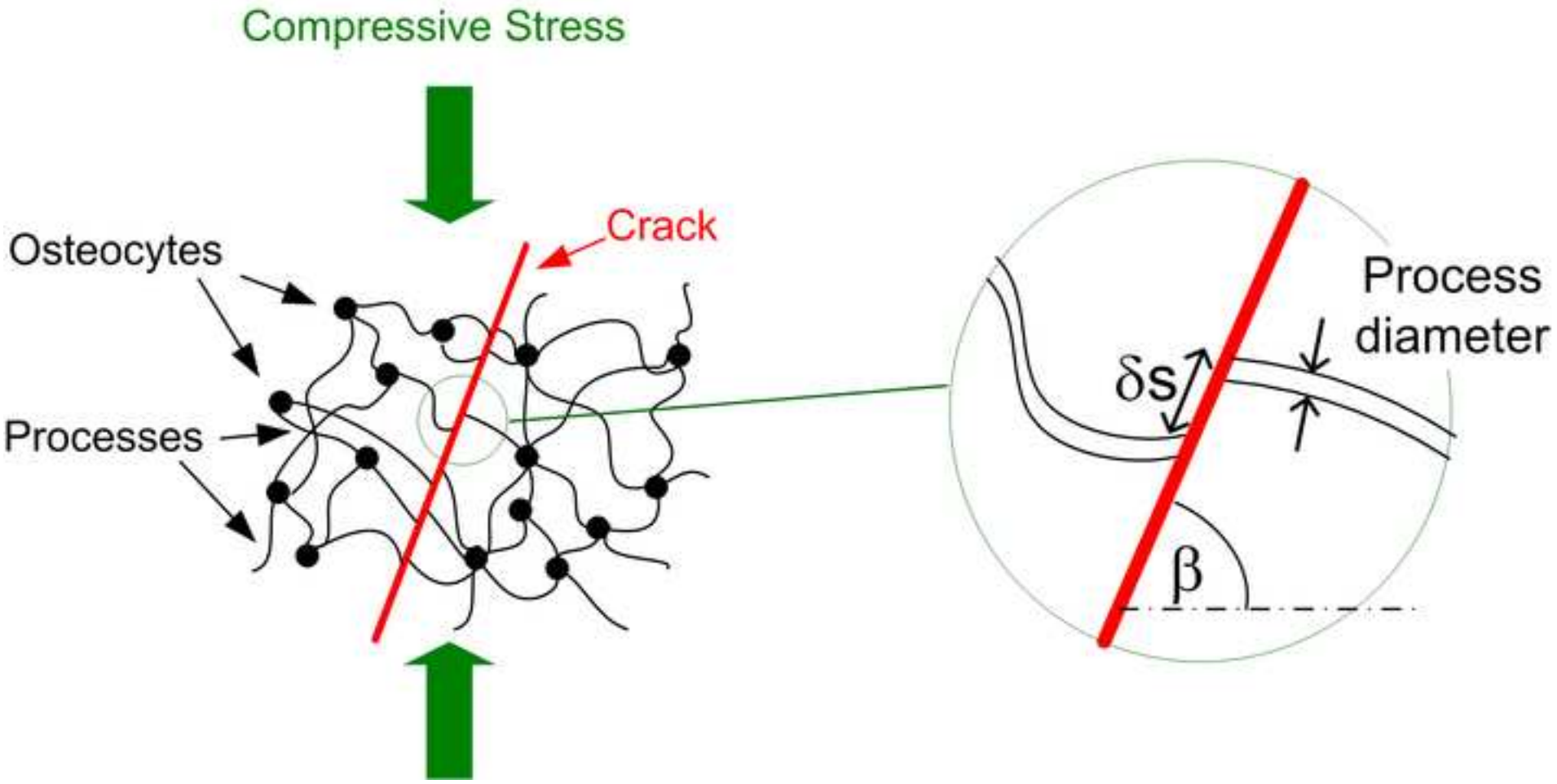

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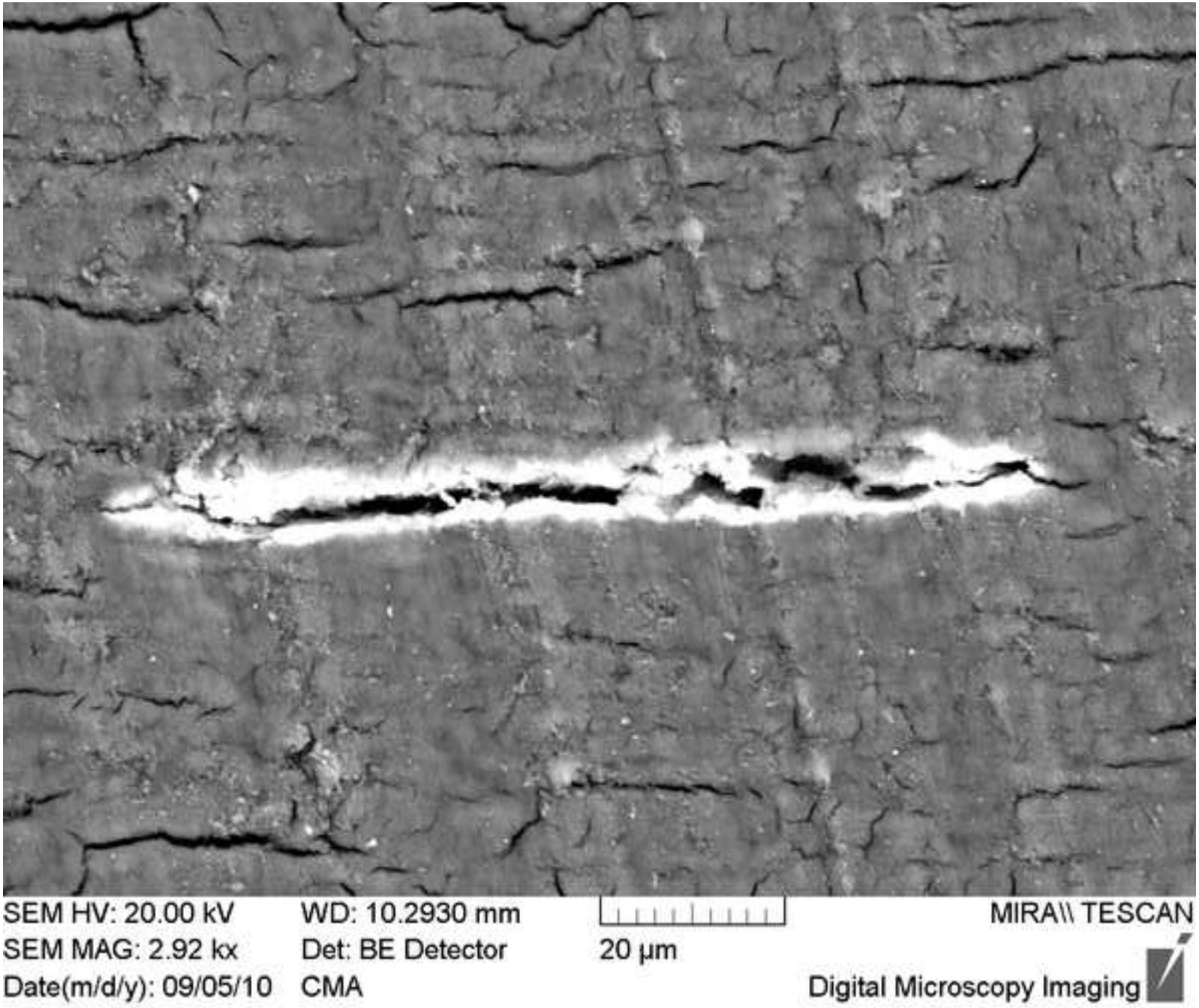

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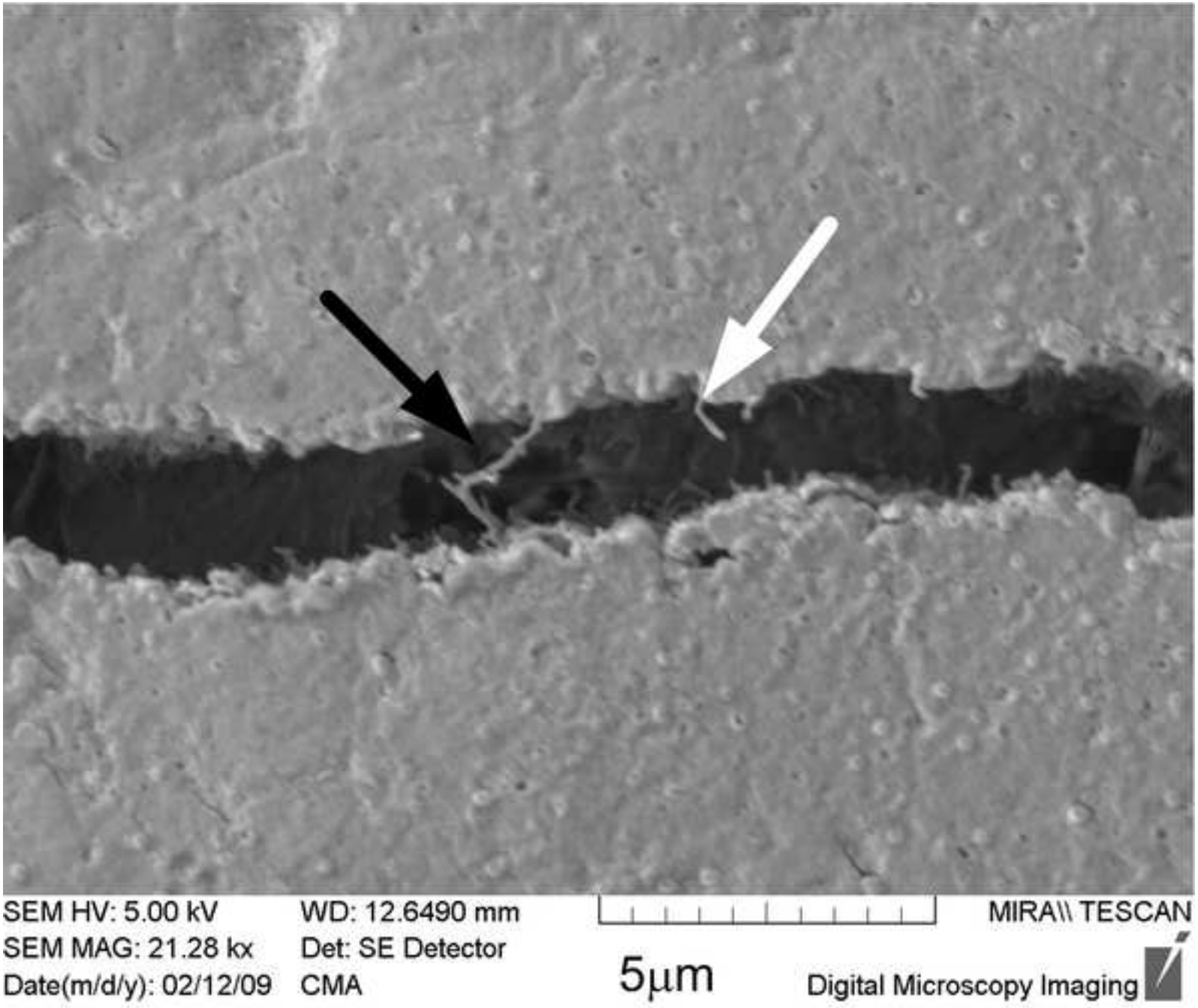

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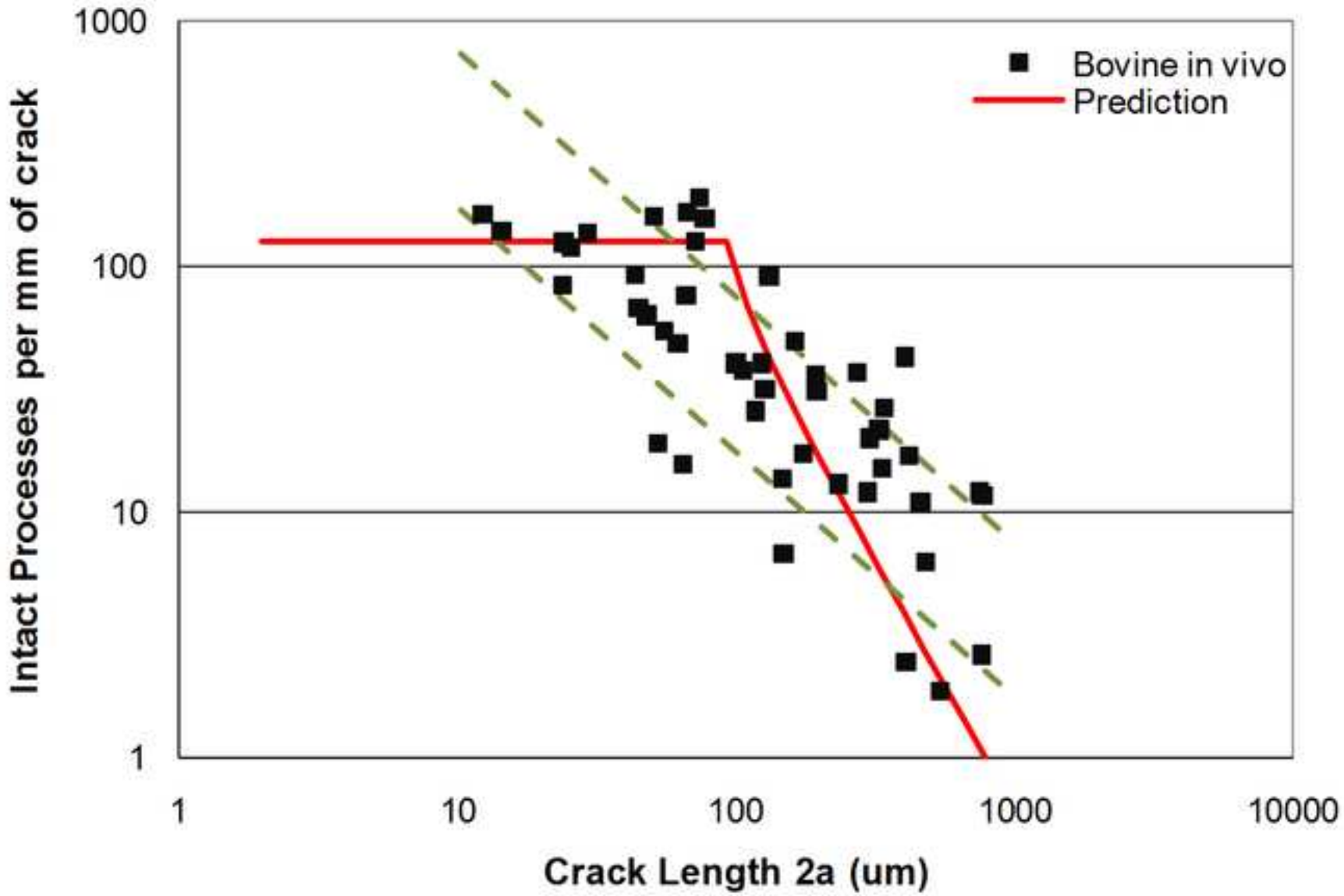

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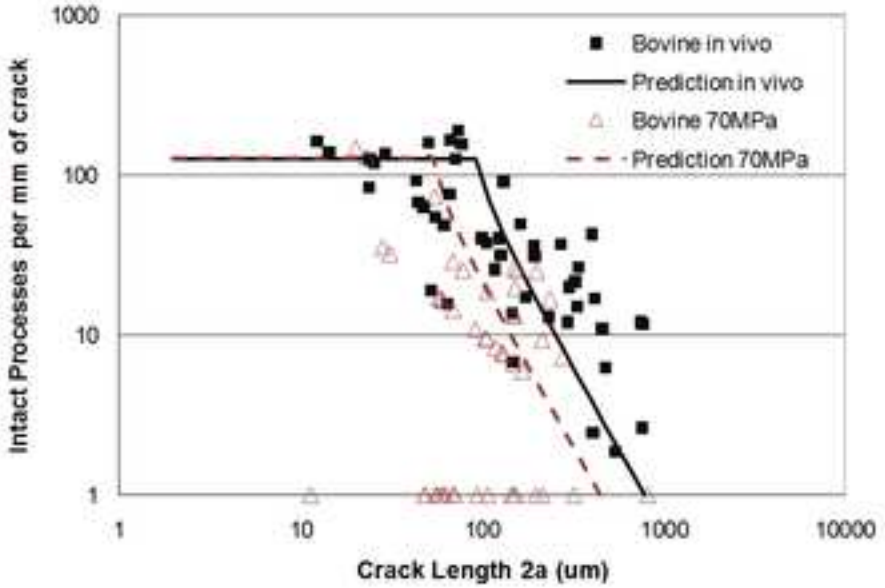

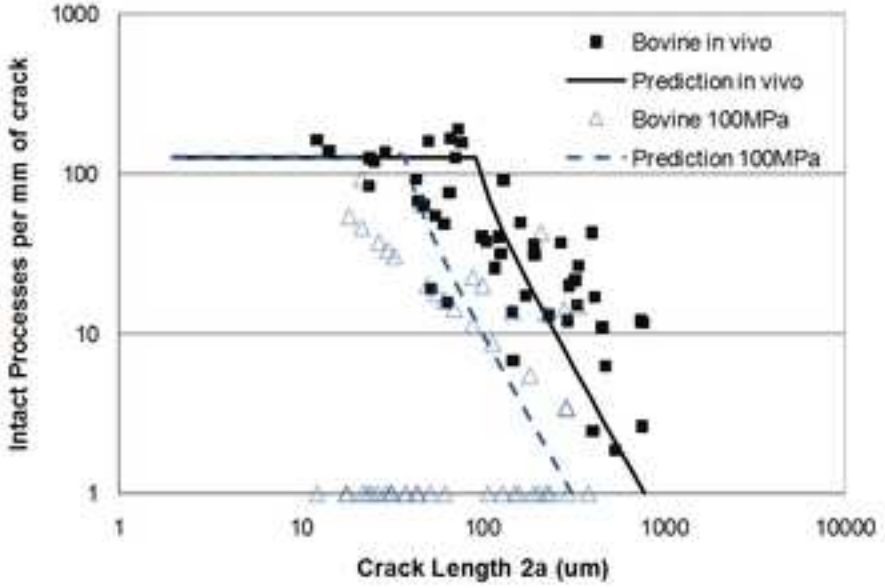

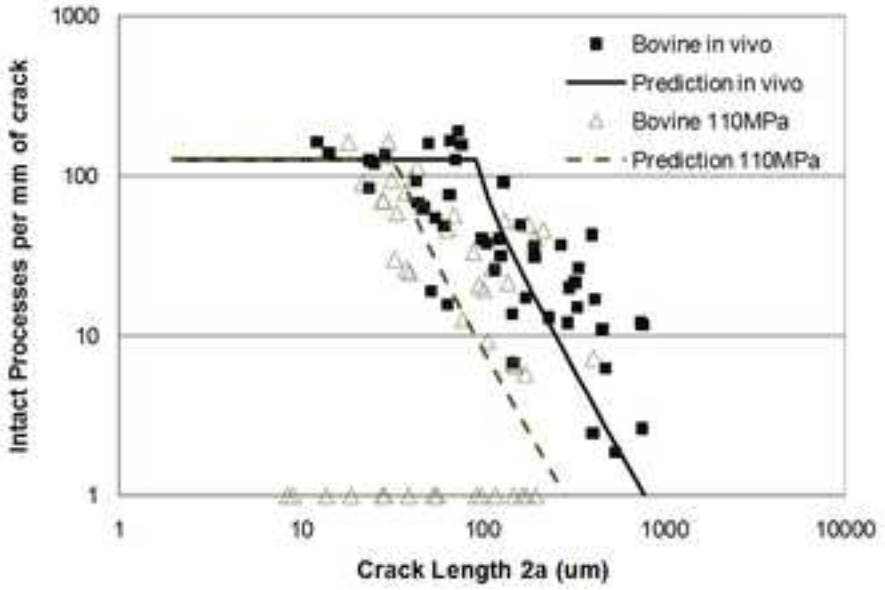

Figure 6

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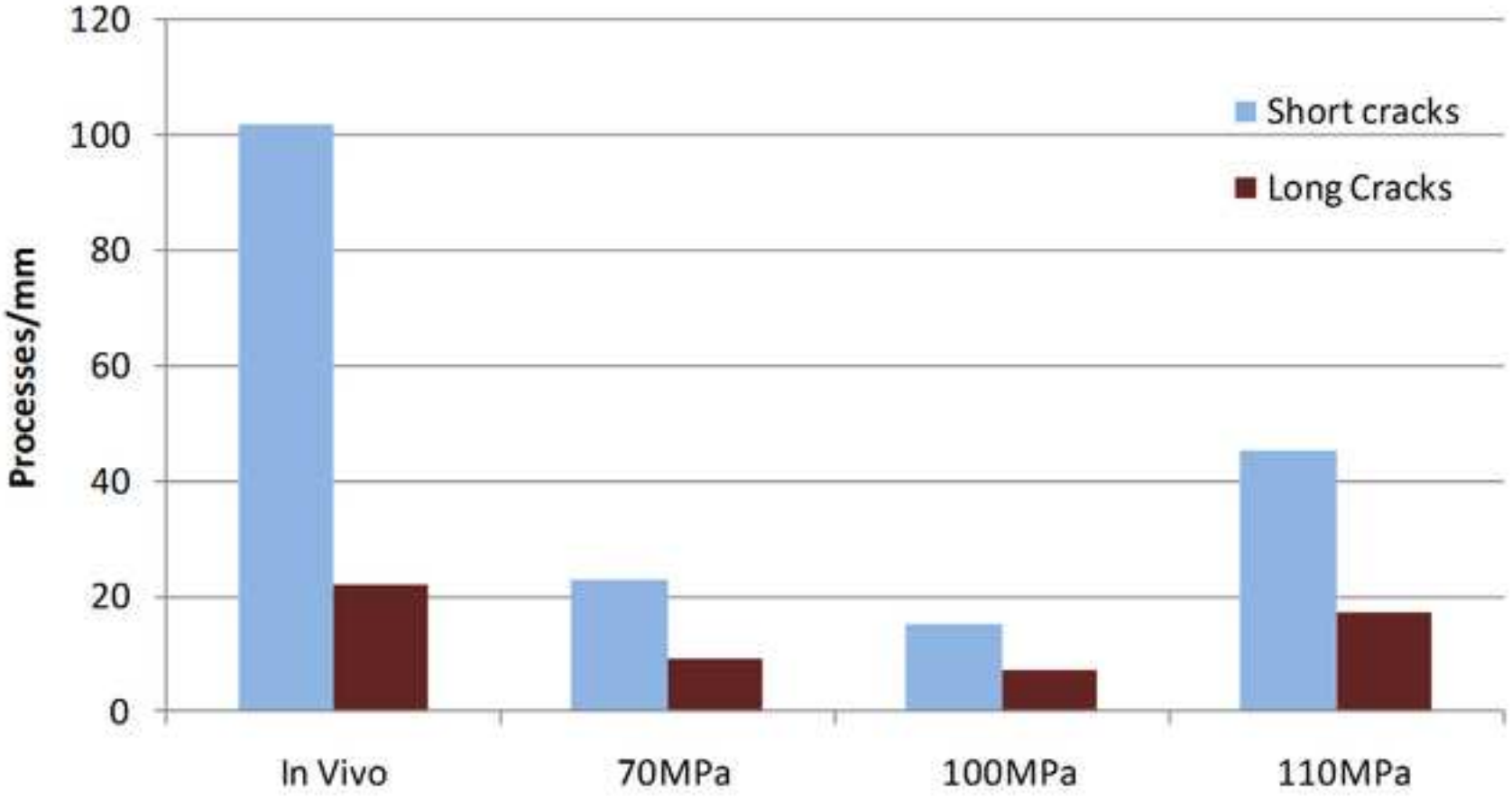

Figure 7
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