

# **Bone mineral density (BMD) and tissue mineral density (TMD) calibration and measurement**

**Method note  
MCT-009**

## Foreword and notice:

### New BMD phantom set, May 2021

In May 2021 Bruker changed its bone mineral density calibration phantom set to a new and improved version. The phantom material remains fine calcium hydroxyapatite (CaHA) powder embedded in plastic resin. However the main difference is that the concentrations of CaHA are changed, giving a wider range of values and therefore improved accuracy of calibration. In addition, the new phantom material has improved uniformity and a lower incidence of defects such as cracks and pores.

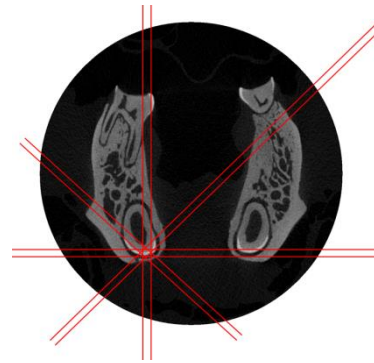
Here are the essential changes that come with the new paired phantom rods:

- **The new paired concentrations are 0.3 and 1.25 g/cm<sup>3</sup> of CaHA.**  
The previous concentrations were 0.25 and 0.75 g/cm<sup>3</sup> of CaHA.
- The rod pair diameters remain the same as before: 2, 4, 8, 16 and 32mm.
- For some pairs such as the 2mm rod pair, the length of the rods is slightly increased (for convenience of handling).
- The phantom sets remain the same as before. The “small” set comprises the 2mm and 4mm rod pairs. The “medium” set comprises the 8mm and 16mm rod pairs. The “large” set comprises the 32mm rod pair. The embedded foil trabecular thickness phantom remains a part of each BMD phantom set.
- The BMD calibration method in CTAn (preferences) remains unchanged. Only the reference BMD values of the new phantoms are now different.
- So when doing BMD calibration – please check that you are using the correct reference BMD values for your phantom set.
- Solid bone has mineral density of about 1.1-1.4 g/cm<sup>3</sup>. Thus a calibration value range of 0.3-1.25 is better than 0.25-0.75 for encompassing natural BMD values. It will also improve densitometry of denser tooth enamel.
- **If you wish to get the new enhanced BMD phantom set, contact us!**

## 1. Introduction

### 1.1. *What is “density” measured by x-ray micro-CT?*

A micro-CT 3D image of an object is basically a 3D map of local x-ray absorption in the object. The reconstruction from multiple angular views provides information on how much x-ray absorption is happening within each cubic voxel element of the scanned volume.



It is important to understand what is meant by “density” in a microCT image. The reconstructed grey-scale intensity of each image voxel does not relate directly to mass density alone, thus do not expect it to correspond with measurements of weight per volume, e.g.  $\text{g.cm}^{-3}$ . The primary entity that is measured is x-ray absorption, defined as the attenuation coefficient in units of  $1/\text{distance}$ , specifically,  $\text{mm}^{-1}$ . This is determined both by mass density and elemental composition of the material.

However, when we know that the x-ray absorption of a material is dominated by one specific material, then we can relate the measured x-ray attenuation coefficient (AC) to the mass density of that material. The calibration of bone mineral density (BMD) is an example of this. We assume that the x-ray attenuation within mineralised tissues such as bone, dentine and enamel is dominated by, and can be approximated as, the x-ray attenuation of the mineral compound calcium hydroxyapatite (CaHA) which has the formula  $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ .

Then we obtain “phantoms” with two or more known mass concentrations of CaHA. By linking these mass concentrations of CaHA with measured x-ray AC in the microCT

image, we can establish calibration linking the two quantities, allowing us to infer from the microCT measured attenuation coefficient in a mineralised tissue, the density of CaHA in  $\text{g.cm}^{-3}$ .

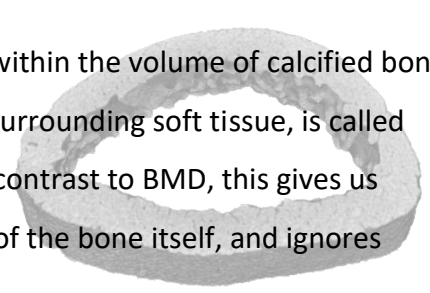
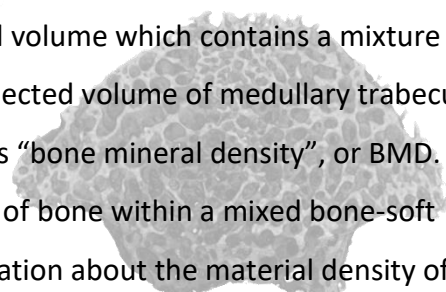
Note that this calibration system could be used to calibrate measurement of other substances apart from CaHA and bone, where x-ray absorption is known to be dominated by a certain substance and phantoms can be prepared with known concentrations of that substance.

### 1.1.1. Definitions of BMD and TMD

Bone mineral density (BMD) is defined as the volumetric density of calcium hydroxyapatite (CaHA) in a biological tissue in terms of  $\text{g.cm}^{-3}$ . It is calibrated by means of phantoms with known density of CaHA.

Bone mineral density can refer to two different measurements:

- the combined density of a well-defined volume which contains a mixture of both bone and soft tissue, such as a selected volume of medullary trabecular bone in a femur or tibia, is measured as “bone mineral density”, or BMD. This parameter BMD relates to the amount of bone within a mixed bone-soft tissue region, but does not give information about the material density of the bone itself.
- the density measurement restricted to within the volume of calcified bone tissue, such as cortical bone, excluding surrounding soft tissue, is called “tissue mineral density” or TMD [1]. By contrast to BMD, this gives us information about the material density of the bone itself, and ignores surrounding soft tissue.

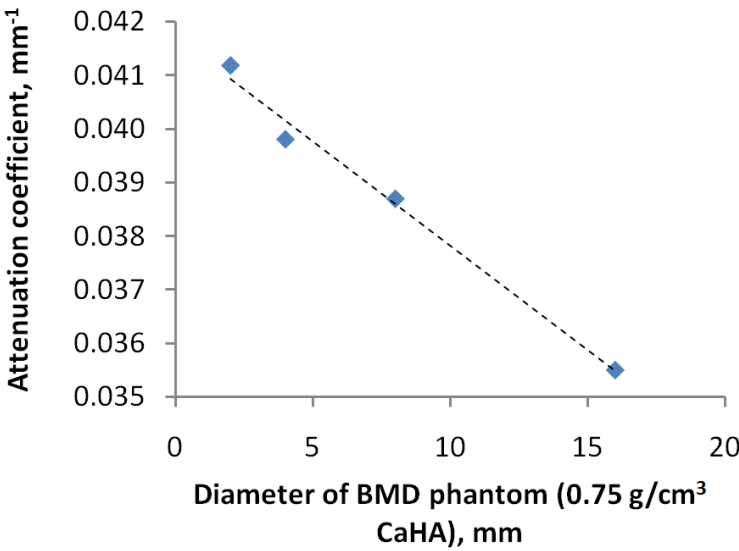


Typically trabecular bone is assessed for BMD, averaging bone and marrow within the medullary volume of interest. By contrast, for cortical bone, TMD is measured. Note that in the case of trabecular bone, partial volume effect compromises the measurement of TMD except at very high micro-CT resolutions. As a general rule, resolution should be such that the thickness of a bone structure should equal 10 pixels or more, in order for TMD to be measured.

*1.1.2. Bruker-MicroCT BMD/TMD calibration phantoms: size matching of phantom to sample*

Bruker-MicroCT BMD calibration phantoms are in pairs, with concentrations of CaHA of 0.25 and 0.75 g.cm<sup>-3</sup>.

Due to beam hardening effects, size of an object slightly affects measured density, as shown in figure 1 for rods of 0.75 g.cm<sup>-3</sup> CaHA (in epoxy resin) at thicknesses of 2-16 mm.



**Figure 1.** Increasing diameter rods of the same material (with 0.75 g.cm<sup>-3</sup> CaHA) show a small decline in measured attenuation coefficient, due to beam hardening.

This means that it is important to use a calibration rod which approximately matches

the cross-sectional area of calcified tissue of the bone or tissue sample within which you wish to measure bone mineral density.



**Figure 2.** Bruker-MicroCT BMD calibration rod pairs are provided in a range of diameters (small set 2-4mm, medium set 8-16mm, large set 32mm) to match the size of the scanned bone.

Bruker-MicroCT provides calibration rod pairs, composed of epoxy resin with embedded fine CaHA powder at concentrations of 0.25 and 0.75 g.cm<sup>-3</sup>, and at diameters of 2mm, 4mm, 8mm, 16mm and 32mm. The size of phantom to be used for different bones is suggested in table 1 below.

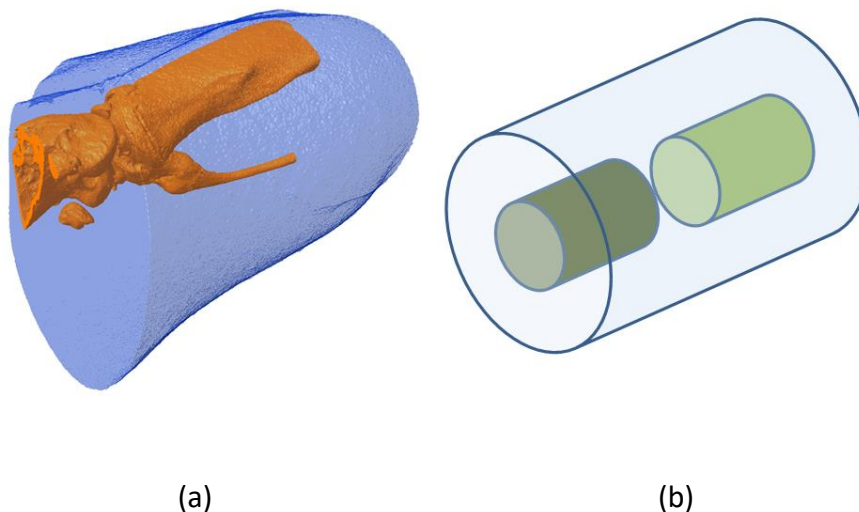
**Table 1.** The five diameters of Bruker-MicroCT BMD calibration rod pairs are recommended for bones of corresponding size.

BMD phantom rod pair diameter	Appropriate bone types
2mm	Mouse bones (femur, tibia, vertebra etc.)
4mm	Rat bones (femur, tibia, vertebra etc.)
8mm	8mm trephine biopsy cores, rabbit bones, human teeth, bones of primates, dogs
16mm	Larger bones such as sheep bones
32mm	Bones above 3cm in diameter, such as human bones, bovine, etc.

### 1.1.3. *Surrounding material around scanned bone must be simulated in calibration phantom scans*

Not only does the thickness of a scanned material such as bone affect its measured density. Soft tissue such as muscle, fat and skin around a bone will also significantly affect the measured density of the bone.

Therefore, if rodent limbs are scanned *in vivo*, the corresponding BMD calibration phantoms should be scanned in a tube of water (e.g. eppendorf) approximately matching the width of the animal's leg (figure 3). Water closely imitates the x-ray absorption of soft biological tissue in general.



**Figure 3.** An *in vivo* scan of a rodent limb (a) should be calibrated for BMD measurement by an equivalent scan of the BMD rod pair of appropriate size (mouse 2mm, rat 4mm) with the rods scanned inside a tube of water (b), the tube matching approximately the animal's leg diameter. This is to simulate x-ray absorption of the surrounding tissue, for improved accuracy of BMD measurement.

The water tube diameter for example should be about 6-8 mm for the mouse knee, 10-15 mm for the rat knee. Note that it is not too important for the BMD rods to be

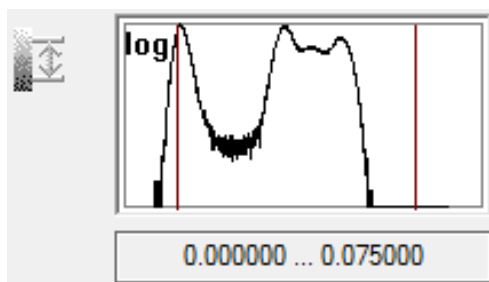
in the center of the water tube – they can lie against the side, the effect of the water on x-ray absorption averaged over 180 or 360 degrees of scanning will be similar.

The strong effect of surrounding medium on reconstructed attenuation was shown experimentally in exhaustive measurements with different thicknesses of aluminium phantoms (proxy for bone) and surrounding water [2] – demonstrating the context sensitivity of microCT densitometry and emphasising the need to mimic approximately, in the BMD calibration phantoms, both bone thickness and thickness of surrounding medium.

#### 1.1.4. Calibration of BMD from attenuation coefficient or from Hounsfield units

Attenuation coefficient (AC, or  $\mu$ ) is the unit of exponential x-ray attenuation in a material. ( $I_0$  and  $I_1$  are X-ray intensity before and after attenuation,  $e$  is the exponential coefficient,  $t$  is material thickness.) Cone-beam tomographic reconstruction such as by Bruker-MicroCT NRecon calculates AC for every dataset voxel. (This is the unit with which you define the contrast range in the displayed histogram at the NRecon output tab.)

$$I_1 = I_0 \cdot e^{(-\mu \cdot t)}$$

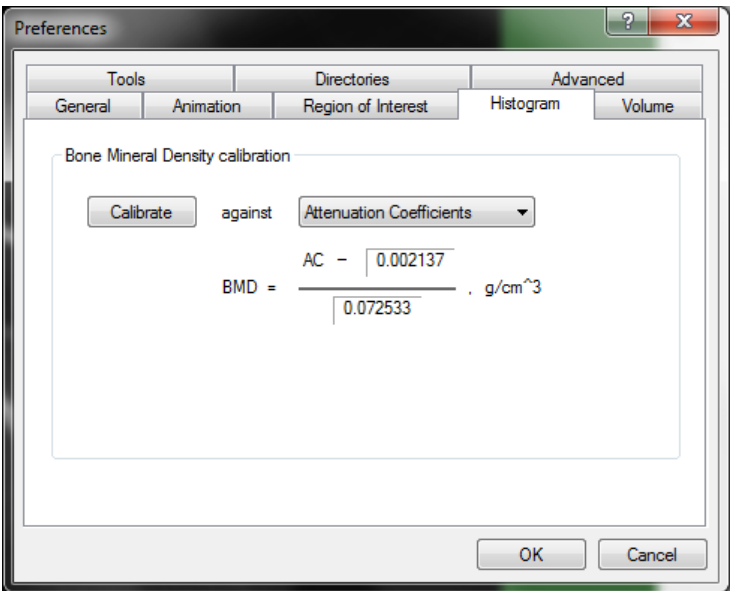
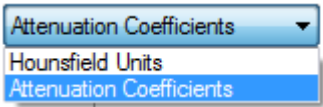


**Figure 4.** The contrast range in NRecon is set in units of attenuation coefficient.



Hounsfield units (HU) are a standard unit of x-ray CT density, in which air and water are ascribed values of -1000 and 0 respectively. It has been found over several decades of CT imaging to be a useful general CT density calibration system. HU is an appropriate density scale for soft tissue densitometry in vivo. The calibration of HU in CTAn is described in a separate method note.

In Bruker-MicroCT CT-Analyser ("CTAn"), BMD can be calibrated from either AC or HU. In preferences, under the "histogram" tab, you can choose to calibrate BMD against either AC or HU (figure 5, below).



**Figure 5.** In CTAn under preferences / histogram calibration of BMD can be made from input data in units of either attenuation coefficient or Hounsfield units.

The calibration of BMD against AC is simpler than against HU. AC is calculated in reconstruction by NRecon and requires no calibration (except for the scanner flat field correction – which should be done daily – which re-normalises the ambient air density). HU requires calibration and thus calibrating BMD from HU requires two

calibration steps – first for HU, then BMD against HU. Calibrating BMD from AC requires only a single calibration step.

This document will describe the calibration of BMD against attenuation coefficient. (To calibrate against HU the method is the same except that you input the densities of the phantoms in units of HU instead of AC. HU must be correctly calibrated as described in a separate method note.)

## Steps for implementing HU and BMD calibration of bone micro-CT scans.

### ***1.2. Issues of scan methodology and sample mounting relating to BMD measurement***

#### ***1.2.1. Scan with a filter, to reduce beam hardening***

A major source of error in density measurement by micro-CT is “beam hardening”. This results from the x-rays in micro-CT scanners being polychromatic – having a mix of higher energy (“hard”) and lower energy (“soft”) x-rays. As the x-rays pass through a bone or other sample, soft x-rays are absorbed faster than the hard x-ray photons. This results in an artefact of non-uniform density in the reconstructed image with artificially higher density on the surface.

Beam hardening can be approximately corrected in reconstructed images using the “beam hardening correction” parameter in Bruker-MicroCT NRecon software. However beam hardening must also be reduced during the scan by using a metal filter, such as aluminium or copper (or both). The thickness of filter will depend on

your sample. Typically, mouse bones should be scanned with 0.5mm Al filter (or possibly 0.25mm Al in the SkyScan 1174 scanner). Rat bones should be scanned with 0.75mm-1mm Al filter. Larger bones may require even more filtration. However if no filter is used for a bone scan, then *densitometry cannot be performed* since beam hardening in the images will be too high to be adequately corrected in reconstruction.

### 1.2.2. *Storage of bones prior to micro-CT densitometry: alcohol a problem*

If bones are to be microCT scanned for BMD or TMD, then they cannot be dehydrated, since this changes the density especially of medullary trabecular bone regions. So removal from alcohol storage and scanning while dry is not an option. However if bones are stored in alcohol, as is frequently practiced, then they should be re-hydrated prior to microCT scanning by immersion overnight or over a weekend in 0.9% physiological saline.

The acceptable options for storage of bone and preparation for microCT scanning for micro-densitometry are:

- Harvest without chemical fixation and store frozen at -20 C either in tubes of saline or wrapped in saline-soaked gauze.
- Fix and store in phosphate-buffered saline. However take care that formaldehyde is adequately buffered in the case of long term storage so that partial change to acetic acid does not occur and result in demineralisation of the bone.

- Fix (alcohol or buffered formalin) and then store in alcohol: however, prior to scanning, rehydrate by overnight storage in 0.9% physiological saline.

### *1.2.3. Maintain normal hydration of the bone during scanning*

As mentioned above, bone cannot be scanned dry if one wishes to measure mineral density.

One option for measuring BMD in *ex vivo* scans of bone is to place the bone samples into a tube of water for scanning, and also placing the BMD phantom rods into an identical tube for the calibrating scan. This allows the HU density value to be (optionally) independently calibrated for each individual bone scan. If this approach is used, the water tube should be of the minimum diameter possible. Note: the tube should be plastic, not glass – polypropylene is a suitable low density plastic.

However it is not necessary to scan bones (or other objects) fully immersed in water or saline for density measurement. This can introduce added complication, such as how to immobilise the samples. A good alternative is to wrap the bones in paper tissue, load the tissue-wrapped bone into a suitable plastic tube, then moisten the paper tissue around the bone with water or saline (figure 6).

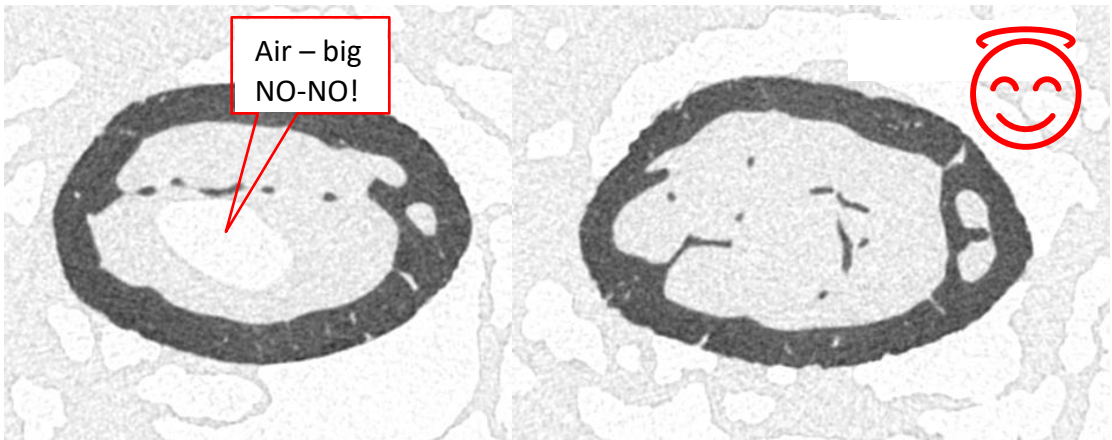


**Figure 6.** To maintain hydration of bones during a scan, one option is to wrap the bone in paper tissue for placement in a suitable plastic tube holder, then moisten the paper tissue with tap water or alternatively saline. This will effectively keep the bone moist during a scan.

Another alternative is to wrap the bones in plastic film, either parafilm (image, right) or “cling-film” obtainable in a supermarket. Note – if cling-film is used, take care to avoid the type containing PVC, this will have too high an x-ray density: ensure you use non-PVC cling-film.

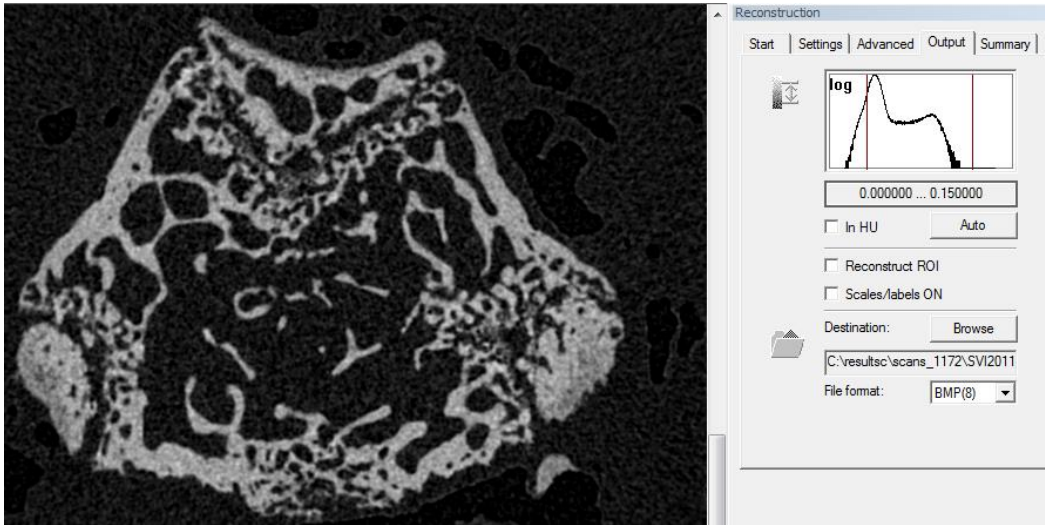


**IMPORTANT: THERE MUST BE NO AIR IN THE BONE MARROW!**



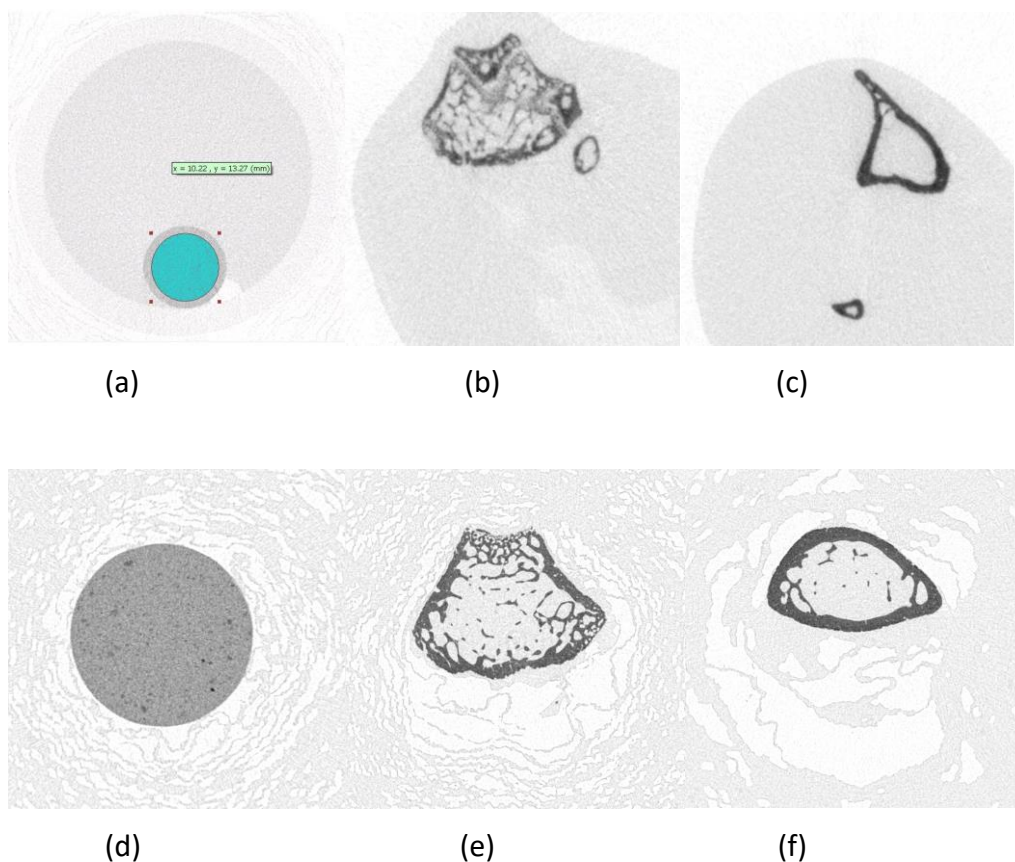
#### 1.2.4. *Scanning the bones and phantoms for calibration of BMD/TMD*

1. Obtain phantom rods for BMD measurement with a diameter selected to approximately match the cross-sectional thickness of calcified bone in the bones you are measuring (see section 1.3 above). These rods can be purchased from Bruker-MicroCT.
2. Scan one or more of your experimental bones, *in vivo* or *ex vivo*. Then scan two BMD rods (under the same scan settings as the bone scan) with BMD values of 0.25 and 0.75 g.cm<sup>-3</sup> CaHA.  
Pay attention to the issues of sample storage and mounting, and the size and mounting of the calibration rods (e.g. put BMD rods in a water tube for calibrating *in vivo* scans) as discussed in section 1 and 2.1.
3. Reconstruct the scan of the bone in the water tube. Select and optimise the reconstruction parameters for this bone scan, taking care to find a good value for beam hardening correction. Also take care with the selection of the lower and upper contrast limits. The lower limit should be zero or a small negative value. The upper limit should be slightly higher than the top end of the brightness spectrum for the bone (see figure 7). For this purpose it is best to view the density histogram in the “output” tab of NRecon in the log scale view – double click on the histogram to switch from linear to log. All the reconstruction parameters you select must be applied identically to all bone scans and to the calibration scan of the BMD rods (with the exception of the post-alignment correction).
4. With the scans and reconstructions of the bones and the calibrating phantoms complete, implement the BMD/TMD calibration in the program CT-analyser. This is described in the next section, 2.3.



**Figure 7.** The contrast limits for bone should be set appropriately for density measurement. The lower limit should be zero (or slightly negative). The upper limit should be a little higher than the upper (right hand) end of the density distribution representing the highest bone CT density value. View the histogram in log scale (double-click on histogram window).





**Figure 8 a-c:** *In vivo* bone micro-CT densitometry: (a) a BMD calibration rod water tube of appropriate diameter, (b) mouse femur at metaphysis scanned *in vivo* for trabecular bone, (c) mouse tibia at a cortical region.

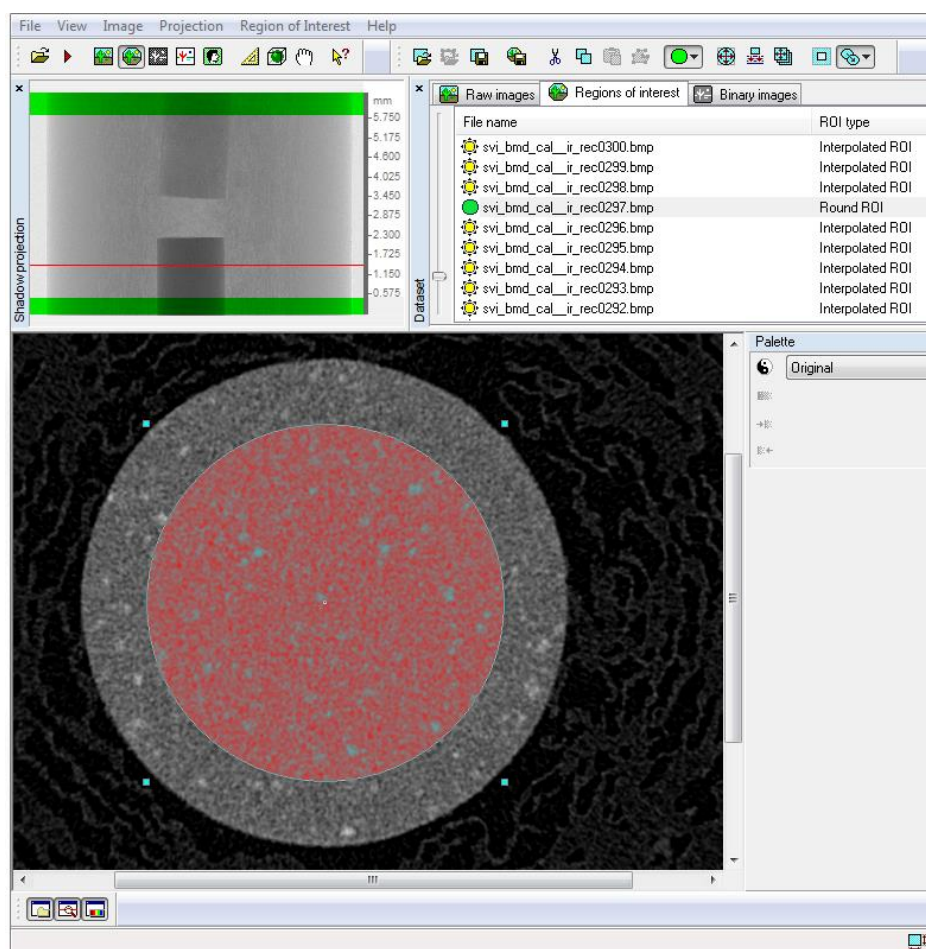
**Figure 8 d-f:** Calibration and sample scans *ex vivo*: (d) *ex vivo* BMD rod scan, (e) trabecular metaphysis region the femur is wrapped in moistened tissue paper; (f) cortical metaphysis-diaphysis, also wrapped in moistened paper tissue.



### 1.3. Calibration of BMD/TMD in CT-Analyser

#### 1.3.1. Open calibration dataset and set two ROIs for the two phantoms

- (1) Open in CT-Analyser ("CTAn") the dataset of the BMD phantom scan with the two calibrating rods, with 0.25 and 0.75 g.cm<sup>-3</sup> CaHA concentrations (in epoxy resin).



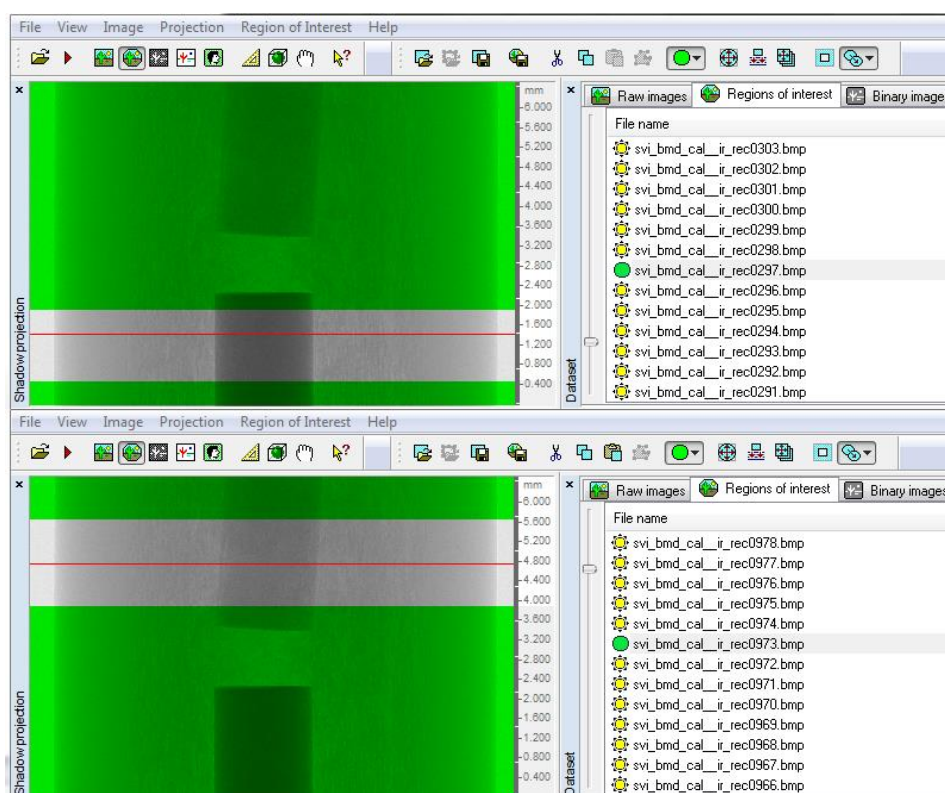
**Figure 9a.** The cross-sectional ROI for each phantom ROI should be a circle, excluding the outer margin of the phantom rod.

**1.3.2.** *Go to the second, region of interest (ROI) page of CTAn, and choose ROIs within the two phantoms.*

The crosssectional ROIs should be set as a circle, smaller than the phantom outline to exclude the exterior margin (figure 9a).

The vertical ranges of the ROIs for both phantoms should exclude the part near the ends (interface with water) and need not run the whole length of the phantom – just one or two hundred crosssections is sufficient (figure 9b).

Save the .roi file for both these VOIs for the two phantoms – however it is not in this case necessary to write ROI datasets for the two VOIs.



**Figure 9b.** The phantom ROIs' vertical extent should exclude the top and bottom ends of the rod, and include about 100-200 slices.

*1.3.3. Measure the attenuation coefficient value of the two phantom VOIs, and enter these values in the preferences-histogram calibration table*

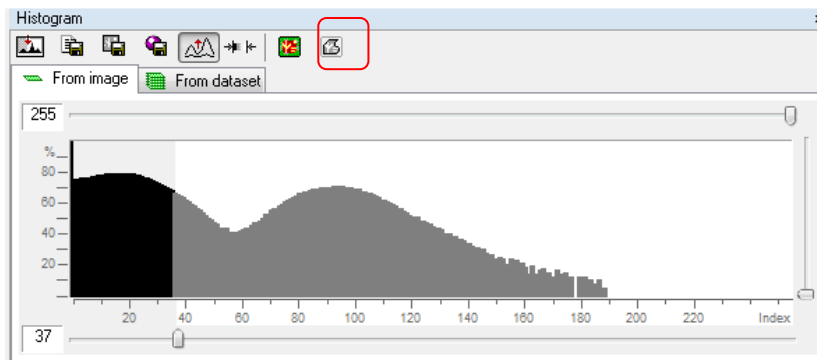
- (1) First check in the file menu/preferences and “histogram” tab that it is selected to calibrate BMD against attenuation (not Hounsfield units) – refer to section 1.4 above.

Bone Mineral Density calibration

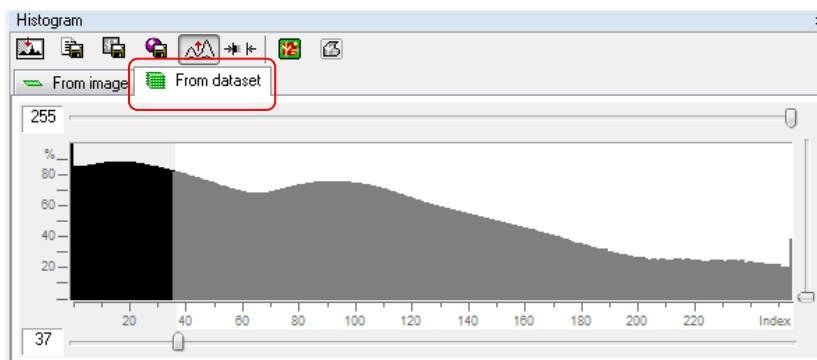
against

$$\text{BMD} = \frac{\text{AC} - 0.00111}{0.0333} \text{ , g/cm}^3$$

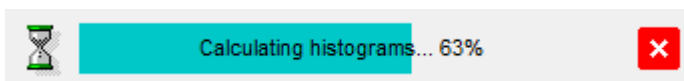
- (2) At the ROI page load one of the phantom ROIs, then move from the ROI page to the binary page of CTAn. A density histogram is shown, with two tabs above it, “from image” and “from dataset”. By default “from image” is selected, thus the histogram displayed is for the current cross-section level only (image a, below). Note that the button for a log y axis should be pressed (red square).



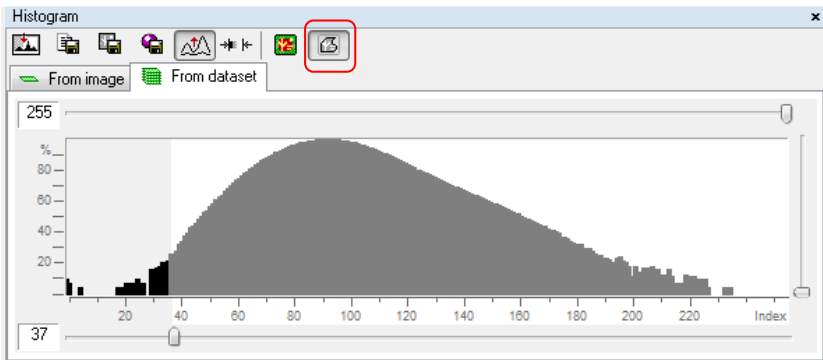
- (3) Now click on the tab “from dataset”. It is important to do this since this will integrate the density histogram over the whole open dataset (“b” below).



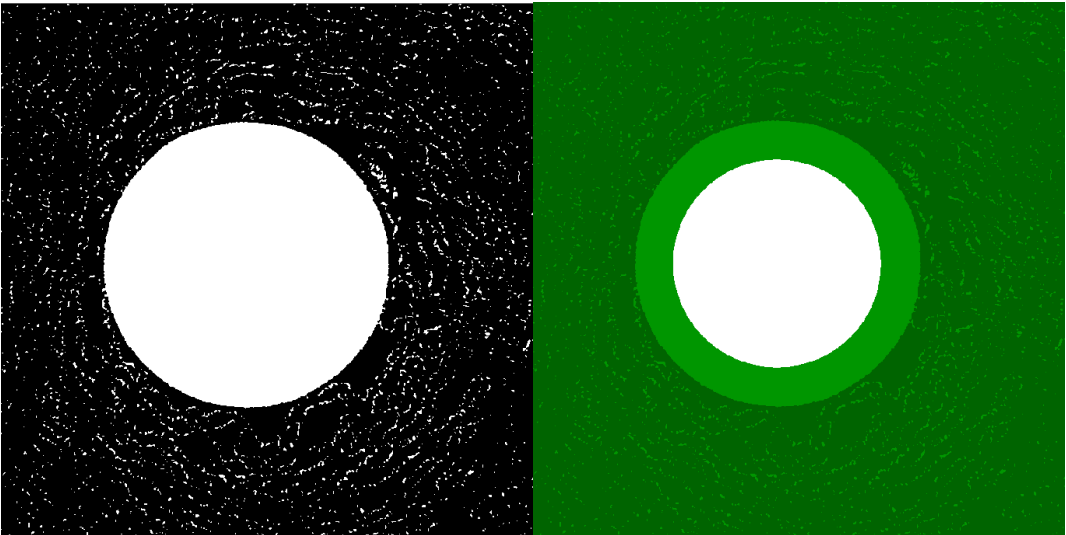
When you first press on “from dataset” a progress bar will run, indicating the integration of voxel greyscale data from the whole dataset:



- (4) However we require the density histogram from within the VOI only, integrating over all the VOI slices, not from the whole dataset, as shown in “b” above. Therefore, now click on the button on the far right under the heading “histogram” – this button will “toggle VOI view” and will restrict the density histogram to the contents of the VOI only (“c” below):



The displayed binary image will change in appearance depending on your selection of the “toggle VOI view” button.



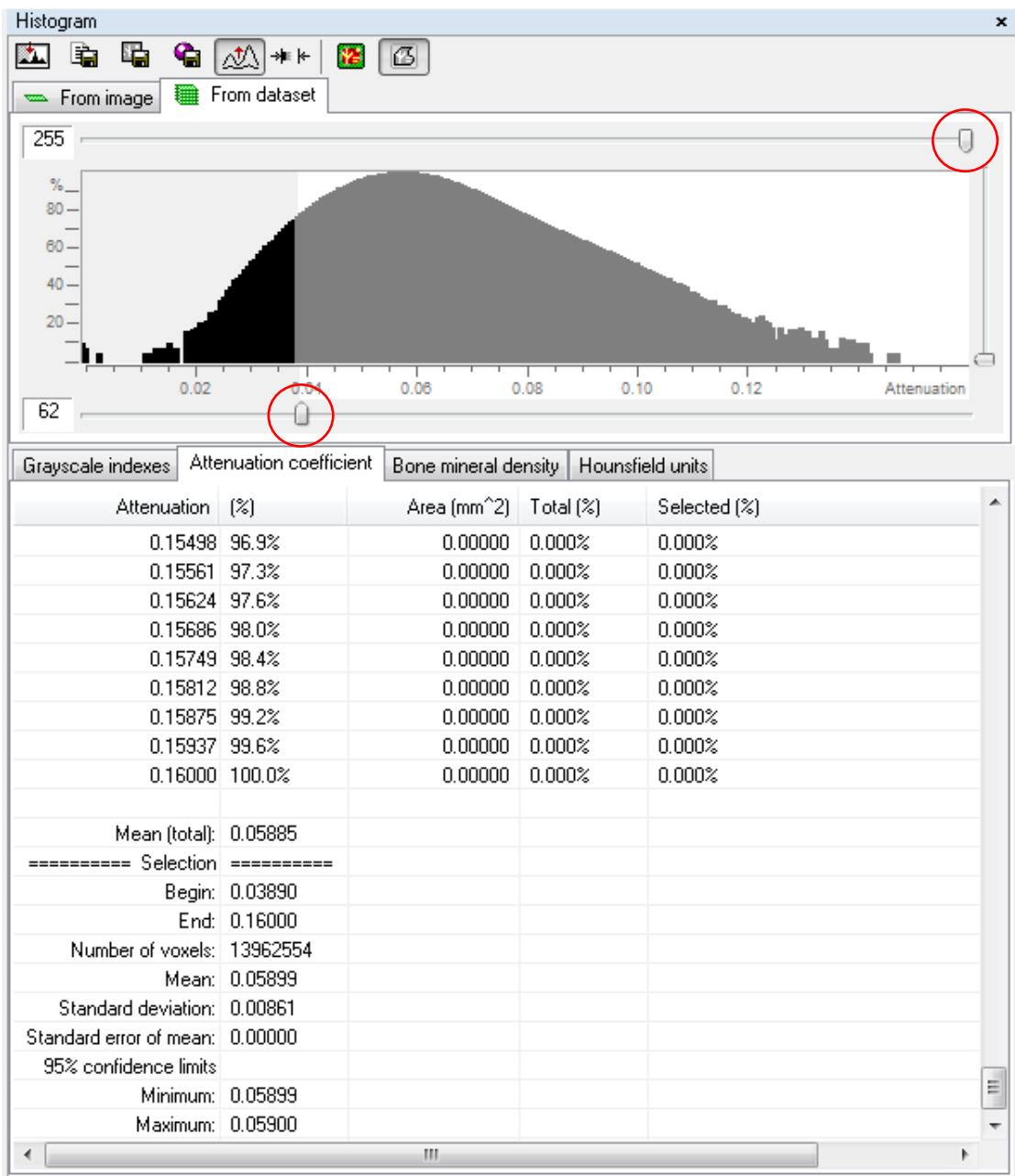
With “toggle VOI view” not selected, the binary image is all black and white.

With “toggle VOI view” selected, the part of the image outside the VOI is green-shaded, and only the part inside the VOI is black or white.

PLEASE NOTE: it is very important that both the “from dataset” tab and the “toggle VOI view” button are pressed, to obtain the density from within the phantom VOI.

- (5) With the ROI (VOI) for one of the BMD phantoms loading in CTAn (ROI page), and also with both the “from dataset” tab selected and the “toggle VOI view” button pressed (Binary page), now select the tab below the histogram entitled “attenuation coefficient”.

Below the histogram you will see a table display of the histogram values.



Note that along the top and bottom of the density histogram there are two sliders, shown by red circles in the above image (added here for illustration only). These sliders are for thresholding – otherwise called “segmentation”, where you choose to binarise a limited part of the density range. Often for instance we wish to binarise the solid object such as bone to white, and surrounding material or space to black.

At this stage in the calibration method the threshold values i.e. position of the sliders is not important. We need to measure the mean density of all the voxels within the phantom rod volumes of interest (VOIs) so threshold values are not important.

There are five columns in the histogram table – these columns are:

- **Attenuation:** The first column shows the value of the density bin or interval, in the selected unit of density, such as attenuation (this means the attenuation coefficient in  $\text{mm}^{-1}$ )
- **%:** The density value from column 1 expressed as a % of the maximum
- **Area:** The number of pixels / voxels within the image (if the “from image” tab is chosen) or the whole dataset or VOI (if the “from dataset” tab is chosen) with the given density (e.g. attenuation) value, expressed as an area based on the pixel size.
- **Total %:** This is the area data expressed as a % of the total area or volume. (In the case that “from dataset” is selected, then “area” actually means volume).
- **Selected %:** This is the area/volume of voxels with a given density value, but as a percentage of all pixels within the selected density range

It is important to note that the term “selection” here refers to the density range selection implemented by the histogram sliders. It does not refer to the region or volume of interest selection.

Scroll to the bottom of the histogram table and you will see a statistical summary of the histogram data, shown in the image above. Examples of this summary table are shown below for our two calibration phantoms with 0.25 and 0.75 g.cm<sup>-3</sup> of CaHA.

- The first value in the summary table is “mean (total)”. This is the mean density value – in the chosen unit such as attenuation – of all the voxels within the VOI.
- Next in the table is the heading “selection”. All the data below the selection heading refer only to the pixels that are within the selected density range (i.e. between the histogram sliders), that is, the pixels that are binarised to white in the displayed image.

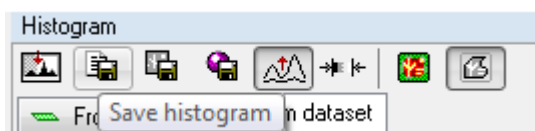
Mean (total):	0.02532	Mean (total):	0.05885
===== Selection =====		===== Selection =====	
Begin:	0.03890	Begin:	0.03890
End:	0.16000	End:	0.16000
Number of voxels:	1074697	Number of voxels:	13962554
Mean:	0.05164	Mean:	0.05899
Standard deviation:	0.01402	Standard deviation:	0.00861
Standard error of mean:	0.00001	Standard error of mean:	0.00000
95% confidence limits		95% confidence limits	
Minimum:	0.05161	Minimum:	0.05899
Maximum:	0.05167	Maximum:	0.05900

Above image: Attenuation summary data for the 0.25 (left) and 0.75 (right) g.cm<sup>-3</sup> CaHA phantoms.

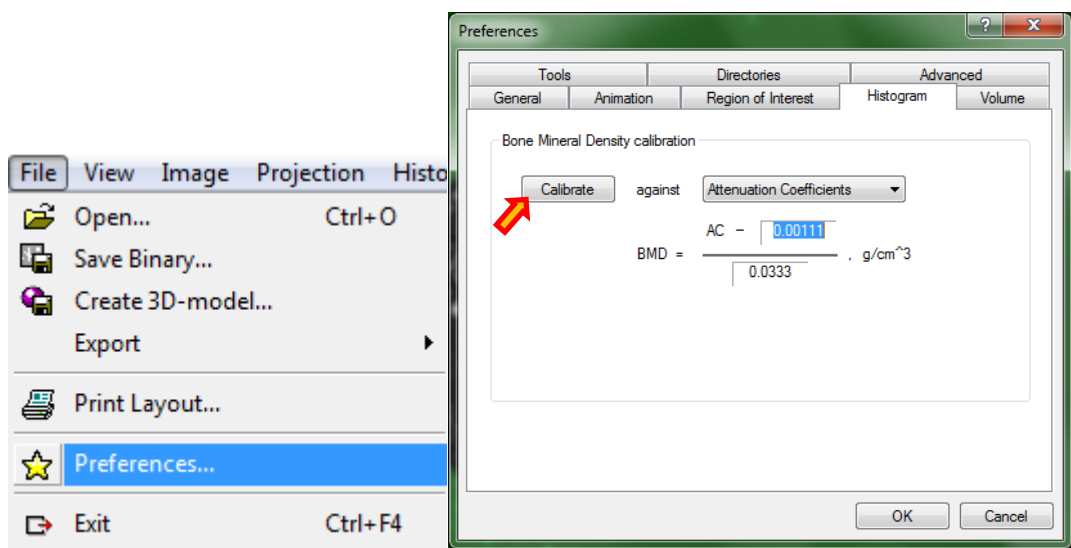


In the above images we can see that the “mean total” attenuation coefficient values for the two calibration phantoms, with 0.25 and 0.75 g.cm<sup>-3</sup> respectively, are 0.02532 and 0.05885.

Finally, with this data correctly displayed, the displayed histogram table including the summary table at the end can be stored as a comma-delineated ASCII text file. To do this, click on the “save histogram” button, the second button from the left on the button bar under the “Histogram” heading:



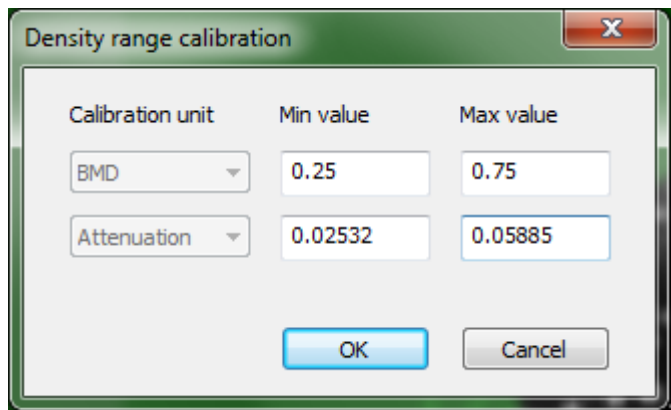
- (6) These two attenuation values for the two BMD phantoms (0.02532 and 0.05885) are all the data we need to apply the BMD calibration. It is recommended that you make a record of this data alongside your experimental data, for instance in a text file.
- (7) Now go to the file menu of CTAn and “preferences”. Open the tab entitled “histogram”.



Note that in part 2.3.2 (1), page 13, we have selected to calibrate BMD against attenuation coefficients, not – in this case – Hounsfield units.

Now click on the “calibrate” button (red arrow). A table will appear as shown below. In this table, in the top row, enter the two BMD concentrations for the two phantoms that we already know, i.e. 0.25 and 0.75 – the unit is g.cm<sup>-3</sup> of calcium hydroxyapatite.

In the second row, enter the two values that we have measured and recorded for the attenuations of the two phantoms, that is, 0.02532 and 0.05885.

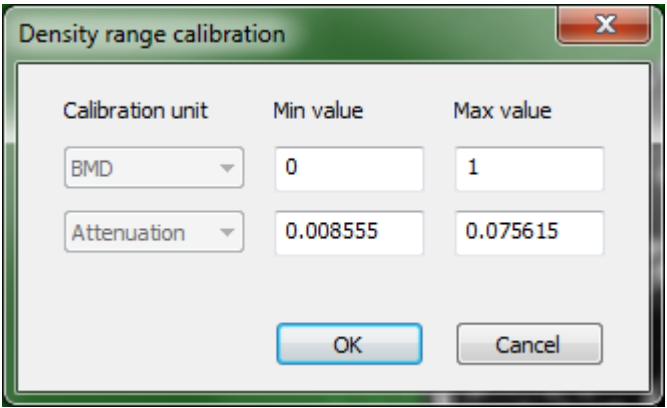


Then click “OK”. Note that after entering these values, the formula displayed in the histogram window will change to the following:

$$\text{BMD} = \frac{\text{AC} - 0.008555}{0.06706} \cdot \text{g/cm}^3$$

This formula shows the straight line relationship between BMD and attenuation coefficient. With this formula updated, calibration of BMD (against attenuation) is complete. The calibration values are permanently saved in the computer (written to the registry file) so that, even if you restart the computer, the calibration remains in place.

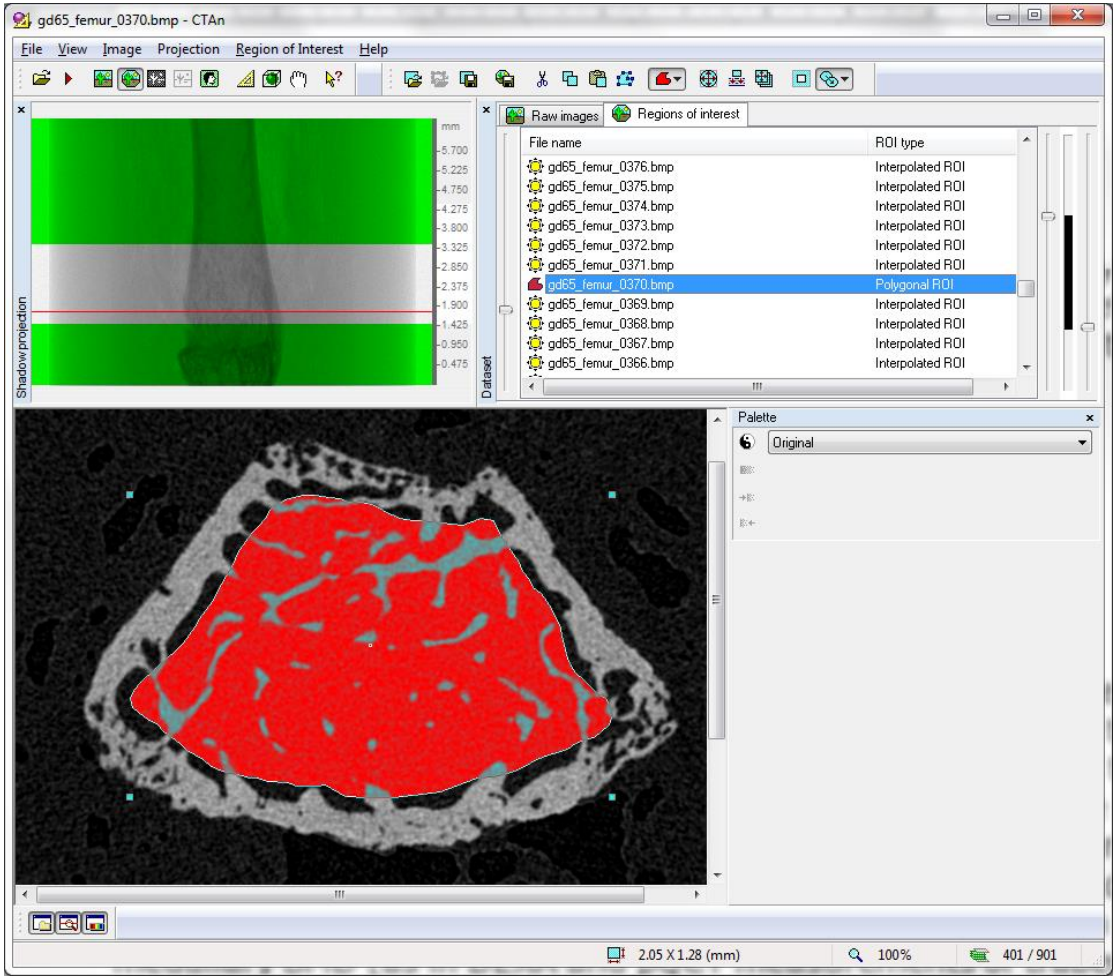
Finally, if you now click again on the “calibrate” button you will see different, re-normalised values:



Please note here that the calibration we have just entered has not changed, the displayed values have just been re-normalised to show the attenuation values corresponding to BMD values of 0 and 1 g.cm<sup>-3</sup>. The BMD-AC formula as shown above will still be the same.

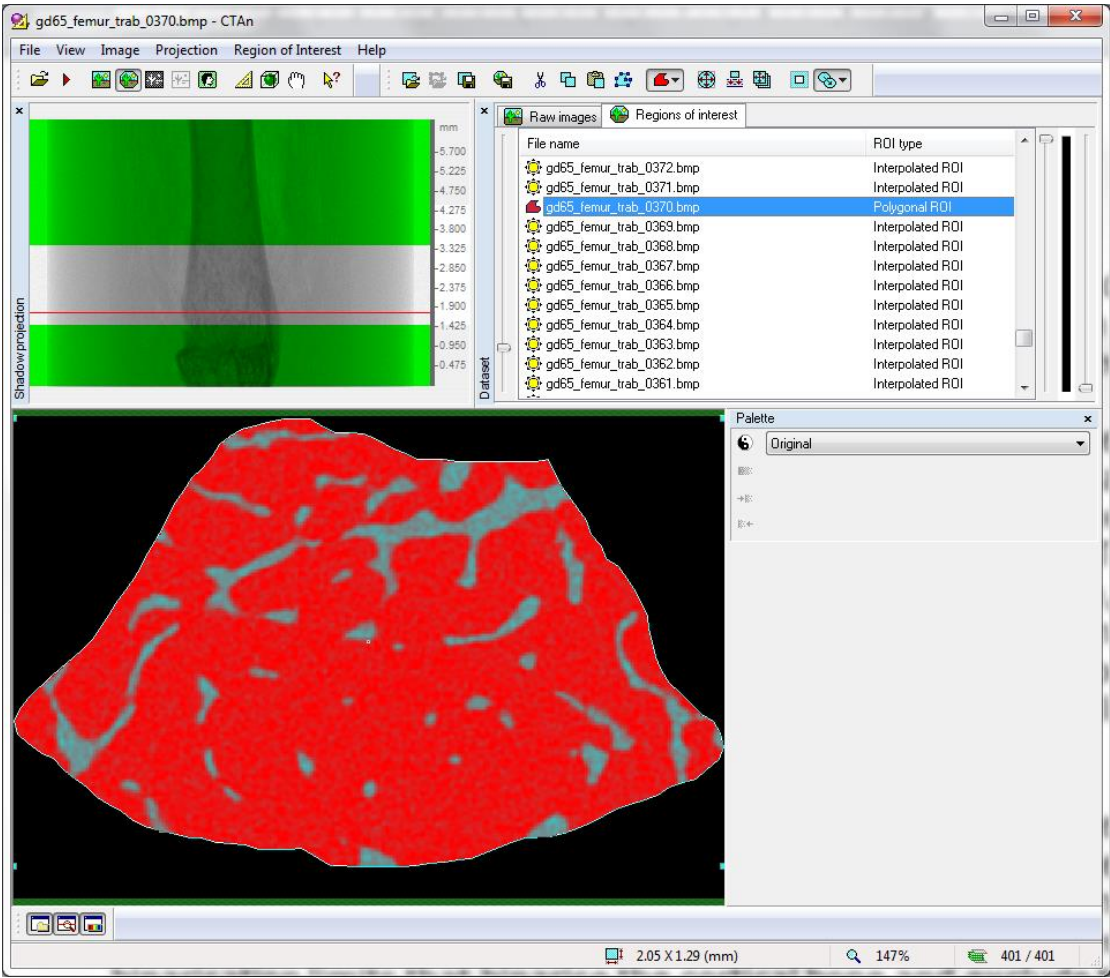
### 1.3.4. Measuring calibrated BMD for trabecular bone

- (1) Open a scan dataset in the same experimental study series, taking care that the scan and reconstruction parameters (except misalignment correction) should all be identical for the phantom scans and bone scans.
- (2) Select the trabecular bone VOI in the bone dataset.



For analysis purposes it is convenient and recommended to perform analysis by opening and using the “ROI dataset”, i.e. the new dataset saved containing only the contents of the VOI (see image below).

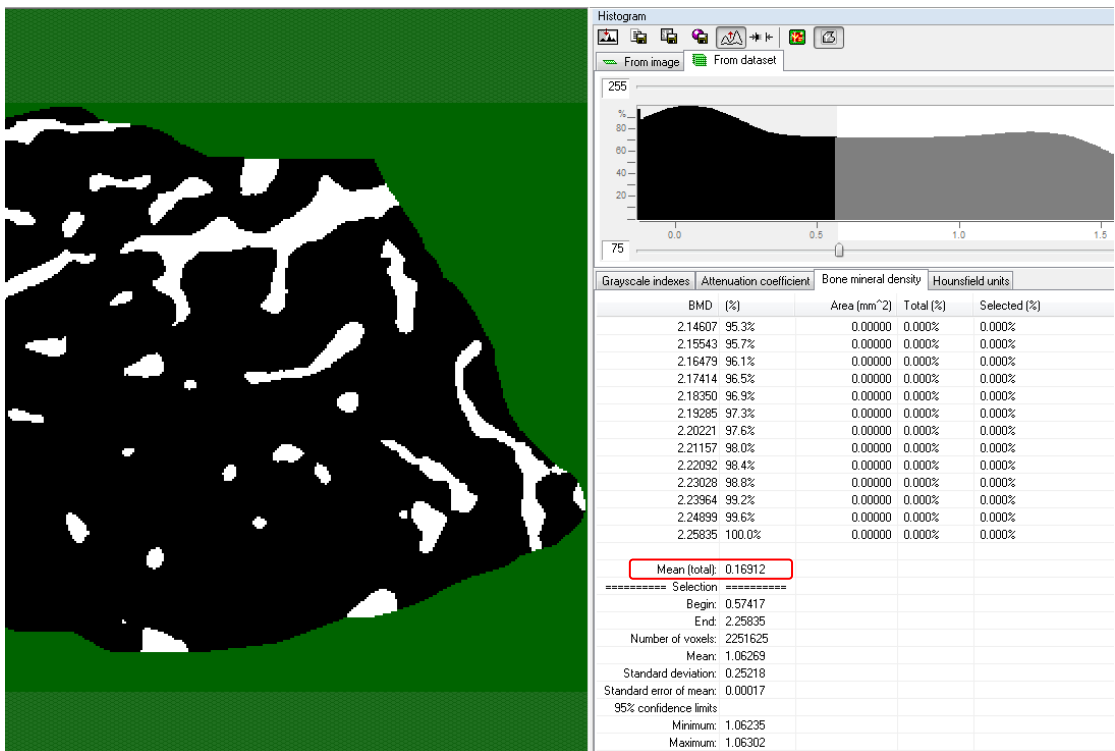
Remember to apply the VOI by loading the ~.roi file, to make sure the BMD measurement is made within the VOI only, not including background black pixels in the areas excluded from the VOI.



- (3) With the trabecular VOI dataset loaded and the ROI correctly applied, move to the binary page of CTAn, and remember to click on the “from dataset” tab above the histogram, so that density is integrated over all cross-section levels within the VOI. Also, the “toggle VOI view” button (far right) should be pressed.
- (4) With this done, the binary image will appear with black and white pixels restricted to within the ROI only and green pixels in the outside part. (See image below.)

Click on the “Bone mineral density” tab under the histogram image, to display the density histogram tabular display in units of BMD, as shown in the screen shot below. As before, at the bottom of this list there is the summary table.

The value after “mean (total)” under the BMD tab is 0.16912, in units of  $\text{g.cm}^{-3}$ . This is the BMD of our trabecular VOI.



For trabecular bone mineral density (BMD) the parameter that we read is the “mean (total)” which is the mean greyscale of all voxels within the VOI, this is unaffected by the binary threshold. Here trabecular BMD is 0.169 g.cm<sup>-3</sup>.

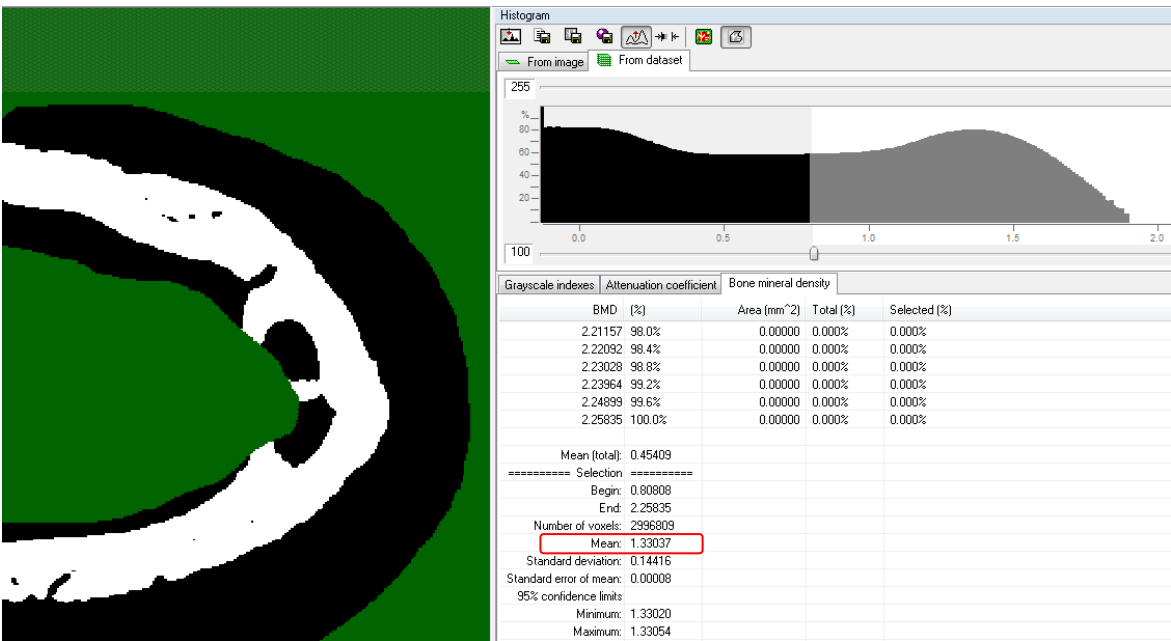
At this point, recall the discussion above in section 1.2, page 2, of the two different definitions of bone density:

- Bone mineral density, or BMD, means the mean density of bone and bone marrow within a medullar region containing trabecular bone. For trabecular bone, BMD is the parameter we normally measure.
- Tissue mineral density, or TMD, means the density specific to the bone mineralised tissue itself, excluding any surrounding non-bone tissue. TMD is the density parameter appropriate for cortical bone. (TMD can be measured for trabecular bone, but only at high spatial resolution such that the minimum trabecular thickness is at least equivalent to 10 pixels).

The main reason for this difference in parameter definition is that, with standard resolutions typical of in vivo and much ex vivo microCT scanning, trabecular bone – especially of the mouse – is too thin for the density of the fine trabecular structures to be an accurate reflection of the real material density. (However if you scan mouse trabecular bone with <3 micron pixel, then TMD can be measured.)

1.1.1. Measuring calibrated TMD for cortical bone.

Load the cortical bone ROI dataset. At the ROI page load the cortical ROI file (~.roi). Then go to the binary page, and as usual select the “from dataset” tab and make sure the “toggle VOI view” button is switched to ON.



For cortical bone tissue mineral density (TMD) the parameter that we read from the summary list is the mean under the “Selection” heading – this means the greyscale density of only the pixels that are binarised to white. Here it is 1.33 g.cm<sup>-3</sup>.



In the case of cortical bone, unlike trabecular bone, you DO need to select a threshold value suitable to binarise the cortical bone. In the above example the threshold is 100-255. Choose a threshold high enough to binarise the porosity in the cortical bone as black pixels (but not too high so that noise appears as black pixels). Normally for analysis the grey threshold for cortical bone should be higher than that for trabecular bone.

With the cortical bone thresholded, click on the “Bone mineral density” tab under the histogram image and above the histogram tabular display. At the bottom of the tabular display is the summary data.

Under the heading “Selection” the fourth line is “Mean”. This is the mean greyscale density – calibrated here to BMD – for all the pixels which are binarised to white; that means here the cortical bone pixels.

Thus in our case above, the tissue mineral density (TMD) of the cortical bone is 1.330 g.cm<sup>-3</sup>.

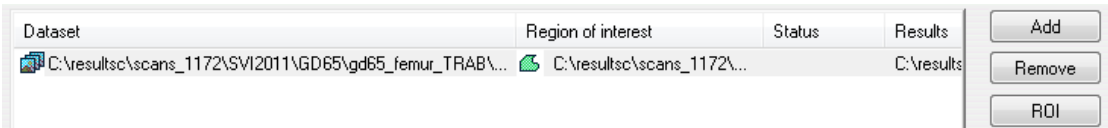
It is important to understand what is happening here. The density measurement does not take white (grey=255) as the density of these pixels – in this case it would tell us nothing about the object density. Instead, for each pixel binarised to white in the image, it is reading the greyscale density in the original reconstructed image – the image we see at the “raw images” page of CTAn.

This procedure – using a binary image to define a region of interest for density measurement – is an important one, and it can be used also at the custom processing page of CTAn, using logical functions under the “Bitwise operations” plug-in. However here in the binary page, and under the “selection” heading, the use of the binary image as a region of interest is done in real time.

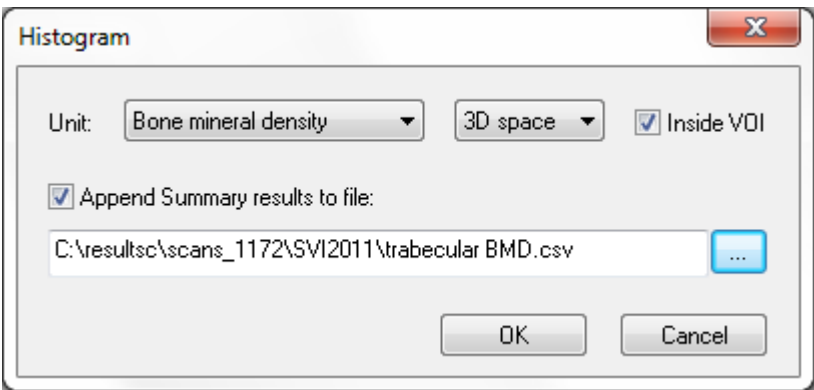
**1.4.    *Output of trabecular BMD and cortical TMD from CTAn custom processing and BatMan***

**1.4.1.    *Trabecular BMD from custom processing / BatMan***

Load the trabecular ROI dataset, and then load the ROI file. For custom processing this is done at the ROI page. If this is being done in BatMan, then load the dataset with the “add” button and the ROI with the “ROI” button so that the listed “Region of interest” icon with the dataset changes from the red “default” to the green symbol.



The task list here consists of only one item, the plug-in “histogram”. This outputs the mean density in chosen units of all voxels within the VOI – it is equivalent to “mean (total)” in the binary page. Since the trabecular VOI is already set, only the histogram plug-in is needed to output BMD.



To configure the plug-in:

- Set unit to BMD
- Select 3D space (not 2D)
- Select “Inside VOI”
- Select “Append Summary results to file” and click on the “...” button to set up a destination text file for the summary table results.

The result file will look like this:

```
-----
[ 07/05/12 18:05:33 ] Histogram (3D space) inside VOI

Unit:,BMD

Dataset
name:,C:\resultsc\scans_1172\SVI2011\GD65\gd65_femur_TRAB\gd65_femur_trab_
-0.12757,-0.11822,-0.10886,-0.0995,-0.09015,-0.08079,-0.07143,-0.06208,-0.05272,-0.04336

548797,137372,165824,196633,231311,268784,310721,353945,399299,446501

Mean,Standard,deviation,Standard error of mean,95% confidence limit (minimum),95%
confidence limit (maximum)

0.16912,0.32755,0.00007,0.16898,0.16926

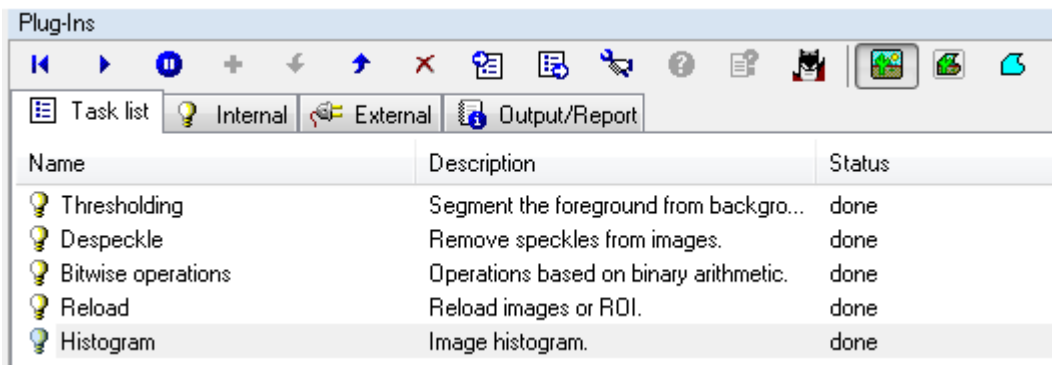
<<<<< End of task (fcc085d0-05e0-44a8-ab39-8ec2655be528) >>>>>
```

The first number on the last line is the mean BMD for the trabecular VOI, here 0.16912, the same result we obtained from the binary images page above.

1.4.2. Cortical TMD from custom processing / BatMan

To output cortical TMD from custom processing, we need to binarise the cortical bone, convert that binary image to our ROI. Then we re-import the grey voxels to the cortical ROI using the “reload” plug-in, and finally run the histogram plug-in, once the cortical (binary-based) ROI has been correctly set.

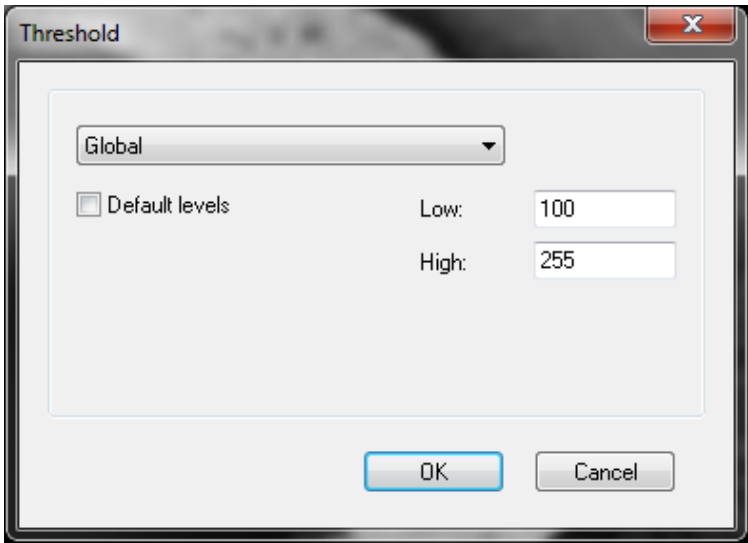
The task list is shown below:



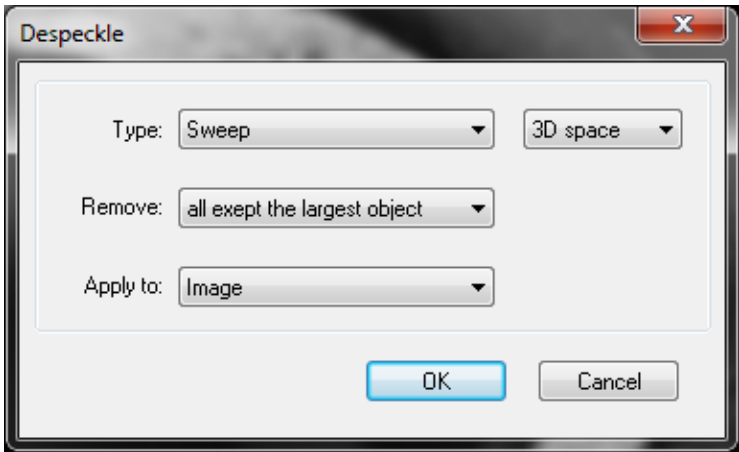
Name	Description	Status
Thresholding	Segment the foreground from backgro...	done
Despeckle	Remove speckles from images.	done
Bitwise operations	Operations based on binary arithmetic.	done
Reload	Reload images or ROI.	done
Histogram	Image histogram.	done

The task list items are described in detail as follows:

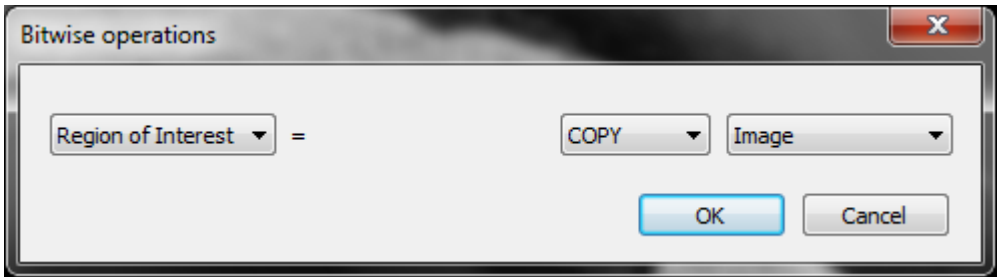
- (1) Thresholding: A global threshold is fine for cortical bone, in our case we use 100. (Note the cortical threshold is higher than what we would use for trabecular bone.)



- (2) Despeckle: Under despeckle, choose “sweep”, set to 3D and “remove all except the largest object”, and apply to image.



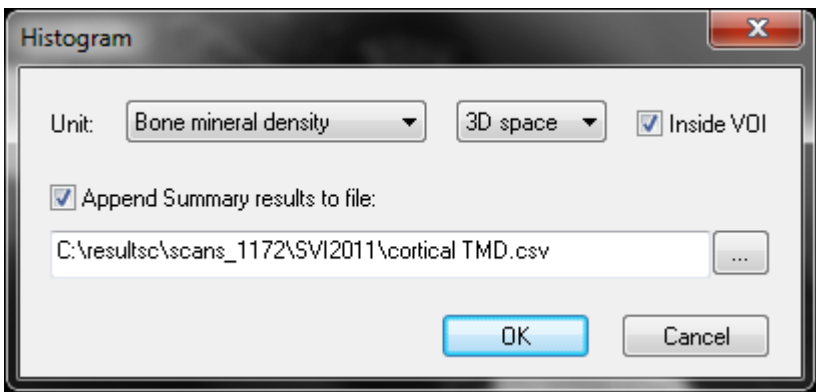
- (3) Bitwise operations: set the expression ROI = COPY IMAGE; this will make the ROI become the binary image of the cortical bone, so that density is only measured for the bone pixels. Thus we will measure TMD, not BMD.



- (4) Reload: After copying the ROI from the cortical binary image, the plug-in reload must be run so that the density measured is of the greyscale voxels, not of white voxels.



- (5) Histogram: the histogram plug-in is set up in the same way as for trabecular bone, but specifying a different summary results file for cortical TMD.



The output results from the histogram plugin have the same format as in the case of trabecular bone:

```
-----

[ 07/05/12 18:46:54 ] Histogram (3D space) inside VOI

Unit:,BMD

Dataset
name:.,C:\resultsc\scans_1172\SVI2011\GD65\gd65_femur_CORT\gd65_femur_cort_
-0.12757,-0.11822,-0.10886,-0.0995,-0.09015,-0.08079,-0.07143,-0.06208,-0.05272,-0.04336

0,0,0,0,0,0,0,0,0,0

Mean,Standard deviation,Standard error of mean,95% confidence limit (minimum),95%
confidence limit (maximum)

1.33038,0.14415,0.00008,1.33021,1.33055

<<<<< End of task (2af87993-1440-4e37-8d99-fa5274a0ea86) >>>>>
```

The important thing is to have the ROI correctly set, in this case as the cortical binary mask, so that the mineral density value will have the correct meaning, i.e. TMD (density of mineralised bone tissue only) for cortical bone.

Conversely, with a trabecular VOI selected, the histogram plug-in will output BMD (average of bone plus marrow) for trabecular bone.

Examples of published papers where the above methodology is correctly applied are given in references [3] and [4].

## References

1. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Müller R, Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res.* 25(7): 1468-86. 2010.
2. Salmon PL, Liu X (2014) MicroCT Bone Densitometry: Context Sensitivity, Beam Hardening Correction and the Effect of Surrounding Media. *The Open Access Journal of Science and Technology* Vol. 2 (2014), Article ID 101142, 25 pages. doi:10.11131/2014/101142
3. Srivastava K, Khan K, Tyagi AM, Khan MP, Yadav DK, Trivedi R, Maurya R, Singh D, Chattopadhyay N (2013) Greater Skeletal Gains in Ovary Intact Rats at Maturity Are Achieved by Supplementing a Standardized Extract of *Butea monosperma* Stem Bark that Confers Better Bone Conserving Effect following Ovariectomy and Concurrent Treatment Withdrawal. *Evidence-Based Complementary and Alternative Medicine* Volume 2013, Article ID 519387, 12 pages, <http://dx.doi.org/10.1155/2013/519387>
4. Kumar Rai R, Barbhuyan T, Singh C, Mittal M, Khan MP, Sinha N, Chattopadhyay N (2013) Total Water, Phosphorus Relaxation and Inter-Atomic Organic to Inorganic Interface Are New Determinants of Trabecular Bone Integrity. *PLoS ONE* 8(12): e83478. doi:10.1371/journal.pone.0083478