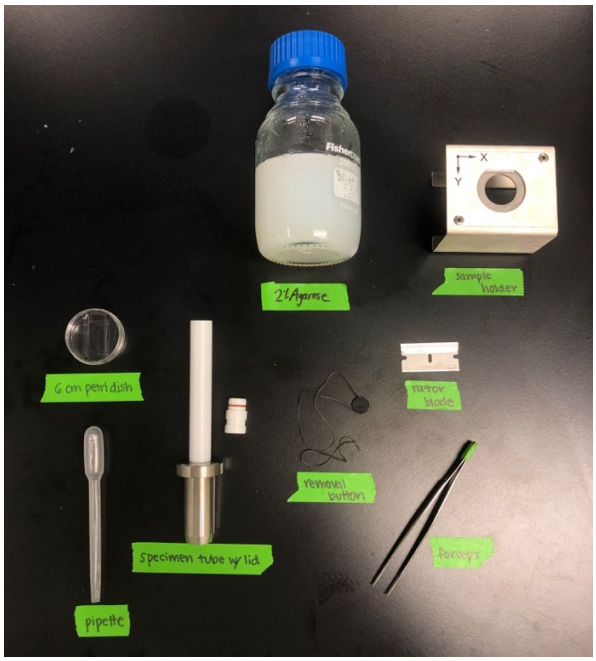
Title:	Performing Micro-Computed Tomography (microCT) Scanning and Analysis	Revision Date:	Aug 2021

Notes: The purpose of this document is to present detailed descriptions of the methods used in performing Micro-Computed Tomography (microCT) scanning and analysis, beginning with the preparation of a sample.

I. Preparation of a Sample for microCT

Materials

- SCANCO microCT 50
- Sample holder
- 6 cm petri dish (60 x 15 mm)
- Razor blade
- Forceps
- Pipette
- SCANCO Specimen tube with a lid
- Removal button
- 2% agarose



1. Microwave a jar of agarose for as long as is needed to liquefy it. Put agar to the side until it is cool enough to be comfortably held in one's hand without burning (about 38ºC). If the agar used to load the samples is too hot, you risk cooking the sample and later disrupting the histological analysis.
   Recommended time to microwave: 1 minute
   **If no jar of agarose is available, add 90.5 mL of water to 1.5 g of agarose powder and boil using a microwave/hot plate until solution is transparent.*

2. Start by pipetting warm agar onto a Petri dish, coating the bottom so as to make a base layer. Let gel cool until firm.

3. When placing bones on top of the first layer, stay consistent in your orientation of proximal and distal ends and try to make them all as vertically straight as possible. The bones need to be close enough together so they ultimately fit inside of a 14mm tube (the inner diameter is 12mm).
   **In the case of femurs, we position the distal end on the bottom and the proximal end (denoted by the femoral head) toward the top.*

4. Try to align the mid-points of the bones and be sure to keep track of the specimen numbers and specimen lengths corresponding to the positions you are loading in your sample. We recommend filling out the "Sample Information" section of the SCANCO uCT50 Bone Scanning Procedure sheet as you load the bones, however, you may also make note of this by making a sketch in your lab notebook. This step is crucial for later microCT analysis and for keeping track of the bones during and after scanning.

5. Pour a layer of agarose over the first layer to make a barrier between the first and the second layer. To the best of your ability, make the layer even as bumps will make it more difficult to place samples in the following row. Allow layer to cool.

6. Place two more bones on the barrier, substituting a toothpick for the third spot in the row. The toothpick will serve as a fiducial marker to help locate which bone is where in the scan. Add agarose to cover.

7. After agarose has solidified, use a razor to cut a block containing the samples to be loaded into the microCT50. For scanning a block of 1-5 femurs, we recommend using a 14 mm diameter specimen tube. You may choose a smaller or larger tube depending on your sample size.
   **Consult the lab manager for assistance in choosing the right sized specimen tube for scanning your sample.*

8. Cut around the bones as necessary to get the sample to fit snugly in the tube.

9. We recommend placing the specimen tube in the sample holder to prevent the tube from falling over while inserting the sample. Before inserting the agar block into the specimen tube, however, place the removal button in the tube with strips hanging out. Neither the removal button nor its strings will show up in the final scan.

10. Place agar block in the tube. By convention, we position the distal end toward the bottom of the tube and the proximal end toward the top. Push the agar block and removal button to the bottom of the tube.
    **If sample is loose in the tube, add agarose to the bottom or sides to keep it in place while scanning.*

11. Tuck in the strings so that nothing is sticking out on the outside of the tube that could possibly get caught on the inside of the microCT50 while scanning. Finally, cap the tube with a lid provided by SCANCO. Your sample is now ready to be loaded into the microCT50.

II. Loading the microCT50

1. **Before loading any new samples, check to make sure the door is open.** If door is locked, navigate to the microCT program dock on the lower left of the screen of the computer to the right of the microCT50. Single click *Button 2* to operate the measurement program. The door should now be open. If the door is still locked after clicking *Button 2*, select 'No' when prompted and hit 'Unload.' The door should now be open and you are ready to load your sample.

2. Lift door to the microCT carefully and insert the tube into one of the 12 positions on the carousel, orienting the flat side of the metal base so that it is in contact with the flat side of the dodecagon on top of the carousel.

3. If loading more than one tube onto the carousel at once, be sure to keep track of which tube is which.
   WARNING: *While running, microCT 50 must always have at least one vacant position on the carousel in case of emergencies. Never load more than 11 samples at a time.*

4. Once all samples are loaded, gently close door and navigate back to the Measurement program to begin scanning.

III. Starting a New Experiment

1. Prior to scanning any samples, the system needs to have a sample number that is system generated, under which all measurements for your experiment will be stored. The sample number is unique and can feasibly be used for hundreds of measurements, however, it is good practice to generate a new sample number for each new experiment.

2. Navigate to the microCT program dock on the lower left of the screen of the computer to the right of the microCT 50. This dock represents the workflow you'll be using when making a sample, scanning, and analyzing your data. You should see the following on your screen:

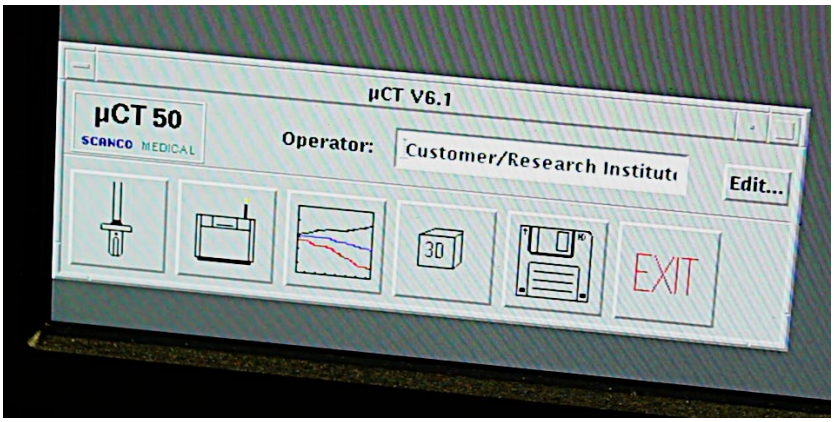


Figure 1.
MicroCT50 Program Dock

3. Single click *Button 1* on the bottom left to create a new sample.

4. Each sample number requires a name, formatted thusly:
   PILASTNAME_nameofuser_projectname
   NOTE*: There are no dates included in the sample name. The goal of formatting the sample names in this way is so that someone 5 years from now could understand it.*

5. Hit save. The sample number will automatically be generated. Write down this number as you will need to reference it when conducting your analysis or for scanning additional items.
   NOTE: *You do not need to enter any information into the 'DOB' or 'Remarks' fields to create a sample number.*

IV. Starting a Set of Scans

1. Once samples are loaded into the microCT and the sample number has been obtained, click *Button 2* to operate the measurement program.

2. When prompted, say "Yes" that you would like to do a scan.

3. Enter the sample # you will be using for your experiment.

4. Before adding any new scans to the task list, click 'Task List…" and clear the task list if there are any existing tasks. Click 'Close Window' once the task list is clear.

5. The machine will automatically check to see which locations on the carousel are occupied and highlight the number next to diamonds that have a sample. Click on the diamond next to the carousel position corresponding to the sample you would like to scan first.

6. Type in and select the controlfile you intend to use for your experiment. **Controlfiles should be set per experiment under the advisement of the lab manager**. The controlfile tells the machine (1) what size tube you're using, (2) the resolution you're using, and finally (3) the energy settings used for the X-ray.
   NOTE: *You should be using the same controlfile, without edits, for your entire experiment.*

7. Once the controlfile is selected, click 'Scout View.' A default range of scanning from the controlfile will be selected. We recommend scanning the entire length of the tube.

8. Once the range is set, click 'Scout View' on the right. The machine will then grab the selected tube, place it in the sample holder, and perform the scout scan—a low-resolution 2D projection image of the specimen holder. This can take up to a few minutes in the case the x-ray tube has to warm up.

9. Once the scout scan is complete, click 'Reference line' to begin selecting your region of interest for scanning.

10. The solid green line will indicate where a scan will start while the dotted green line will indicate where a scan will end. To adjust the space between the two lines, hold down the left shift key and move

mouse up or down to increase/decrease the number of slices included. To lock green lines into place, click the mouse button. To change the position of the lines after locking, hit 'Reference line' again.

11. It is good practice to make note of the scanning parameters used for each scan you complete in the lab. We recommend filling out the "Scanning Parameters" and "Measurement Notes" sections of the SCANCO uCT50 Bone Scanning Procedure sheet—specifically recording the controlfile #, the # of slices, and the time required to complete the scan. These notes will help you replicate the results of your experiment at a later date, if necessary. See the lab manager for help in filling out the rest of the worksheet.

12. Once you have set the reference lines and made note of the necessary information for your scan, press 'Add scan'. The scan is now in the task list, awaiting a final command to be processed.
NOTE: *Each scan will result in a measurement. Measurement numbers will not show up until the scan is completed, therefore, you will only make note of the measurement number in the final step.*

13. Repeat steps 5-12 for any other tubes you are scanning in this experiment. If multiple samples are stacked in one tube, repeat steps 9-13 for the second region of the tube to be scanned. Be sure to keep track of which scan corresponds to which samples.

14. Once all scans have been added, click 'Ok' on the scout view window.

15. Check the task list one last time to make sure all scans have been added to the queue, then press 'Submit batch scans.'
NOTE: *The Measurement program will close once a batch scan is submitted. This does not mean that the program has crashed.*

16. It is good practice to check the results of the scans after they are completed by navigating to the Evaluation software. You will also be able to make note of the measurement # at this time. The measurement numbers will be listed in the order in which the scans were submitted. Once you have identified your most recent scan results, go back to the "Measurement Notes" section of the SCANCO uCT50 Bone Scanning Procedure worksheet to take note of which measurement #s correspond to which scan.

V. Analyzing Your Scans

1. Click *Button 3* to activate the evaluation program.

2. Select a measurement from your sample number and double click.
**WARNING: *When selecting a measurement, never hit 'Cancel.' If you hit cancel and run an evaluation, your data will be invalid. If you have already chosen your measurement of interest and wish to stay on this measurement, simply double click the measurement again.*

3. If conducting cortical analysis, proceed to the following section, A. If conducting trabecular analysis, proceed to section B.

A. Cortical Analysis

1. In order to perform cortical analysis, we must first identify the cortical region of the bone. If there is more than one bone in the scan, refer to the sketch you should have made illustrating the position of the bones and the toothpick, and begin with the bone in position 1.
   NOTE: *The following instructions assume you scanned the entire bone length.*

2. Identify the slices at the start and end of your bone of interest. Identification will vary from user to user; therefore, it is important to be consistent across measurements. Subtract the starting slice # from the ending slice #, divide by two, and add this number to the starting slice #. You have now successfully identified the midpoint of your bone of interest.

3. For cortical analysis, we recommend conducting the evaluation over a 1mm length of the diaphysis at a 7.4 μm resolution. This is equivalent to about 135 slices, where the cortical region corresponds to about 67 slices greater than and 67 slices less than the identified midpoint. We denote the starting slice of this region as 67 slices less than the midpoint.

4. Go to the "Zoom" menu and select a magnification of your choice to zoom in on your bone of interest.
   **Suggested magnification: 4x*

5. Select the starting slice of the cortical region and click on the "C…"

6. Choose "Forwards" in the selection column.

7. To draw a contour around the surface of the bone, first hit the "lasso" button on the top left, hold down the mouse and trace near the outer (periosteal) surface in a COUNTERCLOCKWISE motion. Release mouse once tracing is complete.

8. Found in the Contouring window are the recommended contour thresholds. For cortical analysis, these thresholds are around 30 and 500 for the outer and inner thresholds, respectively. Contour thresholds aid the computer in integrating our area of interest across the many slices of the bone measurement and save the user from going through each slice and drawing the contour every time.
   NOTE*: Contour thresholds are different from evaluation script thresholds and do NOT need to be the same between bones.*

9. Double click anywhere in the box—see Figure 2—so that the evaluation software may automatically adjust the trace you drew to the contour of the bone.

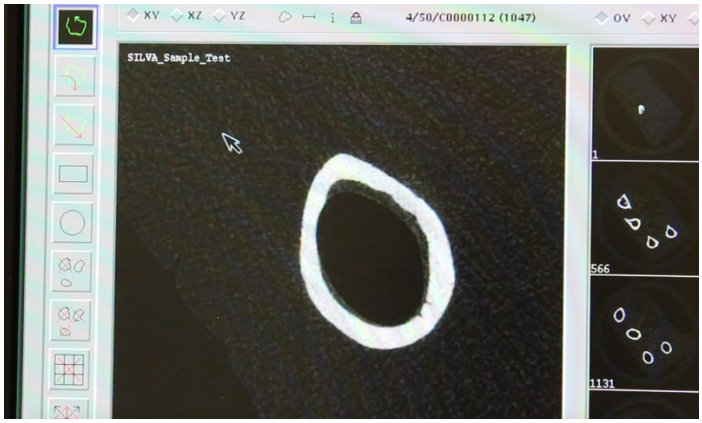


Figure 2.
Screenshot taken from Cortical Analysis SOP

If there are any loops or other discrepancies present where the generated contour deviates from the bone surface, you may double click again or manually fix the error, again tracing counterclockwise.

10. Once you are satisfied with your contour, click on “Iterate forwards.”

11. Iterate for 135 slices, then hit “Stop” in the contouring window to terminate iteration. If you mistakenly iterate past 135 slices, simply select the contours you’d like to erase and click “Delete.” Hit “Yes” to delete the selected contours when prompted.

12. After all automatic contours have been drawn, scroll through the slices to check for contour accuracy, going in to fix any errors as needed.

13. Now that iteration is done, click “T…” on the bottom left.

14. A window titled, ‘3D Evaluation,” will pop up where you must choose an evaluation script that specifies what is and what is not going to be evaluated as bone in the image analysis. **Evaluation scripts should be set per experiment under the advisement of the lab manager.** Note that, unlike the contour thresholds, the same evaluation thresholds must be used for all measurements in the analysis. We will have a separate instructional video explaining the process of choosing appropriate evaluation thresholds that will favorably align with all samples in an experiment, not just a single measurement.

15. Once the evaluation script has been chosen, hit ‘Default VOI’ then ‘Start Evaluation.’ Defaulting the volume of interest is extremely important as forgetting this step will stall your analysis because the software will try to analyze a square of black space, searching aimlessly for bone that it will never find. Further, when evaluating other samples in the same measurement, this step is important to adjust the analysis region to your current bone of interest from the previously contoured and analyzed bone.

16. Hit 'Yes' to save all contours when prompted. Note the specimen # and the time at which you complete this step in your lab notebook as this information will be crucial in locating the results of your analysis in FileZilla later.

17. You can check the status of the analysis by typing 'que' in the DECTerm window which should already be open in the background, see Figure 3.

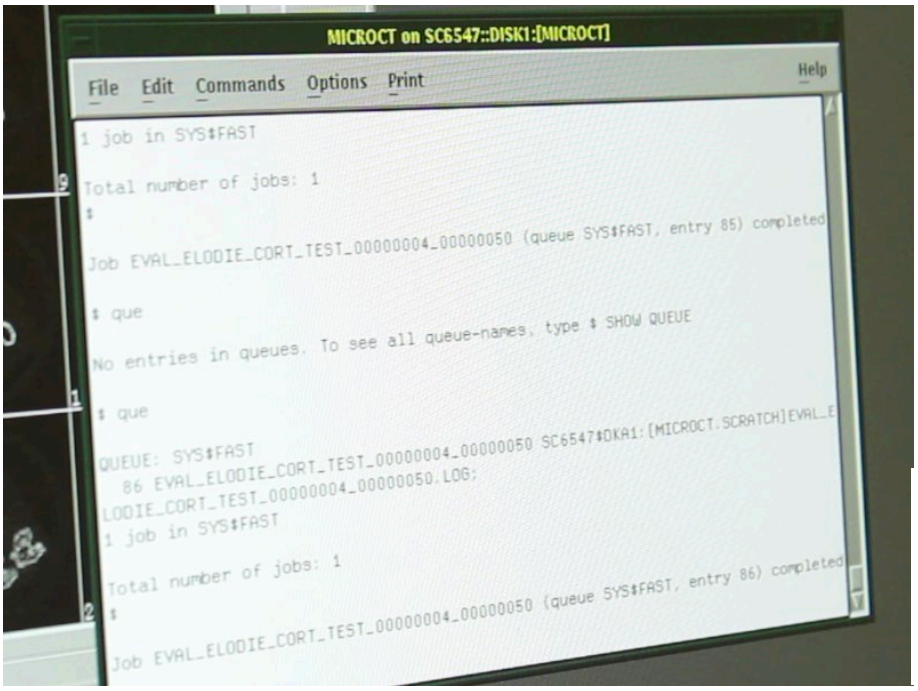


Figure 3. DECTerm window where a user may check the status of their analysis.

In this window, you would also find a completion message once the analysis of that specific bone in the measurement has finished.
NOTE: *If the DECTerm window is not already open, navigate to the toolbar at the top of the screen, find 'Applications,' and finally select 'DECTerm' from the dropdown menu.*
**IMPORTANT: *Do not begin the contour tracing and analysis of another bone in the same measurement while an analysis is already taking place. Wait until the previous analysis has finished, then move onto the next bone.*

18. Once completed, clear previous contours by hitting the  button to remove all contours from all slices. Click 'Yes' to confirm.
    NOTE: *When performing this step, you are not erasing the contour data from the previous bone analysis, but rather clearing the canvas to make way for the next set of contour iterations. We will have a separate instructional video explaining the process of retrieving previous contour data after clearing.*

19. Move on to the next bone in your measurement and repeat steps 3-18. Don't forget to hit 'Default VOI'!

20. Repeat steps 1-19 if multiple measurements need to be analyzed within the same experiment. Repeat this procedure if multiple measurements need to be analyzed within the same experiment.
    NOTE: *You may complete these analyses in different user sessions.*

B. Trabecular Analysis

1. In order to perform trabecular (cancellous) analysis, we must first identify the trabecular region of the bone. If there is more than one bone in the scan, refer to the sketch you should have made illustrating the position of the bones and the toothpick, and begin with the bone in position 1.
   NOTE: *The following instructions assume you scanned the entire bone length.*

2. For trabecular analysis, we recommend conducting the evaluation over a 1.5 mm length of the metaphysis at a 7.4 μm resolution. This is equivalent to about 200 slices directly adjacent to the metaphyseal growth plate. That being said, the first step in identifying the trabecular region is identifying the location of the growth plate.

3. To do so, we look for the following progression, illustrated in Figure 4:

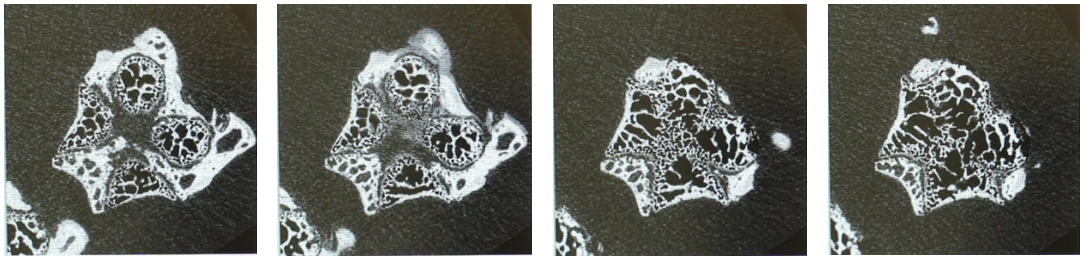


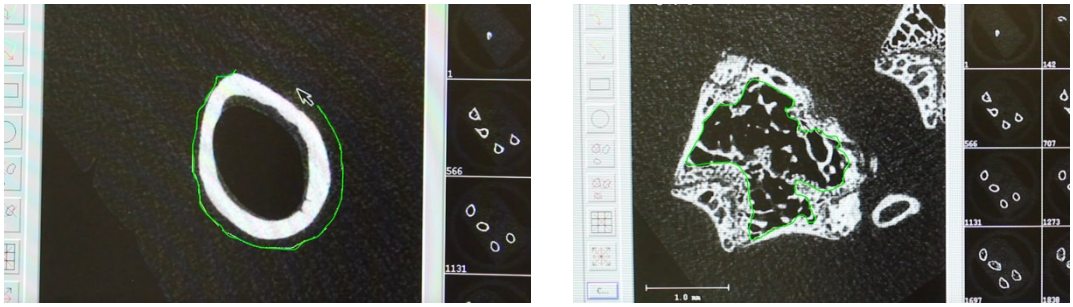
Figure 4. *The progression of the metaphyseal growth plate disappearing to make way for the metaphyseal region.*

As can be seen in the picture on the far left, the growth plate can be recognized by the four ovals that constitute the crown of the plate. As we get closer to the metaphyseal region, the ovals begin to merge, and we can see above how the trabeculation in the bone progressively increases as well. In the picture on the far right, we can see that the middle of the bone has opened significantly since the first picture—we denote this slice as the starting slice of the metaphyseal region. The transition between the last two stages can often be tricky to distinguish, and for this reason, the identification of the starting slice will vary from user to user. For more help in identifying the growth plate, the metaphyseal region, or the starting slice, see the lab manager.

4. Once the trabecular region has been identified, go to the "Zoom" menu and select a magnification of your choice to zoom in on your bone of interest.
   Suggested magnification: 4x

5. Select the starting slice and click on the "C…"

6. Contrary to cortical analysis, we will now be drawing our contour on the inner border of the shell of the metaphyseal cavity as opposed to tracing the outer surface. See Figures 5a & 5b.



(a) (b)

Figure 5. *(a) Contour drawn along outer bone surface in Cortical Analysis.*
(b) Contour drawn along inner bone surface in Trabecular Analysis.

7. To draw a contour, first hit the "lasso" button on the top left, hold down the mouse and trace in a COUNTERCLOCKWISE motion. Release mouse once tracing is complete.

8. Because the contours are now being drawn on the inner border, the thresholds need to be flipped. For trabecular analysis, the recommended contour thresholds (which can be found in the 'Contouring' window) are around 500 and 30 for the outer and inner thresholds, respectively. Contour thresholds aid the computer in integrating our area of interest across the many slices of the bone measurement and save the user from going through each slice and drawing the contour every time.
   NOTE: *Contour thresholds are different from evaluation script thresholds and do NOT need to be the same between bones.*

9. Once you are satisfied with your thresholds, choose 'Backwards' in the selection column and click 'Iterate Backwards' to begin iterating the trabecular region. The contour may misbehave slightly white iterating; if so, stop the iterations, go back to the slice with the error, and use the freehand adjustment tool (button below the lasso) to correct the mistake before continuing to iterate. Hit 'Iterate Backwards' to resume iteration.

10. In the case the bone does not allow you to use the automatic iteration tool, draw contours by hand intermittently (e.g. every 10 slices), and then use the morph tool (found in the

'Contouring' window) to fill in the gaps. You may need to increase or decrease the frequency of drawing contours by hand depending on the size and shape of the bone. Try to hit key frames where the shape of the bone starts to change.
NOTE: *Interpolated slices will show up as red. This doesn't mean they are bad, just interpolated.*

11. Once you have drawn all your contours and morphed or iterated as needed, hit "Stop" in the contouring window to terminate iteration. If you mistakenly iterate past your desired number slices for the trabecular region, simply select the contours you'd like to erase and click "Delete." Hit "Yes" to delete the selected contours when prompted. Finally, click "T…" on the lower left.

12. A window titled, '3D Evaluation," will pop up where you must choose an evaluation script that specifies which pixels are going to be evaluated as bone in the image analysis. **Evaluation scripts should be set per experiment under the advisement of the lab manager.** Note that, unlike the contour thresholds, the same evaluation thresholds must be used for all measurements in the analysis. We will have a separate instructional video explaining the process of choosing appropriate evaluation thresholds that will favorably align with all samples in an experiment, not just a single measurement.

13. After the evaluation script has been chosen, hit 'Default VOI' then 'Start Evaluation.' Defaulting the volume of interest is extremely important as forgetting this step will stall your analysis because the software will try to analyze a square of black space, searching aimlessly for bone that it will never find. Further, when evaluating other samples in the same measurement, this step is important to adjust the analysis region to your current bone of interest from the previously contoured and analyzed bone.

14. Hit 'Yes' to save all contours when prompted. **Note the specimen # and the time at which you complete this step in your lab notebook as this information will be crucial in locating the results of your analysis in FileZilla later.**

15. You can check the status of the analysis by typing 'que' in the DECTerm window which should already be open in the background, see Figure 3. In this window, you would also find a completion message once the analysis of that specific bone in the measurement has finished.
NOTE: *If the DECTerm window is not already open, navigate to the toolbar at the top of the screen, find 'Applications,' and finally select 'DECTerm' from the dropdown menu.*
**IMPORTANT: *Do not begin the contour tracing and analysis of another bone in the same measurement while an analysis is already taking place. Wait until the previous analysis has finished, then move onto the next bone.*

16. Once completed, clear previous contours by hitting the  button. Click 'Yes' to confirm.
NOTE: *When performing this step, you are not erasing the contour data from the previous bone analysis, but rather clearing the canvas to make way for the next set of contour iterations. We will have a separate instructional video explaining the process of retrieving previous contour*

data after clearing.

17. Zoom in on the next bone and repeat steps 3-16. Don't forget to hit 'Default VOI'!

18. Repeat steps 1-17 if multiple measurements need to be analyzed within the same statistical analysis.
    NOTE: *You may complete these analyses in different user sessions.*

VI. Exporting Data to Excel

1. On your desktop or laptop computer, open FileZilla. FileZilla is a program intended to allow remote users to transfer files between computers over FTP. In this lab, it is often used to transfer single or few files, like TIF images generated with Scanco software.

2. To connect, type the appropriate host IP, username, and password. See the lab manager for log-on information. After typing in all relevant information, hit quickconnect (You can also save a profile in the site manager tool if desired).

3. By default, you'll be connected to disk1:[microct]. We typically have people save TIF images to the scratch folder, at disk1:[microct.scratch]. Result files from cortical and trabecular analysis will be found in the disk1:[microct.results] folder.

4. Double click the 'Results' folder. In this folder you will find two types of files: files beginning with UCT_LIST_MOILOG and UCT_LIST_3DLOG. The latter corresponds to results from trabecular analysis while the former corresponds to results from cortical analysis. The name that comes after UCT_LIST_MOILOG or UCT_LIST_3DLOG is what we refer to as the export file name. The export file name is set by the lab manager when the evaluation script for your experiment is being created and it should closely resemble the name of the evaluation script.

5. Once you have located the files in the results folder of interest to you, the data can be copied over to the desktop by dragging and dropping to a folder, or by right clicking and selecting 'Download.' Note which folder is selected in the download pane on the left to be able to track down the downloaded data later. Also, when transferring data from the remote machine, make sure that 'Transfer à transfer type' is set to 'Auto.'

6. After moving to your folder, files will still be in a VMS style. Remove ";1" from the file names to be able to open and view data. Note, cortical data gives 2 outputs, two-dimensional and three-dimensional—we usually ignore the 3D output.

7. Hit "Yes" to rename the files when prompted.
   *NOTE: *If you have many files to rename at once, the lab manager can use a script to assist with the process.*

8. Finally, drag renamed files to an Excel worksheet to open and view. Listed in the following table are the relevant Cortical and Trabecular (Cancellous) parameters:

Table 1. *Recommended Parameters for Cortical and Cancellous Results*

Recommended Set of Cortical Parameters	Recommended Set of Cancellous Parameters
• Total Area (TA) • Bone Area (BA) • Medullary Area (user calculated: TA-BA=MA) • Cortical Thickness (Ct.Th: from DT-Ct.Th column) • Polar Moment of Inertia (pMOI) • Tissue Mineral Density (TMD: from the Mean2 column)	• Bone Volume (BV: from the VOX-BV column) • Total Volume (TV: from the VOX-TV column) • Bone Volume over Total Volume (BV/TV: from the VOX-BV/TV column) • ConnectivityDensity (Conn.D) • Structure Model Index (SMI: From the TRI- SMI column) • Trabecular Number (Tb.N: from the DT-Tb.N column) • Trabecular Thickness (Tb.Th: from the DT-Tb.Th column) • Trabecular Separation (Tb.Sp: from the DT-Tb.Sp column) • Volumetric Bone Mineral Density (vBMD: from the Mean1 column)

**See "Understanding uCT Outcomes" on the WUSTL MRC Core B webpage for an explanation of these parameters and their significance in your experiment.*

9. We will now go over **how to locate the results of a particular specimen from your experiment**. To do so, check the timestamp located in the column titled, "ListDate," and cross reference with the time you should have made a note of for your specimen of interest after saving your contours during the analysis stage.
   *If you are still having trouble locating the results of your experiment, see the lab manager. If you need a better understanding of Filezilla, please go to https://filezilla-project.org/ or https://wiki.filezilla-project.org/Documentation.

VII. Generating 3D Images from Quantitative Scans

1. Navigate to the microCT program dock.
2. Click *Button 4* to activate the 3D viewer software.
3. Navigate to your sample and measurement numbers the same way you would in the Evaluation software.
   NOTE: *Only measurement numbers for which a 3D object exists will be displayed so not all your measurements may show up.*
4. By default, the 3D viewer will only display the most recently created 3D object which aligns with the most recently completed numerical evaluation.
5. To display a previous 3D object, select a sample number, select 'All Files,' and double click the _SEG.AIM file corresponding to the 3D object of interest.
   NOTE: *The semi-colon and number following the file name indicate the order in which the 3D objects were generated where ";1" is first, ";2" is second, and so on. This is another reason why it is important to make note of the order in which you run your analyses.*
6. Once you have found your 3D object of interest, click once anywhere on the blue screen or click 'Start' on the lower left. The software will now begin to do a 4-pass render to complete a drawing of the 3D object.
7. To rotate the object, you can interrupt the render at any time by dragging the mouse in the blue space. This will cause the software to redraw the 3D object in its new orientation once you release the left mouse button. How you are moving the object will be indicated by the white rectangular box that appears when the mouse is clicked.
   NOTE: *You may also use the sliders on the left of the screen for more precise image adjustment—both for object rotation and lighting adjustment.*
8. **You cannot alter a 3D object**, so feel free to play around as much as you'd like. To reset the object to its original settings, simply close the 3D viewer and re-open the object you're interested in.
9. Once you have oriented the object to your liking, you can remove portions of the bone to get a better look at the inside or to reduce the amount of bone shown. To do this, click 'Subdim' on the top dropdown menu and then subdim again to bring up a set of sliders that represent the boundaries of what will be drawn in the X, Y, and Z-directions of the scanner-defined space.
   NOTE: *The xy-, xz-, and yz-planes CANNOT be adjusted to the orientation of your bone, hence why we encourage users to orient bones as straight and close to vertical as possible in the tube.*
10. To cut your image, move the sliders in your direction of choice and then release. If the image does not update automatically, click in the blue area to refresh. Like with tweaking the orientation and lighting

settings, any changes made to the amount of bone rendered will not be permanent and do not alter the original 3D object.

11. Once you have oriented and cut away bone to your liking, you can save the image by fist allowing the current rendering to finish all its passes. Then go to **File --> Print**.
    NOTE: *If for some reason 'Print' is unable to be selected, it usually means that you have not allowed the rendering to finish all of its passes (i.e. finish loading). To fix this error, wait until the red timer on the blue screen disappears and try again.*

12. Once you have successfully navigated to the 'Ray: Print' window, click TIFF and Scratch/Temp. Finally, give the file a name that has the following format: firstname_anotheridentifierofmeaningtoyou

13. Click 'Ok' when done. The picture will then save to the scratch directory. See SOP video on how to export data to Excel for help in locating the image after it has been saved.
    NOTE: *If the following warning, "ERROR: Error Creating TIFF-Image," comes up, you have likely surpassed the maximum # of characters allowed for a file name. To fix this error, simply make the file name shorter and try to save again.*

VIII. Choosing Evaluation Thresholds

1. Evaluation script thresholds determine which voxels in your scan will be counted as bone during analysis. This data is then used to calculate trabecular thickness, bone area and volume, and more. Unlike contour thresholds, the same evaluation thresholds must be used for all measurements in the analysis. Therefore, it is extremely important that the user chooses values for the threshold that will favorably align with all samples, not just a single measurement.

2. To determine these values, navigate to the "3D Evaluation" window. On the right you will find the evaluation script settings which include the Upper and Lower thresholds along with the gauss filter settings. To suppress noise, we apply a gauss filter. Gauss sigma increases the weight of neighbor pixels while Gauss support controls the number of used neighbor pixels. You will usually not need to worry about the gauss filter as the standard for sigma and support are 0.8 and 1, respectively.

3. Back to determining the appropriate threshold values. Toggle back and forth between "Preview" and "Grayscale" to see what is being masked as bone and what is not. We recommend using a pencil or some other marker that you can hold up to the computer screen to compare Preview and Grayscale images.

4. In the following figures we will demonstrate how to distinguish between what is too low or too high of a lower threshold value for a trabecular analysis.
    NOTE: *There will rarely be a time where you adjust the upper threshold; You will usually always be adjusting the lower one.*

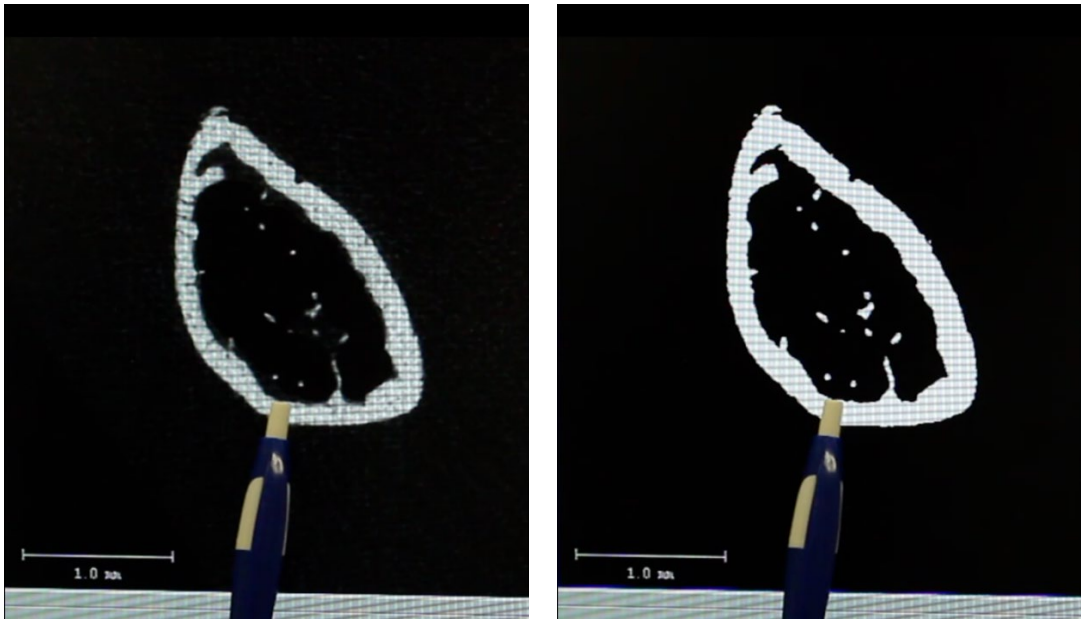


Figure 5. Comparison of Grayscale (left) and Preview (right) images at a lower threshold value of **158**.

Above is an example of OVER-masking, i.e., the chosen lower threshold value is too low, and more bone is being incorporated into the Preview than is present in Grayscale.

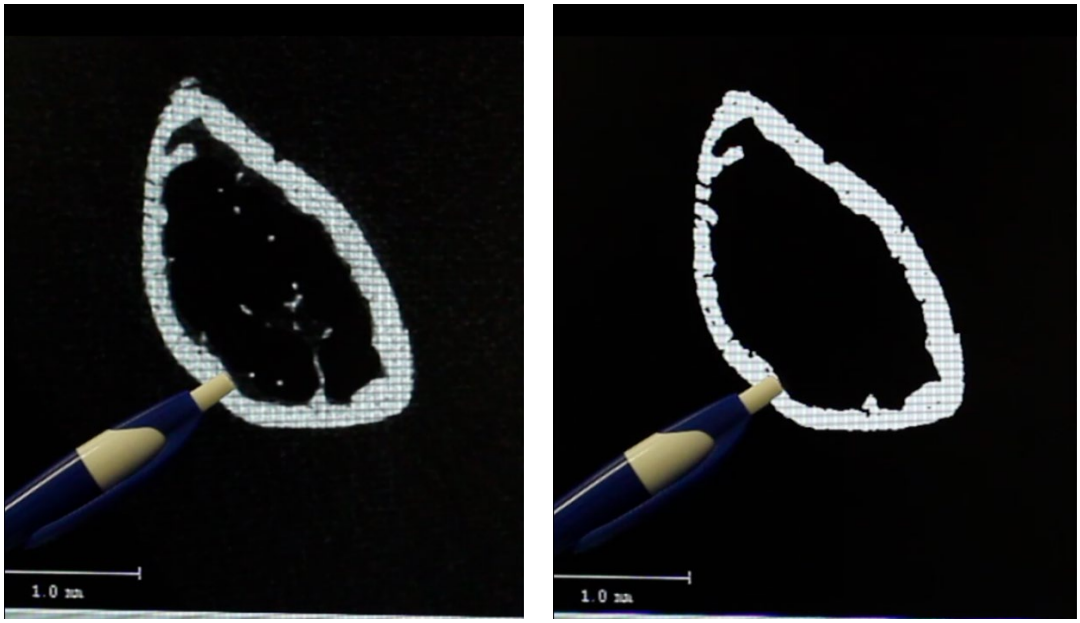


Figure 6. Comparison of Grayscale (left) and Preview (right) images at a lower threshold value of **395**.

Above is an example of UNDER-masking, i.e., the chosen lower threshold value is too high, and more bone is being excluded from the Preview than is present in Grayscale.

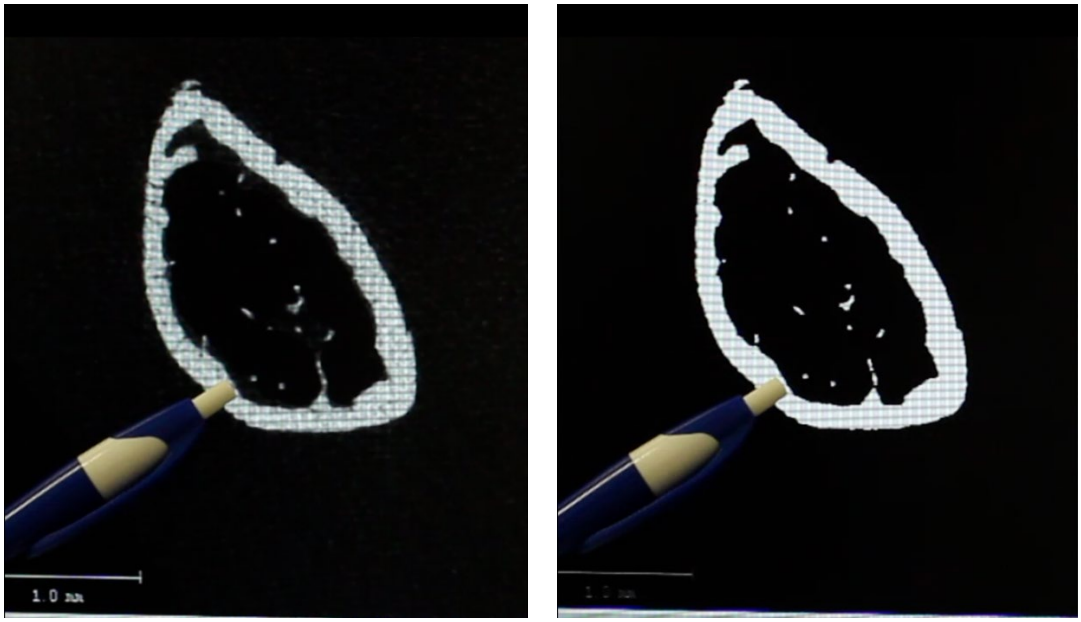


Figure 7. Comparison of Grayscale (left) and Preview (right) images at a lower threshold value of **263**.

Above is an example of ideal masking, i.e., the chosen lower threshold value is just right, and about the same amount of bone is being incorporated into the Preview as is actually present in Grayscale.

IN SUMMARY: If not enough is being masked (i.e. preview is incorporating more bone than is actually present into analysis), RAISE the lower threshold. If too much is being masked, LOWER the lower threshold.

5. To verify that the thresholds will hold for other bones in the analysis, compare thresholds for 3 animals per sample group.

6. Once you are satisfied with the chosen thresholds, see the lab manager for help in creating a new evaluation script for your analysis.
   **IMPORTANT: Do not edit any existing evaluation script as you risk disrupting another lab user's analysis and compromising their data.