

Chang 7/26/2010

# Trabeculae Analysis Protocol for BioQuant OSTEO 2010

## Novack's lab

### General Info (READ FIRST)

- This protocol only covers a few features in the BioQuant, which are the histomorphometry analysis for rodent trabecular bone.
- There is much subjectivity during analysis, such as choosing ROI, outlining bone, and defining osteoblasts/osteoclasts. The position at which you cut bone into slices adds variance too. Those factors are not a big issue as long as you can be consistent during the measurements and have enough samples. In order to be more objective, BioQuant users should not know which slice they are working on.
- The trabeculae underneath the growth plate in the proximal end of tibia are the recommended area for the analysis of rodent trabecular bone.
- To set the objective of the microscope, usually 4× is for tissue volume, 10× is for trabecular bone and osteoclasts (OC), and 20× is for osteoblasts (OB).
- Stitched high magnification image is highly recommended as opposed to manual move mode because of the following reasons: (1) do not need to worry about positioning errors, such as bumping stage accidentally or clicking wrong buttons; (2) you have a image file that can be stored and measured again; (3) orientation of bone slices can be easily modified to facilitate the measurement.
- All the measurements only apply to the current ROI (Region of Interest) area. You will need to redefine it often.
- The system files (e.g., data volume file, array list file, and calculation set file) used in this protocol are customized for routine rodent trabeculae analysis. They are manually defined by users and may not be found in other BioQuant systems.
- It is recommended to follow the order of the procedures in Analysis section shown below because of the following reasons: (1) some parameters are used to calculate some of the later parameters; (2) you only can undo some of the procedures; (3) the program may freeze from messy procedures. Once you find you cannot draw anything to the image, you have to exit and restart the program.
- Use a right click of your mouse to get out of the *Image Window* if your cursor gets stuck.
- *Clear Overlay* button  in the Shelf window doesn't erase data, just clears the Image Window. You will need it to hide the markings/tracings.

- *Toggle Display Threshold* button  (or Ctrl + D) in the Shelf window will be useful if you do not want the solid yellow threshold of bone to get in the way when you define OB/OC surface.
- *System Redraw* button  (or Ctrl + R) in the Shelf window puts the markings/tracings back. You will need to redraw often.

## **Reservation**

There are 3 BioQuant systems on medical campus. If you want to use the one in Dr. Deborah Novack's lab (Yalem 602), please make a reservation in Google Calendar. Account: Histo.BioQuant@gmail.com, password: pathoquant10. If you have to stay late or work in weekend, you can ask for a key to enter the room. In addition, please sign in a register sheet beside the microscope.

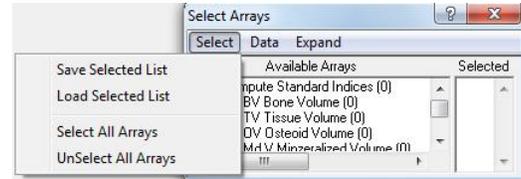
## **Contact**

If you encounter problems, please contact Deb Novack (454-8472, novack@wustl.edu) or Chang Yang (454-5975, cyang@dom.wustl.edu).

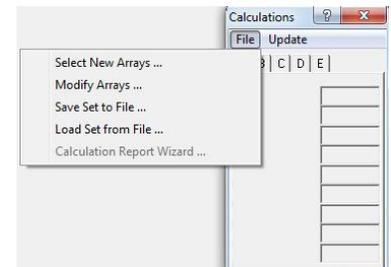
## Set Up Working Environment

1. Make sure you read and understand the General Info in the first page.
2. **Launch BioQuant OSTEO 2010** from desktop.
3. **Get image from microscope:** in main menu of BioQuant, go to *Image -> Open QImaging Camera...* If microscope is on, this should turn the *Image Window* white instead of black. If not, check the output from the microscope (top right, round slider), which should go to camera or eyes+camera. For bright field, check that dial on front of microscope is on #1. Use #4 for tetracycline/calcein fluorescence. These microscopic settings only apply to the one in Novack's lab.
4. **Adjust image:** place your slide on the scope. Along the top bar of icons, click *Auto Expose* button . Now your tissue should show. Focus to screen and put a field with some or all white (no tissue) on the screen and choose *White Balance* button . If you want to adjust the image manually, click *Image -> Camera Setup...* and you can have more control in the *Camera Settings* window. Click *Enable Large Image Capture* button  if you want to save 1280 × 960 image and it is recommended.
5. **Make stitched image:** take a series of high magnification pictures with about 20% overlaps to cover the whole area to be analyzed, and then save them individually (*File -> Save Image(s) -> Save Single Image...*). The dimension of each picture should be 1280 × 960. Stitch them together in a third-party software. If you use Photoshop CS2, use *File -> Automate -> Photomerge...* Once you get the big stitched image, rotate it to an orientation in which the horizontal axis of the growth plate is parallel to the top edge of the image by using *Image -> Rotate Canvas -> Arbitrary....* Adjust color, brightness, or contrast if necessary. Open it in BioQuant by clicking *Load Image* button  on main toolbar or *File -> Load Image(s) -> Load Single Image...* You will see your stitched image in the *Image Window*. A *Large Image Navigator* window will show up and click *Shrink/Expand Image* button , now you can see the whole image in the *Image Window* and it is ready to define a ROI of tissue volume.
6. **Choose data volume:** click *Open Data Set* icon  on main toolbar or *File -> Open Data Set...* from main menu. Highlight *General Bone Volume* in *Data Volumes* box and *General Bone Master* in *Data Sets* box, and click *Open* button. Data volume defines what kind of measurements will be used in the following analysis and can be seen as a project name, so stick to the same one for same experiments.
7. **Choose data set:** click *Quick Data Set* button  on main toolbar or *File -> New -> Quick Data Set...* from main menu. Name your data set and leave *Current Data Set* selected. Click *Create* button. Data set is where the results from a specific sample store, so slide ID works well.

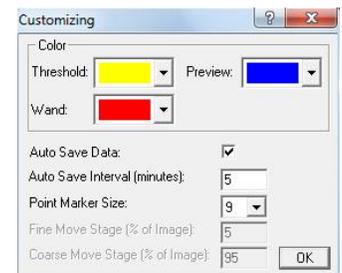
8. **Choose arrays for measurement:** in *Select Arrays* window, under *Select* menu, *Select All Arrays* if you need all of them or *Load Selected List* then choose *General Bone* in pop-up window if your purpose is trabecular bone analysis. If the *Image Window* screen turns solid yellow after you load the arrays, use the *Clear Overlay* button  in *Shelf* window to see your image. Selected arrays here define what kind of parameters you want for the following measurements. The number in the parenthesis at the end of every array denotes how many values are measured by it.



9. **Choose calculation results:** Find *Calculations* window, click *File* -> *Load Set from File...*, and choose *General Bone* in the *BQ Open File* window. Different measurements are found under the A, B, and C tabs. The items in the tabs of *Calculations* window are the items you will see in your final results spreadsheet. Each item equals a parameter of measurement.



10. **Choose color assignment (optional):** click *Option* -> *Customizing...* on the main menu. In the color box of *Customizing* window, make sure *Threshold* and *Preview* are assigned to different color. For later on, if you see nothing change when you click the *Preview* button, you might need to go back here to change it, as you will need that feedback. Yellow Threshold and blue Preview are recommended.

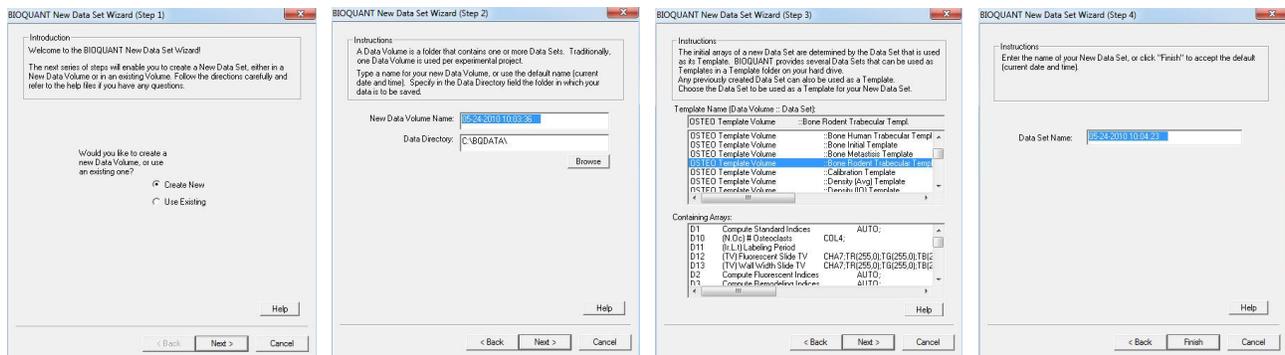


## Define Your Own System Files

*General Bone Volume* and *General Bone Master* in step 6, *General Bone* file in step 8, and *General Bone* file in step 9 are created manually by users for trabeculae analysis. You can make your own system files to match your need and facilitate your analysis.

### Define your own data volume and data set

Click File -> New -> New Data Set... from the main menu. A wizard called BIOQUANT New Data Set popup. There are 4 steps to create your own data set. In step 1 window, you can choose to either create a new data volume or modify an existing data volume. In step 2 window, name your own data volume. In step 3 window, select your own template. For the purpose of rodent trabeculae analysis, Bone Rodent Trabecular Templ. is recommended. In step 4 window, name your data set. Now, your own data volume and data set will show in the *Open a Data Set* window.

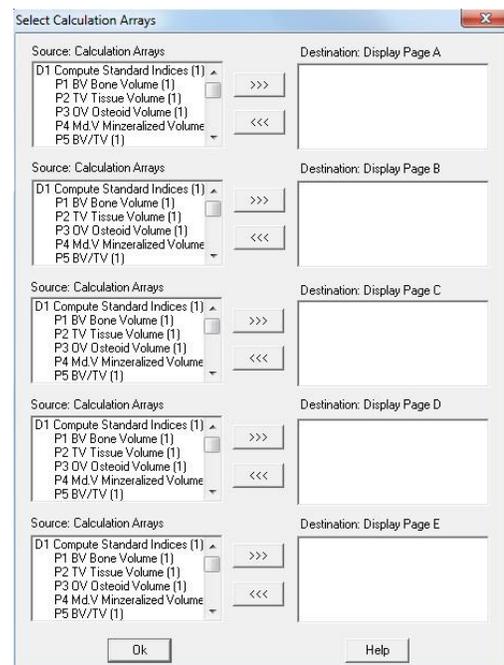


### Define your own array list

Follow these steps: in *Select Arrays* window, *Select -> UnSelect All Arrays ->* double click the arrays you want in the *Available Arrays* box -> repeat until all the arrays you want are in the *Selected* box -> *Save Selected List ->* name your bqa file. By default, your own array list file will be saved to C:\BQOSTEO\Selected Lists.

### Define your own calculation set

In *Calculations* window, choose *File -> Select New Arrays...* A window called *Select Calculation Arrays* shows up, put the parameters you want in to different pages. There are 5 pages you can choose, and normally 3 pages are enough. The parameters in page A will show in tab A in the *Calculations* window. Once you are done with selecting, save your own calculation arrays by choosing *File -> Save Set to File...* -> name your bqc file. By default, your own calculation set file will be saved to C:\BQOSTEO\Calculation Lists.



## Analysis

1. **Set magnification and topography:** In *Parameters* window, make sure the microscope objective in use matches the magnification selected. If a stitched high-power image is loaded, set the *Mag* accordingly. Start with the 4x objective to select your area of measurement. Check the box for *Save Topo* as all the topographic info will be saved (saved in array D5 by default) and showed in *Overview* window.

2. **Set a consistent ROI** for tissue volume measurement. Click the *wrench* icon  on main toolbar if *ROI Tools* window is not there. In *Create* tab of *ROI Tools* window, choose *Rectangle* from *Type* drop-down menu. Now ignore the green box that appears in the *Image Window*. Set the height and width. Click *Define ROI* button, cursor will show up in the *Image Window* in the shape of a cross with a dark-blue rectangle box round it. When you are satisfied with the position of ROI, left click to fix it and right click to get out.

A consistent ROI for all the bone slices is critical for the whole analysis and how to set it is somewhat tricky. To balance the pros and cons between irregular ROI tracing growth plate and rectangle ROI with fixed size, a rectangle ROI with a fixed height and distance to growth plate and a variable width might be practical.

Maintaining the horizontal axis of ROI parallel to the growth plate helps to maintain consistency among samples. A constant distance to growth plate helps too.

Although bone slices can vary, it is recommended to have a ROI with enough height and about 100  $\mu\text{m}$  distance between the top edge of the ROI and bottom edge of the growth plate. Stamp Tools can help you to measure the distance. Click the *stamp* icon  on main toolbar, choose *Diagonal* line type and set *Distance* in the *Stamp Tools* window. Click *Define* button to draw a line in the *Image Window*, which will give you an idea of distance. *Erase All* button can delete defined lines.

3. **Measure tissue volume (TV).** Highlight *D6* in the *Selected* box of the *Select Arrays* window. Now, your ROI turns solid yellow with a green edge. Find *Auto Measure* window (or you can click the *compass* icon  on the main toolbar if it is not there), click *Preview* button in *General* tab. Your ROI will have an extra blue edge which is the color set in the *Customizing* window. Then click *Measure* button to obtain tissue volume (TV). Now ROI turns light blue. You will see a box that appears in the *Overview* window. The TV value will appear in the *List Current Array* window and the number in *D6* parenthesis will turn to 1.

4. **Change to higher magnification.** Once you get the TV value in low magnification field, change to high power to measure other parameters if necessary. To set the environment for higher magnification,

If you use a stitched image, click *Shrink/Expand Image* button  in the *Large Image Navigator* window. The stitched image switches to full size and you can only see the upper-left part of it. Drag the small blue box in the *Large Image Navigator* window, which corresponds to the field shown in the *Image Window*, to the upper-left corner of previous defined tissue volume ROI. You need to click *Redraw* button or hit Ctrl+R, you will see the tissue volume ROI in a grey box. The full size

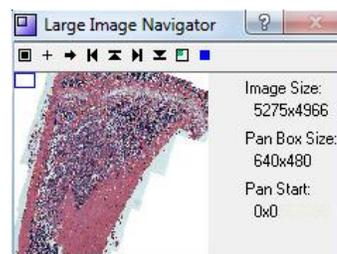
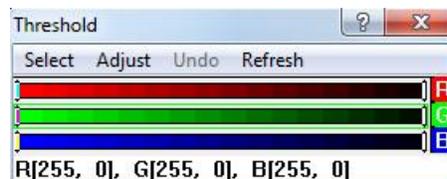


image will move accordingly. The size of the blue box is proportional to the field in the *Image Window*. It can be changed to facilitate the measurements if the features in the *Image Window* can be easily identified in lower magnification. Click  button, choose *Pan Box Size* among 1:1, 1:2, and 1:4 to zoom in/out.

Click *Move Clicked Point To Upper Left* button  in the *Large Image Navigator* window, then cursor will appear in the *Image Window* as a blue arrow head with a red dot at the top. Align the red dot to the upper-left point of the grey ROI box, left click. Now, current field in the *Image Window* is aligned to the upper-left corner of the tissue volume ROI, as you can see in the *Overview* window. In ROI Tools window, choose *Full Screen* or an appropriate type to redefine the current ROI. Only the features in current ROI box (not the previous defined tissue volume ROI) will be measured.

In the *Overview* window, you will see 2 boxes. The dotted-line box represents your current location of the field in the *Imaging Window*, and the solid-line box is the ROI defined for tissue volume.

5. **Measure bone volume (BV).** Highlight *D7 (bone volume)* in the *Select Arrays* window. In the *Threshold* window, click    buttons to deselect them. If they turn grey, automatic thresholding will not get in the way.



In *Threshold Tools* section in the *Shelf* window, click *Draw Threshold* icon  to draw the outline of trabecular bone. You can change the way of drawing by toggling *Keyboard Termination*, either hit Ctrl+K, click  in the *Shelf* window, or click *Measure -> Keyboard Termination*. Once you close the outline, click *Fill Holes* icon  and then click anywhere inside the outline to fill bone area. The outlined trabecular bone area will turn yellow. If you have multiple bone areas, outline and fill them individually; if you have a hole inside a bone, outline the hole as well, and it will be left blank when you fill the bone area. Click *Dilate* button  once in the *Shelf* window (you MUST click it if you want bone perimeter). You'll benefit from it as it'll make sure your BV touches the current ROI edge. Click *Preview* button in the *Auto Measure* window (make sure *All* is checked), then click bone area in the *Image Window*. When you see the outline turn blue, click *Measure* button. Bone volume values will show up in the *List Current Arrays* window. Now you can see the BV/TV value in the *Calculations* window.

There are a couple of ways to define bone volume. For TRAP or H&E – stained bone slices, manually drawing the outline would be a good way. Make sure your outlines touch the edge of current ROI, so that they will be ignored automatically from the bone surface calculation. Making changes to preview outlines by using *Line Edit Tools* is not recommended in this situation.



6. **Measure Ob.S/BS (osteoblast surface/bone surface) and Oc.S/BS (osteoclast surface/bone surface).** Highlight *L9 (Automatic Bone Perimeter)* in the *Select Arrays* window. In the *Auto Measure* window, click *Preview* and click bone in the *Image Window*. Trabecular bone will be outlined in a different color. Now, make sure you check the *Overview* window. The bone outlines in pink should not appear on the edge of the white box, which means this part of the bone will be ignored in bone surface calculation. To define

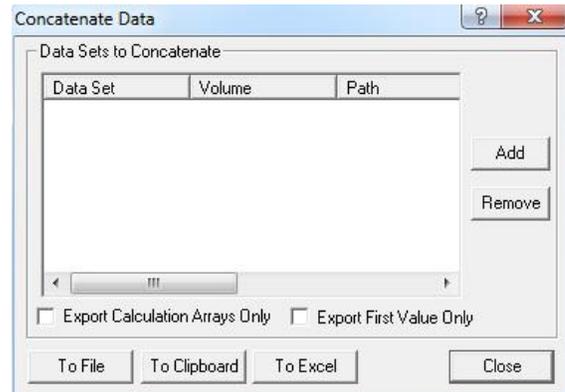
OB/OC surface, click *Segment Assign* button in the *Auto Measure* window. When your cursor appears in the *Image Window*, move counterclockwise to click on both edges of an OB/OC. After you define both ends of an OB/OC, hit 1 for OB or 4 for OC. In both the *Image Window* and the *Overview* window, the defined line for OB/OC will be shown in a different color.

If the bone outlines touching ROI show up in the *Overview* window, it may be because the bone outlines do not really touch ROI. Instead of re-outlining the bone, clicking *Dilate* button  in the *Shelf* window may be helpful. Maximizing the *Overview* window will make the lines easier to see, and click *Resize To Fit* button  to automatically fit the enlarged window.

7. **Measure N.Ob/BS (osteoblast number/bone surface) and N.Oc/BS (osteoclast number/bone surface).** Highlight *D9 (N.Ob, # Osteoblasts)* or *D10 (N.Oc, # Osteoclasts)* in the *Select Arrays* window. BioQuant will calculate N.Ob/BS and N.Oc/BS by using previously defined bone surface. To obtain the best visual of the field, click *Clear Overlay*  or *Toggle Display Threshold*  in the *Shelf* window if necessary. Choose manual measure mode by clicking *Manual Measurement* button  on main toolbar or clicking *Measure -> Manual Measurement...* from main menu. Click *Measure* button in the *Manual* window, and then click on each OB/OC in the *Image Window*. You will see symbol  $\times$  is assigned to each OB/OC. *Overview* window will show them as well if you check *Options -> Draw Point Mark*.
8. Once you finish all the measurements in the current field, move to next field. The way to move depends on the stitched image or manual move mode you are using (see page 10 for the details). Move several times to cover the whole tissue volume ROI and do measurements to every field. The measurements results will be updated automatically in the *Calculations* window.
9. In a situation that one mouse has several slices which are stained differently, data from different slices can be stored into the same data set. For example, if you have a calcein double labeling slice in addition to a TRAP stained slice from the same mouse, you can add a new measurement to current data set by choosing *Arrays -> Modify Data Set...* from main menu. In *Modify Data Set* window, highlight *Topo* in *Primary Arrays* box, click *Add Primary Array* button ONCE. Now you will find an extra array in the *Topo Array* drop-down menu of *Parameters* window, which can be used to store the info of the new slice. If you have 2 more slices, click *Add Primary Array* button twice, so BioQuant will create two *Topo* arrays for you. Make sure you remember which *Topo* Array corresponds to which slice.
10. To **export the results into an Excel file**, choose *File -> Save Data*, then *File -> Data Manager*. In the *BIOQUANT Data Manager* window, highlight *BQDATA* folder in the left box as shown below



Click *Search for BIOQUANT Volumes* button  on main toolbar, you will see all the data sets in the middle box. Icon  represents the data volume and icon  shows the data sets. Highlight them, and you'll see the results appear in the right box. Click *File -> Concatenate Data...*, when you see the *Concatenate Data* window appears, highlight the data set you want to export in the middle box, click *Add* button in the *Concatenate Data* window. You'll see your data set show up in the *Data Sets to Concatenate* box. Click *To Excel* button to export the data to excel spreadsheet file. If you do not want to see blank arrays, check *Export Calculation Arrays Only* box.



## Moving to Next Field

### For stitched image mode

1. In the *Large Image Navigator* window, click  button on the upper-left corner and choose *Pan Box Step Size...*, and make sure the value is *100*. 100 means current field of view will move to next field in a 100% manner without any overlap. Move current field of view to next field by clicking *Move Left* , *Move Up* , *Move Right* , *Move Down* . Pay attention to the blue box in the *Large Image Navigator* window and the blue/white boxes in the *Overview* window when you're moving the field, make sure they align well.
2. When you move to a new field, change ROI as needed because only the features in current ROI box will be measured. When you click *Redraw* in the new field, if you see the blue box of tissue volume ROI inside the field, you have to draw a new ROI which is exactly the same as the blue box. To redefine a rectangle ROI, there is a better way other than typing in height and width values: choose *Rectangle* type, click *Define ROI* button, cursor switch to a dark blue box with a cross in the middle within the *Image Window*, hold down Shift key and move mouse to remove the box. Now use the cross to redraw a current ROI with left button, which should be within the tissue volume ROI.

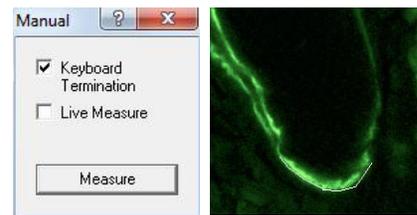
### For Manual Move mode

1. Highlight *D17*. Your current ROI will turn yellow. Select *Full Screen* type in *ROI Tools* window. Click *Preview* button in the *Auto Measure* window, then click in the *Image Window*. Right click. Click *Measure* button in the *Auto Measure* window, then click in the *Image Window*. You will see a red box that outlines the field. Right click. Click the *camera* icon  to get a live image.
2. Use manual move  feature to move to new field of the blue ROI, using white box in the *Overview* window as guide. Use *redraw* to help match things up if you make a mistake with manual move (clicking twice ends the function, so if you double click before your move, you are unable to complete the manual move alignment).
3. Once you are in the field you want to measure, click *Redraw*. You should see the boundary of what you have measured in Red and the boundary of your ROI in blue. To form the ROI for this field, select *Irregular* ROI from the *ROI Tools* window. Click *Redraw*. A teal box is now added in addition to the red and blue boxes. Click *Keyboard Termination*  in the *Shelf* window, and click *Define ROI* in the *ROI Tools* window. Use cursor and left clicks to draw the new ROI by following the boundaries of the screen and the red and blue ROIs. For the final segment, use spacebar to allow the program to close the ROI. If you make a mistake, hit *Define ROI* again and try again.

## Calcein double labeling measurement

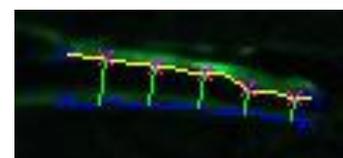
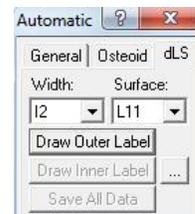
1. *MAR (Mineral Apposition Rate)* and *BFR (Bone Formation Rate)* are the two major parameters to measure the bone formation activity. MAR measures the activity at the labeling area, whereas BFR calculates the activity from the overall bone surface. In order to monitor the overall bone formation rate, BFR divided by bone surface (BFR/BS) is better than BFR alone. The calculation of BFR involves single and double labeling, which means you have to outline both of them to get an accurate result.
2. Define TV, BV and BS like we discussed above because you need comparable TVs and BS for calculation. Normally you can't see nice parallel labels very much because of the curvature of trabecular bone. Only measure the parallel part for the double labeled surface and try your best to be consistent on how you choose that part.
3. **Define Labeling Period.** Highlight *D11 (Ir.L.t, Labeling Period)* in the *Selected* box of *Selected Arrays* window. In *List Current Array* window, click *Display List Menu* button , and choose *Edit Menu -> Append Data*. Type in labeling period (days) in the *Value To Append* box. In *Calculations* window, find *MAR* and *BFR/BS* to see the results. *MS/BS (mineralization surface / bone surface)* is used for calculating BFR/BS from MAR.

4. **Measure Single Labeled Surface.** Highlight *L10 (sLS, Single Labeled Surface)* in *Selected Arrays* window. Choose *Manual Measurement* and make sure *Keyboard Termination* is checked. Click *Measure* button, then click along the single labeled surface. Hit the space bar to terminate the measurement.



5. **Measure Double Labeled Surface.** Highlight *L11 (dLS, Double Labeled Surface)* in *Selected Arrays* window. Choose *Automatic Measurement*, click *dLS* tab, and make sure *Width* is *I2* and *Surface* is *L11*. Make sure *Keyboard Termination* in the *Shelf* window is checked.

In *dLS* tab, click *Draw Outer Label* button, and cursor will appear in the *Image Window* as a red arrow head. Click to define the outer labels' contour. Click *Draw Inner Label* button, define the inner labels' contour. Once you right click to exit, you will see lines between the double labeling automatically drawn by the program. Click *Save All Data* button. Check *Calculations* window for results. Make sure your click on the same side of the double labeling.



6. You can change the way program draws the double labeling lines by clicking  button in the *dLS* tab. In the most cases you don't need to make any change to them, as the default setting works well.
7. Move to next field as explained before and do the measurements. Keep doing this until you cover the whole tissue volume ROI.

## **Some useful features**

### **Save tracings on image**

For some situations, it's better to show bone slice picture along with its markings/tracings. For example, in a bone resorption experiment, as the staining backgrounds are usually very strong, they are much easier to see if the edges of pits are highlighted.

To do this, choose *Option -> Save Tracings On Image* from main menu before making any markings/tracings. Once you done with drawings, choose *File -> Save Image(s) -> Save Single Image...*

### **Measure resorption/metastasis area**

There are no pre-defined resorption or metastasis arrays in BioQuant, you can either manually define a pair of measurement array and calculation array, or simply consider BV (bone volume) as resorption/metastasis area.

To do this in a simple way, define a TV (tissue volume) first, and then draw resorption/metastasis areas as what you do for BV. For the BV/TV value, it can be seen as resorption area / total area in the case of a pit experiment, or as tumor volume / tissue volume in the case of a bone metastasis experiment.