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TURNING IMAGES INTO KNOWLEDGE

Working With: newCAST™

Training manual
2013-08-16

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CONTENTS

INTRODUCTION

The scope of this manual is to 1) introduce basic concepts of stereology and to 2) in detail describe how to use the newCAST™ software to obtain stereological estimates from tissue sections.

To obtain unbiased stereological results, it is required that the correct stereological methods are used during tissue processing and sectioning. First of all, the sections must be obtained using correct sampling methods; the most used method is *Systematic Uniform Random Sampling* (SURS). Some endpoints, such as individual volumes, surfaces, and length, require either, *Isotropic Uniform Random* (IUR) sections or *Vertical Uniform Random* (VUR) sections. It is beyond the scope of this manual to go into detail with this so please consult the stereology literature on the subject. We have included a list of relevant references at the end of the manual.

The newCAST™ software is designed to assist the user in obtaining unbiased estimates such as *total number, length, surface area or volume.* It is important, however, to emphasize that the final responsibility for obtaining unbiased results remains with the user, as the groundwork for getting stereological unbiased results (sampling, staining, sectioning and so forth) is done before the sections are analyzed.

A complete stereology system consists of a microscope equipped with a motorized stage, a digital camera, a high-precision microcator for measuring the height position of the stage, a PC and the newCAST™ stereology software. The PC with the newCAST™ software communicates with and controls the microscope, stage, camera and microcator. If you do not use thick sections for the optical disector – the microcator can be omitted.

Using a slide scanner – you will only need the virtual slides (images acquired at the slide scanner) and a PC with the whole slide stereology newCAST™ package (deployed stereology) or a cloud access to the software. Please visit our homepage for more information: www.visiopharm.com.

Many steps in the specific analysis are the same for various endpoints. A generalized workflow consists of the following steps:

1. **Create a super image**
2. **Delineate region(s) of interest**
3. **Setup probes for analysis**
4. **Setup and start sampling**
5. **Count in each Field of View**
6. **Save data in the database**
7. **Analyze data**

GENERALIZED WORKFLOW

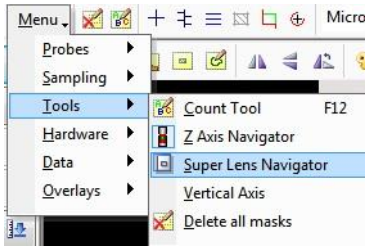
In this section, we will explain the individual steps of a normal workflow. All steps are also explained in the *Help* files in the software (F1) or from http://www.visiopharm.com/manual/.

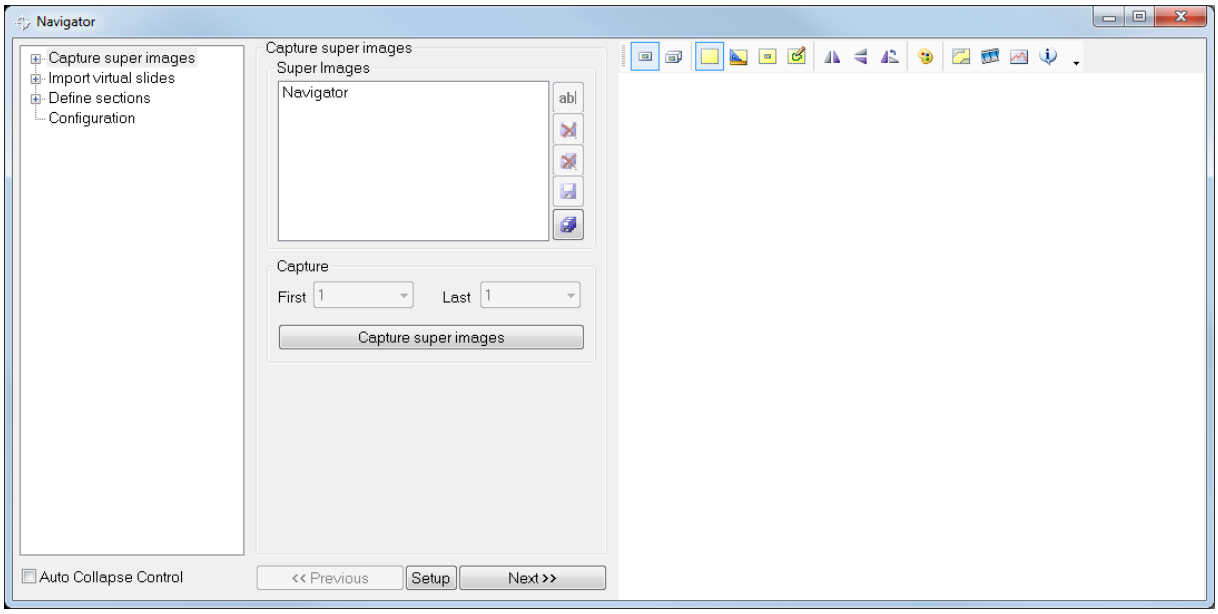
1) CAPTURE SUPER IMAGE

The super image is a low resolution image of the entire slide. It allows you to see you current sampling position on the whole section and to easily maneuver around your tissue section(s).

A super image is generally not required, except for the physical disector, but many users find it very helpful to have during the analysis.

1. **Open the Super Lens Navigator from: Menu/Tools/Super Lens Navigator ( ).**

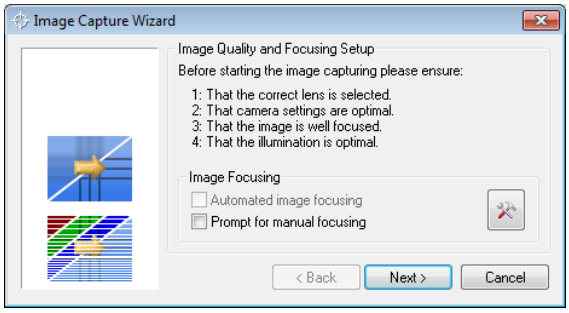




Customers who use virtual slides can import super images directly by clicking them in the database, or import them from *Import virtual slides.*

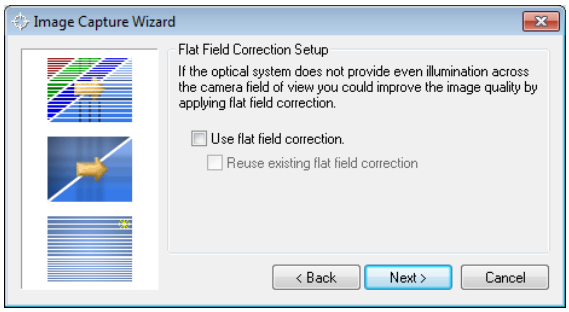
For microscope users:

2. **Activate the Image Capture Wizard by clicking: *Capture super images*.**



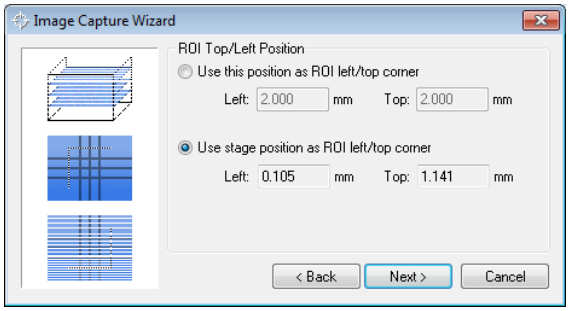
Generally you do not need automated refocus as the images are acquired using the smallest lens in your set-up i.e. 1.25, 2 or 4x objective that all have a rather low numerical aperture.

3. Flat field correction



The image is not equally illuminated which means that you will be able to see shadows when stitching the super image from individual images. To correct for this you can use Flat Field Correction. You will have to move the stage to a position with only background pixels i.e. no tissue. After clicking next an image is taken and the software compensates the darker edges in the images acquired for the super image

4. Define the top left corner of the section.

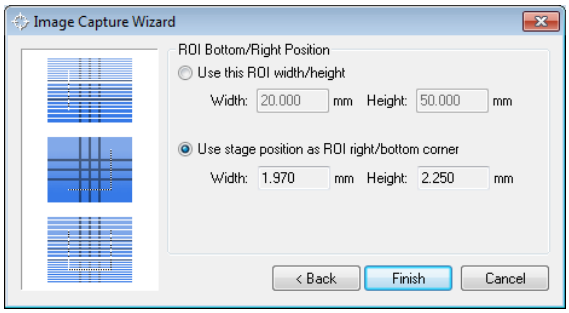


Check: *Use stage position as ROI left/top corner*.

Here you define the position of the top left corner: on the monitor - not in the microscope! The top left corner of the field of view will be the top left corner on the super image.

When you have positioned the stage in that position – click next.

5. **Define the bottom right corner of the section.**



Check: *Use stage position as ROI right/bottom corner*.

Here you define the position of the bottom right corner (on the monitor not in the microscope). The bottom right corner of the field of view will be the bottom right corner on the super image.

When you have positioned the stage in that position – click next. The images are now being sampled.

6. **If captured image is too large.**

If the size of the specified image exceeds 100 MB a warning will appear, giving three options of how to solve the problem.

For super image capture resampling it is highly recommended to:

- Use resampling for all captures of this session - Using this option will resample the image. The wizard will compute what minimum resampling is needed to get below 100 MB. This option will reduce the image size but will also reduce the resolution.
- Use individual resampling for captures of this session - Using this option will use an individual resampling at each slide position. The wizard will ask how much resampling to do at each slide position.
- Adjust lens, camera binning etc. - Using this options will restart the wizard, push the button and adjust the settings.

You will now have a Super image automatically labeled for example SI 00000001 with a yellow box indicating the position of the live view (i.e. stage position). You can save the image in your database using the floppy disc icon  .

When basic image viewing is selected () a double click on any position on the Super Image will move the stage automatically to this position.

Note: Keep the Navigator dialog open if you wish to use this during sampling - it can be minimized but do not close it.

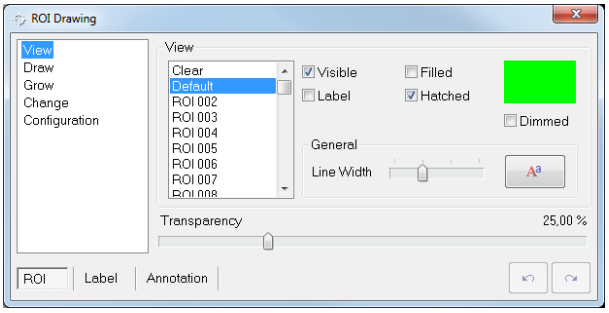
2) DELINEATE REGION OF INTEREST (ROI)

The user must delineate the region(s) containing the tissue of interest. This can be done either on the Super Image or on the live view at the lowest possible magnification. The outlined regions are used to guide the sampling process – so only fields of view (FOV) inside the ROI are sampled.

Important: Each image window (live view and Navigator window) contains a Layer drawing tool for drawing the ROI specifically on that image. Consequently:

- If the ROI will be drawn on the Super image - activate and use the Layer drawing dialog in the Super Lens Navigator.
- If the live view is used for the ROI drawing - activate and use the Layer drawing tool on the live view.

Activate Layer Drawing  **in the image window you wish to draw the ROI in**



In the default setting you are now ready to delineate the ROI (use ROI not Label or Annotation). If you want to know more about the settings please consult the Help.

1. **Delineate the Region of interest.**
2. **Left click and move the mouse pointer to a new position and left click and move the mouse pointer again etc. until the tissue of interest is inside the ROI.**
3. **The ROI outline is finished by a double click.**

Guidelines for delineating region of interest:

- It is important that all tissue of interest is *inside* the ROI.
- It is not a problem if the ROI is too large - better too large than too small.
- The user should not spend too much time meticulously delineating the tissue.

Hints for delineating region of interest:

- You can add to your existing ROI simple by drawing again having some point inside the ROI.
- You can "subtract" from your ROI by using the ROI *Clear*. Everything outlined will not be part of the ROI.
- You can have multiple ROIs for example: several ROI 2s if you have several similar tissue sections on one slide.
- You can have different ROIs in one tissue section delineating different areas within one structure (for example cortex/medulla).

If the tissue sample is too large to fit into the live field of view displayed on the monitor, the motorized stage will automatically move when a click is near the border of the image. The stage moves so that the clicked point is centered in the image and the user can continue marking the outline.

3) SET-UP PROBES

The choice of probes, also called test system, depends on the desired end-point. For each probe there are a number of adjustable parameters such as density (number of probes on the screen), groups, orientation, color, etc. The parameter settings can be stored in configurations and recalled for later use. Setting up the probes is the process of specifying the parameters for the active probes.

1. **Change the magnification to the desired magnification to be used for the analysis.**
2. **Activate the probe from: Menu/Probes.**
3. **Set up the probe according to your experimental set-up. Please consult the Help guidelines and read through the individual endpoints in this paper.**

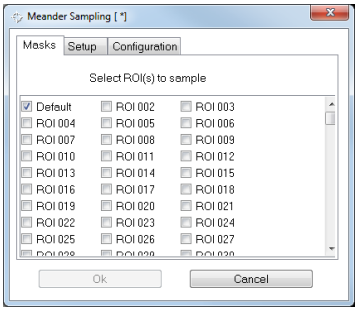
Note: You can easily have more than one probe active at the same time.

Main probes overview		
Volume		**Probe**
Total Volume		Points
Fractional volume		Points
Mean particle volume	Volume weighted	Point sampled Intercepts
Individual volume	Number weighted	Nucleator and Rotator
Surface		**Probe**
Surface area density		Lines
Area		**Probe**
Area		Points
		2D nucleator
Length		**Probe**
Length density	Thin sections	Counting frame
	Thick section	Virtual planes
Number		**Probe**
Physical disector	Thin adjacent sections	Physical disector and Counting frame
Optical disector	Thick sections	Counting frame

4) SET-UP SAMPLING

A systematic uniform random sampling (SURS) in newCAST™ should be performed inside a region of interest (ROI).

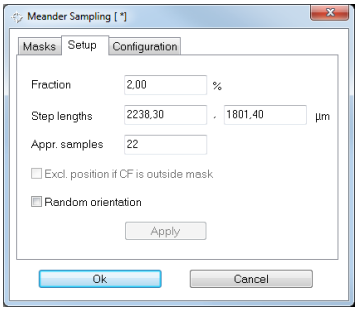
1. **Activate the Meander sampling from Menu/Sampling/Set-up Meander Sampling ( ).**



You can choose to sample in different ways:

- one ROI - sampling is performed inside the ROI
- two or more ROIs of the same type - sampling is performed inside each ROI. Data are saved as sampling 1, sampling 2 etc. in the database.
- two or more different ROIs- sampling is performed inside each ROI. Data are saved as Default, ROI 2 etc. in the database.

2. **Set up the sampling parameters.**



A given fraction, step length or appr. samples can be entered and the other two parameters are calculated.

In general, during the experiment a fixed fraction or step length should be used for all sampling. Approximate samples are used almost exclusively for pilot studies.

3. **Start on a new datasheet: Menu/Data/ New Data Sheet (and Save Current).**

Make sure to reset all data that might be in the system before starting a new sampling.

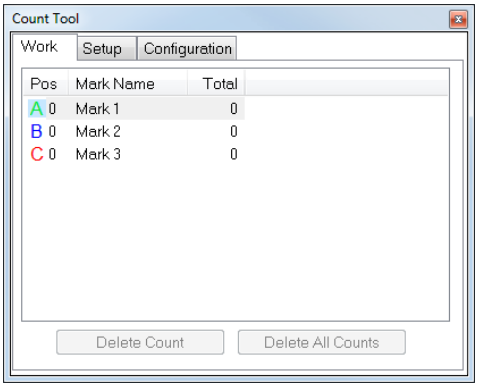
4. **Click ok to start the sampling.**

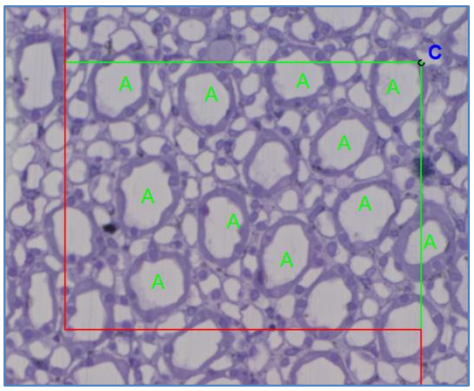
Note: If a counting frame is not active at the time the sampling is started, the sampling fraction is calculated from the area of the field of view. Otherwise the sampling is based on the counting frame size. This also means that the counting frame size should not be altered when a sampling has been initiated.

5) COUNT IN EACH FIELD OF VIEW

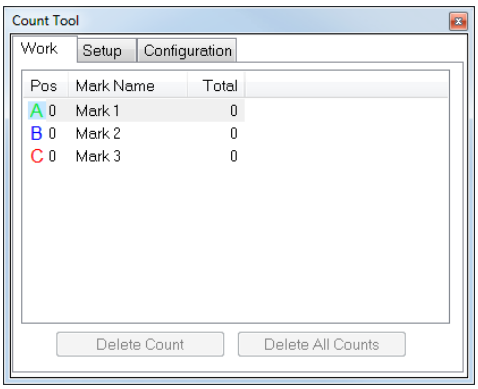
The first sampling field of view is now presented. The user should now mark the number of counting events in the field of view. The actual event, and how to mark it, will depend on the probes – here are a few examples:

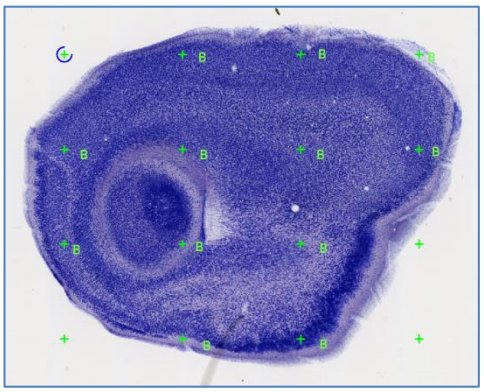
- **Count: Count using the count tool.**



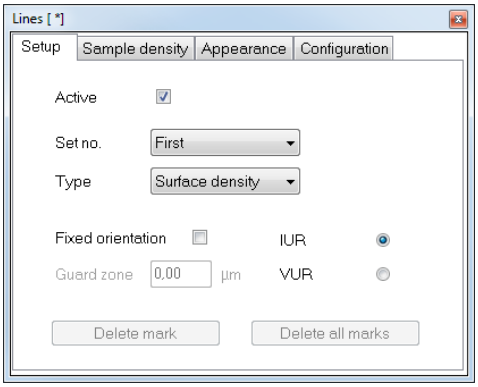


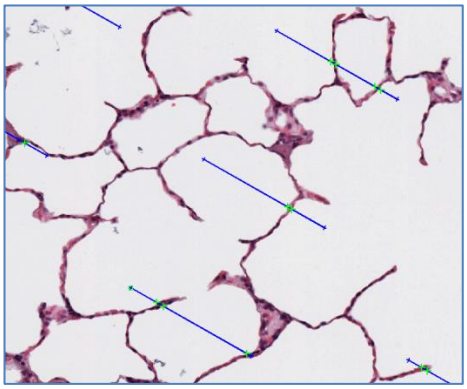
- **Points: Count using the count tool.**



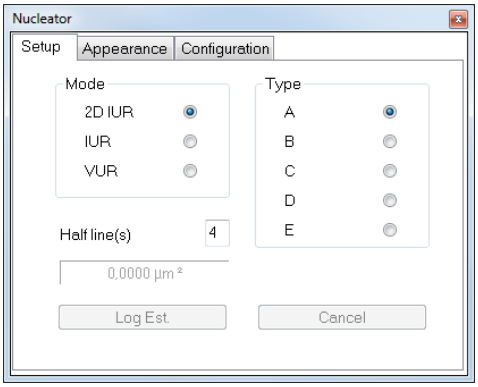


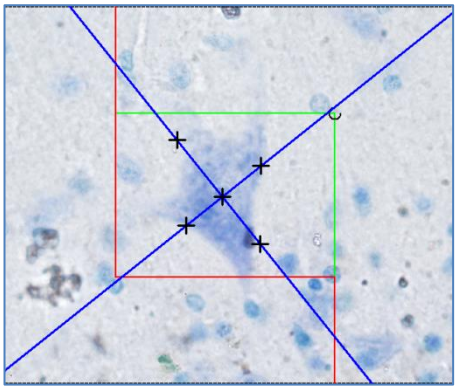
- **Lines: Mark Intersections.**



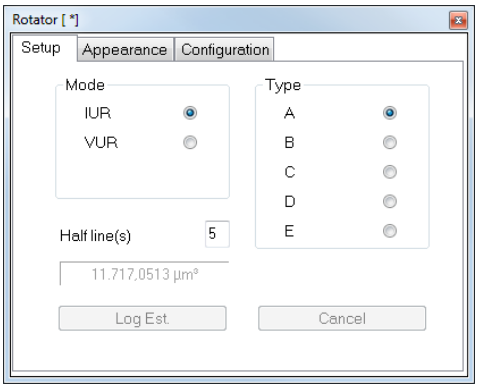


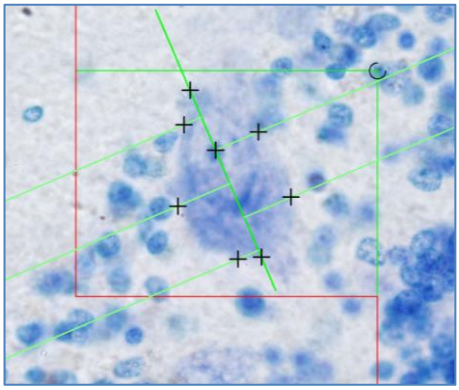
- **Nucleator: Mark intersections.**





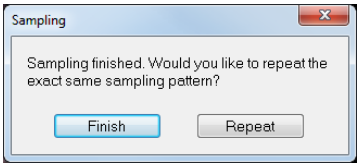
- **Rotator: Mark intersections**





6) SAVE DATA IN THE DATABASE

When you are done sampling after the past field of view you are asked to finish the sampling.



The data are now currently in the random access memory (RAM) of the PC.

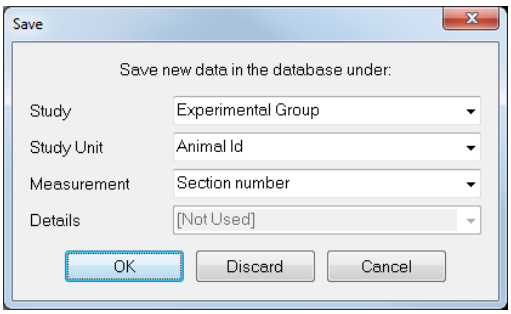
If you need to sample more section on the same slide you can continue and start delineating the new section. The next data set will appear in the same database location as Sampling 2.

If you are done with the section and wish to save you should:

1. **Save the data: From Menu/Data/ New Data Sheet (And Save Current) ( ).**

A dialog window will appear:

2. **Label the data entries and click ok.**



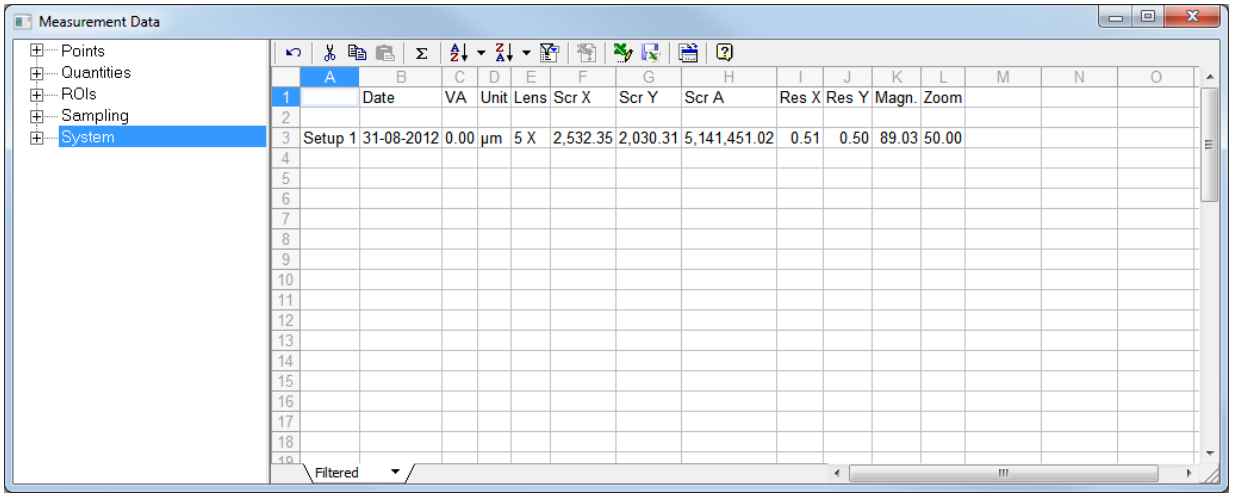
7) ANALYZE DATA

DATABASE VIEWER

You can see the stored data by clicking the database entry and then choose to see the data from: Study, Study Unit or Measurement level:

- Menu/Data/ View Study ( )
- Menu/Data/ View Study Unit ( )
- Menu/Data/ View Measurement Data ( )

This opens up the database viewer:



	A	B	C	D	E	F	G	H	I	J	K	L
1		Date	VA	Unit	Lens	Scr X	Scr Y	Scr A	Res X	Res Y	Magn.	Zoom
2												
3	Setup 1	31-08-2012	0.00	µm	5 X	2,532.35	2,030.31	5,141,451.02	0.51	0.50	89.03	50.00

SYSTEM:

This category contains data about the system and how it has been set up.

- Date: The date the data was logged.
- VA: Vertical axis, angle in radians.
- Unit: The distance unit.
- Lens: The name of the lens used to capture the image.
- Scr X, Scr Y: Width and height of the image.
- Scr A: Area of the image.
- Res X, Res Y: The resolution of the image (pixels/‹unit›, e.g. pixels/µm).
- Magn.: Magnification of the image (ResX x ResY) from the physical size on the slide to the physical size on the particular screen, used when logging, including zoom.
- Zoom: Image screen magnification in %.

SAMPLING:

This category contains data about the sampling.

- Configuration: The name and path of the configuration loaded when logging the sampling.
- Sampling Name: Name of the sampling. Auto assigned to newCAST ‹number› e.g. newCAST 1 for a regular online sampling. For offline sampling the name is user defined.
- Percentage: Fraction being sampled in %.
- Samples: Number of sample positions.
- Random: Whether or not the sampling grid is randomly rotated.
- Step X, Step Y: Sampling step length in the x and y direction.
- Step A: Sampling step area.
- Offline: Yes if the sampling is offline, not visible otherwise.

QUANTITIES:

This category contains data about the measurements in the sampling(s). The data is structured so there is a subcategory for each ROI. Each sampling performed within a ROI has a subcategory under the ROI, within which

it is performed. In a specific sampling, the sampling positions are shown and within these the placed ROIs can be seen. The Mark categories contain, the following data.

- Pos X, Pos Y: The coordinates of the center of the sampling-position.
- Time: Time when the sampling position was visited.
- Pos: The number of the sampling-position.
- Image Name: Name of image. Used to specify the name of the sampling position image in offline samplings.
- Configuration: The path of the configuration loaded when logging the count tool.
- X, Y, X: The absolute position of the mark.
- Factual pos: Y/N, yes if the measurement was logged by clicking at a given position in the image, no if it was logged using a keyboard short cut.

There are two view settings: Raw, where all data is shown on all levels, and Filtered, where the levels below the current are not shown. Filtered view is normally used. This also sums up the number of counts and calculates the mean value of local probes. The values can be viewed per measurement, ROI, sampling or sampling position.

- To change filtering-mode push the tab-button in the bottom-left corner of the Excel-sheet, reading either Filtered or Raw.
- In Raw mode it is possible to see columns named system ref., ROI ref., sampling ref. etc. These indicate the number of the setup which was used in each category.

ROI:

This category contains data about ROIs in the image.

- Configuration: The name and path of the configuration loaded when logging for the ROI.
- Outline(s): The number of outlines for the specified ROI type.
- Area: The collected area of the ROI. Total for all outlines of this type.
- Boundary: The circumference of the ROI. Total for all outlines of this type.

PROBES:

All activated probes will have a specific folder containing all relevant data

COUNTING FRAMES:

- Configuration: The name and path of the config. loaded when logging the counting frame.
- Relative: Yes if using a size relative to the image (%), no if absolute size.
- A(group): Area covered by each group in μm^2.
- A(primary), A(secondary), A(tertiary): Area covered by each primary-, secondary- and tertiary-frame.
- Groups X, Groups Y: Number of groups in the x and y direction.
- Groups T: Number of groups in total.
- Frames X, Frames Y: Number of frames in the x and y direction.
- Image Aspect: Aspect ratio of the counting frame. Follows aspect ratio of the image (Y/N).
- Aspect Ratio: The actual aspect ratio (W/H) of the frames.
- Artificial edges in study: Yes if the user specified that the study included artificial edges.
- Unit: The unit.

POINTS:

- Configuration: The name and path of the configuration for the points.
- A(group): Area covered by each group of points (encircled).
- A(point): Area covered by each point.
- Groups X, Groups Y: Number of groups in the x and y direction.
- Groups T: Number of groups in total.
- Points X, Points Y: Number of points in the x and y direction.
- Points T: Number of points in total.

LINEPROBE

- Configuration: The name and path of the configuration for the lines.
- Measure type: Type of measurement used
- Line groups: Number of line groups used.
- Lines: Number of lines used.
- Line spacing: Distance between lines
- Length/point: Length of line per sample point.
- Use segments: Yes if segments were used.
- Segment groups: Number of segment groups.
- Segments: Number of segment.
- Segment length (%): Length of segments in %.
- A(Group): Area per group point.
- A(Point): Area per point.
- Random rotation: Yes if random rotation was used.
- Guard zone size: Length of guard zone (if used)
- Use sample points: Yes is sample points were used
- Sample point density: Density of sample points.
- A(Sample point): Area per sample point
- Unit: The unit.

VIRTUAL PLANES:

- Unit: The unit.
- Configuration: The name and path of the configuration for the virtual planes.
- Planes Separation Distance: The perpendicular distance between planes.
- Planes Orientation: Orientation of the planes.
- No of Help Lines.
- BoxWidth: Width of the counting box (x).
- BoxHeight: Height of the counting box (y).
- BoxDepth: Depth of the counting box (z).
- BoxVolume: Volume of the counting box.

NUCLEATOR:

- Data are shown directly under quantities.

ROTATOR:

- Data are shown directly under quantities.

From within the database viewer you can export all data to Excel by clicking the icon.

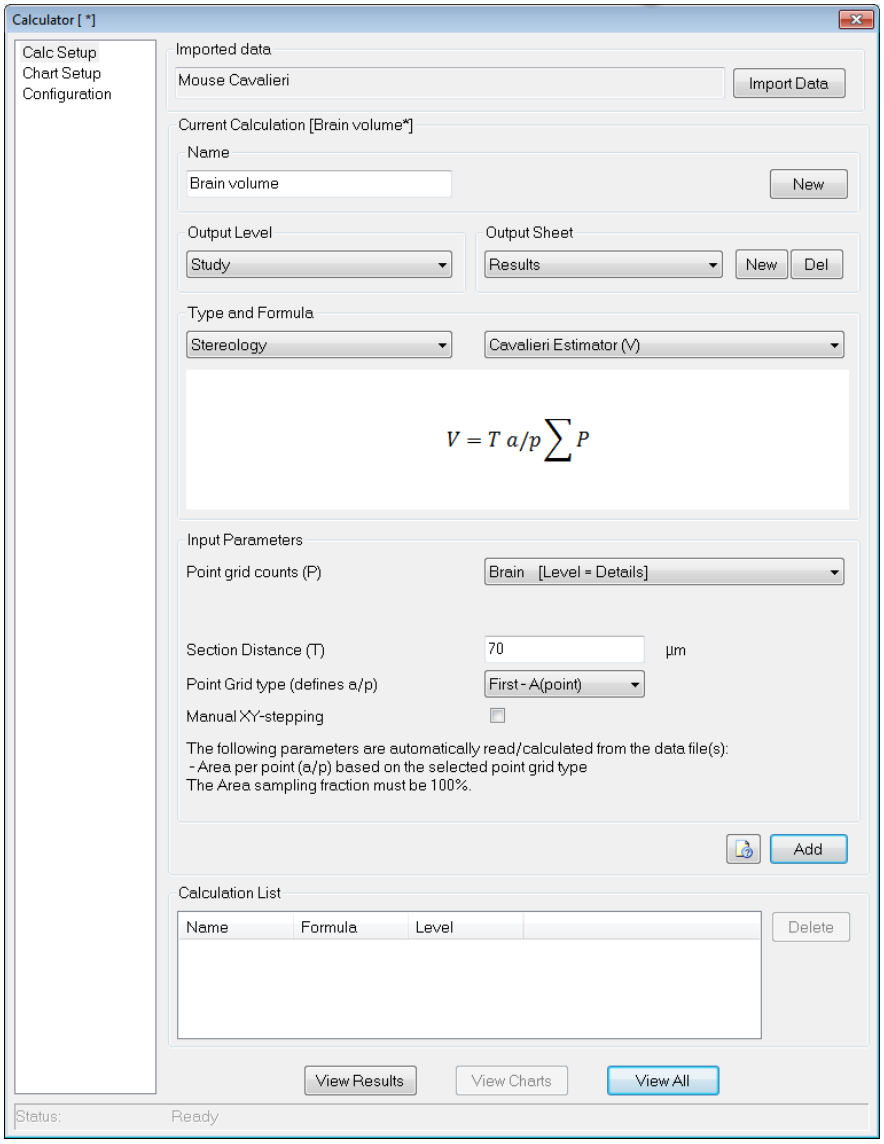
CALCULATOR:

When all data are collected you can also choose to use the Calculator to do the stereological analysis.

In short:

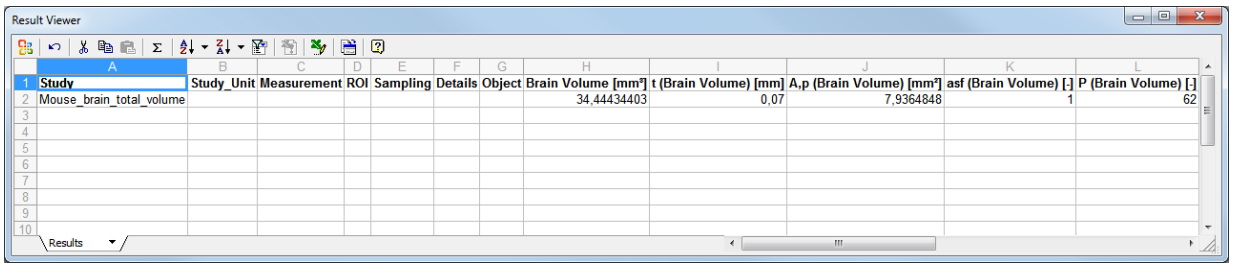
- Open up the calculator tool from the calculator icon:

- Import the data for calculation by selecting data in the database (multiple select is possible).
- Label the specific calculation
- Output level:
  Choose the database level for your output: Measurement, Study Unit or Study.
- Type and formula:
  Stereology
- Choose the relevant estimator
- Fill in all relevant information e.g. which point grid was used
- Add the calculation to the calculation list
- View results



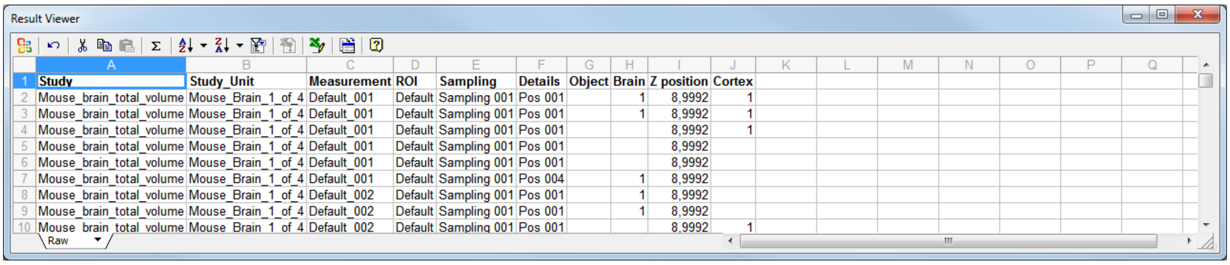
VIEW CALCULATOR RESULT:

- By clicking view **results** you will get all data presented in the result viewer:

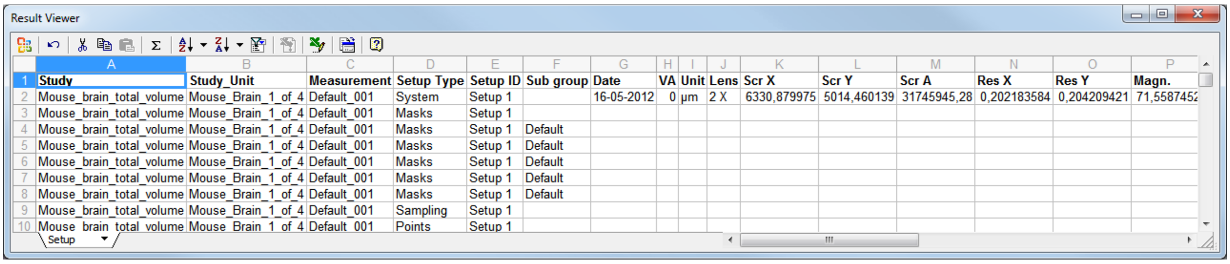


Calculations are added as columns so the other 3 calculations in this example are further on the right.

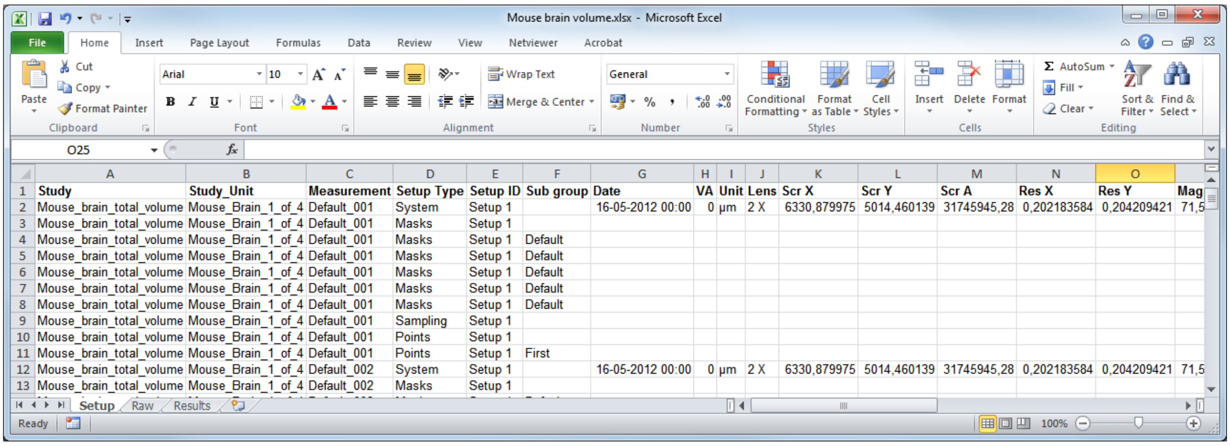
You can also see the raw data going into the calculations by selecting **raw**:



You can also see the general setup by selecting **Setup**:



To export all data to Excel please click the Excel icon



For further information on the Calculator for example how to make calculations and charts please visit www.visiopharm.com/manual.

TOTAL VOLUME ESTIMATION

Volume estimates are generally divided into global or local estimates, measuring the total volume of an organ or substructures therein or individual cells/objects, respectively. The methods for estimating global and local volumes are very different and require fundamentally different approaches.

CAVALIERI VOLUME ESTIMATE - THEORY

The Cavalieri principle provides an unbiased estimate of the volume of any object. Starting at a random position, the entire object is cut into parallel slices of a known and fixed thickness T. The volume is estimated by the product of thickness T and the total cut surface area A (one side of all slices).

$$Volume = T \cdot \sum A_i$$

The area of the individual slices *i* is normally estimated using a point-grid in which each point represents a known area termed the area per point a(p):

$$A_i = a(p) \cdot \sum P_i$$

Combined the total volume is consequently estimated as:

$$Volume = T \cdot a(p) \sum P$$

In addition, to get the total area of any section, the 2D Nucleator can also be used. This will give an estimate of the area directly of each section; hence, the volume is simply the product of the sum of all areas and the section thickness:

$$Volume = T \cdot \sum A_i$$

GUIDELINES:

One should use 6-10 sections per animal depending on the complexity of the organ. Count approximately 200 points or a little more if high precision is biologically relevant or the tissue has a complex shape i.e. has a high shape factor ($\text{boundary}/\sqrt{\text{area}}$).

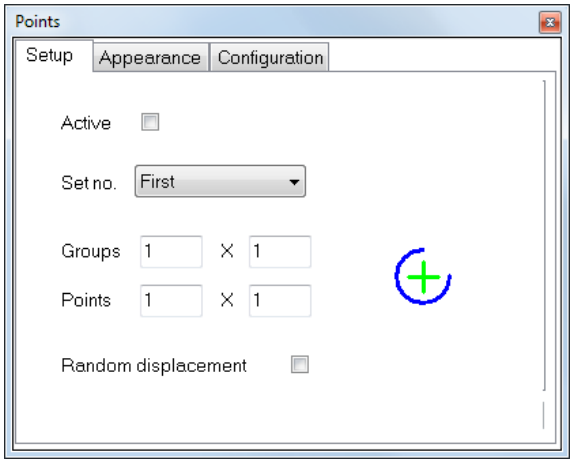
- cut the organ into parallel sections of a fixed distance, t, of $\sim 1/10$ length of organ. The first cut must be at a random position within the range from 0 to t. All cut "right-sided" surfaces of the slices are used for area determination by point counting.
  or
- cut the organ from one end to the other with a larger number of sections, n. Take every m^{th} section for area determination by point counting. The first section must be taken at random among the first m sections and the subsequent sampled systematically.

CAVALIERI VOLUME ESTIMATE – USING NEWCAST

1. **Create a super image**
2. **Delineate region of interest**
3. **Setup probes for analysis**

For Cavalieri volume estimate one need to use a point grid to estimate the area of each section. You will need to change the magnification to the lowest possible where you can recognize the structure.

- Select the lens you want to use for the analysis.
- Activate the point grid: *Menu\Probes\Points* ( ).



***Active*:** Activate the point probe

***Set no.*:** Choose up to three separate point grids

***Groups*:** Number of group points in the X and Y direction, respectively.

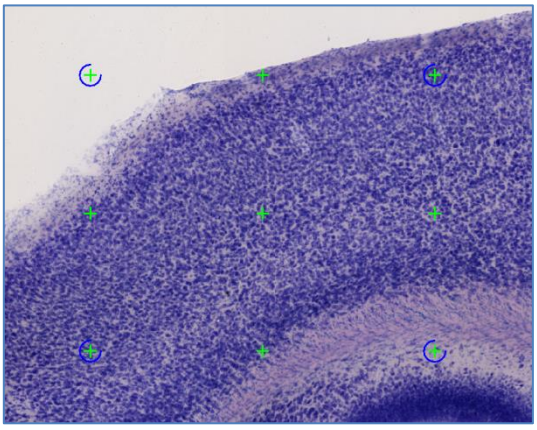
***Points*:** Number of Points per Group point in the X and Y direction, respectively.

***Random displacement*:** Choose to offset the points randomly on the screen.

Groups and points are used to estimate separate volumes at the same time. The groups which occur as encircled point will typically be used for the overall volume and the more dense points for substructures within the reference volume.

For each quantification, you will need to count around 200 points in each individual (all sections; see in theory section above). If you have 10 sections, this means that you should on average count 20 points overlaying the structure in each section.

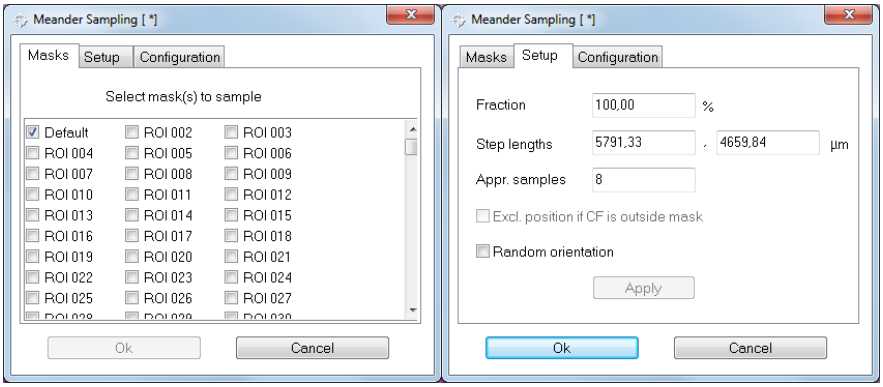
EXAMPLE:



4. **Setup and start sampling from Menu/Sampling Setup Meander sampling ( )**

For the Cavalieri estimate you should sample 100% at the analysis magnification.

- Activate Meander sampling from: *Menu\Sampling\Setup Meander Sampling*.
- Select the Region of interest
- Setup the sampling



5. **Count in each Field of View**
6. **Click Finish when the last FOV is counted**
7. **Save data from Menu/Data/New Data Sheet (and save current)**
8. **Analyze data**

REVIEW DATA FROM MENU\DATA VIEW:

The data can be seen at 3 different levels:

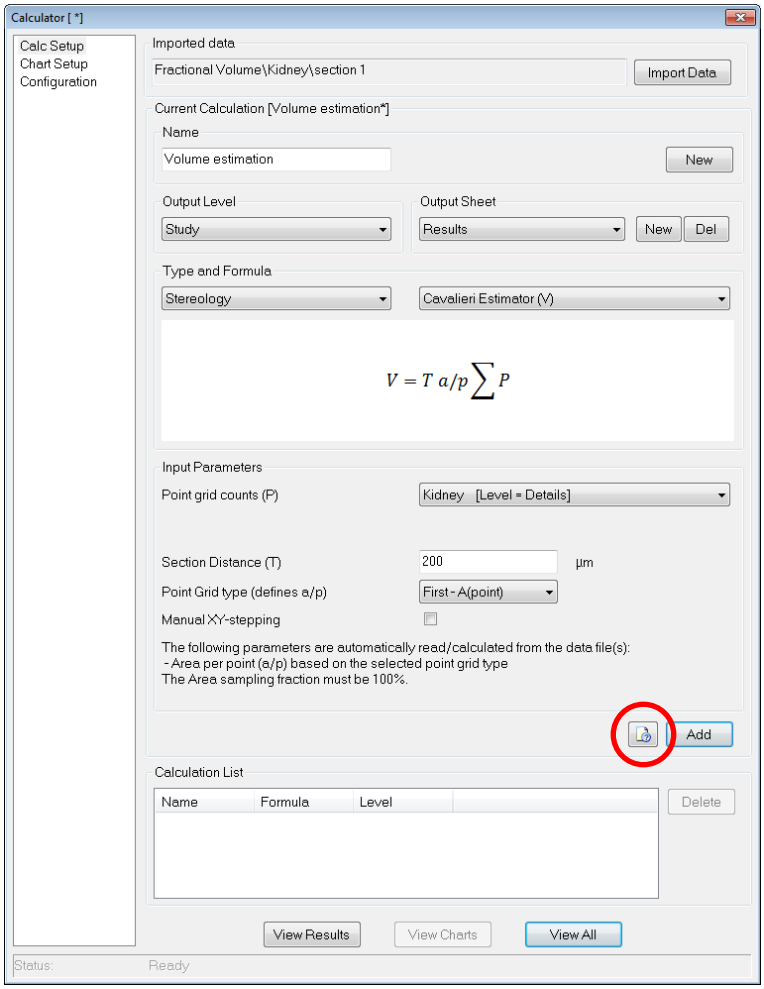
- Study ( )
- Study Unit ( )
- Measurement ( )

ANALYZE USING THE CALCULATOR

In the calculator, you can estimate the total volume using the Cavalieri estimator.

Start the Calculator from the Σ icon.

- Import the data for calculation by selecting data in the database (multiple select is possible).
- Label the specific calculation
- Output level: Choose the database level for your output: Measurement, Study Unit or Study.
- Volume estimate:
  Type: Stereology
  Formula: Volume (Cavalieri Estimator) (V)
- Select Point grid Counts
- Write in the section thickness
- Choose the relevant point grid
- Then press add



For the Coefficient of Error of the Cavalieri estimate:

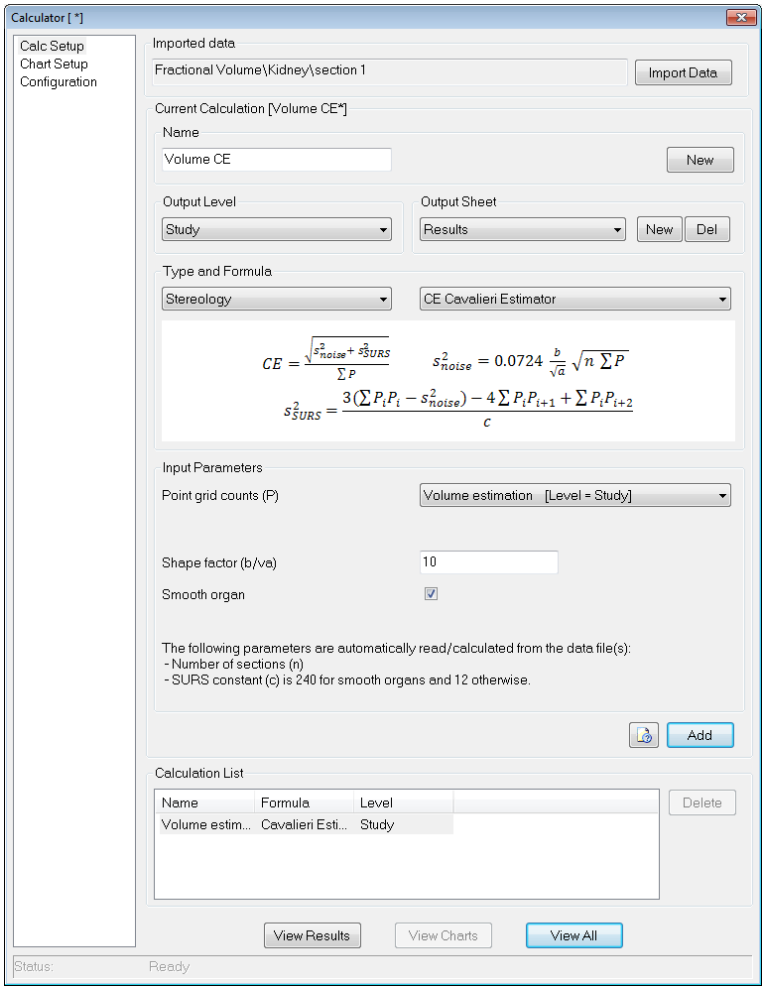
Type: Stereology

Formula: CE Cavalieri Estimator

Shape factor: A numerical value on the complexity of the tissue. It is defined as:

$$\frac{B}{\sqrt{A}} = \frac{Boundary}{\sqrt{Area}}$$

Please find more help using the “Get additional information on the currently selected calculation”-button (red circle).



FRACTIONAL VOLUME

It is quite easy to estimate the volume of a given substructure as the volume fraction V_V is equal to the area fraction A_A which, in turn, is equal to the point fraction P_P. In short, this means that you can get the volume fraction of any given substructure by:

$$V_V = A_A = \frac{\sum P_{substructure}}{\sum P_{organ}}$$

Multiplying this with the total volume of the organ you will get the total volume of the substructure:

$$V_{substructure} = V_{organ} \cdot \frac{\sum P_{substructure}}{\sum P_{organ}}$$

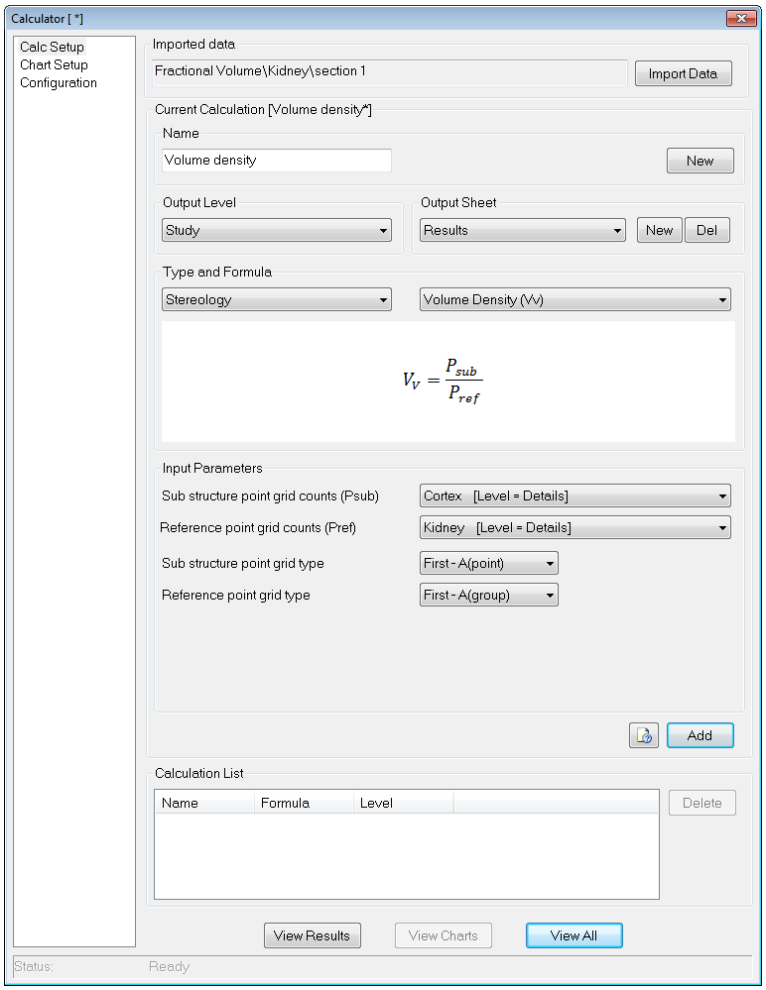
ANALYZE USING THE CALCULATOR

In the calculator, you can estimate the fractional volume.

Start the Calculator from the Σ icon.

- Import the data for calculation by selecting data in the database (multiple select is possible).

- Label the specific calculation
- Output level: Choose the database level for your output: Measurement, Study Unit or Study.
- Volume estimate:
  Type: Stereology
  Formula: Volume Density (Vv)
- Select Sub structure Point grid Counts
- Select Reference structure Point grid Counts
- Select Sub structure Point grid type
- Select reference Point grid type



LOCAL VOLUME ESTIMATION

It is important to realize that the estimate of individual volume of any given particle or cell from sections is dependent on the orientation of the section. Therefore, local volume estimates require that the sections are randomly rotated in 3D space obtained through isotropic uniform random sections (IUR) or vertical uniform random sections (VUR).

NUMBER-WEIGHTED VOLUME ESTIMATE - THEORY

To get a number-weighted volume, the particle or cell have to be sampled using a disector setup (physical or optical) and contain a unique point. Hence, to get the number weighted-volume of cells you would need:

- Sections are randomly oriented either as IUR or VUR sections
- Cells are sampled according to the disector principle either by the physical or optical disector.
- The cell contains a unique and recognizable point for example the nucleolus. If that is not possible it is necessary to measure in the section plane were the cell is widest. In an optical disector that will be where the cell is in focus. In a physical disector, one will need 3-4 samples depending on the size of the particle/cell compared to the section thickness. The measure is then performed were the cell appear largest.

INDIVIDUAL ESTIMATE OF NUMBER-WEIGHTED VOLUME:

When having sampled the cell correctly, you can estimate the volume using one of two approaches:

- Nucleator
- Rotator

Both use the relationship that:

$$\overline{V_N} = \frac{4\pi}{3} \cdot \overline{\ell_n^3}$$

- $\overline{\ell_n^3}$: Mean length of the line from a
  - Nucleator: centrally unique point to edge of the cell
  - Rotator: centrally placed line to the edge of the cell

With these estimates you can create volume distribution graphs.

GLOBAL ESTIMATE OF NUMBER-WEIGHTED MEAN VOLUME:

It you are interested in getting the mean volume of a given cell type, you can count the cells using the disector method, and estimate the total volume of the cell type using a Cavalieri design and point counting. The mean volume can then be calculated as:

$$\overline{v_N} = \frac{total\ cell\ volume}{total\ cell\ number}$$

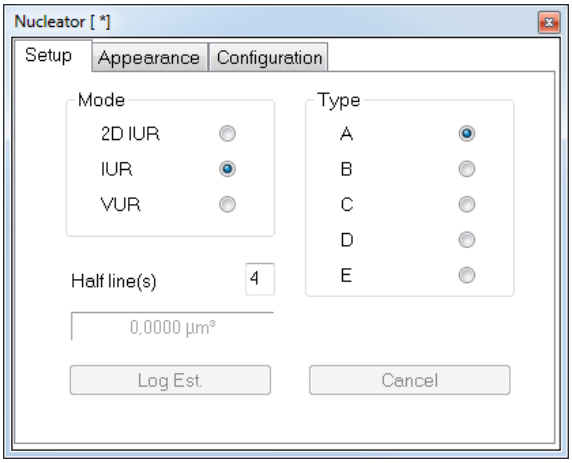
NUMBER-WEIGHTED VOLUME ESTIMATE – USING NEWCAST

A **disector** is needed to unbiasedly sample the cells for volume estimation (see *Number*) – please follow the guidelines there.

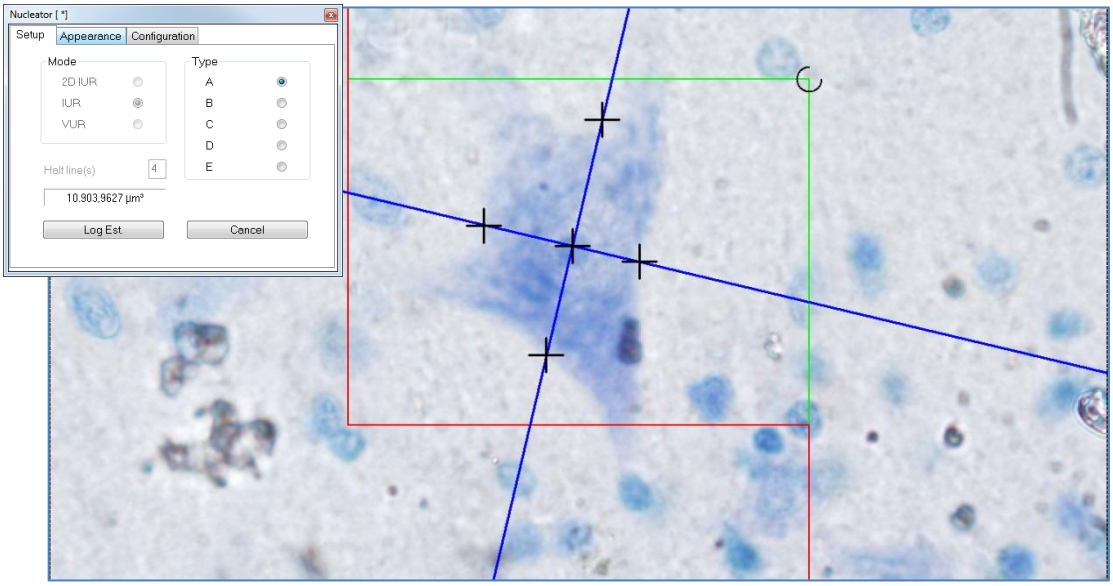
When a cell is sampled you can estimate the volume:

Nucleator:

- Activate from Menu/Probes/Nucleator (+)

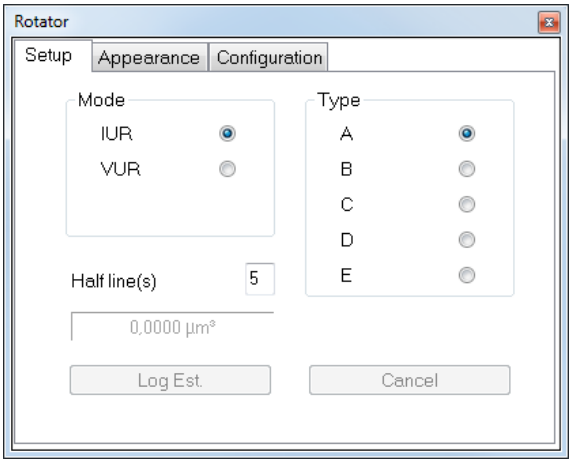


- Choose the type of sections used isotropic uniform random (IUR) or vertical uniform random (VUR)
- Choose the number of half lines. 4 is enough for simple structures – more complex structures requires more lines
- Click on the central unique point
- Mark the intersections between the lines and the cell edge
- Log Est. to store the data in the database as the calculated volume

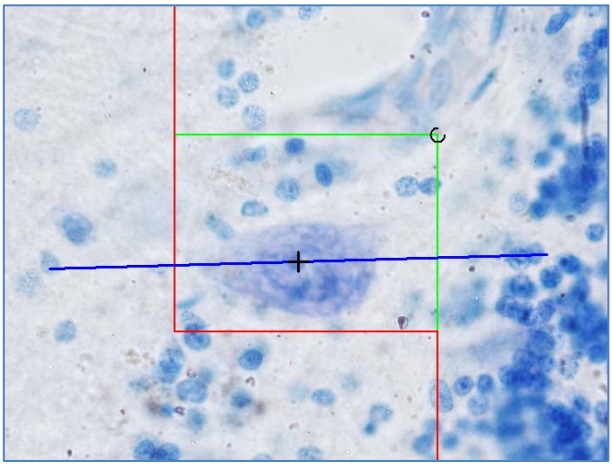


Rotator:

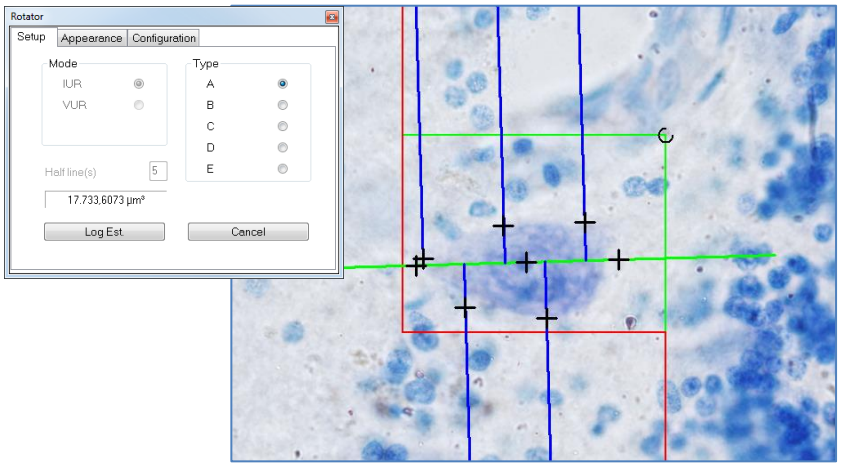
- Activate from Menu/Probes/Rotator ( )



- Choose the number of half lines. 4 is enough for simple structures – more complex structures requires more lines here we have chosen 5.
- Click on a central point and pull the out to align the line with the long axis of the cell click again when done:



- Click on the intersection between the cell and the edge of the line. A number of lines perpendicular to the first line appears.
- Mark the intersections of the cell with the lines.



- Log Est. to store the data in the database as the calculated volume

VOLUME WEIGHTED MEAN VOLUME – POINT SAMPLED INTERCEPTS - THEORY

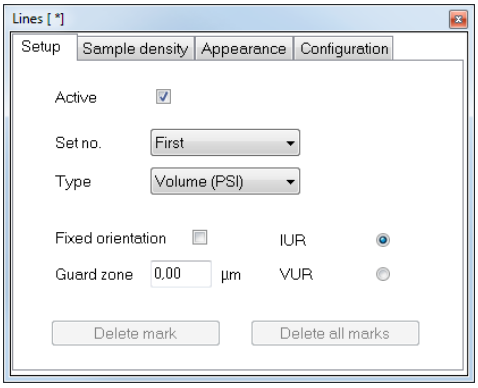
The volume weighted mean volume in which cells are sampled according to size, you need to use point sampled intercepts. If a random point on a random section hits a particle profile one may measure the length ℓ_0, of the linear intercept across the profile and through the point.

If the direction of the intercept is isotropic in 3-dimensional space then the length of the intercept raised to the third power multiplied by $\pi/3$ is an unbiased estimator of the volume-weighted particle volume v, irrespective of the shape of the particle. The method can only be used to estimate the volume weighted mean volume:

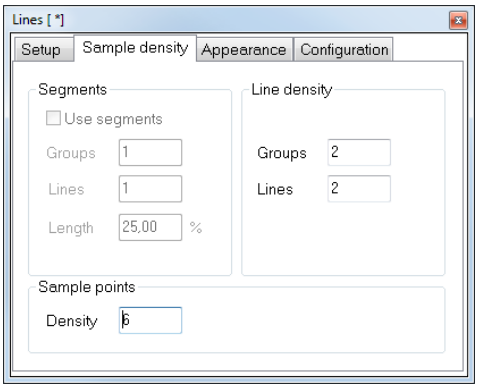
$$\overline{v_V} = \frac{\pi}{3}\overline{\ell_0^3}$$

VOLUME WEIGHTED MEAN VOLUME – POINT SAMPLED INTERCEPTS – USING NEWCAST

1. **Create a super image**
2. **Delineate region of interest**
3. **Setup PSI probe for analysis from Menu/Probes/Lines choose Volume (PSI) ( )**



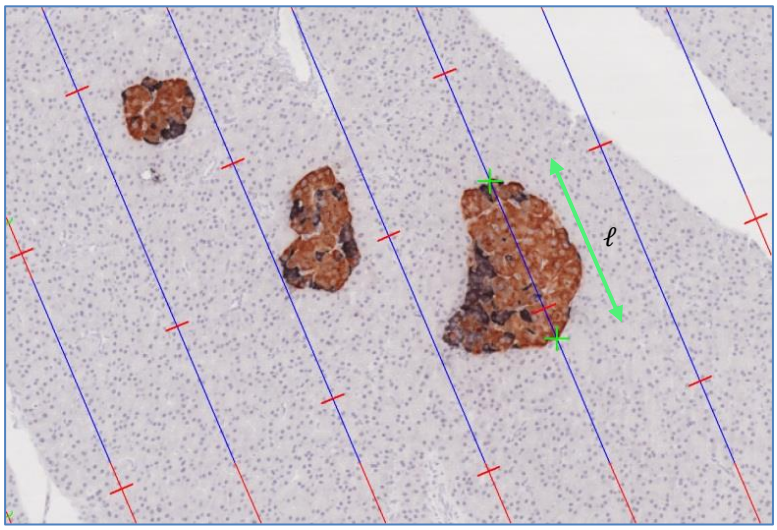
4. **Choose Isotropic or vertical sections (IUR or VUR, respectively)**
5. **A guard zone will be set to ensure that all sampled cells can be estimated (not disappearing out of the FOV)**
6. **Setup the sampling density**



7. **Setup the Meander sampling from Menu/Sampling Setup Meander sampling ( )**

8. **Count in each Field of View**

Each cell that is hit by a test line:



- Click on each edge of the cell thereby measuring ℓ

9. **Click Finish when the last FOV is measured**
10. **Save data from Menu/Data/New Data Sheet (and save current) ( )**
11. **Analyze data**

REVIEW DATA FROM MENU\DATA VIEW:

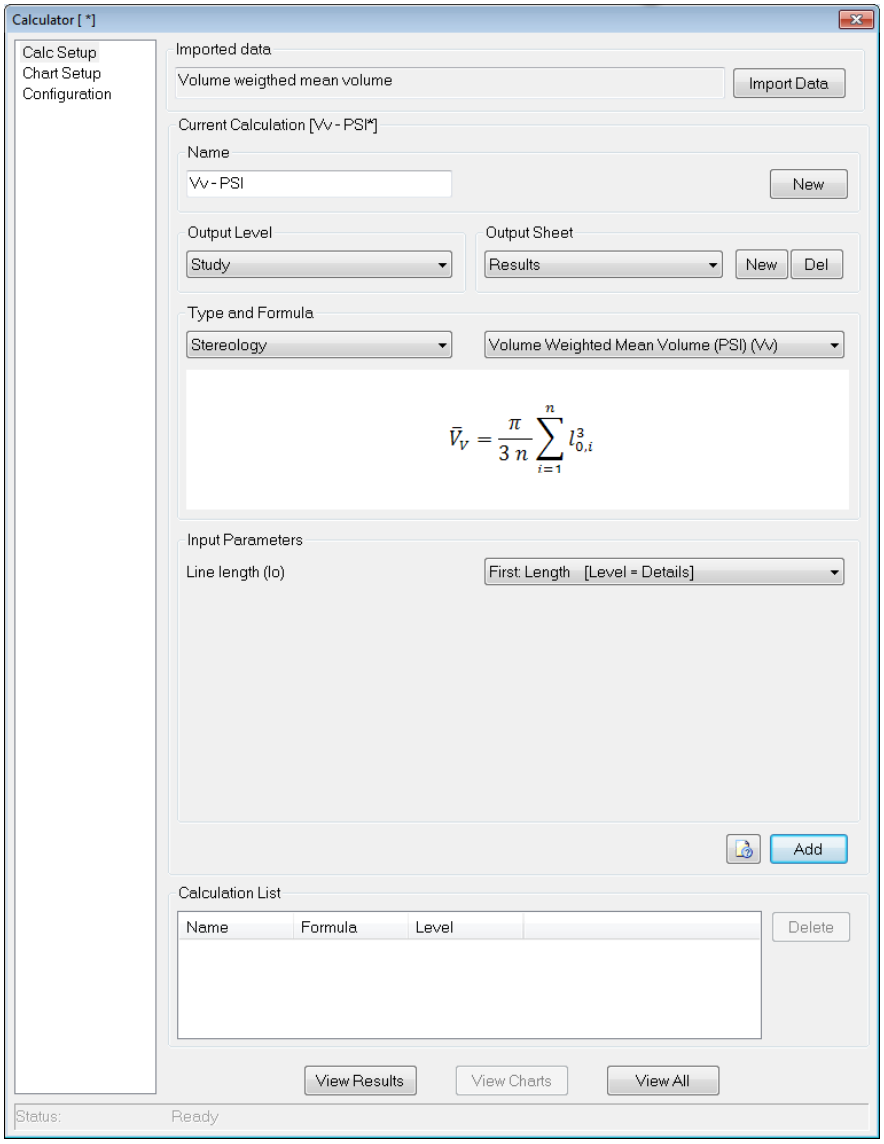
The data can be seen at 3 different levels:

- Study ( )
- Study Unit ( )
- Measurement ( )

ANALYZE USING THE CALCULATOR

Activate the Calculator using the Σ icon.

- Import the data for calculation by selecting data in the database (multiple select is possible).
- Label the specific calculation
- Output level: Choose the database level for your output: Measurement, Study Unit or Study.
- Type: Stereology
- Formula: Volume weighted mean Volume (PSI) (Vv)
- Choose Line length parameter
- Then press add



NUMBER ESTIMATION

It is important to acknowledge that the number of profiles seen in a section plane is not the same as number of cells contained in 3D space! If the cells become larger more cells will appear in a given section and elongated cells will appear proportional to the cut direction.

The solution to this well-known problem is to count in a known volume in two consecutive thin sections termed the di-sector (Sterio, 1984). In short, an unbiased counting frame is superimposed on the sections and cells that appear in one section (sampling section) but not in the next (look-up section) are counted. This disector was further developed to be used on thick section where cells are counted if they come into focus within a defined disector height. This approach was called the optical disector and the original approach called the physical disector.

SAMPLING DESIGN

There are two different sampling designs that will allow you to estimate the number of cells/particles from sections:

- Fractionator design: In which the number of cells are counted in a known fraction of the entire organ (Gundersen, 1986). Using this approach, researches will often term the stereological design:
  - Physical Fractionator
  - Optical Fractionator
- NV design: Here the number of cells/particles are counted in a known volume (Number per volume; NV) and multiplied with the reference volume to get the total number. This is often referred to as:
  - Physical Disector
  - Optical Disector

PHYSICAL DISECTOR AND FRACTIONATOR - THEORY

To count the number of cells/particles using the physical disector/fractionator one needs two consecutive sections: reference and look-up – a disector pair. There are a few guidelines for the method:

- The sections should be systematic uniform random.
- The section should generally be thin (2-5 µm) and around ⅓ to ¼ of the minimum diameter of the cells to be counted. If the cells are large one can skip sections and create a disector pair looking at section 1 and 5, for example remember that the disector volume is now 4x the cut section thickness (distance between the cut surfaces.
- You should count "both ways" meaning that you should also count cells that appear in the look-up section and not in the reference.

PHYSICAL FRACTIONATOR DESIGN:

The total number is given be (see Dorph-Petersen and Lewis, 2011):

$$N := \frac{1}{bsf} \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \sum Q^{-}$$

- bsf, block sampling fraction: What fraction of blocks were sampled for analysis
- ssf, section sampling fraction: What fraction of sections were sampled for analysis
- asf, area sampling fraction: What fraction of the section area was used to count given by: $asf = \frac{a(counting\ frame)}{(A)}$, where A = area of the basic tile in the sampling grid.

PHYSICAL DISECTOR N_V DESIGN:

The total number is given be (see Dorph-Petersen and Lewis, 2011):

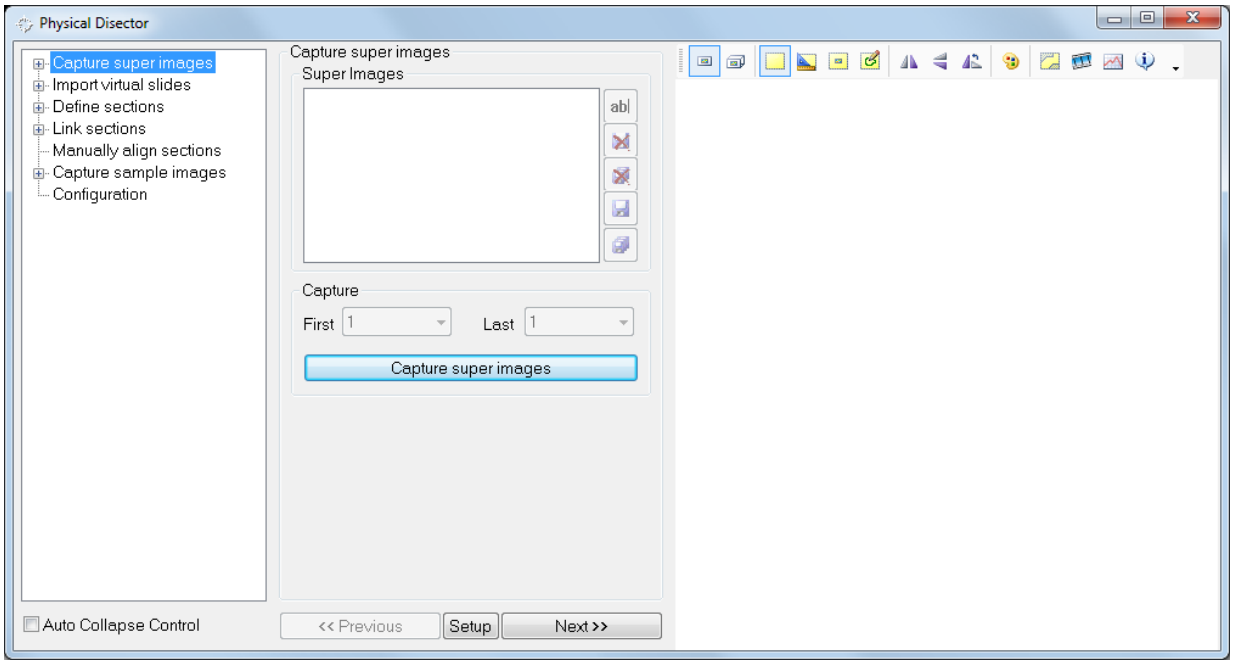
$$N_V := \frac{\sum Q^{-}}{n \cdot BA \cdot (a/p) \cdot \sum P}$$

- n: number of sections between reference and look up section (consecutive sections: n=1)
- BA: Block Advance: the cut thickness of the section on a calibrated cutting device
- (a/p): a is the area of the counting frame, $p\ is\ the$ number of points associated to the frame (normally 1 - using the upper right corner of the counting frame).
- ΣP: sum of counting frame corner points hitting reference tissue

Disector Sections: To estimate number using the physical disector, it is required to have (disector) section pairs. On a microscope based system it is required that both tissue sections are on the stage at the same time. This means that if you have a 1-slide stage you will need both tissue sections on one glass slide, whereas, if you have a 2, 4 or 8 slide stage you can have the sections on separate glass slides.

On a whole slide system, you can load in all the images you need and the disector sections can be on separate images.

1) **Launch the physical disector dialog from:**

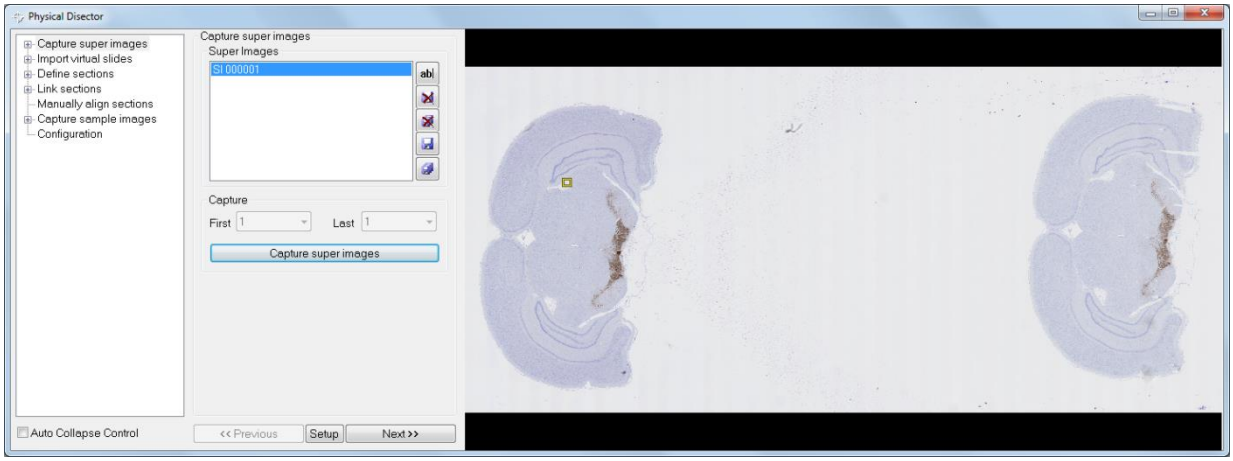


Depending on your installed modules the dialog might look a little different.

The next steps are more or less to follow the list of options given in the physical disector dialog. Click the appropriate point to get started – to go to next point either click the point or click *Next*.

2) **Capture super image**

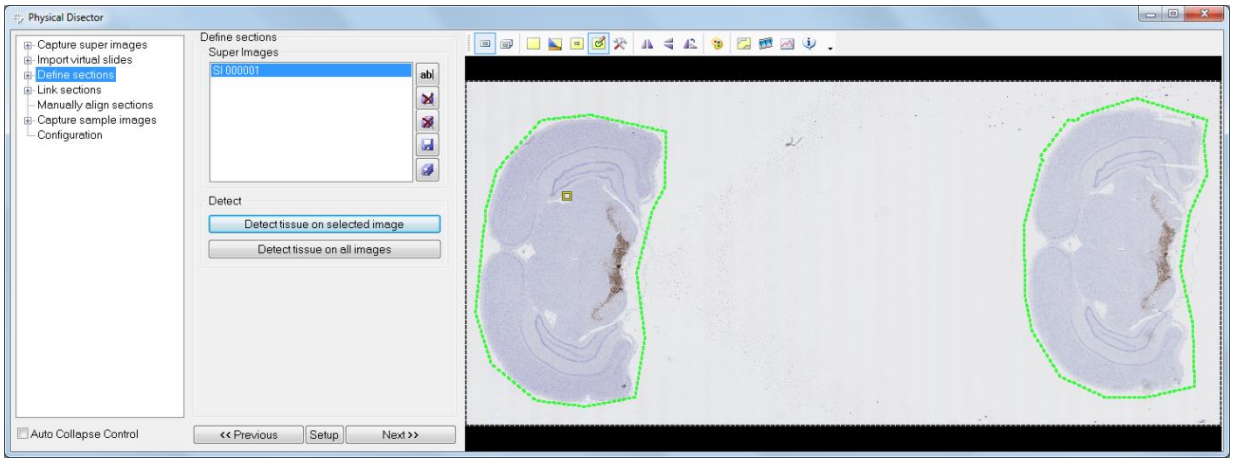
You will need to take Super images of both disector sections either on one glass slide or two separate glass slides. Using virtual slides import the section(s) containing the disector pair by clicking the appropriate images in the database.



3) Define sections

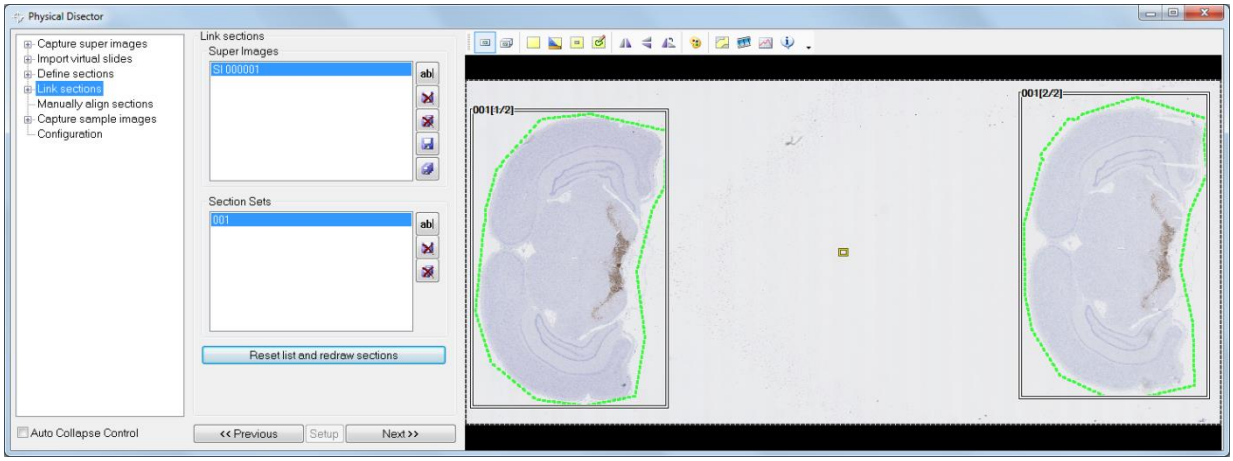
Delineate the sections that constitute your disector pair using the ROI tool from Layer drawing. This will automatically be activated when clicking *define sections*.

This delineation is used for the aligning process so please delineate the entire section even if the region of interest is a sub-compartment of the sections.



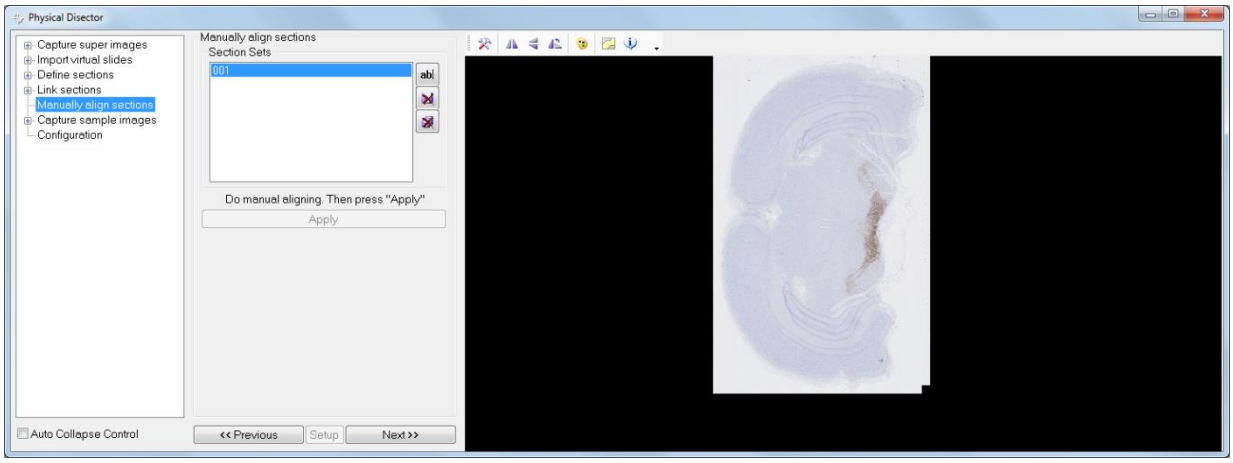
4) Link sections

All tissue sections that were outlined in 3) will now be boxed and ready to link. To link, simple click the sections that is part of the disector pair. More than one section pair can be linked if necessary. If the sections are on separate Super images, simply choose the super image and click the section.

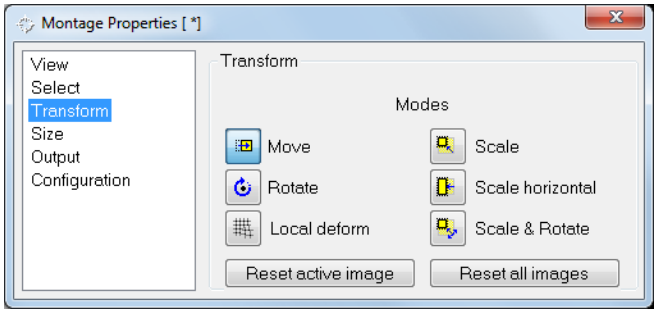


5) Manually align sections

After clicking this point – the sections are placed on top of each other with a transparency of 50 / 50%.



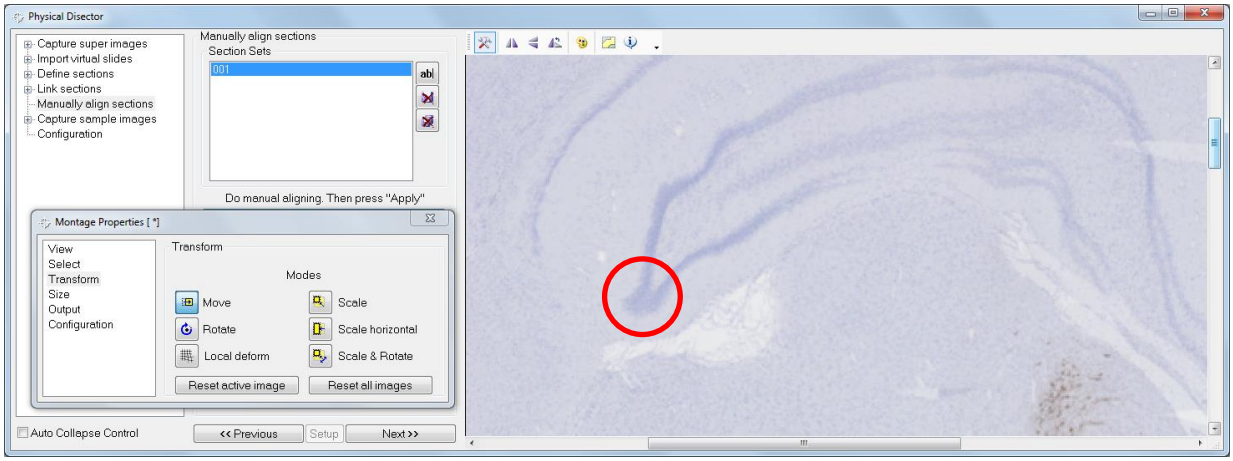
To align click the toolbox icon 



Please consult Help on how to use the montage for tissue aligning.

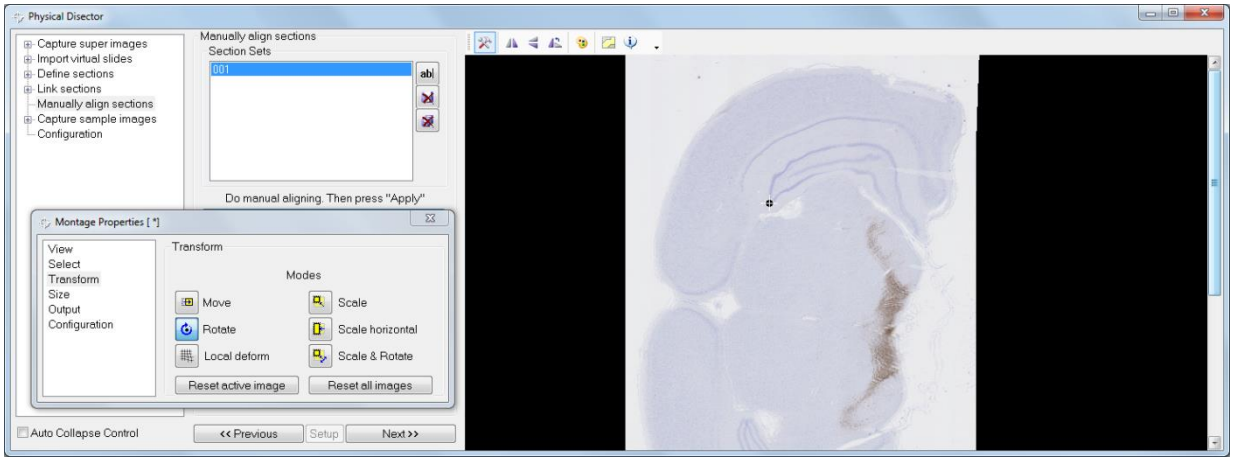
Note: Most sections are rotated – to align you should:

a) Move the section until a unique point is perfectly matched on the two sections. Zoom in if necessary:



b) Select Rotate and double click on the unique point

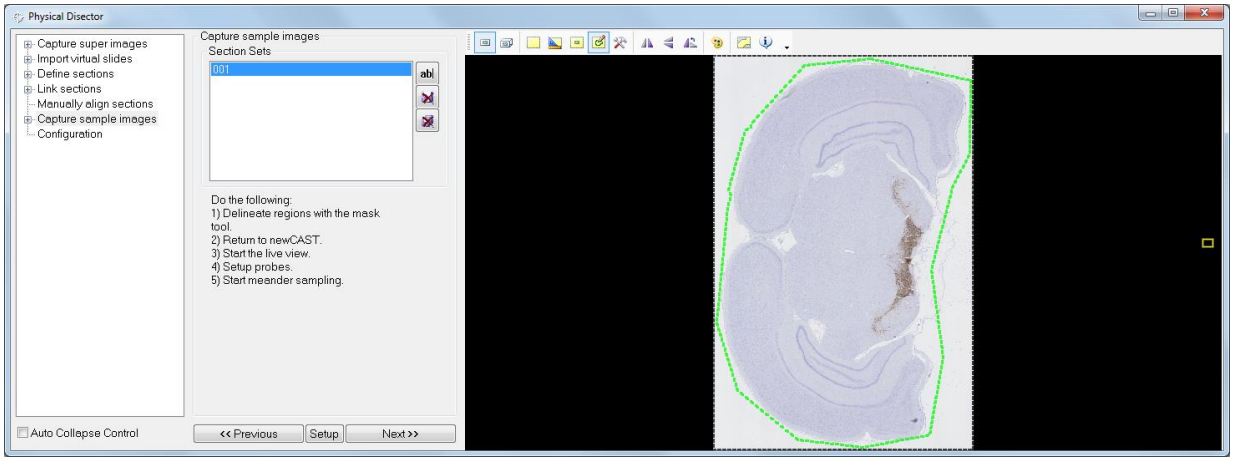
c) Rotate until the sections are matched



6) Click *Apply* when the aligning is done

7) Capture sample images

The proposed ROI is now shown on the first section. This is based on the tissue delineation used to link the sections.

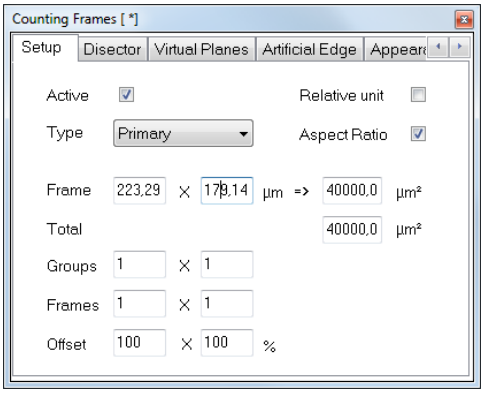


Follow the instructions given in the dialog box:

8) **Delineate Region of interest**
9) **Return to newCAST**
10) **Start the live view**
11) **Setup probes**

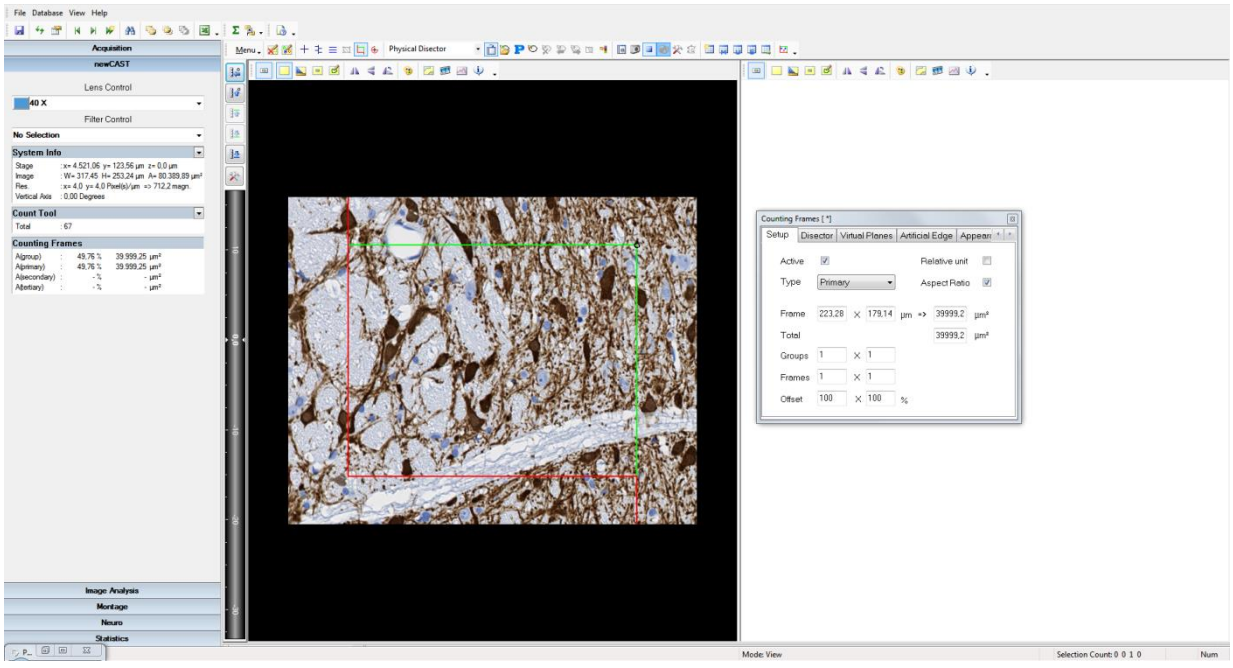
For number estimation, a counting frame is the appropriate probe.

12) **Activate the counting frame from Menu/Probes Counting frame ( )**



Adjust the counting frame to the wanted size.

IMPORTANT: Relative unit should not be selected, as the image on the right hand side is be default (but adjustable) 50% larger than the image on the left hand side. Relative unit will consequently result in counting frames of different size.



13) **Setup meander sampling from Menu/Sampling Setup Meander sampling ( )**
14) **Start sampling**

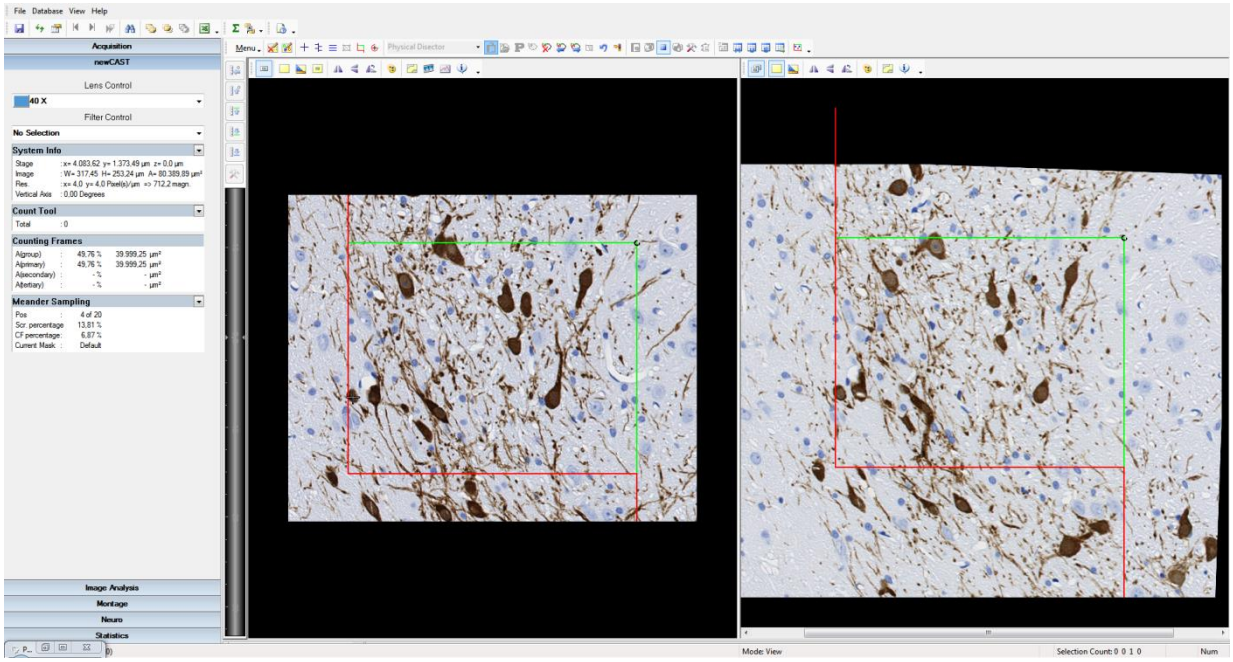
For each sampling position you are asked to:

a) **Focus the image**
b) **Re-focus on the other section and click next**

Two corresponding images are now presented. Before counting:

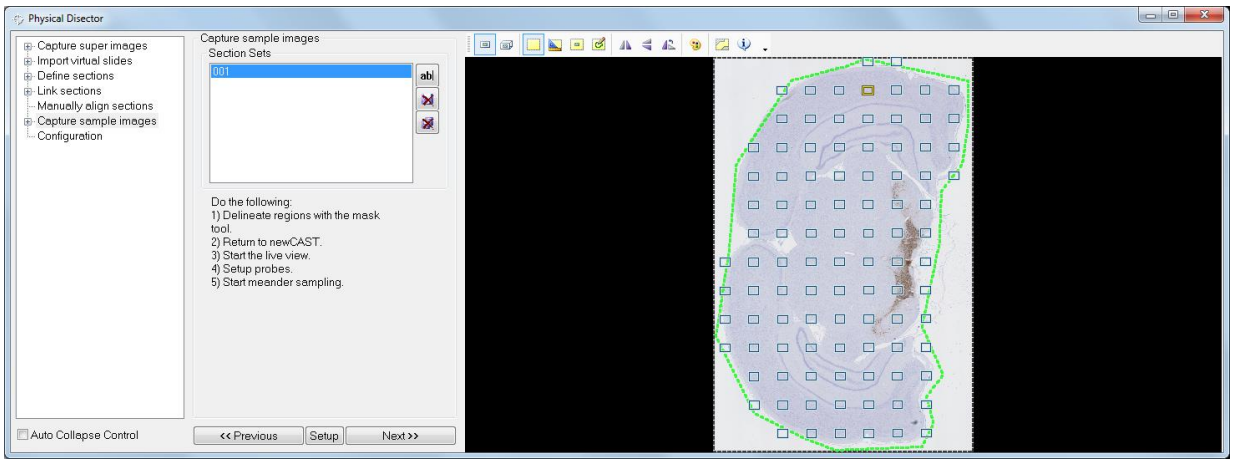
15) **Move the image on the right side to be perfectly aligned**

Hint: You can select a unique cell or structure on the right side and move the image. There is a corresponding cross on the left image - move the image to perfectly match the images.



16) **Count using the count tool**

All sampling positions can be seen in the physical disector dialog including the actual position:



17) **Click Finish when the last FOV is counted**

18) **Save data from Menu/Data/New Data Sheet (and save current) ( )**

19) Analyze data

REVIEW DATA FROM MENU\DATA VIEW:

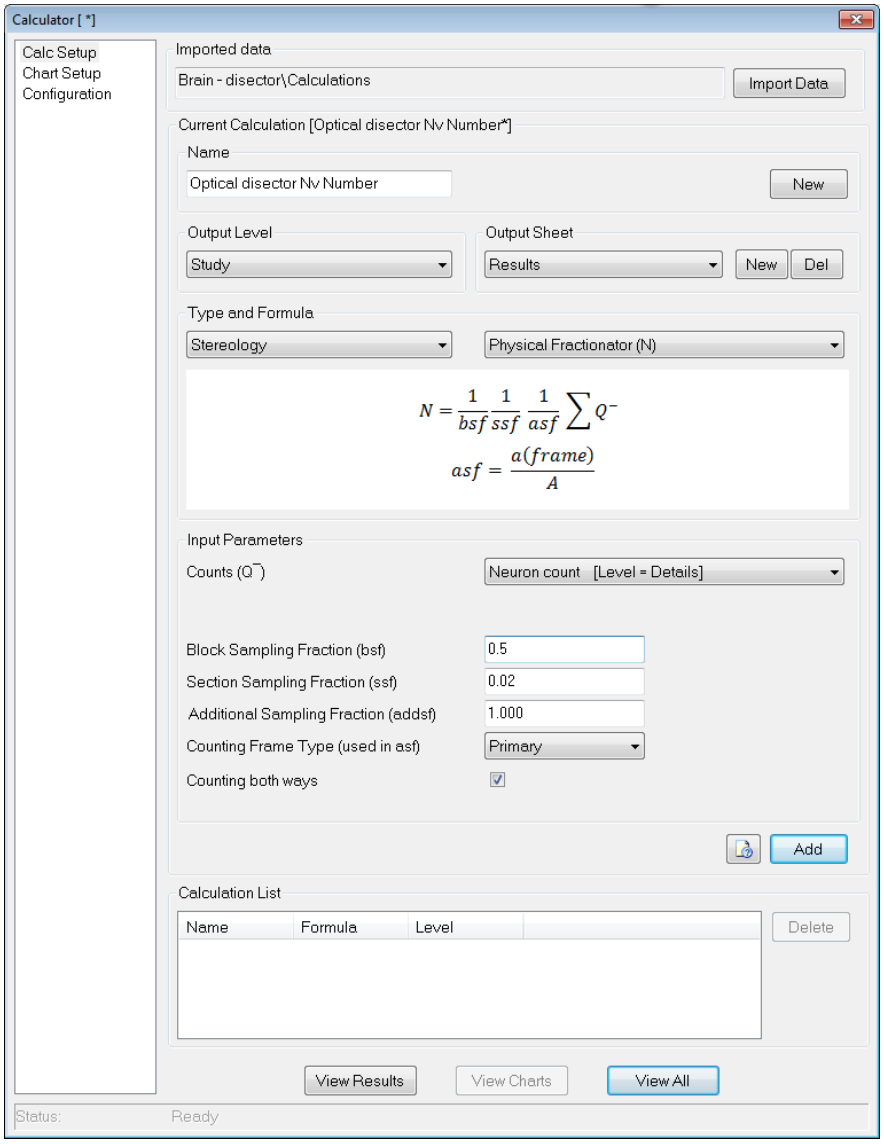
The data can be seen at 3 different levels:

- Study ()
- Study Unit ()
- Measurement ()

ANALYZE USING THE CALCULATOR

Start the Calculator from the  icon.

- Import the data for calculation by selecting data in the database (multiple select is possible).
- Label the specific calculation
- Output level: Choose the database level for your output: Measurement, Study Unit or Study.
- Type: Stereology
  Formula: Physical Fractionator (N)
- Counts: here Neuron count
- Block Sampling Fraction (bsf): Fraction of Blocks used here 0.5 write in as fraction (NOT %)
- Section Sampling Fraction (bsf): Fraction of Section used here 0.02 write in as fraction (NOT %)
- Additional Sampling Fraction (addsf): Additional Fractions used here 1 write in as fraction (NOT %)
- Counting Frame Type (used in asf): Chose the counting frame used
- Counting both ways: De-select if you have only counted one way (reference – look-up)
- Then press add



OPTICAL DISECTOR AND FRACTIONATOR - THEORY

To count the number of cell in an optical disector approach thick sections are needed. The end height at the microscope should generally be at least 20 µm. In addition, a microcator mounted on the microscope measuring the actual z-height is needed as well as an oil-lens with a high numerical aperture typically 40x, 60x or 100x. Using systematic uniform random sampling, fields of view are presented and, using an unbiased counting frame, the researcher counts all cells that come into focus within the disector height.

There are some important challenges that need to be addressed before the counting process can be started:

STAINING PENETRATION:

To be sure that you have full staining penetration, a **z-axis distribution** should always be carried out (Andersen and Gundersen, 1999; Dorph-Petersen et al, 2001). To do this, you sample, from a few animals, all cells from the very top to the very bottom of the section. You need around 300 cells to then plot the histogram showing the

number of counts in different depths (increments of 2 µm for example). When your stainings are working perfectly, a constant cell count is observed in the center part of the section. If you see a lower count (or none!) in the center part – you do not have staining penetration and the sections cannot be used for counting.

GUARD HEIGHT

The number of cells at the very top and bottom will be lower than in the center part of the section because cell might have fallen out during the cutting procedure. From the z-axis distribution, mentioned above, you can set the correct guard height for your experiment. If you have not made the z-axis distribution you will not be able to set the height as you have no knowledge of the degree of lost caps (depending on the mounting media). In addition, you do not know if you have staining penetration.

TISSUE SHRINKAGE

The N_V design is very sensitive to tissue shrinkage as the number of cells per volume is estimated. Therefore, you always have to take the shrinkage into account before reporting data obtained with a N_V design. You should consult Dorph-Petersen and co-workers (2001) for and in depth analysis of the issue.

The fractionator design is not sensitive to shrinkage as the number of cells within a given fraction is not affected by shrinkage.

OPTICAL FRACTIONATOR DESIGN:

The total number is given be (see Dorph-Petersen and Lewis, 2011):

$$N := \frac{1}{bsf} \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{hsf} \cdot \sum Q^-$$

- bsf, block sampling fraction: What fraction of blocks were sampled for analysis
- ssf, section sampling fraction: What fraction of sections were sampled for analysis
- asf, area sampling fraction: What fraction of the section area was used to count given by: $asf = \frac{a(counting\ frame)}{(A)}$, where A = area of the basic tile in the sampling grid
- hsf, height sampling fraction: $hsf = \frac{h}{\overline{t_{Q^-}}}$, where $\overline{t_{Q^-}} = \frac{\sum_i (t_i \cdot q_i)}{\sum_i (q_i)}$, h= disector height, t_i=measured section height at position i and q_i = number of counts at position i

OPTICAL DISECTOR N_V DESIGN:

The total number is given be (see Dorph-Petersen and Lewis, 2011):

$$N_V := \frac{\overline{t_{Q^-}}}{BA} \cdot \frac{\sum Q^-}{h \cdot (a/p) \cdot \sum P}$$

- $\overline{t_{Q^-}}$: is the number-weighted mean section thickness: $\overline{t_{Q^-}} = \frac{\sum_i (t_i \cdot q_i)}{\sum_i (q_i)}$, t_i=measured section height at position i and q_i = number of counts at position i.
- BA: Block Advance: the cut thickness of the section on a calibrated cutting device
- h: disector height.
  - (a/p): a is the area of the counting frame,$p\ is\ the$ number of points associated to the frame (normally 1 - using the upper right corner of the counting frame)

- ΣP: is the sum of points hitting reference tissue.

OPTICAL DISECTOR – USING NEWCAST

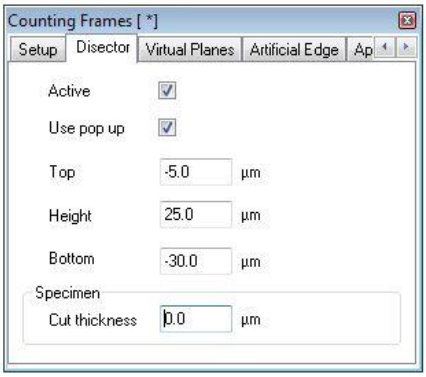
The optical disector follows the generalized work flow

1) **Capture Super Image**
2) **Delineate Region of Interest**
3) **Activate the Optical disector from Menu/Probes/Counting frame ( )**

When the counting frame has been activated and given the appropriate size:

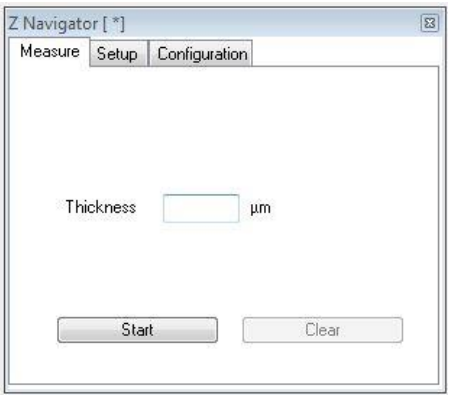
4) **Select the disector menu within the counting frames dialog**

Use Pop-up:	Lines changes color when at the start of the disector.
Top:	Indicates the top of the disector. Here 5 µm below (-) the top of the section.
Height:	Disector height (from Top).
Bottom:	Calculated bottom of disector.
Cut thickness:	For visual purposes only - will show the thickness of the section on the Z-navigator.



5) **Setup meander sampling from Menu/Sampling Setup Meander sampling ( )**
6) **At each sampling position:**

- Count the top right (encircled) corner point if it overlays the tissue with the count tool. You could rename one of the marks to Corner Point
- Find the first position in the z-axis where something comes into focus in the center of the counting frame. If nothing comes into focus - jump to next position.
- Set current position to 0 (**1**)
- Start the Z navigator (**2**) and press start



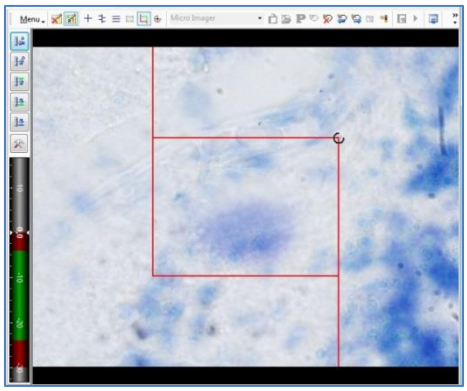
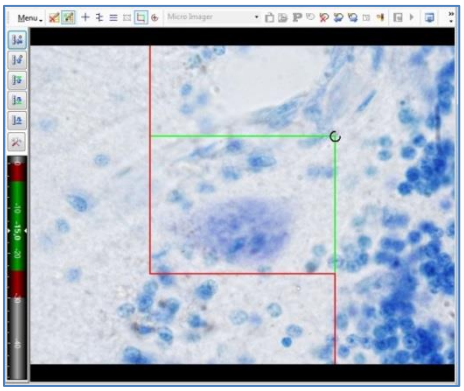
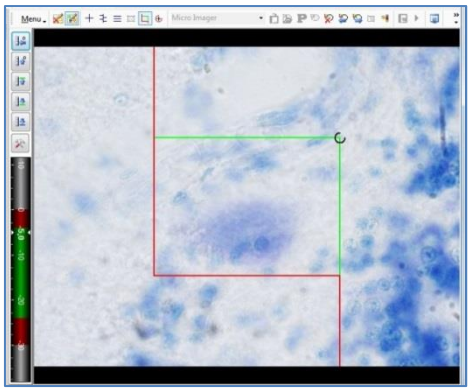
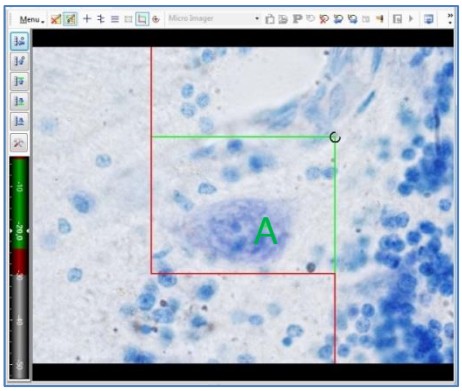
- Focus down and start counting objects that comes into focus within the disector (green).
- Continue to where nothing is in focus (bottom of the section) – and push stop on the Z navigator.

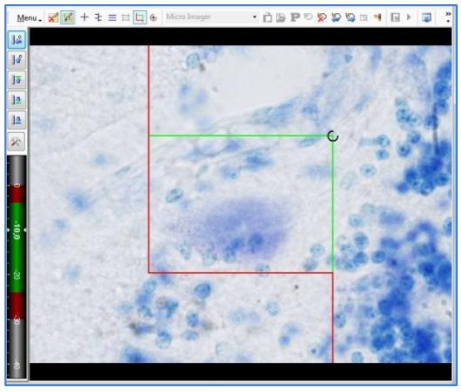
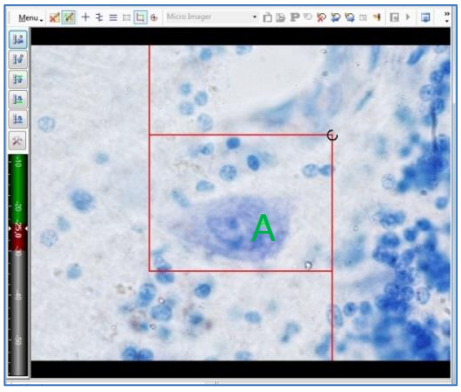
7) Click Finish when the last FOV is counted

8) Save data from Menu/Data/New Data Sheet (and save current) ( )

9) Analyze data

EXAMPLE

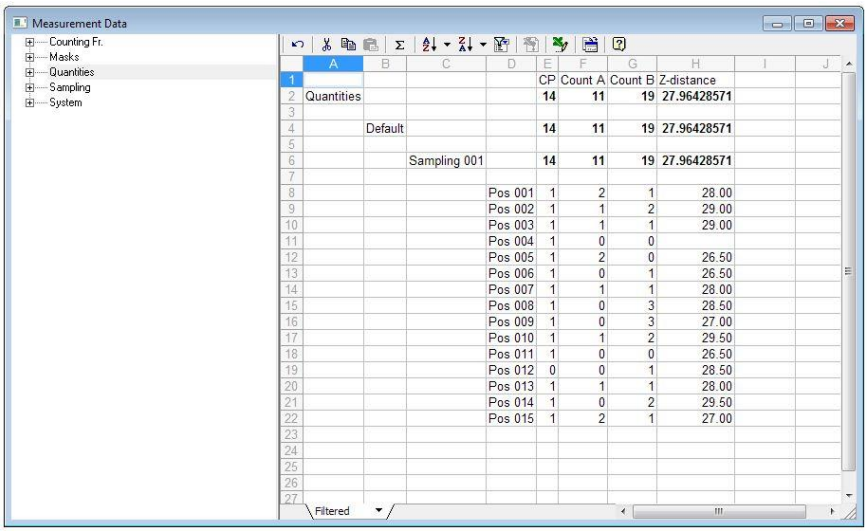



Images taken at every 5µm down through a thick section. At -20 µm (20 µm below the top of the section) a neuron is counted marked A (counting unit: the nucleolus)

REVIEW DATA FROM MENU\DATA VIEW:

The data can be seen at 3 different levels:

- Study ( )
- Study Unit ( )
- Measurement ( )



	A	B	C	D	E	F	G	H
1					CP	Count A	Count B	Z-distance
2	Quantities				14	11	19	27.96428571
4		Default			14	11	19	27.96428571
6			Sampling 001		14	11	19	27.96428571
8				Pos 001	1	2	1	28.00
9				Pos 002	1	1	2	29.00
10				Pos 003	1	1	1	29.00
11				Pos 004	1	0	0	
12				Pos 005	1	2	0	26.50
13				Pos 006	1	0	1	26.50
14				Pos 007	1	1	1	28.00
15				Pos 008	1	0	3	28.50
16				Pos 009	1	0	3	27.00
17				Pos 010	1	1	2	29.50
18				Pos 011	1	0	0	26.50
19				Pos 012	0	0	1	28.50
20				Pos 013	1	1	1	28.00
21				Pos 014	1	0	2	29.50
22				Pos 015	1	2	1	27.00

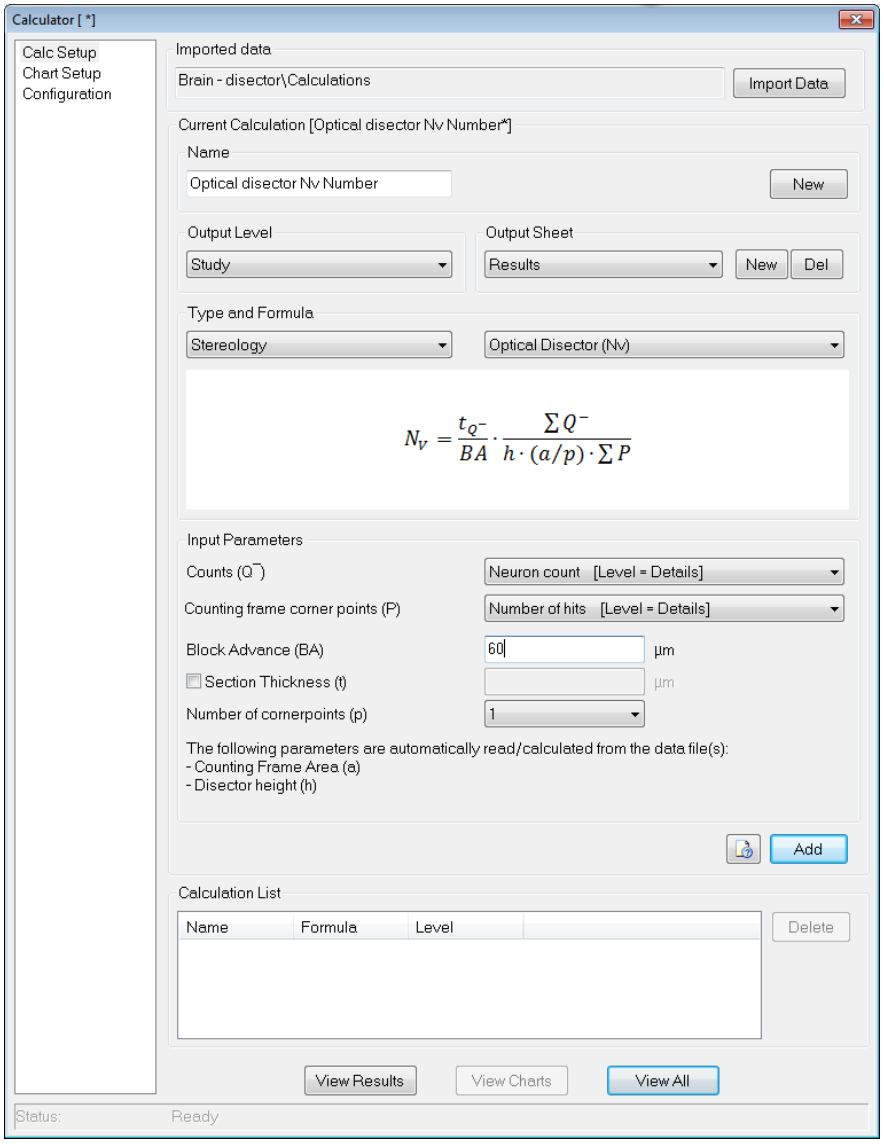
Here: Measurement level, Cell counts A and B; CP = counted corner points; Z-distance = measure of Z-height

All data can be exported directly to excel using the Excel icon in the tool bar.

CALCULATIONS USING THE CALCULATOR:

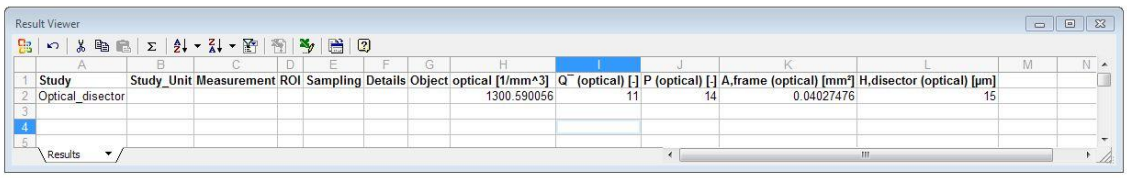
Activate the Calculator using the Σ icon.

- Import the data for calculation by selecting data in the database (multiple select is possible).
- Label the specific calculation
- Output level: Choose the database level for your output: Measurement, Study Unit or Study.
- Type: Stereology
  Formula: Optical Disector
- Counts: here Neuron Count
- Corner points: here Number of hits
- Block Advance: the cut thickness in µm
- Section thickness: If you have not measured the section thickness during the sampling (as recommended) you can subsequently measure the thickness and enter the mean value here. This will replace the t_Q- that is normally calculated in the Calculator.
- Then press add



The calculation is now added to a calculation list. You can add several calculations to one calculator configuration.

The results are presented like this (click *view results*):



In this example, there were 1300 cells (A) per mm^3. This should be multiplied by the reference volume to get the total number of cells in the organ.

NOTE: This result is from one section only – there should be 6-10 sections from each animal and the total count (A) in one animal should be around 200.

SURFACE

Surface area (and surface density) is strongly depended on the orientation of the section. Therefore, to get an unbiased estimate of the surface area of any structure, it is required that test lines are randomly rotated in 3D space. This can be achieved using:

- IUR, isotropic uniform random sections
- VUR, vertical uniform random sections

SURFACE DENSITY - THEORY

The surface density is defined as:

$$S_V := \frac{Area\ of\ interface\ in\ reference\ space}{Volume\ of\ reference\ space}$$

And consequently the total surface area is estimated as:

$$S = V_{ref} \cdot S_V$$

ESTIMATION OF S_V:

To estimate the surface density a set of isotropic linear test lines are used. If IUR sections are used the test lines are truly isotropic if VUR sections are used the lines are either sine-weighted according to the vertical axis or a cycloid grid is used (Baddeley et al., 1986). An intersection count occurs when the line probe crosses the surface in question. The surface density is then calculated as:

$$S_V = 2 \cdot I_L$$

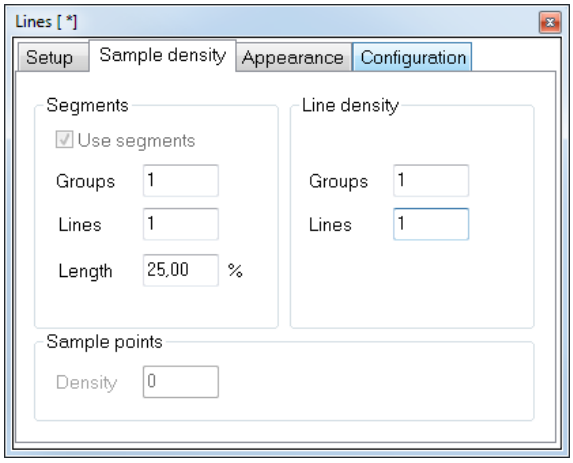
Were I_L is the mean number of intersection points per unit length of test line in reference space given by:

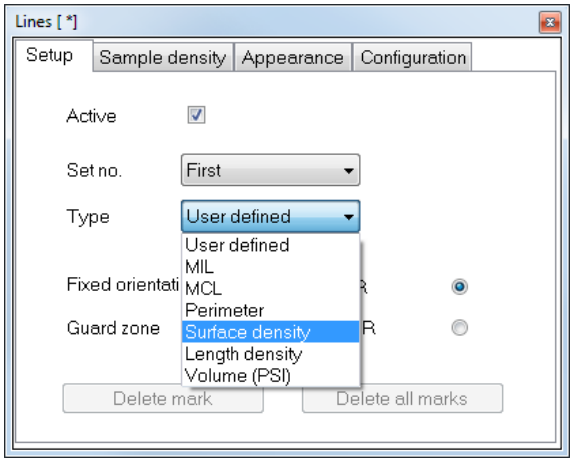
$$I_L = \frac{\sum I}{l_P \cdot \sum P_l}$$

- l_P: length per line end-point
- $\sum P_l$: sum of line end-points hitting reference space
- $\sum I$: sum of intersections

SURFACE DENSITY – USING NEWCAST

1) **Capture Super Image**
2) **Delineate Region of Interest**
3) **Setup the line probe from Menu/Probes/ Lines ( )**





- Segments
  For surface density line segments are automatically used.
- Groups - Change the number of groups in the X direction.
  Each group of segments contains the number of segments listed in the Lines value. Groups make it possible to organize multiple line probes on the screen at once.
- Lines - Change the number of segments per group in the X direction.
- Length - Change the length of the line segments.
  The length of segments is set in percent of the image width.
- Line density
  - Groups - Change the number of groups in the Y direction. Each group of lines contains the number of lines listed in the Lines value.
  - Lines - Change the number of lines in the Y direction.

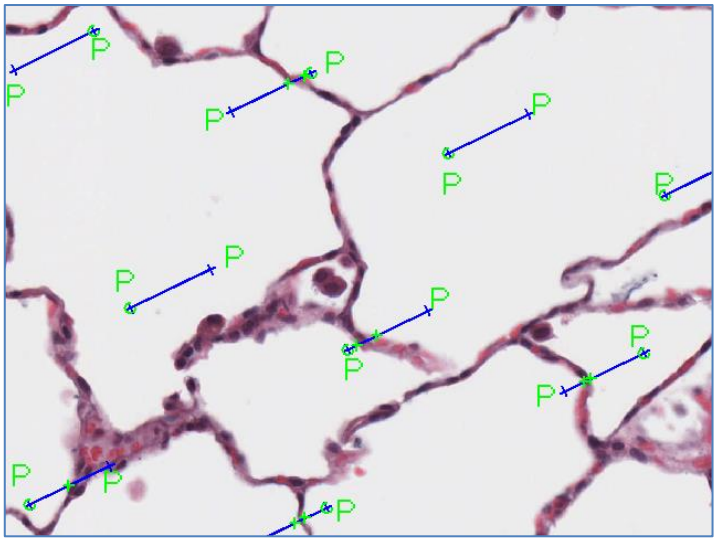
4) **Setup Meander Sampling from Menu/Sampling Setup Meander sampling ( )**
5) **Mark intersections between the lines and the surface**

When counting the intersections please have the lines probe activated.

Note: The active mode is shown in the Status bar in the lower part of the screen:

6) **Count number of points hitting the reference space using the count tool.**
7) **Click Finish when the last FOV is counted**
8) **Save data from Menu/Data/New Data Sheet (and save current) ( )**

EXAMPLE:



Alveolar surface density: The number of intersections (I) between the test lines and the alveolar wall is counted (green crosses) as well as the number of points in references space (P).

9) Analyze data

REVIEW DATA FROM MENU\DATA VIEW:

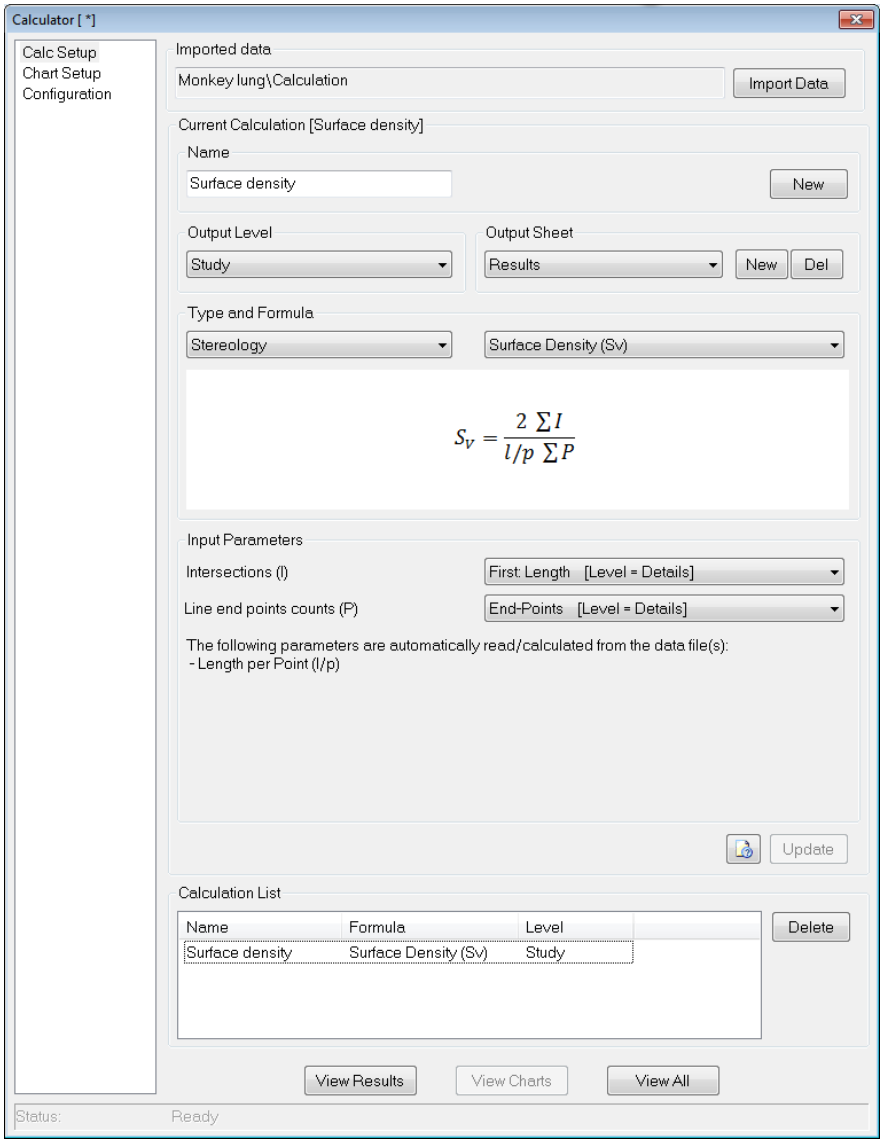
The data can be seen at 3 different levels:

- Study ( )
- Study Unit ( )
- Measurement ( )

ANALYZE USING THE CALCULATOR

The surface density can be automatically calculated using the Calculator Σ

- Import the data for calculation by selecting data in the database (multiple select is possible).
- Label the specific calculation
- Output level: Choose the database level for your output: measurement, study unit or study.
- Type: Stereology
  Formula: Surface Area Density
- Choose parameter for Intersections
- Choose parameter for Line end Points Count
- Then press add



To get the total surface area you should multiply by the reference volume obtained using the Cavalieri principle.

If you have the data from the Cavalieri estimator:

1) **Add the Cavalieri estimate for total volume in the calculation list**
2) **Multiply the two calculations to get total surface area**
    a. Type: Math
    b. Formula: Multiply
    c. X1: Reference volume
    d. X2: Surface Area Density

If you have the reference volume from another source (eg. Tissue weight or water displacement)

1) **Multiply the Surface Area density with the Reference volume**
    a. Type: Math
    b. Formula: Multiply
    c. X1: As a constant – remember to use the same unit.
    d. X2: Surface Area Density

LENGTH

The length of capillaries and other tubular structures can be estimated using stereological methods under the mild assumption that the length is much larger than the width (approx. > 10x). As with surface area, length (and length density) is strongly depended on the orientation of the section. Therefore, to get an unbiased estimate of the length of a structure, it is required that the section planes are randomly rotated in 3D space. This can be achieved using:

- IUR, isotropic uniform random sections
- VUR, vertical uniform random sections, Vertical projection
- Isotropic virtual test planes in an arbitrarily cut thick section, global spatial sampling with virtual planes

LENGTH DENSITY - THEORY

The length density is defined as:

$$L_V := \frac{Length\ of\ feature\ in\ reference\ space}{Volume\ of\ reference\ space}$$

And consequently the total surface area is estimated as:

$$L = V_{ref} \cdot L_V$$

ESTIMATION OF L_V ON THIN IUR SECTION

To estimate the length density on thin IUR sections, unbiased counting frames are put on the section in a systematically uniform random way. The number of profiles sampled by the counting frame is counted and the length estimate is:

$$L_V = 2 \cdot Q_A$$

Were Q_A is the number of profiles per unit area of test probe given by:

$$Q_A = \frac{\sum Q}{a_f \cdot \sum P_f}$$

- a_f: area of the counting frame
- $\sum P_f$: sum of counting frame corner points hitting the reference volume
- $\sum Q$: sum of profiles within the counting frame

ESTIMATION OF L_V ON THICK ARBITRARY SECTIONS – VIRTUAL PLANES

Another approach is to make isotropic virtual test planes within an arbitrarily cut thick section. A box if defined (size in x, y, z) and the intersections between the structure and the virtual test plane are counted:

$$L_V = 2 \cdot Q_A = \frac{2 \cdot P_{box}}{avg\ a(plane)} \cdot \frac{\sum Q}{\sum P_{ref}}$$

- P_{box}: Number of box corners.
- $avg\ a(plane)$: Average of the total area of planes confined inside the virtual box. It is calculated by taking the volume of the box and divide by the distance between the planes.
- ΣQ: Total number of transects with the structure.
- $\Sigma P(ref)$: Total number of box corners that hit reference space.

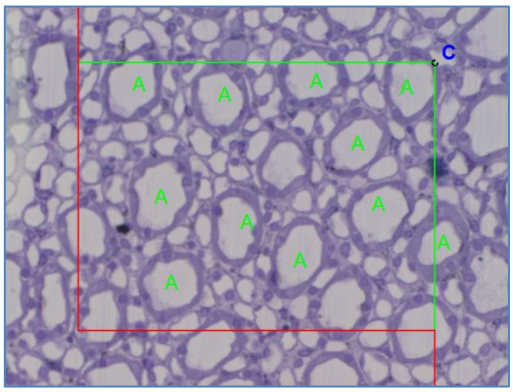
Note: To get the total length density from one animal this calculation should be done with the total number of intersection from all sections and the total number of box corners hitting reference space from all sections. One cannot take the average L_V from each section!

LENGTH ESTIMATION - THIN IUR SECTION - NEWCAST

1) **Capture Super Image**
2) **Delineate Region of Interest**
3) **Setup the counting frame from Menu/Probes/Counting frames ( )**
4) **Setup Meander Sampling from Menu/Sampling Setup Meander sampling ( )**
5) **Count number of profiles in each Field of View (A)**

Count the number of profiles inside the counting frame or touching the green inclusion lines but not touching the red exclusion lines.

6) **Count the number of counting frame corner points hitting reference space (C).**



7) **Click Finish when the last FOV is counted**
8) **Save data from Menu/Data/New Data Sheet (and save current) ( )**
9) **Analyze data**

REVIEW DATA FROM MENU\DATA VIEW:

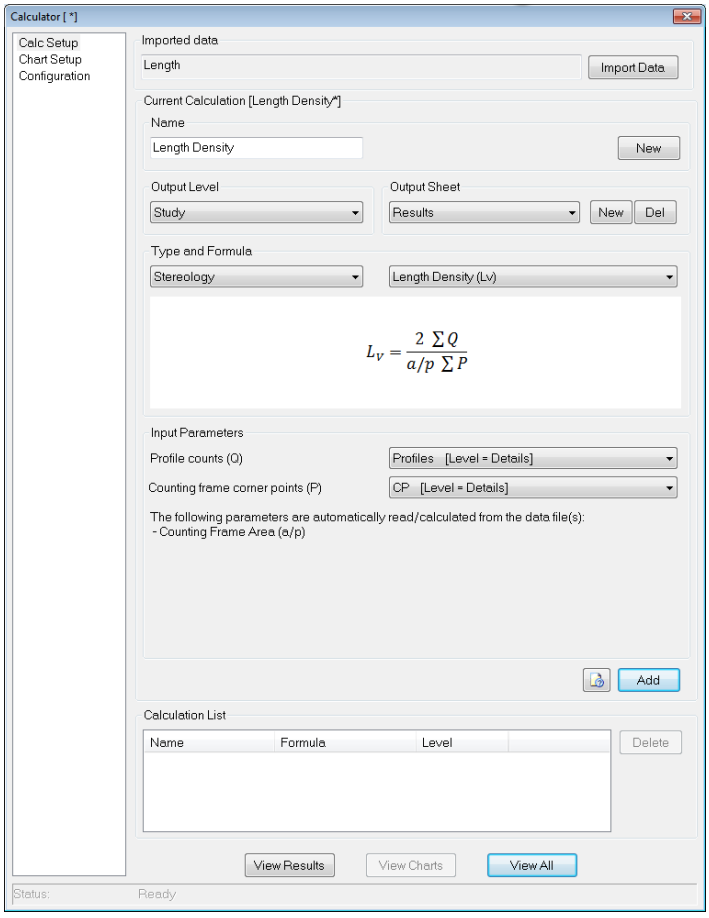
The data can be seen at 3 different levels:

- Study ( )
- Study Unit ( )
- Measurement ( )

ANALYZE USING THE CALCULATOR

The length density can be automatically calculated using the Calculator

- Import the data for calculation by selecting data in the database (multiple select is possible).
- Label the specific calculation
- Output level: Choose the database level for your output: Measurement, Study Unit or Study.
- Type: Stereology
  Formula: Length Density (Volume Weighted) (Lv)
- Choose parameter for Profile counts
- Choose parameter for frame corner counts
- Then press add



To get the total length you should multiply by the reference volume obtained using the Cavalieri principle.

If you have the data from the Cavalieri estimator:

1) **Add the Cavalieri estimate for total volume in the calculation list**
2) **Multiply the two calculations to get total surface area**
    a. Type: Math
    b. Formula: Multiply
    c. X1: Reference volume
    d. X2: Length Density

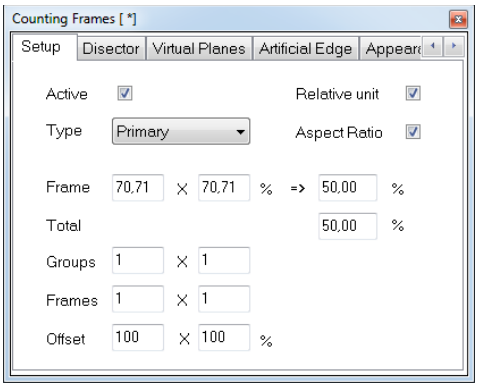
If you have the reference volume from another source (eg. Tissue weight or water displacement)

1) **Multiply the Surface Area density with the Reference volume**
    a. Type: Math
    b. Formula: Multiply
    c. X1: As a constant – remember to use the same unit.
    d. X2: Length Density

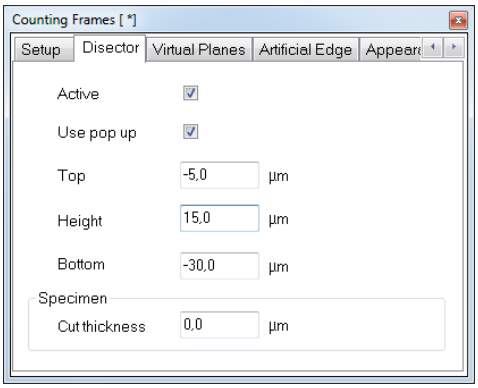
ESTIMATION OF L_V ON THICK ARBITRARY SECTIONS – VIRTUAL PLANES – NEWCAST

1) **Capture Super Image**
2) **Delineate Region of Interest**
3) **Setup the line probe from Menu/Probes/ Counting frame ( )**

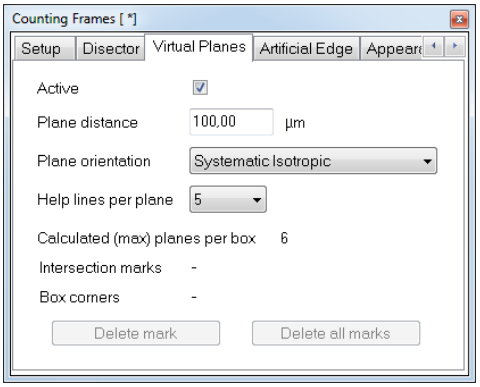
- **Activate the counting frame and set the size**



- **Activate the disector and set the depth of the box for virtual planes counting**



- **Activate the Virtual planes and set up the parameters**



- **Plane distance** - Sets the perpendicular distance between each plane. This will define the amount of planes pr. box: the lower the distance the more planes. Remember that setting the distance very low will only produce a small increase in the quality of data and does not compensate for the increase in work load. It is only necessary to have approx. 150-200 positive counts per animal.
- **Plane orientation** – Set the orientation of planes (default IUR across FOVs)
- **Help lines per plane** - Sets the number of help lines pr. plane. The help lines show the positions of the focal plane and virtual plane intersections in the given tissue (i.e. green or red lines) as the focal plane descends or ascends.
- **Calculated (max) planes per box** - This number indicates the maximum number of virtual plane and focal plane intersections (i.e. green or red lines). The maximum number of lines are only reached if the virtual plane is close to vertical (90º).

4) **Setup Meander Sampling from Menu/Sampling Setup Meander sampling ( )**
5) **Mark number of intersections between the virtual planes and the structure in each Field of View.**

- **Box corners:** By default the box corners are green inside the ROI and Red outside and are automatically counted

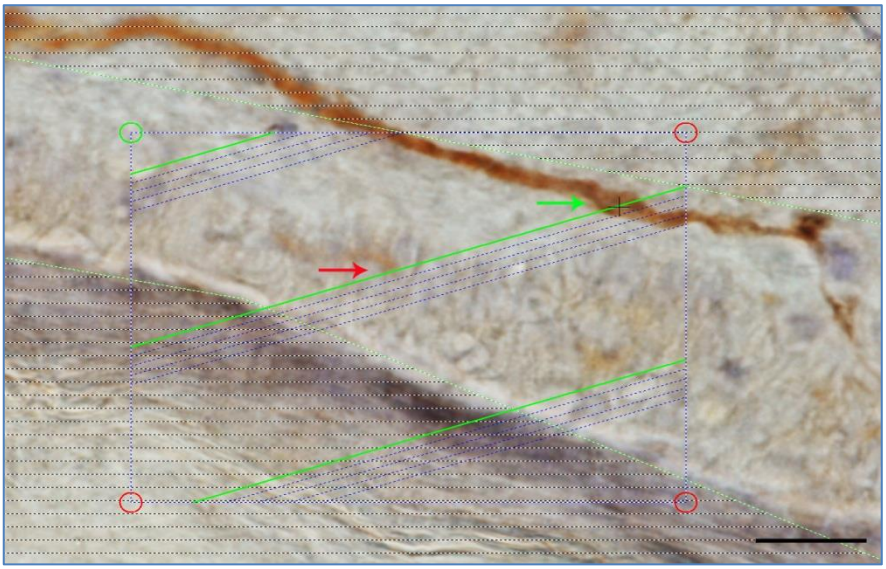
If a corner inside the ROI does not touch the reference space you should click the green box and it will change to red.

If a box corner outside the ROI touches the reference space you should click the red box and it will change to green.

- **Intersections:** Focus slowly down the tissue and mark whenever a virtual plane touches the structure in focus.

For further guidelines on this please consult: *Virtual test systems for global spatial sampling in thick, arbitrarily orientated uniform, random sections*. Larsen, J.O. and Gundersen H.J.G. Acta Stereol, 18(3): 381-388, 1999.

EXAMPLE:



Estimating nerve fibers length from a 3 mm punch skin biopsy taken at the distal leg in a healthy subject. The three green are representation of where the focal plane and the virtual plane intersect. When a green line crosses a fiber in focus, the fiber should be marked (cross at the green arrow). If the fiber is not in focus no mark should be added (red arrow). The box corners are green if they are within the ROI (here indicating the epidermis)and red circles indicate corners outside epidermis. Scale bar = 0.05 mm

10) **Click Finish when the last FOV is counted**
11) **Save data from Menu/Data/New Data Sheet (and save current) ( )**
12) **Analyze data**

REVIEW DATA FROM MENU\DATA VIEW:

To calculate the Lv you need to add up all data from one individual: these can be found in the data viewer from: Menu/Data/view measurement data.

The calculations are straight forward:

$$L_V = 2 \cdot Q_A = \frac{2 \cdot P_{box}}{avg\ a(plane)} \cdot \frac{\sum Q}{\sum P_{ref}}$$

- P_{box}: Number of box corners.
  - = 4
- $avg\ a(plane)$: Average of the total area of planes confined inside the virtual box. It is calculated by taking the volume of the box and divide by the distance between the planes.
  - Found under: *Quantities - Raw*
- ΣQ: Total number of transects with the structure.
  - Found under: *Quantities - Filtered*
- $\Sigma P(ref)$: Total number of box corners that hit reference space.
  - Found under: *Quantities – Filtered*

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- Quantitative Methods in Neuroscience: A Neuroanatomical Approach by Stephen M. Evans, Ann Marie Janson, and Jens Randel Nyengaard (2004)
- Stereology for Statisticians (Monographs on Statistics and Applied Probability) by Adrian Baddeley and Eva B. Vedel Jensen (2004)

GENERAL STEREOLOGY:

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