CAST is a Computer Assisted Stereological Toolbox for PCs running Microsoft® Windows™.

CAST is an interactive, user-friendly, data collection program to be used for obtaining efficient and unbiased estimates of cell number, cell density, cell size and all other stereological quantities in 2-D and 3-D.

CAST supports several sampling schemes and includes numerous grid types and all known unbiased estimators.
**Sampling**

In order to apply the geometrical probes used in stereology on an object of interest, the sampling must be randomized in order to give every item of a population the same chance of being sampled. Also, the sampling scheme must be comprehensive enough to show how each item was sampled.

Despite common belief, ALL of sampling theory boils down to two possibilities for “correct” or unbiased sampling:

- either sampling is uniformly random in the complete organ (in which case the actual sampling probability may be unknown),
- or the sampling probability must be known exactly and be greater than zero for each and every sampling item.

In stereology systematic, uniform random sampling is almost always performed. In systematic uniform sampling, one decides on a sampling interval, takes the first sampling item at random in the first interval, and all the following sampling items at a fixed distance (the interval) to the previous one. Systematic uniform sampling is used because it is very easy to perform and much more efficient than simple uniform random sampling. In systematic sampling, the sampling items (sections or fields of vision) are dependent (in all ordinary, simple uniform sampling the items must be independent). This has the drawback that all ordinary static is invalid; more sophisticated methods are necessary for computing estimator precision.

**Geometric probes**

Consider an object in a room filled with a systematic, uniform random array of points which are strictly systematic in the array and the whole array is randomly positioned. The number of points which fall inside the object is proportional to the volume, i.e. the larger the volume of the object the greater the probability of a point hitting it and the more points will fall inside it. If the volume associated with each point is known
then an estimate of the volume of the object is simply the number of points falling inside the object multiplied by the volume associated with each point, provided that the initial position of the regular array of points was randomized.

The purpose of choosing a uniform random start is to give all parts of the object an equal probability of being sampled, i.e. for sampling to be unbiased, and is a general requirement of all stereological methods.

Notice that in moving from a 3-D object to a set of 2-D sections, the object’s volume is represented by or is reduced to an area, its surface to a boundary and, if it is a very long object, its length is represented by a number of profiles - see the figure below.

Orientation of tissue

The above deals with the random position of all probes. For a number of important estimators it is equally important that the 3-D structure or the probes have systematic, uniform random orientation, called isotropy, in 3-D space. Prime examples are estimators of surface area and length, but also most of the very potent cell size estimators require isotropy. Unbiased estimators of total volume, number and connectivity do not require isotropy. In order to fulfill these requirements, all existing methods require that the tissue is given random orientation. Besides simply rotating the organ or the block of tissue randomly before sectioning, which is called IUR sampling, there is fortunately a simpler and often better way, namely to rotate the organ or block of tissue around a single, identifiable axis of the experimenter’s own choice, the so called uniform vertical design. Vertical designs most often allow identification of important layers and regions on the proper sections, but require special test systems, all of which are implemented in the CAST software.

Sampling and estimation: Dimensions

A random test point will fall inside an object with a probability proportional to the object’s volume, hence its use to estimate volumes. A random test line will hit an object’s surface with a probability proportional to the object’s surface and a section will contain an object’s profile with a probability related to the object’s length or mean height. These relationships are summarized in the following tables:

<table>
<thead>
<tr>
<th>Stereological probe</th>
<th>Geometric characteristic of 3-D object</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Measure</td>
</tr>
<tr>
<td>Point</td>
<td>Number</td>
</tr>
<tr>
<td>Line</td>
<td>Length</td>
</tr>
<tr>
<td>Plane</td>
<td>Area</td>
</tr>
<tr>
<td>Disector</td>
<td>Volume</td>
</tr>
<tr>
<td>Name</td>
<td>Measure</td>
</tr>
<tr>
<td>Volume</td>
<td>Volume</td>
</tr>
<tr>
<td>Surface</td>
<td>Area</td>
</tr>
<tr>
<td>Curve</td>
<td>Length</td>
</tr>
<tr>
<td>Cardinality</td>
<td>Number /Connectivity</td>
</tr>
</tbody>
</table>

From the tables a pattern emerges: the dimension of the probe plus the dimension of the geometric characteristic of the object equals 3 so that the geometrical characteristic which is being estimated is meaningful in 3-D space. These are the principal relations when estimates are obtained by counting events.
The stereological estimators

Stereology may be thought of as a tree with two main trunks: global and local estimators. The two trunks branch off into various geometric quantities one may estimate: volume, surface area, length, number and connectivity.

The global estimators are used to estimate geometric quantities of “large” structures, e.g. entire organs and regions therein. The local estimators are used to estimate geometric quantities of individual “small” structures, i.e. cells. As an extra benefit, one therefore often has free-of-charge access to important information about the size distribution of these cells.

For decades, mostly global stereology has been a valuable tool in biology, material sciences and in geology for estimating compositions of organs, metals and rocks. Collectively, these estimators are now known as “classical” stereology.

In 1984 a new estimator named the “disector” was published. This was the starting signal for a long line of new estimators tuned to the estimation of 3-D characteristics in biological materials, mostly developed by a group of Danish stereologists with Professor Hans Jørgen Gundersen. The collective name for these estimators is “new” stereology.

Sampling using CAST

When using the grids or the local estimators of the CAST software, it is usually required that the objects under investigation are sampled in an uniform fashion. That is, given a certain area with objects to be sampled, every object must have an equal probability of being sampled.

Conventional methods require that the investigator keeps track of the current position in the microscope slide along with counting with grids or using the estimators.

With the use of a software controllable specimen stage, it is possible to merely enter the outline of the area to be investigated and the desired number of sampling steps and leave the sampling procedure to the software.

In the CAST software this sampling procedure is called “meander sampling”.

Meander sampling

When using meander sampling the area under investigation is delineated at a low magnification. Multiple inclusion and exclusion areas can be delineated.

After delineation and input of the approximate number of sampling steps, step size or sampling fraction, sampling (and probing) is often performed at a higher magnification.

While sampling is in progress, a map of the delineated areas and the sampling positions therein may be displayed.

X-Y stepper

In situations where the area of interest is easily recognized at any power of magnification, sampling may be done using the X-Y stepper instead of meander sampling.

After defining the x- and y-step lengths or sampling fraction, a random position inside the area of interest is chosen by the investigator. Movements to other sampling positions are done by pressing the arrow keys.

Probing using CAST

The CAST software allows for numerous grid types to be superimposed onto the video image for the purpose of collecting data for the global estimators. The local estimators are performed using functions that, in addition, control the sampling interface (orientation of counting regions and intersection lines) themselves.

The composition of the grids is completely controlled through a setup dialog.

The following grid types are used for the global estimators: points, encircled points, line segments, counting frames, secondary frames, cycloids and lines.

The lines, spatial distribution and guard area grid types are used for the local estimators, but their size is controlled from the grid’s dialog box.

Global estimators

Most of the global estimators use grids for the collection of the data needed for the estimates. The relevant grid types, the information they carry and their purpose is:

<table>
<thead>
<tr>
<th>Points</th>
<th>Information:</th>
<th>Purpose:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area/unit, area/point.</td>
<td>Estimation of profile area, volume fractions and total volume (using Cavalieri’s principle).</td>
</tr>
</tbody>
</table>

Integrated point sets

<table>
<thead>
<tr>
<th>Information:</th>
<th>Purpose:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area/unit, area/point.</td>
<td>Used for the same purpose as - and in conjunc-</td>
</tr>
<tr>
<td>tion with - point grid in situations where different objects are counted at different grid densities.</td>
<td></td>
</tr>
</tbody>
</table>
### Line segments

**Information:** Area/point, area/unit, length/point, length/unit.  
**Purpose:** Estimation of profile boundary length, shape factor and surface area on IUR sections along with profile area and volume fractions on vertical and IUR sections using the two point sets at the ends of line segments.

### Counting frames

**Information:** Area/unit, area/frame.  
**Purpose:** Counting of profiles for length density of tubules, fibres and capillaries. Estimation of number in combination with the optical disector. Selection of profiles for measurements using the local 2-D area or 3-D volume estimators.

### Secondary counting frames

**Information:** Area/unit, area/frame.  
**Purpose:** Same as - and in combination with — counting frame but in situations where sets of objects with different numerical densities are counted or sampled at different fractions of the screen area.

### Cycloids

**Information:** Area/cycloid, length/cycloid.  
**Purpose:** Estimation of surface area on vertical sections along with volume fractions using the point sets at the end of the cycloids.

### Lines

**Information:** Area/point, length/point.  
**Purpose:** Estimation of surface density on vertical or IUR sections and local volume using PSI.

For the purpose of estimating the total volume of an object using Cavalieri’s principle, there are two alternatives to point counting. The first is to use the 2-D nucleator function, the second is to delineate the area to be estimated. Either function must be applied to each of the selected sections.

#### The disector

The disector is a simple and unbiased 3-D probe for estimating number. Since number is a zero-dimensional structural characteristic, it can only be sampled and estimated unbiasedly in 3-D probes, i.e. using a sampling volume. Two sections are used to create the sampling volume with the lower reference section, containing a sampling (counting) frame. The sampling volume is the area of the frame multiplied by the distance between the upper surfaces of the two sections. The counting (sampling) rules associated with the counting frame are:

- Every profile completely inside the frame is counted.  
- Profiles that touch only the inclusion lines (green lines) are counted.  
- Profiles that touch the exclusion lines (red lines) or their extensions are not counted.

The disector 3-D counting rule:

A particle is counted (sampled) if it appears in the sampling frame in the lower reference section, its profile does not touch any of the exclusion lines, and it does not appear in the upper section at all.
The optical disector

The optical disector is a very efficient method for implementing the disector principle. By using a lens with a short focal depth (high numerical aperture) on thick (~25 - 100 µm) sections, the two sections of the disector can be contained in a single slide. Furthermore, this principle eliminates any risk that objects between the disector sections can be lost.

First the top and the bottom of the disector are determined, then sampling is performed by starting at the top and focusing towards the bottom while counting all new particles that come into focus in the section. This means that particles already in focus in the upper focal plane are not counted, while particles in focus in the lower, reference focal plane are counted. This is analogous to the concept of inclusion/exclusion lines in the counting frame.

Integration of the geometric probes

It is a most useful feature of the CAST software that all the geometric probes and the grids may be combined or integrated (that is why they are strictly organized in tessellations of so-called units). Instead of studying the same section five times in order to estimate five interesting quantities, one studies the section once using the test system composed by the user for this particular purpose. The customized test system may be stored and later recalled in the CAST software.

Local estimators

The selection of objects to be used for local volume estimation is most often done by using meander sampling and counting frames or optical disectors.

The grids dialog box shown on the previous page allows for the definition of an additional grid type to be used by the local estimators:

Spatial distribution

Information: The area of each of the four zones, a(1) through a(4).
Purpose: Estimation of the spatial distribution, e.g. attraction/repulsion of cells.

The CAST software currently supports local volume estimators, surface area estimators, length estimator and number estimator.

The volume estimators, which are all available in versions for either vertical or IUR sections, are:

- Point Sampled Intercept (PSI)
- 3-D Nucleator
- Planar Rotator
- Optical Rotator
- Spatial Rotator

The first three calculate the estimates based on planar (2-D) information and can thus be used on thin as well as thick sections, whereas the last two use spatial (3-D) information and are optimized for thick sections in which they give more accurate estimates than the planar estimators do.

The rotator estimators are usually the most efficient individual cell volume estimators. PSI provides a volume-weighted volume estimate which has a very useful application in cancer diagnostics and objective cancer grading.

The figure below illustrates the rotator estimator in the version for IUR sections. In a cell which is sampled using the disector, a unique point, the nucleolus, is indicated...
by the user. Three lines will randomly be placed through the cell and the user must now enter the intersections between these lines and the border of the cell being examined. Finally the volume estimate is calculated by the software.

The area estimators are:
- 2-D nucleator for 2-D areas
- Optical rotator for 3-D surface areas

The 2-D nucleator operates on planar information, whereas the optical rotator operates on spatial information.

**Simple 2-D measurements**
The CAST software allows for a few simple 2-D measurements on objects seen on the screen. These are: distance, boundary as well as area and are carried out by drawing, either free-hand or in steps, by the mouse.

**Additional features in CAST**
CAST allows for acquired data to be logged for subsequent processing in spreadsheets.

Images can be saved and loaded thus allowing for images from alternative sources (CT scanners and electron microscopes) to be analyzed. CAST can work with multiple images.

CAST has the physical disector – split screen disector – as standard in its software packages.

Counting of numerous different cell types or occurrences is easily handled by the use of a counting tool dialog box and the numeric keypad.