

The Disector Counting Technique

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ABSTRACT

Number of particles in an organ or region is a valuable data as well as the volume. Many techniques have been developed for the estimation of total number or numerical density of cells in tissues and organs. The quality and reliability of each method has constantly been increased compared to its predecessors. Numerical density is one of the parameters that are used to assess the association between a structure and its function. It should be kept in mind that reliability of biological comments derived from the data is dependent on the method that is used for quantitative estimation of parameters. As stated previously, one of the most important morphometric parameters in biological studies is the 'number'. Since the 'number' has no-dimensions (i.e. is not bounded by any dimensional property) it could not be directly estimated using a 2D section plane. A good alternative is to use a "volume probe" generated by two consecutive sections, which is called the disector. The disector counting method that was developed by Sterio in 1984 is the most efficient and unbiased solution for particle counting. In this review, we will summarize the basic principles of the disector approach and give some examples focused especially on some basic topics in neuroscience research.

Key Words: disector, counting, numerical density, stereology, neuroscience

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1. Introduction

The disector is a simple method that allows us to count particles under the microscope unbiasedly in a virtual 3D volume generated by two consecutive physical or optical sections separated by a distance "t" (Sterio, 1984). Although, the basic logic rules behind the method were first described by William R. Thompson in 1932, this method was not attracted much attention of the scientists for a long time. Later years, the method was redefined by Cruze-Orive (1980) and it was recognized as the first design-based

stereological study in the literature (Sterio, 1984). With the use of such an approach, De Groot and Bierman (1983) showed in practice that without the requirement of observation for every component of a structure of interested, quantities can be estimated unbiasedly and truly in biological environment by taking serials sections.

The disector method was developed and re-described for the particle counting by Stero in 1984. Therefore, the birth date of the disector in modern way is generally accepted as 1984 by researchers (Geuna, 2005). Stero, who did not wish his/her name to be associated in perpetuity with the method given in the paper, published a paper in the Journal of Microscopy entitled "The unbiased estimation of number and sizes of arbitrary particles using the disector," hiding his/her identity under the nom de plume of D.C. Sterio, which is an anagram of "disector" (1984). He/she described a three-dimensional counting rule for obtaining unbiased estimates of the number of arbitrary particles and, in combination with other methods, of various mean particle sizes (Sterio, 1984; Geuna, 2005). The method was based on counting

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particles, whose transects are completely or partially inside the counting frame, on sets of pairs of parallel sections separated by a known distance. In practical terms, only those particles that appear in one of the two histological sections (the reference section) and not in the other (the look-up section), are counted (Geuna, 2005).

2. Variants of disector method

In 1988, the first article of disector was published comparison of two physical sections (Pakkenberg and Gundersen, 1988). Two commonly used variants of disector method are known as physical and optical disector. Using the disector method, one can obtain an unbiased estimate of any numerical quantity without affected by the factors arising from different causes including particle size, particle distribution, and orientation of particles, section thickness, and tissue shrinkage or swelling (Hatton and von Bartheld, 1999; Gardella *et al.*, 2003; Kaplan *et al.*, 2010; Odaci *et al.*, 2010; Tümkaya *et al.*, 2010). Due to above mentioned properties, optical and physical disector counting techniques are the most efficient and unbiased methods in the estimation of particle numbers (Howard and Reed, 1998).

Structures contain a small number of particles, say, up to hundreds; can be counted with the disector method which will be detailed in the text later. To count the number of particles in thousands or more, sampling is required in order to estimate the total number (Gundersen and Jensen, 1987). Different versions of the disector method have been developed for counting total numbers. One of them is the sampling application using fractionator approach. In this method, particles within the area of interest are counted in a specific fraction with the help of data obtained from a preliminary (pilot) work. Total number of particles can be estimated by evaluating the data obtained from the fraction to the whole structure. Results will be true and reliable if the sampling interval is dense enough for the fractionation (Gundersen and Jensen, 1987; West, 1993). Another common approach used for estimation of total number is a two-step process that requires counting the number of particles within the unit volume (N_v , Numerical Density) and then multiplying it with the total volume of the structure (V_{ref} , Reference Volume) (Gundersen, 1986). When the disector method proceeded alone, it

gives us the number of particles in the unit volume, in other words, numerical density (N_v) (Howard and Reed, 1998).

3. Basic rules of disector method

To understand the relationship between tissue and its function based on quantitative data, it is important to have the quantities as accurate as possible. Morphological data like volume, surface area, number, length etc. is obtained from biological materials contributes to understanding of a structure as well as the relationship between its function and structure. For instance, the number of mitochondria in a muscle cell may tell us about the amount of energy that is requested by the cell. The disector method enables us to determine the number of particles in a known volume independent from their size, shape and orientation by a three dimensional probe created using two dimensional consecutive sections (Gundersen *et al.*, 1988; Mayhew and Gundersen, 1996). Therefore, this approach based on two parallel section planes that are separated each other by a precise "t" interval. The rules of disector counting are very straightforward. Particles are counted only if their profiles, which is a two dimensional image of a tissue component's in sections, are only seen in one of those two section planes. In the stereology terminology, particles that are counted using the disector counting technique is known as the "disector particle" and is denoted by the symbol "Q-" (Sterio, 1984; Coggeshall, 1992; Howard and Reed, 1998) (Fig. 1).

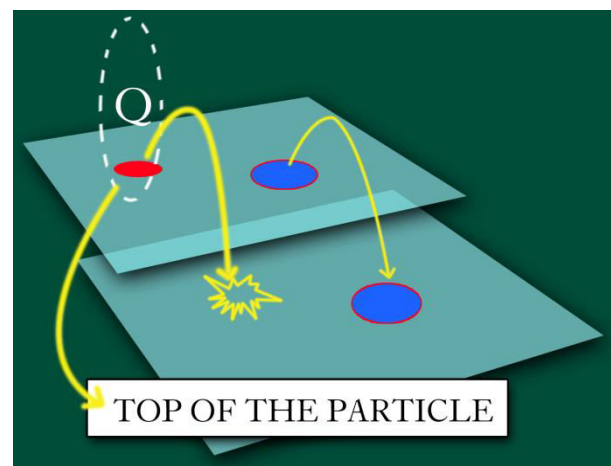


Figure 1: The red profile seen in one of two section planes. Thus the particle represented by this profile can be regarded as a disector particle (Q^-) for this disector volume.

The principle of the disector method is simply to count particles within an imaginary

volume created by a distance between two adjacent sections. Multiplying the distance that is seen between two adjacent sections by area of counting frame that are superimposed on the corresponding areas of both sections would give us the disector volume (Fig. 2). The number of particles within a unit volume, i.e. the numerical density, can be determined if the counted total numbers of particles in a disector volume is divided by the disector volume (Gundersen, 1986; Howard and Reed, 1998). Briefly, the disector method is an opportunity to estimate the numerical density of particles in an unbiased manner. In addition, if we have the unbiased estimated total volume value of the structure that we work on, then we can estimate the total particle number (N) contained by the structure by multiplying the particle density estimated using the disector (Nv) by this volume. This method is also known as the Cavalieri-disector combination (Gundersen, 1986; Howard and Reed, 1998).

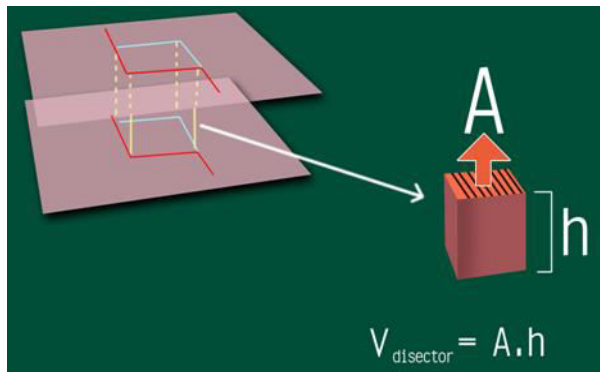


Figure 2: A known distance between disector pairs and the counting area delineated with the unbiased counting frame. Multiplication of these two values gives us the disector volume per each disector pair.

4. Application of the disector method

Important points that should be taken into consideration for an accurate disector application are listed below.

4.1. Preliminary Study

An important feature of the stereological methods is the efficiency, i.e. to obtain more reliable results in relatively short time and with relatively less work. Efficiency, when united with phenomenon of being unbiased, the power of stereological methods comes in sight. To be unbiased, one should not have any pre-assumptions about the tissue geometry and let the sampling and estimation procedures yield the most accurate data.

(Howard and Reed, 1998; Gundersen *et al.*, 1999; Dorph-Petersen *et al.*, 2000).

On the other hand, efficiency is based on adjustable sampling and measurement density, according to the amount of error acceptable for the work. The amount of error arises from different sources. The most important of all is the quantitative variability between the study samples (i.e. animals) which is the major contributor to the overall error of the study. Secondly, the sampling steps, sectional areas investigated and the counting areas decreasingly contribute to the total variation, respectively (Gundersen *et al.*, 1999; Dorph-Petersen *et al.*, 2000).

As a general rule, the 'coefficient of error' (CE) which is the statistical term for the total amount of error in a subject, can be controlled by sampling frequency and by manipulating the measurement density (Gundersen, 1987; Schmitz, 1998; Schmitz *et al.*, 1999; Hof and Schmitz, 2000). The only requirement for such manipulations to work is that the methods used in the study should be truly 'unbiased'.

In general a CE value less than 0.05 values are accepted as a valid amount of error (Gundersen, 1987). To ensure that, it should be known that how much each sampling step contributed to the total variation. Prior to each study, such contributions from several error sources must be determined using a pilot study and then the optimal scheme must be applied to the real study without changing the parameters during the entire course (Howard and Reed, 1998).

The most convenient way to reduce the total variation is to add as many individuals (samples) as possible. For most of the animal studies, 8-10 individuals are acceptable as the lower limit. The number of individuals has the crucial importance, since the quantitative difference between individuals is the major contributing source of variability to the total variation. Moreover, sampling more sections, recruiting more counting areas and counting more particles by larger counting frames also increase the accuracy by reducing the total variation in some degree. The most important thing to do is to determine the most appropriate sampling scheme by taking all the mentioned factors into account. Thus, the significance of the preliminary work comes into play at this point. Preliminary work is done to determine the most appropriate statistical approach that will be applied during

the actual study, and according to the results of this preliminary study, a sampling scheme is decided (Gundersen, 1977; Howard and Reed, 1998; Ünal et al., 2002). It is a serious advantage to have some previous stereological reports in hand on the structure that we interested in (e.g. an area in the rat brain) as a starting point. The sampling frequencies used in those studies (if the species of animals, area of interest and the sampling scheme are clearly defined) would serve as a good starting point for your own study design and would enable you to save serious time which otherwise should be spend on a pilot study. (Howard and Reed, 1998; Ünal et al., 2002).

4.2. Histological processes

As previously mentioned, the dissector method requires optical or physical sections that are taken from a tissue block. Physical sections can be used for routine light microscope preparations or electron microscopic images, while optical scanning of a relatively thick tissue section prepared for light microscopy can generate optical sections. Since the dissector method relies only upon the “existence of the particles”, importance of a careful section preparation process is crucial for an unbiased estimate of particle numbers. Therefore accuracy for the preparation of the slides during histological process (i.e. embedding, sectioning and staining) will improve the quality of sections and therefore, the reliability of counting (Howard and Reed, 1998; Ünal et al., 2002; Bas et al., 2009a).

Throughout the sectioning process it is important to take adjacent parallel sections. It is easier to do so, as sectioning is done at the same way in each time. Unparallel sections will result miscalculation of the dissector volumes as well as the particle number (Fig. 3).

4.3. Distance between consecutive sections

Another important point about the dissector sections is the distance between adjacent sectional planes. This distance should be less than 1/3 or 1/4 of the average height of particles. Otherwise, there might be some uncut particles in the tissue and as a result there is a high chance of ignoring some particles off the counting procedure (Fig. 4). Regardless of the section orientation, it is important to keep the orientation constant throughout the study. Since the distance

between sections is important for the determination of the dissector volume, the dissector height and section thickness should be known. As demonstrated below, an error in the calculation of dissector volume will directly affect the number estimations (Gundersen, 1986).

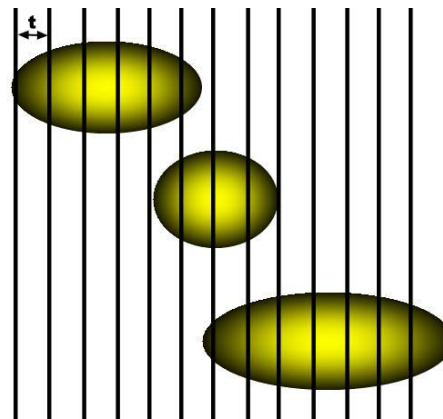


Figure 3. In dissector application we have to take parallel sections, which are taken in a systematic random manner. If section intervals 't' are larger than the mean particle height perpendicular to the section planes, sectional planes may not hit some of the particles, especially smaller ones. This will result in an underestimation of the particle number.

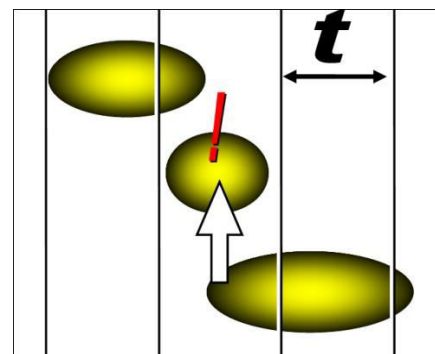


Figure 4. The distance between parallel sections has a critical importance since if we apply an intersectional distance more than the mean particle height, sectional planes may not hit some of the particles and hence we cannot sample all particles uniformly.

Two separate sections are needed for the dissector method. One of those sections is recognized as the reference section while the other will become the look-up section (Sterio, 1984; Geuna, 2005). The basic idea behind the dissector method is to count profile of each particle (cell, glomeruli, etc.) that is seen in one section but not on the other (Sterio, 1984; Geuna, 2005). In general, profiles seen on the reference section is not on the look-up section are counted as a dissector particle. Such an approach is equal to count the “tops” of the particles that have trapped inside the distance between two sections. If each of the particles

has one top towards a direction, which generally is the case, such an approach allows us to determine the particle number in a given (disector) volume in an unbiased manner, since we count each particle “once” (**Fig. 5**). Obviously it is also important to accurately judge the profiles belonging to the same particle or not.

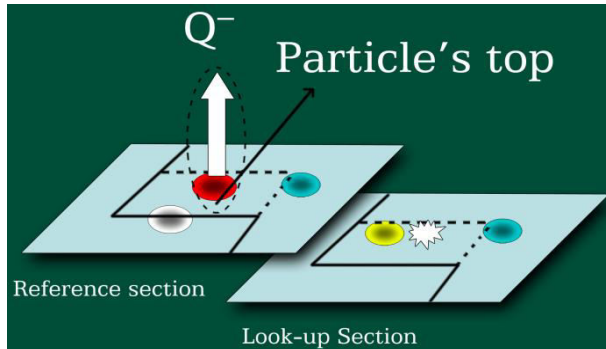


Figure 5. The red profile seen in the section on the left is only visible on reference section and contained in the borders of the unbiased counting frame (see below). Thus the particle represented by this profile can be regarded as a disector particle (Q^-) for this disector.

4.4. Sampling strategy

An appropriate sampling strategy at the levels of section, area and thickness should be determined before analyzing the structure. A commonly used sampling strategy is the systematic random sampling (SRS); which must be determined for each and every sampling level for a particular study before the actual application by means of a pilot or preliminary study. SRS relies upon a basic principle: Samples are chosen with a predetermined fixed interval, selecting the first sample of the set randomly. The first part ensures a systematic sampling while the latter provides randomness. For example, if we decided to select 1/5 of a structure, we should take every 5th section and the following section (to make a disector pair), during the serial sectioning procedure. To make this systematic sampling random, we choose the first disector pair randomly within the first 5 sections, and then we take every 5th pair of sections until the end of structure. A random start point will make the systematic sampling “random”. For example, if we took 2nd and 3rd sections as a disector pair, then the next pair would be 7th and 8th sections, and the next would be 12th and 13th, and so on. After the first random selection, locations of all preceding disector pairs are determined and the whole tissue will be sectioned and sampled accordingly and

exhaustively (Howard and Reed, 1998; Ünal *et al.*, 2002; Bas *et al.*, 2009b; Odacı *et al.*, 2010).

4.5. The distance between the section pairs or the “section thickness”

During the disector application, we may encounter another problem if we are dealing with rather larger particles. For instance, counting of renal glomeruli is a good example for this situation. Because the glomeruli are large structures compared to section thickness, they will appear on numerous sections when they are cut by serial histological sections. In such cases, to use adjacent sections for counting of glomeruli is not suitable in terms of the basic rules of the disector, in which the profiles of the same particle and a profile of other particle must be seen in disector sections. If we use thin adjacent sections for counting of glomeruli, the profiles of the same particles appear in a large number of disector pairs without the profiles belonging to other glomeruli. This prevents the application of disector counting technique. To prevent this unwanted situation, disector sections for glomeruli should be taken in an acceptable distance for the disector counting of glomeruli. For example, 5th and 15th sections could be used as a disector pair. In this way, assessment of unnecessary sections in between will be avoided. Note that, in such a case the disector height will be equal to the distance between chosen sections (the disector height) instead of the section thickness of individual sections. Another rule that should not be missed is that the disector height should not be bigger than 1/3 of the mean particle in diameter or particle “height” (**Fig. 4**). Specific areas are delineated on each section belonging to a disector pair and each of those fields must correspond the area on its counterpart (Howard and Reed, 1998; Ünal *et al.*, 2002; Ragbetli *et al.*, 2007).

4.6. Identifying and limiting the counting area

One of the issues to be considered for the disector counting is the identification of the corresponding areas in the adjacent sections. In order to distinguish the profiles of the same particle and exclude them conveniently from the counting registration of corresponding areas must be performed carefully. Once the areas are determined, confining an area using an unbiased counting frame with an appropriate is another important step in the disector application. To estimate the particle

number unbiasedly, the counting frame and disector rules used in the counting have crucial importance. To our knowledge, the unbiased counting frame of Gundersen (1977) is the most unbiased technique available for areal counting (Gundersen, 1977; Howard and Reed, 1998).

The basic logic behind the disector counting rules aimed to ensure that each particle is sampled only once. In **Fig. 6**, there are particles in different sizes and numbers

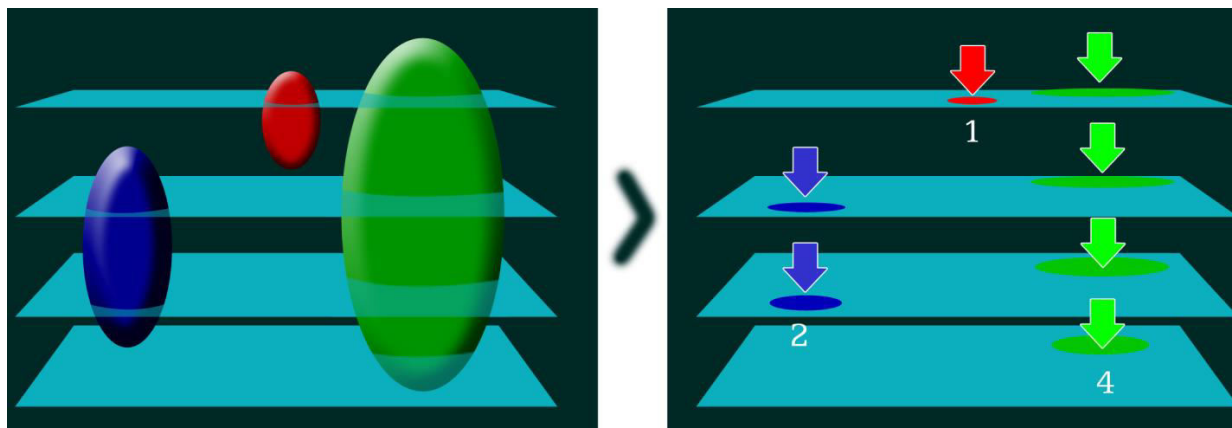


Figure 6. Particle size affects the results if we do not use unbiased counting methods. Particles of different sizes generate different numbers of profiles on serial sections. Bigger particle can be seen more than one section while the smaller one appears less. Counting every visible profile on sections would give an obvious overestimation of particle number, since those profiles represents the sizes of particles rather than the “number” of them.

There are some rules designed to sample each particle only once during the counting using the sectional planes. If the sectional profiles of particle-containing structures have their own natural borders and if all the cells to be counted can be distinguished in terms of location at first glance, there is no need to delineate a border with imaginary rules. One can directly counted each particle on the specimen applying the disector rules (Sterio, 1984). For instance, for the cell counting procedure of renal glomeruli (and in some small brainstem nuclei), if it is possible to see all the cell profiles simultaneously in each section, there will be no need for area delineation or sampling. The chance of a profile being counted in more than one time in such images are considerably low (Sterio, 1984; Howard and Reed, 1998; Ünal et al., 2002).

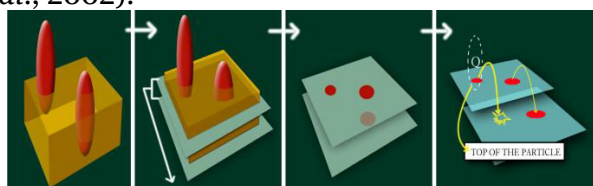


Figure 7. The basis of disector is to count the tips of the particles. If we can count only one tip of each particle in a

given direction, we can obtain an unbiased estimate of the total particle number since generally every particle must have only one tip towards a given direction. Hence at least theoretically, this method must give an unbiased estimate of the total number.

within a tissue block. If all profiles of particles on each of 4 sections obtained from this structure are counted, this would not give the real particle number, i.e. 3 in this case. But instead of counting each profile on sections, one may count the particle ends at one direction (either top or bottom) and thus can achieve the true number of particles (**Fig. 7**) (Sterio, 1984; Howard and Reed, 1998; Ünal et al., 2002; Kozan et al., 2007; Ragbetli et al., 2007).

If we are working in a field containing numerous large profiles or particles in a single image that could not be observed in a general look, limiting a certain area for counting with some specific rules is a necessity. We cannot count each profile in all sections one by one (that is often the case in practice). The problems arising from the artificial borders used to delineate a tissue area are known under the term of “the edge effect” in the stereology, and an efficient solution is offered by the famous Danish stereologist H. J. G. Gundersen (Gundersen, 1977). In the area of neurostereology, we generally do not face such problems since our structures of interest are generally small in size and thus can be mounted and sectioned as a whole in most circumstances (as in the animal studies).

4.7. Unbiased counting frame

Counting frames (**Fig. 8**) are used to limit the counting area on section. It is easy to deduce

that the profiles located inside such a frame are counted and introduce not much problem. But when some profiles located on or hit by the borders of the counting frame, the problem arises: will it be counted or not? Previous studies clearly demonstrated that the use of a “biased” counting frame would lead a serious under-or-overestimation of the particle size; which gets more dramatic as the sample size increases. As shown in the Figure 8, checked

(✓) particles are not only being contained in the current counting frame area but they also belong to the neighboring areas. Whether they are ignored or included in the counting, it will obviously be a biased estimation. Thus, during the sampling, it should be guaranteed that the each particle must be sampled and counted only once (Gundersen, 1977; Sterio, 1984; Ünal *et al.*, 2002).

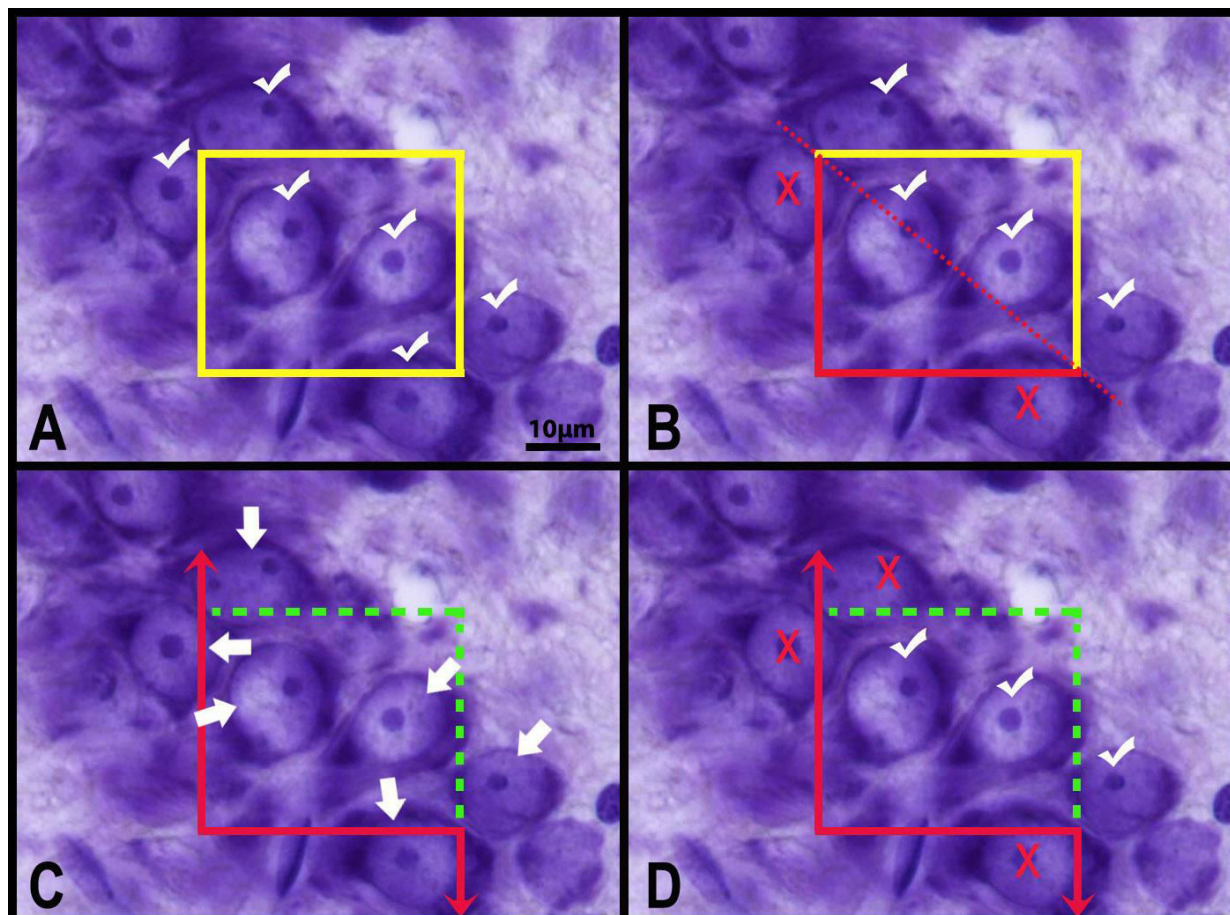


Figure 8. Controversial profiles located on the “borders” of the counting frame. If all profiles are included in the estimation, the result will be an overestimation and if we ignore the ones on the borders completely, it will be an obvious underestimation. In top-left, all the particles associated with the particular frame are shown. On top-right, one can count all profiles associated with the frame, which leads to an overestimation of particle number. On the lower-right, counting frame divided into two equal halves and lower half considered as “exclusion area” and the other half as the “inclusion area”. This approach, despite its intuitive justice, leads to an overestimation of the particle/profile number. We have to achieve an unbiased sampling during the counting. The counting rules of the unbiased counting frame depicted at the bottom are simple: One must count all the profiles trapped completely inside the frame and the profiles hit by the inclusion edges (dashed lines shown in blue). On the other hand any profile hitting the exclusion edges and their extension (shown in red) must be excluded from counting (Gundersen, 1977; Sterio, 1984; Howard and Reed, 1998; Ünal *et al.*, 2002; Ragbetli *et al.*, 2007; Kaplan *et al.*, 2010).

To gain an unbiased count of the profiles appeared on a planar section surface Gundersen developed a counting frame, which is now famously called ‘the unbiased counting

frame’ in 1977 (**Fig. 8**). Counting rules of this frame can be summarized as follows (Gundersen, 1977; Sterio, 1984; Howard and Reed, 1998; Ünal *et al.*, 2002):

1. Profiles completely within the counting frame that does not intersect with any edge or corner are counted;

2. Profiles that intersecting with the lines expressed as 'banned' edges of the frame are not counted.
3. The particle profiles contacting the inclusion-lines are included in counting.
4. Profiles contacting with the extensions of banned lines are not included in counting as well (Gundersen, 1977). The orientation and the function of these extensions are very important. The detailed geometric description of this frame can be found in Gundersen's study (1977).

It is easy to demonstrate the efficiency of the unbiased counting frame and the counting rules as shown by the example depicted in **Fig. 9**. You can test the accuracy and efficiency of the counting frame by dividing the area of interest into a tiling of adjacent sampling areas. Each of these areas represents a complete image area which can be seen under the microscope during inspection and during the inspection of one field, others remain outside our field of vision. In this figure, only five particle profiles are presented in the whole area. However, counting results obtained from sub-areas with different rules, may cause different results (Ünal et al., 2002).

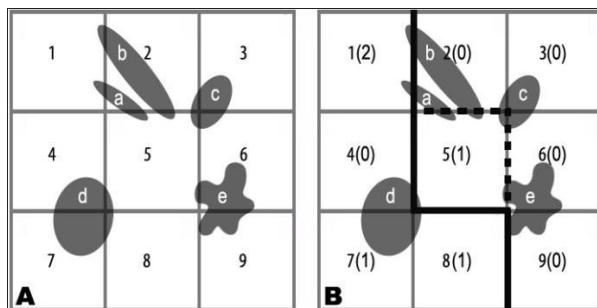


Figure 9. The sampling areas with five profiles are shown in A. The number of profiles obtained in each small square and number counted according to the rule illustrated in B. The sum of the latter figures is independent of the number of small squares into which the large square has been divided. Only the edge effect due to profile "e" is dependent upon non-convexity (Modified from Gundersen 1977).

5. Application of disector method on sections

During the application of the disector method, only the particles belonging to that volume should be included in a particular disector pair. One solution of this problem is the unbiased counting frame, which is detailed above. The basic logic of this frame is to ensure a particle is sampled once and only once in its relevant area. As it can be seen in the **Fig. 5**, in two consecutive sections, some particles found

in the reference section but not in the look-up section is counted as disector particle in the disector volume, as well as an area sampling is performed by an unbiased counting frame located on the image of the reference section and its rules. If any ends of the particle profiles do not touch the forbidden lines, they could be counted as disector particles. In addition, the ones are completely inside the frame are also included (Gundersen, 1977; Sterio, 1984; Howard and Reed, 1998; Ünal et al., 2002).

In brief, if we count the particle profiles in the Figure 5 according to disector counting rules, the blue particle will not be counted whereas the red one will be counted (Sterio, 1984; Ünal et al., 2002).

The main rules for the disector counting method can be simplified as follows;

1. Sampled section pairs should be parallel to each other.
2. The section thickness or the height of disector, which is the distance between two section planes, should be at least 1/3 of the smallest possible particle under investigation.
3. The distance between the surfaces of section pairs should be known.
4. A frame with a known area should be used for counting profiles.
5. The section samples should be chosen systematic random sampling approach.
6. Particles that have profile in the reference section whereas not in the look-up section, and fulfilling the criteria of the unbiased counting frame are counted and others excluded (Sterio, 1984).

6. Estimation of numerical density and total number using disector counting method

6.1. Numerical density

During the application of the disector method, we perform counting on sections with specific sampling steps. Eventually, by using certain number of disector, we count the disector particles trapped inside those disector volumes (Q). As we know the volume of each disector probe (V_{dis}) and the total number of disectors used, we can calculate the total disector volume (ΣV_{dis}) used in the study by multiplying these two. After we have the total disector volume and the total disector particle number (ΣQ) we can calculate the number of particles within a unit volume or, the

numerical density (N_v) as follows (Howard and Reed, 1998; Ünal *et al.*, 2002):

$$N_v = \Sigma Q / \Sigma V_{dis}$$

This formula gives us an unbiased estimate of the particle number per unit volume within the structure. However, specifically in comparative studies, the total number estimations should always be preferred, since the numerical density value does not include volumetric changes about the organ and does not provide enough information.

4.2. Total number

To estimate the total number of particles, we need to know total volume of the structure. For instance, if the number of neurons within a unit volume of Cornu ammonis of the hippocampal formation estimated by the disector method, we need to know the total volume of the hippocampus in order to obtain the total number of particles (neurons). Volume of a specific structure can be conveniently estimated using the Cavalieri's principle. When unbiased volume estimate is multiplied by the numerical density value obtained using the disector method, this will give us an unbiased estimation of the total number of particles in that structure or organ.

$$N = N_v \cdot V_{ref}$$

This method is also known as the Cavalieri-Disector combination (Gundersen, 1986; Howard and Reed, 1998; Ünal *et al.*, 2002, Elfaki, 2011).

Conclusion

'Number' is one of the most important parameters enlighten the relation between a structure and its function. It should be kept in mind that validity and reliability of biological comments are dependent on the method used for quantization. We have known that 'number' is a zero-dimensional parameter, and thus cannot be achieved solely on 2D sectional information. To have an accurate number estimation one needs to use a volume probe that could be made by two adjacent sections, unless the direct, exhaustive counting is possible. We can reliably state that the disector counting method developed by Stereo in 1984 is the most efficient and unbiased solution to count particles (Bradley *et al.*, 1994; Hunter and Stewart, 1993; Geinisman *et al.*, 1996; Lowndes and Stewart, 1994; Sarsilmaz *et al.*, 2007).

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